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Submerged Cultivation of Medicinal Mushrooms: Bioprocesses and Products (Review)

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ABSTRACT: Medicinal mushrooms belonging to higher Basidiomycetes are an immensely rich yet largely untapped resource of useful, easily accessible, natural compounds with various biological activities that may promote human well-being. The medicinal properties are found in various cellular components and secondary metabolites (polysaccharides, proteins and their complexes, phenolic compounds, polyketides, triterpenoids, steroids, alkaloids, nucleotides, etc.), which have been isolated and identified from the fruiting bodies, culture mycelium, and culture broth of mushrooms. Some of these compounds have cholesterol-lowering, anti-diabetic, antioxidant, antitumor, immunomodulating, antimicrobial, and antiviral activities ready for industrial trials and further commercialization, while others are in various stages of development. Recently, the submerged cultivation of medicinal mushrooms has received a great deal of attention as a promising and reproducible alternative for the efficient production of mushroom mycelium and metabolites. Submerged cultivation of mushrooms has significant industrial potential, but its success on a commercial scale depends on increasing product yields and development of novel production systems that address the problems associated with this technique of mushroom cultivation. In spite of many researchers’ efforts for the production of bioactive metabolites by mushrooms, the physiological and engineering aspects of submerged cultures are still far from being thoroughly studied. The vast majority of studies have focused on polysaccharide and ganoderic acid production in submerged cultivation of medicinal mushrooms, and very little has been written so far on the antioxidant and hemagglutinating activity of submerged mushroom cultures. The purpose of this review is to provide an update of the present state of the art and future prospects of submerged cultivation of medicinal mushrooms to produce mycelium and bioactive metabolites, and to make a contribution for the research and development of new pharmaceutical products from mushrooms. A brief overview of the metabolic diversity and bioactive compounds of mushrooms produced by submerged cultures is also given.

KEY WORDS: higher Basidiomycetes, medicinal mushrooms, submerged cultivation, effect of nutrients, physical-chemical factors, fermentation strategies, biologically active metabolites, polysaccharide synthesis, antioxidant activity, lectin activity

ABBREVIATIONS: ACSC: Antrodia camphorata in submerged culture; AOA: antioxidant activity; BAM: biologically active metabolites; CCRD: central composite rotatable design; DMF: dry matter of filtrate; DO: dissolved oxygen; DOT: dissolved oxygen tension; DPPH: 2,2-diphenyl-1-picrylhydrazyl; EC: effective concentration; EPC: extracellular phenolic compounds; EPS: extracellular polysaccharide; GAE: gallic acid equivalent; HA: hemagglutination activity; HWEM: hot-water extracts from dried mycelia; IPC: intracellular phenolic compounds; IPS: intracellular polysaccharide; MEB: methanolic extract from culture broth; MEM: methanolic extracts from dried mycelia; SSF: solid-state fermentation

I. INTRODUCTION

Higher Basidiomycetes represent a taxonomically, ecologically, and physiologically extremely diverse group of eukaryotic organisms. Recently, extensive research on these fungi has markedly increased, mainly due to their potential use in a variety of biotechnological applications, particularly for the production of food, enzymes, dietary supplements, and pharmaceutical compounds.1–3 Medicinal mushrooms belonging to Basidiomycetes are an abundant yet largely untapped source of useful natural products with various biological activities.4,5,6 It is estimated that about 650 mushrooms possess medicinal properties, but only several edible (Flammulina velutipes, Grifola frondosa, Hericium erinaceus, Lentinus edodes, Pleurotus spp., and Tremella spp.) and non-edible mushroom species (Ganoderma lucidum, Schizophyllum com-
mune, and Trametes versicolor) have been investigated. The medicinal properties are due to various cellular components and secondary metabolites, which have been isolated and identified from fruiting bodies, culture mycelium, and culture broth of mushrooms. These biologically active metabolites (BAM) belong to several chemical groups—polysaccharides, proteins and their complexes, and various low-molecular-weight metabolites such as phenolic compounds, polyketides, triterpenoids, steroids, alkaloids, nucleotides, lactones, and fatty acids. Some of these compounds have cholesterol-lowering, anti-diabetic, antioxidant, antitumor, immunomodulating, antimicrobial, and antiviral activities ready for industrial trials and further commercialization, while others are in various stages of development. It is worth noting that some species can possess a high variety of bioactive compounds, and therefore have pharmacological effects. The best example is Ganoderma lucidum, which contains more than 400 different BAM, including triterpenes, polysaccharides, proteins, and other bioactive compounds.

At present, 80%–85% of all medicinal mushroom products are derived from fruiting bodies, which have been either commercially farmed or collected from the wild. Only 15% of all products are based on extracts from mycelia. A small percentage of mushroom products are obtained from culture filtrates. However, the production of medicinal mushrooms’ fruiting bodies usually takes several months, and it is difficult to control the quality of the final product. For this reason, the submerged cultivation of medicinal mushrooms has received a great deal of attention as a promising and reproducible alternative for the efficient production of mushroom mycelium and metabolites. Submerged cultivation of mushrooms has significant industrial potential, but its success on a commercial scale depends on increasing product yields and development of novel production systems that address the problems associated with this technique of mushroom cultivation. In spite of many researchers’ efforts for the production of bioactive metabolites by mushrooms, the physiological and engineering aspects of submerged cultures are still far from being thoroughly studied. Especially significant is the lack of information on submerged cultivation of mushrooms in bioreactors.

The purpose of this review is to provide an update of the present state of the art and future prospects of submerged cultivation of medicinal mushrooms to produce mycelium and bioactive metabolites and to make a contribution for the research and development of new pharmaceutical products from mushrooms. A brief overview of the products from mushrooms that have been produced by submerged culture will be given also.

II. MEDICINAL MUSHROOMS’ POTENTIAL TO PRODUCE BIOACTIVE COMPOUNDS IN SUBMERGED CULTIVATION

Mushrooms have long been appreciated for their flavor and texture. Now, they are recognized as a nutritious food as well as an important source of medicinal and pharmaceutical importance to humankind, displaying a broad range of useful biological activities with less toxic effects. Such medicinally potent compounds have been isolated from fungal fruiting bodies, mycelia, and culture liquids. Recent data indicate that many basidiomycetes are capable of growing in the form of mycelial biomass in submerged cultures. However, the vast majority of studies have focused on polysaccharide and ganoderic acid production in submerged cultivation of medicinal mushrooms, and very little has been written so far on the antioxidant and hemagglutinating activity of submerged mushroom cultures.

A. Polysaccharide Production

In view of the importance of the extracellular polysaccharide (EPS), many attempts have been made to obtain these compounds from submerged cultures (Table 1). Several authors screened a number of basidiomycetes mushrooms belonging to various taxonomic and ecological groups for their capability to produce EPS. Thus, 56 strains of higher Basidiomycetes were screened for the production of EPS and biomass in submerged cultivation in nutrient medium containing glucose-peptone-yeast extract. Results showed that most of the basidiomycetes strains screened are potential EPS producers. The best yield (6.01 g/L, conversion yield Yp/s = 0.761) was produced by Agaricus sp. and Oudemansiella canarii (3.54 g/L, Yp/s = 0.131) after 7 days of incubation. Tricholoma crassum had similar production (3.23 g/L with conversion yield of 0.131) but after 14 days of incubation.
strains of *Schizophyllum commune*, *Pycnoporus sanguineus*, and *Trametes villosa* showed wide diversity in biomass and polymer production in submerged culture.

Exo-biopolymer production yield and mycelial growth kinetics of 19 mushrooms varied widely with respect to the mushroom species and their nutritional status. Among the different media examined, a relatively high level of exo-biopolymer production was achieved in potato malt peptone medium. Among the mushrooms screened, *Ganoderma lucidum* and *Phellinus linteus* showed the best growth and polymer yield (1.17–1.52 g/L).

In another study, eight wood-rotting basidiomycetes strains produced EPS in cultivation on glucose-peptone (0.34–1.31 g/L) and beer wort (0.64–3.05 g/L) media. In our screening studies, the tested mushrooms significantly differed in their capability to grow in 2% glucose–containing medium and to produce EPS in shake-flask experiments (Table 2). The yield of mushroom biomass varied from 5.6 g/L to 12.7 g/L in submerged cultivation of *Pleurotus tuberregium* and *Inonotus levis*, respectively, while that of EPS ranged from 0.5 g/L in cultures of *Lentinus edodes* to 2.2 g/L in cultures of *I. levis*. It is

| TABLE 1. Mushrooms Cultivation Conditions for BAM Production |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Main components of medium (g/L)** | **Cultivation conditions** | **Mushroom** | **Product yield** | **Ref.** |
| Potato dextrose broth, 24; malt extract, 10; peptone, 1 | Shaker, 50 mL medium/250-mL flask, 25°C, 10–15 d | 17 mushroom species | Biomass, 0.4–9.6 g/L; exo-biopolymer, 0.47–1.52 g/L | 13 |
| Lactose, 50; peptone, 5; yeast extract, 10 | Shaker, 50 mL medium/250-mL flask, 30°C, 120 rpm, 17 d | *Humphreya cofeeata* | Biomass, 15.5 g/L; EPS, 6.9 g/L | 27 |
| Glucose, 50; Ca nitrate, 5; FeSO₄, 1; nicotinic acid, 1 | Shaker, 100 mL medium/250-mL flask, 28°C, 150 rpm, 14 d | *Antrodia cinnamomea* | Biomass, 2.6 g/L; EPS, 0.5 g/L | 53 |
| Glucose, 39; peptone, 1; yeast extract, 2 | Shaker, 100 mL medium, 25°C, 150 rpm, 7 and 14 d | *Agaricus sp.* | Biomass, 3.1 g/L; EPS, 6.0 g/L | 12 |
| Glucose, 20; (NH₄)₂SO₄, 2; yeast extract, 3 | Shaker, 150 rpm, 50 mL medium/250-mL flask, 25°C, 8 d | 8 mushroom species | Biomass, 7.7–12.7 g/L; EPS, 1.0–2.2 g/L | 71 |
| Maltose, 30; soy peptone, 2; MnSO₄·5H₂O, 2 mM | 3 L medium in 5-L stirred-tank fermenter, 25°C; 2.0 vvm; 150 rpm | *Laetiporus sulphureus var. miniatus* | Biomass, 8.1 g/L; EPS, 3.9 g/L | 69 |
| Glucose, 20; peptone, 5; yeast extract, 5 | Shaker, 1 L medium/2-L flask, 25–27°C, 150 rpm, 8–14 d | *Agaricus nevoi, Omphalotus olearius, Auricularia auricula-judae* | AOA at ethanolic extract concentration of 2 mg/mL 92.1%, 83.4%, and 80.2%, respectively | 25 |
| Glucose, 10; yeast extract, 3; malt extract, 3; peptone, 5; thiamine, 1 | Shaker, 150 rpm, 50 mL medium/250-mL flask, 25°C, 7 d | *Grifola frondosa* | Superoxide anion scavenging activity and reducing power 98.4% and .95 at 100 μg/mL, respectively | 28 |
| Glucose, 40; yeast extract, 4 | 3 L medium in 5-L stirred-tank fermenter, 22°C; the two-stage aeration rate strategy (1.2–0.6 vvm); 150 rpm, controlled pH 4.0 | *Armillaria mellea* | Biomass, 6.65 g/L; EPS with antioxidant properties, 233.2 mg/L | 63 |
| Milled walnut leaves, 40; NH₄NO₃, 1; yeast extract, 4 | Shaker, 150 rpm, 50 mL medium/250-mL flask, 27°C, 10 d | *Cerrena maxima* | HA, 64103 U/mg | 74 |
interesting that the EPS accumulation in culture liquid rather correlated with mushroom biomass yield; nevertheless, among mushrooms tested, *Agaricus nevoi* had especially high productivity, 0.195 g EPS/g biomass.

These and other literature data\textsuperscript{14–16} indicate that the formation of EPS in submerged cultivation constitutes a common characteristic of different species of higher Basidiomycetes. Moreover, the production of extracellular polysaccharides is a widespread process among basidiomycetes belonging to various taxonomic and ecological groups. This conclusion is not unexpected, taking into account the described roles of EPS as a means of fungi adhesion to the substrate, immobilization of extracellular enzymes, prevention of hyphal dehydration, storage of excess nutrients, and participation in lignin degradation.\textsuperscript{17–19}

**B. Mushrooms’ Antioxidant Activity**

Antioxidant activity (AOA) is one of the important bioactivities revealed in higher Basidiomycetes belonging to various taxonomic groups. Although many researchers have investigated antioxidant properties of a wide spectrum of mushroom fruiting bodies, little attention has been paid to antioxidant production by submerged cultures of medicinal mushrooms. One of the first most comprehensive studies was done by Badalyan.\textsuperscript{20} The tested mycelial samples (cultured liquid, mycelial extract, and biomass suspension) of 14 mushroom cultures (*Coprinus comatus*, *C. disseminatus*, *C. micaceus*, *Hypholoma fasciculare*, *Lentinus edodes*, *Lepista personata*, *Marasmius oreades*, *Pholiota alnicola*, *Pleurotus ostreatus*, *Stropharia coronilla*, *Suillus luteus*, *Schizophyllum commune*, *Trametes versicolor*, and *Volvariella bombycina*) possessed certain antioxidative potentials to inhibit the reaction of free-radical peroxide oxidation of lipids in rat brain homogenate. The level of observed AOA depended on the bio-ecological differences of tested strains (geographical origination, type of wood substrate, mycelial growth rate, and morphology), as well as the experimental conditions. Mycelia of seven screened species (*Pholiota alnicola*, *Lepista personata*, *Trametes versicolor*, *Volvariella bombycina*, *Schizophyllum commune*, *Suillus luteus*, and *Lentinus edodes*) showed more than 20% antioxidant activity.

Song and Yen\textsuperscript{21} compared the AOA and free-radical scavenging effects of dry matter of cultural medium (DMCM), dry matter of filtrate (DMF), and different solvent extracts of mycelia from *Antrodia camphorata* in submerged culture (ACSC). The AOA of ACSC extracts was positively correlated with their ability to scavenge radicals, especially for both DMF and water extract of mycelia (WEM), which showed a potential antioxidant activity. DMCM had a lower free-radical scavenging effect, indicating that the source of antioxidant in the DMF was not the original cultural medium. Authors found that the scavenging ability of DMF and WEM on superoxide was not correlated with their polysaccharide contents. These findings suggested that the polysaccharide content in DMF and WEM was not a major factor contributing to the effective-

### TABLE 2. Mushroom Biomass and EPS Production in Glucose-Containing Medium

<table>
<thead>
<tr>
<th>Species</th>
<th>Biomass, (g/L)</th>
<th>EPS, (g/L)</th>
<th>EPS, (g/g biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agaricus nevoi</em></td>
<td>7.7</td>
<td>1.5</td>
<td>0.195</td>
</tr>
<tr>
<td><em>Cerrena maxima</em></td>
<td>9.5</td>
<td>1.0</td>
<td>0.105</td>
</tr>
<tr>
<td><em>Ganoderma lucidum</em></td>
<td>10.5</td>
<td>1.6</td>
<td>0.152</td>
</tr>
<tr>
<td><em>Inonotus levis</em></td>
<td>12.7</td>
<td>2.2</td>
<td>0.173</td>
</tr>
<tr>
<td><em>Lentinus edodes</em></td>
<td>5.6</td>
<td>0.5</td>
<td>0.089</td>
</tr>
<tr>
<td><em>Phellinus igniarius</em></td>
<td>11.3</td>
<td>1.6</td>
<td>0.142</td>
</tr>
<tr>
<td><em>Phellinus robustus</em></td>
<td>11.8</td>
<td>1.9</td>
<td>0.161</td>
</tr>
<tr>
<td><em>Pleurotus dryinus</em></td>
<td>11.3</td>
<td>1.1</td>
<td>0.097</td>
</tr>
<tr>
<td><em>Pleurotus ostreatus</em></td>
<td>7.1</td>
<td>1.0</td>
<td>0.141</td>
</tr>
<tr>
<td><em>Pleurotus tuberregium</em></td>
<td>5.3</td>
<td>0.6</td>
<td>0.113</td>
</tr>
<tr>
<td><em>Trametes versicolor</em></td>
<td>9.1</td>
<td>1.2</td>
<td>0.132</td>
</tr>
</tbody>
</table>
ness of AOA. They concluded that the polysaccharide in WEM had a higher protein/polysaccharide ratio than in DMF, although no significant difference was observed in their superoxide scavenging effects. In addition, the researchers demonstrated a linear relationship between the inhibition of lipid peroxidation and total polyphenol content. Moreover, they also found high correlation between the inhibition of lipid peroxidation and the crude triterpenoids content of non-aqueous (methanol and ethyl acetate) mycelial extracts. These results indicated that the total polyphenols in the ACSC extracts were an active component involved in the inhibition of lipid peroxidation. However, the authors proposed that the triterpenoids also played a role in the non-aqueous ACSC extracts. In addition, their results indicated that the scavenging effect of crude triterpenoid was dose dependent.

Tsai et al. compared hot-water extracts prepared from *Agrocybe cylindracea* fruiting bodies, mycelia, and fermentation filtrate for their antioxidant properties. AOA of hot-water extracts from fruiting bodies, mycelia, and filtrate were 63.6%, 81.6%, and 56.8%, respectively, at 20 mg/mL. EC$_{50}$ values in reducing power were 2.72, 3.97, and 3.09 mg/mL, respectively, whereas those in scavenging abilities of DPPH radicals were 0.62, 1.66, and 0.82 mg/mL for fruiting bodies, mycelia, and filtrate, respectively. At 20 mg/mL, the scavenging abilities of hydroxyl radicals were 80.1%, 57.0%, and 54.3% for fruiting bodies, mycelia, and filtrate, respectively. From the EC$_{50}$ values obtained, it can be concluded that hot-water extracts from three forms of *A. cylindracea* were effective in antioxidant properties. With regard to EC$_{50}$ values in chelating abilities on ferrous ions, the hot-water extract from filtrate was better than that from mycelia. Total phenols were the major naturally occurring antioxidant components found in hot-water extracts from *A. cylindracea*, in the range of 23.74–30.16 mg/g. Total antioxidant components varied among hot-water extracts and were 30.46, 27.72, and 24.57 mg/g for fruiting bodies, mycelia, and filtrate, respectively. The authors emphasized that the high content of total phenols in all hot-water extracts might explain high antioxidant properties in *A. cylindracea*.

Mau et al. investigated the antioxidant properties of *Ganoderma* species. Hot-water extracts from four forms of *G. tsugae* (mature and baby Ling chih, mycelia, and fermentation filtrate) were prepared, and their antioxidant properties were compared. Hot-water extracts from mature and baby Ling chih showed high antioxidant activities (78.5% and 78.2%) at 20 mg/mL, and had EC$_{50}$ values of 7.25 and 5.89 mg extract/mL, respectively. EC$_{50}$ values in reducing power were 1.12, 1.37, 2.48, and 1.41 mg extract/mL, whereas those with scavenging abilities of DPPH radicals were 0.30, 0.40, 0.72, and 5.00 mg extract/mL for Ling chih, baby Ling chih, mycelia, and filtrate, respectively. At 20 mg/mL, scavenging abilities on hydroxyl radicals were in descending order of Ling chih > baby Ling chih > mycelia > filtrate. Naturally occurring antioxidant components including ascorbic acid, α- and δ-tocopherols, and total phenols were found in hot-water extracts from fruiting bodies, mycelia, and filtrate. Total antioxidant components varied among hot-water extracts and were in descending order of baby Ling chih (44.72) > Ling chih (44.62) > mycelia (41.85) > filtrate (41.53 mg/g).

Evaluation of antioxidant properties of methanolic extracts from *Grifola frondosa*, *Morchella esculenta*, and *Termitomyces albuminosus* mycelia showed high AOA (85.4%–94.7%) at 25 mg/mL. Reducing powers of the three methanolic extracts were 0.97–1.02 at 25 mg/mL. Scavenging effects on DPPH radicals were 78.8%–94.1% at 10 mg/mL. These three mycelia showed no scavenging effect on hydroxyl radicals. Chelating effects on ferrous ions were high (90.3%–94.4%) at 10 mg/mL. Total phenols were the major naturally occurring antioxidant components found in methanolic extracts. Contents of ascorbic acid and tocopherols were similar for these three mycelia. All EC$_{50}$ values were below 10 mg/mL, indicating that the three mycelia had good antioxidant properties except for the scavenging effect on hydroxyl radicals.

Twenty-eight basidiomycetes strains belonging to various taxonomic and ecological groups have been screened for their antioxidant and free-radical scavenging activity after submerged cultivation in a synthetic medium of simple composition. No correlation was revealed among fungi belonging to different ecological groups, but the AOA of the extracts significantly depended on mushroom species. Water extracts from *Coprinus comatus*, *Agaricus nevoi*, and *Flammulina velu-
tipes at a concentration of 2 mg/mL manifested very high AOA (more than 85%), whereas the water extracts from Daedalea gibbosa, Pleurotus citrinopileatus, and Macrolepiota excoriata at the same concentration showed very low AOA (less than 26%). When the ethanol extracts were tested, the highest values of AOA were found in Agaricus nevoi samples, followed by Omphalotus olearius and Auricularia auricula-judae, 92.1%, 83.4%, and 80.2%, respectively, at an extract concentration of 2 mg/mL. In contrast to these fungi, no AOA was exhibited by Coprinus comatus extract at the same concentration, while Phellinus robustus 531 showed only 17.6% inhibition.

The free-radical scavenging activity was also species dependent. The highest activity at a minimal sample concentration of 0.5 mg/mL was shown with water extracts from Ganoderma lucidum (69%) and Daedalea quercina (49%), whereas mycelial biomasses of Pleurotus citrinopileatus, Stereum hirsutum, and Pleurotus nebrodensis showed very weak scavenging ability toward DPPH, only 11%. When the concentration of samples increased to 1.5 mg/mL, the scavenging ability of extracts from G. lucidum and P. cystidiosus rose by 21% and 27%, respectively. Such a sample concentration was sufficient to reveal maximal scavenging ability of the majority of ethanol extracts tested.

Recently, Porras-Arboleda et al. evaluated the AOA of Humphreya coffeata culture filtrates in fungus cultivation in lactose-containing medium. As is shown in Table 3, the percentage of DPPH scavenging activity of H. coffeata culture filtrates increased almost 4.5 times by day 12 of culture as compared with 4-day culture, and later on this value seems to remain constant. In terms of EC\textsubscript{50} of ABTS radical scavenging activity, no significant differences between the EC\textsubscript{50} of culture filtrates at different culture ages have been observed, whereas EC\textsubscript{50} NADH decreased with culture age.

Lin\textsuperscript{27} expressed assayed antioxidant properties of Phellinus igniarius as EC\textsubscript{50} values for comparison of methanolic (MEM) and hotwater (HWEM) extracts from dried mycelia with methanolic extract from culture broth (MEB) after cultivation in a 5-L stirred-tank bioreactor. MEM and MEB exhibited moderate AOA with low EC\textsubscript{50} values of 6.22 and 3.34 mg/mL. However, HWEM showed low activity above 10 mg/mL; MEB and HWEM were comparable in reducing power with approximately the same EC\textsubscript{50} values (about 6.7 mg/mL) of moderate reducing effects, whereas MEM with EC\textsubscript{50} of 9.97 mg/mL, was less effective. For the scavenging effect on DPPH radicals, MEM with EC\textsubscript{50} of 4.96 mg/mL was more effective than MEB (19.93 mg/mL) and HWEM (12.75 mg/mL). The three extracts of submerged culture of Ph. igniarius showed an obvious chelating effect on ferrous ions and exhibited good superoxide radical scavenging activities, as evidenced by their low EC\textsubscript{50} values (<1 mg/mL). Ascorbic acid was mainly found in methanolic extracts from mycelia and broth. However, β-carotene was not detected in such extracts. α-Tocopherol and δ-tocopherol were found only in MEM, whereas γ-tocopherol was detected only in MEB and HWEM. Methanolic extracts were highly rich in total flavonoid as compared to hot-water extracts. Total phenols were the major antioxidant components that appeared in hot-water extracts. The authors concluded that total phenols are the major components responsible for antioxidant properties of extracts from submerged culture of the mushroom. At the same time, antioxidant properties of polysaccharide-enriched extracts from submerged mycelium of Inonotus obliquus\textsuperscript{29} and selenium-containing polysaccharides in liquid culture of Hericeum erinaceus\textsuperscript{30} have been established.

<table>
<thead>
<tr>
<th>Day of culture</th>
<th>Scavenging DPPH (%)</th>
<th>EC\textsubscript{50} ABTS (mg/mL)</th>
<th>EC\textsubscript{50} NADH (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>6.23</td>
<td>29.21</td>
<td>82.12</td>
</tr>
<tr>
<td>8</td>
<td>N.D.</td>
<td>23.89</td>
<td>73.94</td>
</tr>
<tr>
<td>12</td>
<td>28.85</td>
<td>32.43</td>
<td>53.44</td>
</tr>
<tr>
<td>16</td>
<td>22.66</td>
<td>36.60</td>
<td>38.00</td>
</tr>
<tr>
<td>21</td>
<td>32.13</td>
<td>35.36</td>
<td>24.17</td>
</tr>
</tbody>
</table>
Thus, submerged cultivation of medicinal mushrooms is an appropriate approach to obtain significant antioxidant compounds from the submerged mycelium and culture filtrate. However, there are no systematic studies on the physiology of antioxidant production to establish how nutrient medium composition and cultivation conditions affect antioxidant accumulation in biomass and in culture liquid. Only Barros et al.\textsuperscript{31} demonstrated that the bioactive properties and, in particular, AOA of \textit{Leucopaxillus giganteus} depend on the nitrogen source used for mycelium growth. In addition, the submerged cultivation of \textit{Ganoderma lucidum} in the presence of leguminous plants as part of the fermentation medium improved an AOA of broth filtrate.\textsuperscript{32} The active antioxidant compound was found to be protocatechuic acid, which is a powerful antioxidant against human low-density lipoprotein oxidation.

**C. Mushroom Lectin Activity**

Lectins isolated from higher Basidiomycetes have received the most extensive attention of researchers because their anti-tumor, immunomodulating, mitogenic, and several other properties have practical use in clinical medicine.\textsuperscript{3,33–37} Lectins are large and heterogeneous carbohydrate-binding proteins having at least two binding sites and showing no glycosidase activity. The physiological role of lectins in various organisms is extremely diverse; they participate in cell adhesion, recognition, and differentiation, transportation of sugars, and growth regulation. Lectins are also widely used as a biochemical tool in many fields of research, such as medicine, biology, gene engineering, and agroindustry.

Lectins are widely distributed among higher Basidiomycetes; genera of \textit{Lactarius}, \textit{Russula}, \textit{Boletus}, \textit{Phallus}, and family \textit{Hygrophoraceae} are noteworthy for the high percentage of active species, and numerous lectins of different chemical composition, structure, and activity have been isolated from fruiting bodies of various mushrooms.\textsuperscript{33–35} However, in spite of the potential application of lectins in research and medicine, there is still a large number of basidiomycetes that have not been investigated at all. The vast majority of studies have focused on the isolation of lectins from mushroom fruiting bodies. However, yields of lectins from fresh mushrooms are low, e.g., 2–3 mg from 100 g of fresh fruiting bodies.\textsuperscript{38} Dried fruiting bodies of the mushrooms \textit{Russula lepida}, \textit{Pholiota adiposa}, and \textit{Inocybe umbrinella} yielded 39, 70, and 15 mg lectin per 100 g fruiting bodies, respectively.\textsuperscript{39,40} Therefore, production from mushroom fruiting bodies is unpractical.

Very little information is available on mushroom capability to produce lectins in submerged cultures. In particular, hemagglutination activity (HA) was revealed in culture liquid of \textit{Lentinus edodes}.\textsuperscript{41} HA titers of this mushroom in glucose-based medium varied from 4 to 4096; the maximal activity was observed on days 3–7 of culturing. Mikiashvili et al.\textsuperscript{42} showed that nine strains of higher Basidiomycetes have the capability to accumulate lectin activity during their cultivation on defined liquid medium. However, the HA titer varied from 32 to 1024 depending on the mushroom species. Moreover, HA was not only species- but also strain-dependent. Thus, the specific HA of \textit{Pleurotus ostreatus} strains varied from 1939 U/mg to 7062 U/mg.

Twenty-one higher Basidiomycetes strains belonging to 16 species from different taxonomic groups were compared for their lectin activity after submerged and solid-state fermentation (SSF) of 2% wheat bran and 2% mandarin peelings mixture.\textsuperscript{43} Data represented in Table 4 show that the HA titer of tested fungi varied from 0 to 16384 T\textsuperscript{1}. No HA was revealed in both SSF and submerged fermentation of lignocellulosic materials by \textit{Bjerkandera adusta} IBB 47 and \textit{Trametes ochracea} IBB 44. Very low HA was found in extracts from biomasses of \textit{Lentinus edodes} IBB 3721, \textit{Lentinus variabilis} IBB 27, \textit{Pleurotus ostreatus} IBB 2175, \textit{Postia tephroleuca} IBB 50, \textit{Trametes versicolor} IBB 16, and \textit{T. versicolor} IBB 775. Several fungi appeared to be promising producers of lectin. An especially high specific HA activity (166667 U/mg) was revealed in biomass of \textit{Ganoderma applanatum} IBB 25 after SSF of lignocellulose. Biomasses of seven fungi showed more than 3000 U/mg HA. In comparison, the specific HA of crude extracts of \textit{G. capense},\textsuperscript{44} \textit{Lentinus edodes},\textsuperscript{45} and \textit{Pleurotus ostreatus}\textsuperscript{46} fruiting bodies appeared to be equal to 925, 85, and 1083 U/mg, respectively. It is worth noting that the HA is species- and strain-dependent. For example, the specific HA among species of genera \textit{Trametes} varied from 0 to 5556 U/mg. Strains of \textit{Trametes versicolor} and \textit{Pleurotus ostreatus} mani-
fested 0–521 U/mg and 0–2734 U/mg of specific HA, respectively. However, further experiments are needed to establish whether these differences are explained by mushrooms’ peculiarities, culture age, and physiological state. It is possible that the methods of lectin (protein) extraction and precipitation are not equally appropriate for all fungi tested. Furthermore, there is a need to study the profile of lectin accumulation during short-term and long-term cultivation of mushrooms.

In addition to mushrooms’ biomass, the culture liquids of several strains received after submerged fermentation of wheat bran and mandarin peels were also tested for their HA. In this case, the culture liquids of two strains (P. ostreatus IBB 2175 and Ttametes ochracea IBB 44) showed no HA, whereas those received after fermentation of lignocellulose by Cerrena unicolor IBB 301, Trametes versicolor IBB 775, and Pleurotus dryinus IBB 903 had comparatively high specific HA (Table 5). The comparison of HA of mycelial extracts and culture liquids indicates that the HA titers and the specific HA of culture liquids obtained from all tested fungi, with the exclusion of P. ostreatus IBB 2175, appeared to be many-fold higher as compared with the same activities of extracts. Analogically, the culture liquids of all strains of Lentinus edodes had HA titers at least 4- to 32-fold higher than those of the corresponding mycelial extracts.36

III. CULTIVATION METHODS FOR THE PRODUCTION OF MUSHROOM BIOMASS AND BIOACTIVE COMPOUNDS

Many different techniques and substrates have been successfully utilized for mushroom cultivation. For production of mushroom fruiting bodies, various forms of SSF are employed, whereas for mycelial biomass and BAM production, submerged fermentation is preferable to produce a more uniform biomass and pharmaceutical products.

Solid-state fermentation (SSF) is defined as any fermentation process occurring in the absence or near absence of free liquid, employing an inert substrate (synthetic materials) or a natural substrate (organic materials) as a solid support.47,48 SSF is most appropriate for bioconversion of plant raw materials into value-added products, such as

<table>
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<tr>
<th>Species</th>
<th>HA titer (T–1)</th>
<th>Specific HA (U/mg)</th>
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<tr>
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<tr>
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</tr>
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<td>2734</td>
</tr>
<tr>
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<tr>
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mushroom fruiting bodies, fodder, secondary metabolites, and enzymes. SSF has several advantages as compared with submerged cultivation; in particular, with small energy consumption, the nutrient medium is concentrated, and high volumetric productivity can be achieved in a smaller bioreactor. Moreover, in SSF a concentrated product can be obtained from a cheap substrate such as agro-industrial residue. Undoubtedly, the use of natural lignocellulosic materials, especially food-industry residues, as growth substrates for fungi cultivation is the most promising approach, since such residues are rich in sugars and other useful compounds, which are easily metabolized by mushrooms. However, the use of lignocellulosic substrates might make the product purification process more difficult. For this reason, this cultivation technique would be most appropriate for the colonization of growth substrate by mushroom mycelium, when the whole fermented substrate can be used—for example, as with fodder or food supplements. In addition, till now, the major obstacles for the commercial applications of SSF techniques have not been completely overcome. They are related to the design and operation of large-scale bioreactors due to problems concerned with the control of parameters such as pH, temperature, aeration and oxygen transfer, moisture, and agitation.

In contrast to SSF, submerged liquid culture requires large energy expenditures to agitate nutrient medium and to supply oxygen. However, the submerged culture works as a homogeneous system, and the cultivation process control is easy using many on-line sensors. In this case, a very wide range of products can be produced from a wide range of microorganisms with the best productivity, due to medium mixing and unlimited diffusion of nutrients. This approach makes it possible to carry out directed (predominant) synthesis of the target products by establishing appropriate culture conditions. Submerged cultivation of mushrooms permits a fully standardized production of biomass with high nutritional value and other products with predictable composition. Moreover, although the downstream processing after submerged fermentation requires removal of large volumes of water and is more expensive, the product purification may be easier as compared to SSF. Hence, mushroom submerged cultivation has significant industrial potential, but its success on a commercial scale depends on cost compared with existing technology.

Various cultivation techniques and strategies have been used for submerged cultivation of medicinal mushrooms, depending on the fungi physiological and morphological peculiarities and their behavior under different environmental conditions (Table 1). Batch cultivation in shake-flasks and in laboratory fermenters have reportedly been the most frequently used techniques. The advantage of fermenter use is that it is easier to control environmental conditions such as temperature, agitation, dissolved oxygen, and medium pH. However, the extension of the fungal biomass has profound effects on mass transfer, metabolic rate, and product secretion. Fungal mycelia can wrap around impellers, cause blockages, and spread into sampling and nutrient feed lines, as well as increase broth viscosity, which results in mass and oxygen transfer limitations. Because mushroom mycelia and pel-

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<tr>
<td><em>Cerrena unicolor</em> IBB 301</td>
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<td><em>Pleurotus dryinus</em> IBB 903</td>
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<tr>
<td><em>Trametes ochracea</em> IBB 44</td>
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lets are shear sensitive and culture viscosity usually increases during cultivation, the most serious problems in large-scale submerged cultures of mushrooms might be oxygen supply, shear stress, and scale-up. These drawbacks limit the time of operation in bioreactors.

Several authors used fed-batch fermentation for the production of BAM.\textsuperscript{48,51,58} The strategy of fed-batch fermentation is to add one or more of the nutrients during fermentation, based on the possibility that the high concentrations required for high final growth and product yields might inhibit growth if added in total at the start of the fermentation, i.e., this strategy provides a dosed supply of substrates in order to avoid catabolite repression of target compound synthesis. The main advantage of the fed-batch operation is the possibility to control both reaction rate and metabolic reactions by substrate feeding rate, thus avoiding the limitations caused by oxygen transfer and cooling. Potentially, growth and product formation can be extended for long periods compared to normal batch fermentation.

Finally, the immobilization of the mushroom mycelium on different materials to control the growth and BAM production rate may be a possible approach.\textsuperscript{48,56} Immobilized fungal cells have several advantages over dispersed cells. First of all, immobilized cell systems make it easy to separate cells from the liquid medium, which makes repeated batch culture possible and simplifies the operation of both the continuous culture and the subsequent downstream processes. Cell immobilization also decreases the apparent broth viscosity and makes the rheological features more favorable for oxygen supply and mass transfer.\textsuperscript{48,51} In addition, immobilized cultures tend to have a higher level of activity and are more stable to environmental perturbations, such as pH, or exposure to toxic chemical concentrations than suspension cultures. However, there is very scarce information on BAM production by immobilized mushrooms. Yang et al.\textsuperscript{52} introduced a polyurethane foam sheet into the medium of a submerged fermentation in an Erlenmeyer flask. The mycelium adhered to the surface of the foam matrix with almost no mycelia remaining free in the bulk liquid. The biomass density and the amount of EPS obtained were both markedly higher in this culture than in freely suspended cultures.

IV. EFFECTS OF PROCESS VARIABLES ON MUSHROOM GROWTH AND BIOACTIVE COMPOUNDS PRODUCTION IN SUBMERGED CULTURE

Many edible and medicinal mushrooms that produce BAM respond to environmental factors directly, and many studies have shown that mushroom mycelial growth rate and BAM production rate vary with environmental conditions and medium composition, including carbon source, nitrogen source, pH, etc.\textsuperscript{48,53–58} The literature data point out that the correct selection of medium composition and mushroom cultivation parameters is crucial for optimal biomass or metabolite production and for the development of industrial-scale cultures of medicinal mushrooms. Undoubtedly, various physical and chemical factors are interconnected and affect the ability of mushroom culture to produce the target product.

A. Physical Factors

1. Temperature

The effect of temperature on mushroom growth and BAM formation has not been systematically studied, although the cultivation temperature may determine both biomass and target product yield. Thus, to investigate the effect that culture temperature has on mycelial growth and EPS production by \textit{Antrodia cinnamomea}, the fungus was cultivated in the basal medium at temperatures ranging from 20°C to 32°C.\textsuperscript{53} It turned out that the optimum temperatures for mycelial growth and EPS production were 25°C (2.8 g/L and 0.58 g/L, respectively). The production of the mycelial biomass was near its optimal temperature over the range from 25°C to 28°C and declined sharply outside this temperature range, while the EPS production was optimal within the temperature range 23–28°C. Both polysaccharide production and mycelial growth rate of \textit{G. lucidum} were favored at temperatures between 30°C and 35°C, being drastically reduced outside this range.\textsuperscript{54} When \textit{Fomes fomentarius} was cultivated at various temperatures ranging from 15°C to 35°C, both maximum mycelial biomass (7.48 g/L) and EPS (0.81 g/L) were observed at 25°C.\textsuperscript{55} In another study, the antioxidative potency of samples obtained in submerged fermentation of leguminous plants by \textit{Ganoderma lucidum} at 30°C was better than those at 18°C and 24°C.\textsuperscript{32}

In these cases, the temperature optima of
mushroom biomass and BAM production in submerged cultures appeared to be very close. By contrast, the experiment with *Lentinus edodes* showed that the mushroom lectin activity is not a purely growth-associated product, because poor growth at temperatures lower or higher than the optimal value of 26°C was accompanied by a higher lectin activity, and this was true for both culture liquid and mycelial extract. In a shake-flask culture of *Ganoderma applanatum* the mushroom biomass and EPS content in the biomass was highest in cultures grown at 10°C and tended to decrease as the culture temperature increased. The results suggest that the mycelia accumulate polysaccharides in the cell at low temperature. Contrary to this, the yield of EPS increased as the culture temperature increased from 10°C and the highest amount of EPS was obtained in the culture grown at 25°C.

2. Agitation and Aeration

Agitation and aeration intensities are important factors for medicinal mushroom biomass and BAM production, promoting the mass transfer of substrates, products, and oxygen. Successful aerobic fermentation requires the maintenance of an environment sufficient in dissolved oxygen to avoid limitation or impairment of normal respiratory activities. Aeration results in better mixing of the production medium, thus helping maintain a concentration gradient between the interior and the exterior of cells. However, in the cultivation of mycelial organisms, such as medicinal mushrooms, agitation may damage mycelial hyphae and adversely affect growth and product formation. In addition, agitation can cause sticky particles to agglomerate, in either case producing a paste in which O₂ transfer is greatly hindered. The optimum agitation rate represents a balance between oxygen transfer into the medium and shear stress, both of which increase with increasing agitation rate. Therefore, the balance between positive and negative effects of agitation should be established.

In an early study with *Schizophyllum commune*, Rau et al. reported that sufficient oxygen supply resulted in an increase in the specific growth rate and a decrease in the production rate of extracellular glucan. When oxygen partial pressure in the culture broth decreased to almost zero, the fungus responded to this oxygen limitation by reduced cell growth and increased glucan accumulation. Yang and Liau, testing a range of shaking speeds from 50 to 250 rpm with Erlenmeyer flasks in an orbital shaker, obtained the highest biomass density at 100 rpm, but the highest EPS yield occurred at 150 rpm. They suggested that higher shaking speeds favor EPS production because they decrease the adsorption of the secreted extracellular polysaccharides on the cell wall, providing the stimulus for further EPS synthesis. However, a mechanism by which this stimulus would occur was not proposed. In the cultivation of *Fomes fomentarius*, the mycelial biomass and EPS production have no significant differences from 120 to 180 rpm and declined sharply out of this range.

*Ganoderma lucidum* mycelia were found as shear-sensible biomass. Measuring the percentage of cut filaments, related to the impeller speed, it was found that 300 rpm represented a critical impeller speed. Over this limit, a shear field significantly damaged mycelial agglomerates and their peripheral hyphal growth. In another study with *G. lucidum* the production of EPS was higher at a dissolved oxygen tension (DOT) of 10% than when the DOT was 25%. The production of EPS and contents of IPS and ganoderic acid at a DOT of 10% were higher than those at a DOT of 25%. However, the total production and productivity of IPS and ganoderic acid at a low DOT were lower than those at a high DOT, since the biomass growth of *G. lucidum* was significantly inhibited when DOT was controlled at 10% of air saturation; this was due to the oxygen limitation in mycelia aggregates. At an initial volumetric oxygen transfer coefficient *K*ₐ of 78.2/h, a maximal *G. lucidum* dry weight biomass of 15.62 g/L was obtained, as well as a maximal IPS production of 2.19 g/L and a maximal productivity of 217 mg/L per day. An increase of initial *K*ₐ led to a bigger size of mycelia aggregates and a higher production and productivity of ganoderic acid. The ganoderic acid production and productivity at an initial *K*ₐ of 96.0/h was 1.8-fold those at an initial *K*ₐ of 16.4/h.

The effects of various agitation rates on *Tricholoma matsutake* mycelial growth and polysaccharide production were studied. The mushroom was cultivated in the 5-L jar fermenter with a working volume of 3 L and an aeration rate of 1.0vvm. When the agitation level was varied from 100 to 300 rpm, a higher level of mycelial growth was observed at lower agitation speeds. The maximum
mycelial biomass (21.87 g/L) was obtained at 150 rpm. In contrast, the opposite effect was observed in EPS production, where a higher level of EPS production was achieved at the highest agitation speed. The maximum EPS production (8.86 g/L) was obtained at 300 rpm. The authors concluded that higher levels of mycelial growth in *T. matsutake* can be achieved at lower DO levels and that increased EPS production can occur at higher DO levels. In the same study the effects of aeration rate on *T. matsutake* mycelial growth and EPS production were evaluated. The researchers reported that the maximum mycelial biomass (20.85 g/L) and EPS production (8.79 g/L) were observed at aeration rates of 0.5 and 1.5 vvm, respectively. The analysis of mycelial morphology during the mushroom cultivation showed that mycelia were found to form feather-like mycelial clumps at the early stages of culture. Subsequently, during the entire culture period the mycelia were mainly found in the form of pellets.

Production and antioxidant properties of EPS as functions of *Armillaria mellea* culture aeration rate was comprehensively investigated in a 5-L stirred-tank bioreactor. The optimal specific growth rate (0.3/day) and biomass yield (0.22 g/g) were achieved in the culture with an aeration rate of 1.2 vvm. Biomass growth was significantly enhanced from 4.28 to 7.13 g/L when the aeration rate increased from 0.3 to 1.2 vvm, but dropped dramatically to 6.16 g/L at 1.5 vvm. As the aeration rate increased from 0.3 to 0.6 vvm, the maximum EPS production and specific product yield rose sharply, and then declined monotonically above an aeration rate of 1.2 vvm. The optimal maximum EPS production (178.8 mg/L) was found to be at 0.6 vvm. EPS formation was more sensitive to low aeration rates between 0.3 and 0.6 vvm than high aeration rates from 0.6 to 1.5 vvm. Since higher aeration rate (1.2 vvm) favors cell growth, whereas a lower aeration rate (0.6 vvm) enhances EPS production, the authors used a two-stage aeration rate strategy to improve EPS production and productivity. The aeration rate was first controlled at 1.2 vvm for 0–5 days for mushroom growth, and then switched to 0.6 vvm for the next 5 days to improve EPS formation until the end of fermentation. The maximum biomass and EPS concentration achieved in the two-stage culture process were 6.65 g/L and 233.2 mg/L, respectively, which were 1.55- and 2.68-fold enhancements of those fermented at the aeration rate of 0.3 vvm. It is interesting that EPS from the two-stage aeration rate culture exhibited higher AOA than those from other aeration rates. Moreover, the protein/polysaccharide ratio and molecular weight of EPS obtained from different aeration rate cultivations closely correlated with their EC$_{50}$ values in AOA, reducing power, and chelating ability on ferrous ions. The molecular weights of EPS from different aeration rate cultures ranged from 1850 to 2140 kDa, with a maximum obtained in the two-stage aeration rate culture. The authors concluded that the good antioxidant properties of EPS may be attributed to their higher molecular weights and protein/polysaccharide ratios.

B. Chemical Factors

1. **pH of the Medium**

One of the main factors determining the biosynthetic potential of a production method is the pH of the medium, as it may affect cell membrane function, uptake of various nutrients, cell morphology and structure, solubility of salts, ionic state of substrates, enzyme activity, and product biosynthesis. There are a number of studies on pH effects, but the majority of them have been done in Erlenmeyer flasks where the pH is not controlled during cultivation. In this system it is possible to study the influence of only the initial pH on growth and metabolite production.

Fang and Zhong cultivated *Ganoderma lucidum* in synthetic medium containing 35 g/L of glucose, 5 g/L of peptone, and 5 g/L of yeast extract, varying the initial pH from 3.5 to 7.0. Similar pH profiles were obtained after four days of cultivation in all variants; during this period, the pH decreased to 3.2 and then remained constant for one week. After that, around days 10 to 14, when the glucose was almost exhausted, the pH increased rapidly to 7.0. When 5 and 10 g/L glucose was fed on day 8, the pH remained the same until day 14. The authors suggest that the relatively high glucose consumption at 5 and 10 g/L might result in production of certain organic acid(s), which would keep the medium pH at a low value. It is interesting that although the pH profile was almost the same after day 4 irrespective of the initial pH, the mushroom growth and metabolite production did depend on the initial value. Highest yields were
obtained with an initial pH of 6.5 in the case of biomass, 5.5–6.5 in the case of ganoderic acid, 5.5–7.0 in the case of IPS, and 3.5–4.5 in the case of EPS.

To evaluate the effects of the initial pH on mycelial growth of *Rigidoporus ulmarius* the fungus was cultivated in basal medium at different initial pH values (4.0–6.5). Fungus cultivation at the initial pH 4 significantly inhibited mycelial growth, while at a higher pH optimal growth of *R. ulmarius* was observed. No significant differences were found in polysaccharide production among all tested pH values. However, chromatographic characterization of obtained polysaccharides showed that the synthesis of very high-molecular-weight polysaccharides decreased as the initial pH of the medium increased. Also, high-molecular-weight polysaccharides shifted to medium molecular weight with an increase in the initial pH. The synthesis of very low-molecular-weight polysaccharides increased when the initial pH increased. The results showed that growing mycelia in an acid-stressed condition might steer them toward the synthesis of high-molecular-weight polysaccharides.

The optimum pH for EPS and mycelial biomass was different in shake-flask culture of *Armillaria luteovirens*. The highest EPS production was observed at an initial pH of 5.0 after 96 h culture time, whereas the maximum mycelial biomass and two-fold lower EPS yield was found at an initial pH of 4.0. Meng et al. showed that in submerged cultivation *Morchella esculenta* could grow at an initial pH value ranging from 4.5 to 9.5; however, the biomass yield (6.5 g/L) and content of EPS with antioxidant activity (1.98 g/L) reached their maximum at an initial pH of 6.5.

Quite different results were obtained in several other studies. Mycelial growth and polysaccharide production by *Lyophyllum decastes* were significantly affected when the mushroom was cultivated in the basal medium with an initial pH ranging from 4.0 to 9.0. The optimal initial pH for mycelial growth was 8 with mycelial yield 7.1 g/L, whereas EPS and IPS achieved their peaks at pH 7, with a corresponding 1.73 g/L and 320 mg/g dry mycelium, respectively. After pH 7, the maximum of EPS and IPS was achieved from 8 and 6, respectively. Thus, regarding *L. decastes*, a suitable pH was neutral and a slightly alkaline medium for maximum production of polysaccharides and mycelia, respectively. On the contrary, in the testing of *Antrodia cinnamomea* the optimal pH for mycelial growth and EPS production was 5.5; at higher values of pH the mycelial biomass and EPS production declined sharply. An unusual peculiarity compared to other mushroom cultures was revealed in submerged cultivation of *Laetiporus sulphureus* var. *miniatius*. The maximum mycelial growth and EPS production were obtained at an extremely acidic pH of 2.0. Finally, in the cultivation of *Lentinus edodes* the highest lectin activity occurred in medium with initial pH values between 8 and 9. At initial pH values of 2.0 and 2.5 no lectin activity was detected up to days 9 and 12, respectively. At initial pH 3.0, on day 12, the hemagglutination titer was 1/32 compared to the initial value. The addition of a buffer to maintain the pH at 7 did not lead to an increase in lectin activity in the culture liquid, while the addition of 10 mM phosphate buffer containing 0.15 M NaCl decreased lectin activity.

The effects of culture pH ranging from pH 3.0 to 6.0 on *Antrodia camphorata* growth, EPS biosynthesis, and molecular weight distribution were examined both in shake flask culture and in a stirred-tank fermenter. In cultivation in a stirred tank with a controlled pH, the optimum pH for fungus growth was 4.0 with a biomass yield of 0.3 g/g, while that for EPS formation was 5.0 with a product yield of 5.05 mg/g. It is worth noting that a relatively high-molecular-weight EPS with a lower yield was obtained at low pH values, while a relatively low-molecular-weight EPS with a high yield was obtained at higher pH values. A two-stage pH process that maximized product formation was demonstrated, with a high product yield of 148 mg/L with the relatively high average molecular weight of 2.18×10^5.

2. Carbon Source

The carbon source is a major component of nutrient media, which ensures the growth of microorganisms and BAM production. Although most researchers have used glucose as a carbon source, there are some studies that compared the effects of different sugars on mushroom growth and target compound production.

Among the carbon sources (glucose, lactose, and sucrose) evaluated at a concentration of 35 g/L, lactose followed by glucose showed the
highest biomass concentration (11.9 and 10.8 g/L, respectively) in submerged cultivation of *Hum- phreya coffeata*. In sucrose-containing medium the fungus biomass yield reached only 2.9 g/L. The authors suggested that sucrose has an effect on the catabolic repression of cellular secondary metabolism. Moreover, they observed two growth phases in *H. coffeata* kinetics using lactose and glucose as carbon sources. For the first 4 days of culture, *H. coffeata* grew at a specific rate of 0.46, 0.48, and 0.30/day using lactose, glucose, and sucrose as carbon sources. From days 4 to 8, *H. coffeata* stopped growing, and from days 8 to 12, a second growth phase was observed at specific growth rates of 0.18 and 0.17/day for lactose and glucose, respectively; however, no growth was observed in sucrose-based medium.

To find appropriate carbon sources for mycelial growth and polysaccharides production by *Lyophyllum decastes*, Pokhrel and Ohta tested seven different carbon sources at a concentration of 30 g/L in the basal medium. The mycelial growth of this fungus occurred in a variety of carbon sources; however, production of mycelia, EPS, and IPS were quite distinct. Among the sources examined, lactose followed by glucose and fructose yielded the best mycelial growth (6.36–6.73 g/L). The EPS production by various carbon sources ranged from 1.25 to 1.65 g/L. Glucose was the best carbon source for EPS production and did not differ significantly from maltose. Minimum EPS production was attained from a sucrose medium. IPS production ranged from 187 to 317 mg/g biomass. Glucose was found to be the best carbon source to produce a significant increase in the IPS, followed by xylose and sorbitol. Minimum IPS was recorded in fructose-containing medium.

Among the ten carbon sources tested at the concentration of 30 g/L, the maximum mycelial biomass (7.48 g/L) was obtained in the glucose-containing medium, whereas the maximum EPS production was achieved in the lactose- (0.89 g/L) and glucose-based (0.81 g/L) media. *Antrodia cinnamomea* appeared to be able to grow using various carbon sources, but the carbon sources for EPS and biomass production were quite distinct. The highest level of EPS (0.58 g/L) was obtained when glucose was the carbon source. Xylose stimulated the greatest mycelial biomass (9.17 g/L) in *A. cinnamomea*. All of these carbon sources, however, resulted in significantly lower specific product yields, relative to that of the control flask, which lacked a carbon source supplement. Moreover, the profile of EPS production with respect to the carbon source generally was not consistent with that of the mycelial growth of *A. cinnamomea*.

To find out the effect of different carbon sources on the production of *Ganoderma applana- tum* mycelial biomass, IPS, and EPS, five carbon sources were compared in an airlift bioreactor. The yields of mushroom biomass, EPS, and IPS varied according to carbon sources in the media. Fructose (19.4 g/L) followed by maltose (18.9 g/L) and glucose (17.8 g/L) resulted in high biomass, whereas in the presence of lactose and sucrose the biomass yield reached 15.3–15.5 g/L. EPS production was higher in the cultures with glucose (1.25 g/L) and maltose (1.35 g/L) whereas fructose gave the lowest yield of EPS (0.44 g/L). Moreover, the sugar compositions of EPS and IPS varied with the carbon source. The authors assumed that different carbon sources might have different effects of catabolic repression on the cellular secondary metabolism. Furthermore, while the molecular weight of EPS cultivated with glucose or maltose was higher than 2000 kDa, those cultivated with lactose, sucrose, or fructose were lower than 2000 kDa. This indicated that the molecular weight of EPS was influenced by the sugar composition of culture media. Glucose or maltose might be easier to use than any other carbon sources for biosynthesis of EPS since glucose is the main sugar component of EPS. The researchers revealed that the longer the fungus was cultured, the higher the molecular weight of the biopolymer was. At the initial culturing stage, the molecular weight of EPS was lower than 500 kDa. However, at the stagnant phase, it increased to higher than 1000 kDa. At the death phase, the molecular weight was higher than 2000 kDa. This is an important finding to control the EPS type, since the biological activities of the polysaccharide were reported to be affected by its molecular weight.

The effect of the various carbon sources on the yield of *Morchella esculenta* biomass and EPS with antioxidant activity was evaluated. The highest production of EPS (2.3 g/L) was obtained in the presence of glucose as the carbon source in medium, while the biomass dry weight was 7.3 g/L. The yield of biomass (7.5 g/L) in the medium containing...
xylose was a little higher than that in the medium containing glucose, but the EPS content was only 0.84 g/L, significantly lower than that in the medium containing glucose. When studying the effects of nutritional requirements for superoxide anion scavenging activity and reducing power by *G. frondosa*, various carbon sources were used at a concentration of 10 g/L in the basal medium. Among them, sucrose provided the highest superoxide anion scavenging activity (67%), much higher compared with the glucose-containing medium (52%). Xylose produced the highest reducing power compared to other carbon sources. The maximal reducing power in the culture was about 0.682. The fungus demonstrated the lowest reducing power (0.486) when using glucose as a carbon source.

Six mono- and disaccharides have been tested as sources of carbon for their effect on lectin activity of *Lentinus edodes*. The best source of carbon was lactose (HA titer = 4096 on day 3 of culturing); the worst, D-mannose. It is interesting that in the presence of sodium acetate in nutrient medium the HA titer was, at most, 256 during the entire period of culturing.

Seven carbon sources were tested in our study for their effect on the *L. edodes* and *Pleurotus* species growth and EPS formation. The data indicated that the mushrooms were capable of using all tested carbon sources. However, fungal growth and polysaccharide production greatly depended on the compound used in the nutrition medium. All of the fungi showed their highest mycelia dry weights in cultivation in the medium supplemented with glucose or mannitol. Among the mushrooms studied, *Pleurotus eryngii* and *P. ostreatus* strains produced 8.2–9.6 g/L of biomass, while *Lentinus edodes* and *Pleurotus tuberregium* strains accounted for only 5.8–6.5 g/L of biomass. A much lower final biomass was found after mushroom cultivation in the presence of xylose or sucrose. In the case of *L. edodes*, cellobiose and sodium gluconate ensured a comparatively high yield of biomass from these fungi. All mushrooms produced EPS in submerged cultivation in the presence of all tested carbon sources, proving that polysaccharide synthesis in the tested basidiomycetes cultures occurred constitutively. However, EPS formation was strongly affected by the carbon source used. The best EPS yields were recorded in mushrooms cultivated in the media containing sodium gluconate or glucose as a carbon source. However, mannitol appeared to be a preferred carbon source for EPS production by *P. tuber-regium* HAI 737.

Eight higher Basidiomycetes were capable of growing in basal medium supplemented with xylose, glucose, maltose, sucrose, mannitol, or sodium gluconate as carbon sources, accumulating from 5.3 to 12.8 g/L of mycelial biomass. Among them, *Cerrena maxima*, *Phellinus igniarius*, *Pleurotus dryinus*, and *Trametes versicolor* grew almost equally well in the presence of all studied compounds (Table 6). In the cultures of *Ganoderma lucidum*, *Inonotus levis*, and *Pleurotus dryinus* the highest final biomass content (10.5–12.7 g/L) was obtained after mushroom growth in the medium containing glucose. Maltose ensured the highest yield of *Phellinus robustus* biomass accumulation, while mannitol was favorable for growth of *Agaricus nevoi*, *Cerrena maxima*, *Phellinus igniarius*, and *Trametes versicolor*. Xylose, sucrose, and sodium gluconate appeared to be rather poor carbon sources for tested fungi, ensuring the lowest biomass yields after 8 days of submerged cultivation.

Like fungal growth, polysaccharide production was affected by the carbon source used in the nutrition medium (Table 6). *Inonotus levis* appeared to be the most efficient producer of EPS, with a yield of polymers from 1.7 to 2.2 g/L. Glucose was the best carbon source for EPS production by *Ganoderma lucidum*, *Inonotus levis*, *Phellinus robustus*, and *Pleurotus ostreatus*. *Cerrena maxima*, *Phellinus igniarius*, and *Trametes versicolor* produced the highest level of polysaccharide during growth in the presence of maltose, whereas mannitol favored maximum EPS accumulation by *Agaricus nevoi*. The maximum specific EPS yields (0.295 and 221 g/g) were obtained in the cultivation of *Inonotus levis* in the sodium gluconate and xylose supplemented media, followed by *A. nevoi* in a maltose-based medium (0.200 g/g). Cultivation of *C. maxima* and *P. igniarius* in mannitol-containing medium resulted in the lowest specific EPS production (0.062 and 0.050 g/g, respectively). These results indicate that good mycelial biomass does not seem to be a determining factor for high EPS production by several mushrooms.

### 3. Initial Sugar Concentration in the Medium

Substrate concentration is one of the factors that in-
fluence fungal growth and BAM production. Consequently, the effect of varying glucose concentrations in the growth medium on fungus biosynthetic activity was studied.\textsuperscript{16} In general, increased levels of biomass and EPS were obtained when fungi were grown in media containing high glucose concentrations. However, increasing the glucose concentration from 10 g/L to 20 g/L resulted in a 1.2- to 1.7-fold increase of the level of biomass and a 1.6- to 3-fold increase of EPS. A further increase to 40 g/L of glucose did not significantly increase the level of desired products. On the contrary, this concentration of glucose led to the inhibition of \textit{Len
tinus edodes} and \textit{Pleurotus tuberregium} growth, while other basidiomycetes were insensitive to a high concentration of the sugar.

In experiments with \textit{Ganoderma lucidum}, Fang and Zhong\textsuperscript{64} clearly showed that the biomass yield against glucose fell steeply with an increase of initial glucose levels from 20 to 65 g/L. The authors suggested that the cell growth was inhibited by high osmotic pressure in the medium. At the same time, the yields of both intracellular and extracellular polysaccharides rose when initial glucose or lactose concentrations were increased.\textsuperscript{72,73} The highest levels of \textit{G. lucidum} intracellular and extracellular polysaccharides were obtained at an initial glucose concentration of 50 g/L and at an initial lactose concentration of 65 g/L. Initial sugar concentrations also influence pellet size. Pellets of diameters smaller than 1.2 mm predominated at initial glucose concentrations of 50 and 65 g/L, while pellets of diameters larger than 1.6 mm predominated at initial concentrations of 20 g/L.\textsuperscript{72} Since larger pellets are correlated with higher ganoderic acid and lower IPS content, and pellet size is affected by initial sugar concentration, the fermentation conditions can be manipulated in order to favor one product over another as a proportion of the biomass. However, the overall yield of the desired product depends not only on its content in the biomass, but also on the biomass yield obtained. For example, the ganoderic acid content of the mycelium was higher with an initial glucose concentration of 20 g/L than with an initial concentration of 50 g/L. However, the biomass concentration obtained at 50 g/L was sufficiently high to give a higher yield of ganoderic acid per volume of fermentation broth.

To determine the effect of carbohydrate concentration in the medium on \textit{G. applanatum} biomass and polysaccharide production, Lee et al.\textsuperscript{56} cultivated mushrooms under different glucose concentrations, ranging from 10 to 80 g/L. They reported that the higher the concentration of carbohydrate in the media, the more EPS was produced.

\begin{table}[h]
\centering
\begin{tabular}{lcccccc}
\hline
Species & Xylose & Glucose & Maltose & Sucrose & Mannitol & Sodium gluconate \\
\hline
\textit{Agaricus nevoi} & 6.3 & 7.7 & 6.5 & 5.3 & 9.2 & 7.2 \\
\textit{Cerrena maxima} & 8.5 & 9.5 & 10.4 & 9.0 & 12.8 & 8.8 \\
\textit{Ganoderma lucidum} & 5.8 & 10.5 & 10.3 & 7.6 & 10.4 & 8.4 \\
\textit{Inonotus levis} & 7.7 & 12.7 & 9.5 & 10.2 & 10.0 & 6.1 \\
\textit{Phellinus igniarius} & 8.3 & 11.3 & 11.5 & 9.3 & 12.0 & 9.0 \\
\textit{Phellinus robustus} & 8.6 & 11.8 & 12.7 & 8.6 & 9.5 & 6.4 \\
\textit{Pleurotus dryinus} & 9.2 & 11.3 & 10.2 & 10.2 & 10.6 & 8.5 \\
\textit{Trametes versicolor} & 8.6 & 9.1 & 10.5 & 9.6 & 12.5 & 8.0 \\
\hline
\end{tabular}
\caption{Effect of Carbon Sources on Mushroom EPS Production (g/L)}
\end{table}
On the contrary, the IPS content decreased with increasing carbohydrate concentration in the media. Moreover, the authors set different C/N ratio by changing only nitrogen concentrations, with carbon concentrations fixed at 40 g/L in the media. They showed that EPS production did not seem to be affected by variable C/N ratios. In contrast, IPS content steadily increased until the C/N ratio reached 43, and decreased at higher ratios.

**4. Nitrogen Source**

Another factor essential for efficient mushroom growth and polysaccharide production is the nitrogen source used for fungi cultivation. Its nature and concentration have both been reported to be of considerable importance. Nitrogen is a critical factor in the synthesis of some fungal enzymes involved in both primary and secondary metabolism. This element can be supplied to the culture medium in the form of ammonium or nitrate ions, or in organic form (such as amino acids or proteins). It is common to add yeast extract and peptone, either singly or together, each at concentrations from 1 to 5 g/L. Pokhrel and Ohga\(^6\) investigated organic and inorganic nitrogen sources in order to compare mycelia growth and polysaccharide production. Eight various (1% organic and 0.1% inorganic) nitrogen sources were individually employed in the basal medium. Among them, yeast extract yielded the highest mycelia growth with 7.03 g/L, as well as EPS and IPS with 1.76 g/L and 325 mg/g dry mycelia, respectively. Following the yeast extract, mycelial growth was comparatively high in the presence of another organic source, polypeptone. Although a two-fold lower mycelial growth was achieved in medium supplemented with ammonium sulphate, EPS and IPS were second best in the presence of this salt. Variation of yeast extract concentrations from 0.5% to 2% showed that a yeast extract of 1% provided maximum mycelial growth and IPS production, whereas EPS production was further improved by increasing its concentration (2.46 g/L in 2%).

Effects of various organic and inorganic nitrogen sources (5 g/L) on *Grifola frondosa* superoxide anion scavenging activity and reducing power were evaluated.\(^{25}\) The data showed that soytone resulted in the highest level of superoxide anion scavenging activity compared to other sources of organic nitrogen. The maximal ability in culture was 68.5%. Soytone was also the best organic nitrogen source supporting reducing power; its maximum value was about 0.78. In comparison with organic nitrogen sources, inorganic nitrogen sources yielded much higher superoxide anion scavenging capability. Among them, the maximal superoxide anion scavenging activity of 81.6% was obtained in the ammonium chloride–supplemented culture, followed by sodium nitrate (78.3%) and ammonium acetate (77.3%) cultures. Ammonium acetate was the best inorganic nitrogen source supporting reducing power; its maximal value was about 0.46. Amino acids were also tested as nitrogen sources for superoxide anion scavenging capacity and reducing power of *G. frondosa*. The maximal superoxide anion scavenging activity of 68.3% was obtained in the glutamic acid culture. Arginine was the best nitrogen source supporting reducing power; its maximal value was about 0.95.

To investigate the effect that the nitrogen source has on mycelial growth and EPS production, *Antrodia cinnamomea* was cultivated in basal medium containing nine different nitrogen sources at a concentration of 0.5%.\(^53\) Among them, calcium nitrate was the most effective for enhancing EPS production (0.75 g/L). The mycelial biomass appeared to be stimulated by all of the organic nitrogen sources tested, with little or no obvious difference among them. Relative to the organic nitrogen sources, however, the use of inorganic nitrogen sources led to relatively lower mycelial growths. These results indicate that a nitrogen source can be used to improve the production yield of EPS and that good mycelial growth does not seem to be a determining factor for a high production yield of EPS in *A. cinnamomea*. The maximum specific product yield obtained was 0.50 g/g in the calcium nitrate–supplemented culture, followed by the ammonium oxalate (0.48 g/g) and ammonium acetate (0.45 g/g) cultures.

In our work,\(^71\) the effect of different inorganic and organic nitrogen sources (in final concentrations equal to 20 mM of nitrogen) on mushroom growth and EPS synthesis was assessed when fungi were grown in media containing 50 g/L of glucose. All nitrogen-containing compounds showed a significant positive effect on mushroom growth and EPS production, increasing the level of biomass approximately 2.5- to 4.7-fold and the level of EPS 1.5- to 5-fold compared to the control medium.
containing only 3 g/L yeast extract. Maximal biomass and high EPS production was achieved when using organic compounds as the nitrogen source. Among them, corn steep liquor ensured the highest yield of EPS (2.5–3.0 g/L). It is worth noting that the supplementation of media with inorganic nitrogen sources KNO₃ and (NH₄)₂SO₄ also provided comparatively high levels of EPS accumulation by *Agaricus nevoi* and *Inonotus levis*, respectively.

Among six nitrogen sources evaluated for their effect on the yield of *Morchella esculenta* biomass and EPS with antioxidant activity, yeast extract provided the maximum production of EPS (1.73 g/L) and biomass (6.3 g/L) yield, slightly lower than that in media containing peptone (6.3 g/L) and ammonium sulfate (6.5 g/L), respectively. Barros et al. demonstrated that the bioactive properties (antimicrobial and AOA) and nutraceutical production of *Leucopaxillus giganteus* mycelia depend on the nitrogen source used for fungus growth. Among four different compounds tested, (NH₄)₂HPO₄ proved to be the best nitrogen source for the synthesis of phenols and flavonoids, showing the highest content at all growth times. Extracts from mushroom mycelia grown in the presence of (NH₄)₂HPO₄ revealed better antioxidant properties than in samples from other nitrogen sources and correlated with the higher content of phenols and flavonoids.

The dependence of the activity of extracellular lectins on the source of nitrogen in media (sodium nitrate or ammonium chloride) and the C:N ratio was shown in the cultivation of *Lentinus edodes*. The best results were obtained either in culture media with the lowest content of nitrogen (C:N = 152:1) or in the absence of nitrogen.

Thus, mycelial growth appeared to be stimulated by organic sources. Relative to organic nitrogen sources, inorganic nitrogen sources are usually not efficient for mycelial growth, whereas polysaccharide production improved greatly. In general, good mycelial growth does not seem to be a determining factor for high production of polysaccharides.

### 5. Complex Media

Higher Basidiomycetes represent a potential source of BAM with various properties. Therefore, there is a need to select new organisms with significant accumulations of these bioactive compounds and to develop low-cost and competitive technologies for their production. One of the appropriate approaches for this purpose is to utilize the potential of agro-industrial lignocellulosic wastes, many of which are rich with organic compounds insuring abundant growth of fungi.

A study was conducted to determine the effects of including lignocellulosic material, corn stover, on production and antioxidant activity of extracellular (EPC) and intracellular (IPC) phenolic compounds by *Inonotus obliquus* in submerged fermentation. The nutrient medium contained 3% ground corn stover and 3.5% corn flour, and the control medium contained 5% corn flour without corn stover. Under controlled culture conditions, the EPC production reached 34.7 and 42.5 mg GAE (gallic acid equivalents)/L in shake-flask cultures and fermenter runs. In cultures grown in media supplemented with corn stover, the EPC level reached 118.9 and 135.7 mg GAE/L in shake-flask cultures and fermenter runs, respectively. In the control medium, the IPC production maximized at 12.5 and 13.5 mg GAE/g after 72 h of incubation, and then fell gradually to 1.8 and 2.9 mg GAE/g at 288 h in shake-flask cultures and fermenter runs, respectively. In the corn stover medium, IPC production reached 21.2 and 23.7 mg GAE/g after 144 h of incubation, and then fell gradually to 5.8 and 6.0 mg GAE/g at 288 h in shake-flask cultures and fermenter runs, respectively. Both EPC and IPC from the corn stover medium showed a higher scavenging activity against DPPH radicals than those from the control medium during the later fermentation period. In dose-dependent experiments, EPC from the corn stover medium at 216 h demonstrated a significantly stronger free-radical scavenger activity against DPPH and hydroxyl radicals, shown as much lower IC₅₀ values, than that from the control medium and IPC from the two media. The results demonstrated that corn stover is cost-effective as a carbon source and as a phenolic compound production enhancer. It is useful to utilize inexpensive corn stover as a lignocellulosic material for production of active phenolic compounds of *I. obliquus* in submerged fermentation.

Recently, we described the capability of higher Basidiomycetes mushrooms to produce lectin in both submerged and SSF of various lignocellulosic substrates. In submerged fermentation of five tested plant materials the hemagglutination titer of extracts from *Cerrena unicolor* biomass varied from
4 to 128, while the specific HA ranged from 160 to 1785 U/mg protein in media containing wine bagasse and mandarin peels, respectively (Table 7). It is interesting that the culture liquids obtained after fermentation of wheat bran and mandarin peels expressed even higher titer (256) as compared with the respective biomasses, although no HA was revealed in culture broths after fermentation of wine bagasse and wheat straw by *C. unicolor*. By contrast, very low or no HA was revealed in culture liquids after submerged fermentation of lignocellulosic materials by *Fomes fomentarius*, although these substrates significantly promoted lectin production by biomasses.

Subsequently, the HA of three strains of *Cerrena unicolor* and two strains of *C. maxima* was evaluated. An important finding of this study was that lectin production was lignocellulose substrate and strain dependent. The hemagglutination titer in biomass extracts from tested *C. unicolor* strains varied from 0 to 1024. The fermentation of walnut pericarp favored the predominant accumulation of lectin protein in extracted biomasses of all *C. unicolor* strains. Among the lignocellulosic substrates tested, walnut pericarp, followed by mandarin and kiwi peels, provided the highest specific HA of *C. unicolor* IBB 302. It is interesting that the biomass of *C. unicolor* IBB 301 showed low specific HA (769 U/mg) in submerged fermentation of banana peels, while the culture liquid expressed the highest specific HA (5582 U/mg). Among strains tested, *C. maxima* IBB 402 expressed much higher hemagglutination titers (128–2048). Submerged fermentation of walnut pericarp, followed by wheat bran and kiwi peels, provided the highest hemagglutination titers of *C. maxima* IBB 401, while the fermentation of walnut leaves, walnut pericarp, wheat bran, and mandarin peels revealed very high hemagglutination titers in biomasses of *C. maxima* IBB 402. Consequently, the highest HA (8333 U/mg) of *C. maxima* IBB 401 was detected in fungus cultivation in the presence of walnut pericarp on day 10. *C. maxima* IBB 402 appeared to be a more potent producer of lectins, expressing very high specific HA in submerged fermentation of walnut leaves (64,103 U/mg), mandarin (33,333 U/mg), and kiwi peels (28,571 U/mg).

### 6. Vitamins

Scarce information is available on the effects individual vitamins have on mycelial growth and EPS production by medicinal mushrooms. The study of vitamins’ effects on mycelial biomass and EPS production by *Grifola frondosa* showed that riboflavin was the best vitamin source for EPS production (1.39 g/L), followed by biotin (1.19 g/L) and...
nicotinic acid (0.94 g/L). These results indicate that some vitamins can be used to improve EPS production. Among the five vitamins tested, thiamine and riboflavin had increased mycelial biomass. Nicotinic acid, ascorbic acid, and biotin had smaller growing effects than did vitamin-free media, i.e., the supply of vitamins was not an absolute requirement for mycelial biomass of G. frondosa.

In the cultivation of Antrodia cinnamomea, thiamine, riboflavin, ascorbic acid, nicotinic acid, and biotin have been added to the basal medium at a concentration of 0.1%. Nicotinic acid was the best vitamin source for EPS production (0.51 g/L), followed by riboflavin (0.50 g/L). In fact, the differences in EPS production and specific product yield were slight among all of these vitamins. In addition, of the five vitamins tested, only riboflavin increased growth. Thiamine, ascorbic acid, nicotinic acid, and biotin supported slightly less growth than did the vitamin-free medium. On the basis of these results, the authors suggested that the supply of vitamins is not an absolute requirement for the growth of A. cinnamomea. It is possible that this fungus is capable of synthesizing the listed vitamins.

When Grifola frondosa was cultivated in basal medium containing various growth factors (thiamine, riboflavin, nicotinic acid, ascorbic acid, and biotin) at a concentration of 1.0 g/L, nicotinic acid, ascorbic acid, and biotin supported better superoxide anion scavenging activity. The maximal activity of about 69.98% was obtained in the nicotinic acid–supplemented culture, followed by the ascorbic acid (67.29%) and riboflavin (67.08%) cultures.

7. Special Additives

To increase the production of BAM by medicinal mushrooms, many investigators used some stimulating agents, including fatty acids, surfactants, vegetable oils, and organic solvents. These agents are known to mediate cell permeabilization by disorganizing the cell membrane and/or directly affecting the level of enzyme synthesis involved in product formation, thereby contributing to enhanced production of target products. The addition of 0.3% Tween 80 on day 5 enhanced mycelial biomass and EPS production by Pleurotus tuberregium by 51.3% and 41.8%, respectively. The authors observed that the glucose consumption rate was significantly increased after the addition of Tween 80, implying that the nutrient uptake efficiency from the fermentation broth had been increased, which eventually led to an increase of mycelial biomass and EPS production of P. tuberregium. They suggested that the mechanism by which Tween 80 could affect fungal metabolism is associated with the intact structure of and transport activity across the mycelial membrane.

The effects of 0.1, 1, and 2% ethanol, 0.5, 1, 5, and 10% Tween 80, and 1 and 5% oleic acid on EPS production were evaluated in the shake-flask culture of Armillaria luteovirens. Among the agents examined, ethanol caused a negative effect on EPS accumulation and mycelia growth. Supplementation of media with 0.5% Tween 80 or 1% oleic acid displayed only a slight stimulating effect on EPS production, although mushroom biomass yield appeared to be significantly higher than did vitamin-free medium. Chen et al. showed that the polymer additive polyethylene glycol displayed an effective stimulatory effect on both biomass and EPS production in Grifola umbellata submerged culture.

Several plant oils, which can be used as anti-foam agents, may also be beneficial to mushroom growth and BAM production. The effects of soy, peanut, safflower, corn, sunflower, and olive oils were investigated in submerged fermentation, all at a volume fraction of 1%. All the tested oils stimulated growth, with the highest biomass density being obtained with olive oil. The authors proposed that such stimulation is due to a partial incorporation of lipids in the cell membrane, thereby facilitating the uptake of nutrients from the medium. EPS production was highest with safflower oil, slightly inhibited with soy oil, but was not significantly affected by the other oils tested, when compared to fermentation with the same medium but with no oil added.

The addition of sunflower oil stimulated mycelial growth of Hericium Erinaceus in a dose-dependent manner. The addition of Selol (containing selenite triglycerides) at concentrations of 2.5–10 g/L increased the mycelial yield from 3.80 to 5.60–11.25 g/L and 4.97–10.79 g/L for culture media containing Selol2% and Selol5%, respectively. The acceleration of mycelial growth by oil in this experiment might be explained by the partial incorporation of glycerolipids in the cell mem-
brane, which enable the uptake of nutrients from the medium.\textsuperscript{79} Moreover, it was found that the production of EPS with antioxidant properties was significantly increased by the addition of sunflower oil or Selol.\textsuperscript{30} The highest EPS yield (2.25 g/L), 2.5 times greater than that in the control medium (0.91 g/L), was obtained in the presence of 7.5 g/L of Selol\textsuperscript{15}. The EPS production was found to proportionally increase with elevations in sunflower oil and Selol\textsuperscript{2} concentration up to 10 g/L, and its maximum value was 1.54 and 1.83 g/L, respectively. The authors assumed that sunflower oil and its derivative (Selol) serve as either carbon sources or stimulators of biosynthesis of secondary metabolites (including EPS) during submerged cultivation of H. erinaceus. It is generally recognized that vegetable oils and related substances promote excretion of fungal extracellular polysaccharides in liquid culture conditions.\textsuperscript{76,79} It was suggested that the possible mechanism of stimulating effects on EPS production might be related to a modification of structure of the cell membrane, which increases its permeability. Another explanation is that oils directly affect the level of enzyme synthesis involved in EPS formation.\textsuperscript{77}

The use of organic solvents could be a relatively effective method for cell permeabilization; they are less expensive than other stimulating agents and may be eliminated by simple evaporation. Therefore, the influences of four different organic solvents (e.g., toluene, chloroform, acetone, and heptane) on EPS production in Collybia maculata were studied by supplementing 0.3\% of each into culture media on the fourth day of fermentation.\textsuperscript{78} Of the agents examined, toluene, heptane, and chloroform displayed enhanced EPS production with reduced mushroom growth. Acetone did not result in any increase in EPS production, nor in cell growth inhibition. Toluene followed by chloroform was the best stimulant for EPS production, although mycelial growth was inhibited by solvent additions. When the effect of toluene and chloroform concentration on enhanced EPS production was studied using a range of 0.1\%–0.5\%, the highest C. maculata growth and EPS production was observed at 0.3\% toluene and 0.1\% chloroform. The EPS yields achieved by supplementations of toluene and chloroform were 3.48 and 3.0 g/L, respectively. This corresponded to 77\% and 53\% enhancement in EPS production, respectively, when compared with control media. The study of the effect of addition timing of toluene revealed that the late exponential phase was preferred for enhanced EPS production. The highest EPS production was obtained when 0.3\% toluene was added to the production medium at 108 h after the start of shake-flask cultivation, just before terminating fermentation. It is interesting that a significant increase in EPS production (to 4.12 g/L) was achieved when fermentation was prolonged for 12 h. The authors concluded that the enhanced production of EPS was a result of the outer cell structure modifying action of toluene, thereby driving out the polysaccharide entrapped inside the cells. However, cells should be permeabilized without lysis or destruction of the whole inner organization. This is an important reason why solvents have to be supplemented in the late stage of growth phases, as established in this study.

V. Fermentation Strategies

It is known that filamentous fungi have different morphological growth forms, which have a significant effect on the rheology of fermentation broth and, thus, on the performance of the bioreactor. Cultures with filamentous growth usually exhibit a high apparent viscosity and non-Newtonian rheology. At moderate-to-high biomass levels, these broths display shear thinning or pseudoplasticity.\textsuperscript{82} These effects can have a number of undesirable results, such as poor mass transfer, which would decrease overall productivity. In addition, the control of hyphal extension is of great importance to operate the bioreactor in a continuous mode. When mushroom growth occurs in pellet form, the yield of BAM depends on the pellet size, which determines the oxygen diffusion in the pellet center. Pellet size is in itself influenced by variables such as sugar concentration in the medium, agitation regime, and the inoculum density. Unfortunately, many studies devoted to exploring the relationship between growth morphology and metabolite production simply describe the effect of changing a variable and do not elucidate the underlying mechanism that causes the effect.

A. Batch Cultures

The growth and EPS production by Grifola frondosa in an airlift and stirred-tank bioreactors have been compared.\textsuperscript{83,84} Although an airlift bio-
reactor is often better for mycelial growth than a stirred-tank bioreactor due to lower shear forces, the maximum concentrations of mycelial biomass (10 g/L) and EPS (4.5 g/L) obtained in the airlift bioreactor were lower than those in the stirred-tank bioreactor (24 g/L and 6.5 g/L, respectively). It is probable that the stirred-tank bioreactor not only provided better mixing, but also promoted the formation of the desired morphology. Thus, the study of the morphology of growth in stirred-tank bioreactors showed that *G. frondosa* mainly forms pellets with high hairiness, with pellet size increasing rapidly from the beginning of the fermentation and reaching a maximum value at day 4, which corresponded to the time of maximum biomass and EPS concentrations. After this period, the core region became denser and larger, due to a lack of nutrient and oxygen supply. Later, the larger pellets broke into several smaller pellets, but without significant hyphal fragmentation, since there was no significant increase in the concentration of free mycelia. The culture pH, aeration rate, and hydrodynamic behavior affected growth morphology and changes during the culture: compact pellets were formed under low aeration, whereas freely suspended mycelial growth was obtained under high aeration. Maximum mycelial biomass and polysaccharide levels were achieved when the biomass grew as loose mycelial clumps with high hairiness.

The following strategy was used for biomass and EPS production in *Inonotus levis* and *Agaricus nevoi* cultivation in a 13-liter stirred-tank fermenter containing 10 L of medium based on glucose and corn steep liquor. During *I. levis* and *A. nevoi* cultivation for the first 2 and 5 days, respectively, the agitation speed gradually increased from 50 to 300 rpm, while the pH of the cultures was controlled at 5.5 to permit maximal growth of both fungi. When the cultures reached the middle of the logarithmic phase of growth, the pH was controlled at a level of 4.5 to provide conditions favorable for EPS accumulation. *I. levis* developed very rapidly and after 5 days of fermentation the culture reached the stationary phase of growth with a high level of mycelial biomass of 16 g/L. A maximum level of EPS concentration (4.2 g/L) was achieved at the end of fermentation. *A. nevoi* was distinguished by a much lower growth rate and entered the stationary growth phase on day 10. At the end of fungus cultivation the yield of mushroom biomass was equal to 12 g/L, while the EPS yield reached 3.9 g/L.

Since ganoderic acid production was favored at a low oxygen tension, Fang and Zhong undertook a two-stage process in Erlenmeyer flasks. The first stage was realized with agitation in a rotary shaker. After 4, 8, or 12 days the agitation was stopped, and the culture then remained static until the 24th day. A control culture was shaken for the whole time. In static culture glucose was consumed at a slower rate and converted to biomass more efficiently. The highest biomass density was obtained with 4 days of agitation followed by 12 days of static culture. The highest production of ganoderic acid that has yet been reported (582 mg/L at day 12) was obtained in the culture that was agitated for only the first 4 days. The production of ganoderic acid was almost double that in the continuously shaken control culture. A thick layer of mycelium was noted in this culture, which obviously restricted oxygen diffusion into the layer. As a result, low oxygen availability appears to stimulate ganoderic acid production. No thick mycelial layer was found for the culture agitated for the first 12 days and then left static for another 12 days. In this case, the production of ganoderic acid did not increase significantly compared to a shaking culture without any static stage.

A very efficient strategy using bi-stage control of pH for a many-fold increase of EPS production by *Ganoderma lucidum* in batch cultivation in an air-lift fermenter was described by Lee et al. They investigated the effect of pH on *G. lucidum* growth and EPS production in batch cultivation in an air-lift fermenter with controlled and uncontrolled pH. Fermentation at controlled pH 3.0 gave higher biomass densities than at pH 6.0. With respect to EPS production, control at pH 6.0 gave a higher yield than control at pH 3.0. The authors suggested that the better EPS production when the pH was controlled at 6.0 was because pellet growth was maintained. In a cultivation initiated at pH 6.0 without pH control, the pH fell to 2.6 and the morphology changed from pellet to filamentous form in the latter stages of cultivation, leading the authors to suggest that pH influences the morphology, which in turn affects EPS production. Based on these observations, fermentation with bi-stage pH control was proposed, with the pH commencing at 3.0 and then changing to...
6.0 after 2 days. This strategy resulted in the extremely high EPS yield (20.04 g/L on day 6).

B. Fed-Batch Cultures
Shih et al. compared *Grifola frondosa* growth and EPS production in batch and fed-batch cultures in a 5-L jar fermenter under optimal culture conditions. In batch culture without pH control the growth of mushroom biomass increased steadily for the first 9 days, with a corresponding depletion of sugar concentration and dissolved oxygen, and decline of pH. The mycelium was observed to form mainly pellets from the early stage (3 days of fermentation) without notable morphological changes in mycelium during the entire period of fermentation. The growth of cell biomass rose significantly from the 9th day of fermentation, which was accompanied by a rapid increase of EPS and a significant increase of viscosity in the broth. The concentrations of mushroom biomass and EPS at day 11 of cultivation reached 5 g/L and 1.32 g/L, and at day 13 of cultivation they were 6.7 g/L and 3.3 g/L, respectively. A sugar analysis showed that the carbon source was depleted toward the end of fermentation. The authors suggested that the lack of a carbon source prevented EPS production and mycelial growth from reaching their optima at the end of fermentation. The additional carbon source is presumably helpful in enhancing the final cell biomass, and EPS accumulation and productivity. However, it is often seen that the high nutrient concentrations required for final cell growth and product yields inhibit growth if added in total at the start of fermentation. Therefore, the fed-batch strategy, feeding glucose during fermentation, was adopted to avoid the inhibitory effects of nutrient on the biosynthesis while increasing the final cell biomass and polysaccharide accumulation. The fed-batch culture was started when the glucose concentration in the culture medium was lower than 0.5%, which occurred on day 6 of cultivation, where the concentration of dry biomass and EPS was 3.97 g/L and 1.04 g/L, respectively. The feeding solution of glucose was pumped into the fermenter, and each feeding led the glucose concentration in the broth close to 1.5%. In all, six feedings were carried out; they were performed on days 6, 7, 9, 9.5, 11, and 12.5 of cultivation. The concentrations of dry biomass and EPS reached 8.23 g/L and 3.88 g/L on day 13 of cultivation. The results presented have demonstrated that the fed-batch culture was useful for efficient accumulation of *G. frondosa* biomass and EPS. Moreover, the authors pointed out that each pause feeding to raise the concentration of glucose to 1% was sufficient to enhance the accumulation of biomass but insufficient for EPS accumulation. They indicated that each pause feeding to raise the concentration of glucose to 1.5% was necessary and suitable for both biomass and EPS production.

A process for simultaneous hyper-production of *Ganoderma lucidum* polysaccharide and ganoderic acid by fed-batch fermentation was developed. The researchers were motivated by the observation that lactose concentrations above 35 g/L had diminished the production of ganoderic acid. The fermentation was started at 35 g/L lactose. When the sugar concentration fell to between 5 and 10 g/L, sufficient lactose was added by pulse feeding to increase its concentration by 15 g/L. The maximum yields of biomass, EPS, IPS, and ganeric acid reached in a stirred-tank fermenter were 21.89 g/L, 0.87 g/L, 2.49 g/L, and 367.1 g/L, respectively.

C. Multi-Pulse Feeding Integrated Strategy
Recently, Zhang and Tang developed a novel three-stage light irradiation strategy in submerged fermentation of medicinal mushroom *Ganoderma lucidum* for the efficient production of ganoderic acid and polysaccharides. From the viewpoint of total ganoderic acid accumulation, the first stage was 2 days of dark culture, the second stage was 6 days with 0.94 W/m² of white light irradiation, and the third stage was 4.70 W/m² of white light irradiation until the end of fermentation. Then Tang and Zhu developed a multiple-addition strategy of Cu²⁺ by adding 1 mM Cu²⁺ on days 2, 6, and 8, and 2 mM Cu²⁺ on day 4 to enhance the total accumulation of ganoderic acid. Both multiple Cu²⁺ additions and the three-stage light irradiation induced an accelerated transformation of lactose to secondary metabolites. Moreover, multiple Cu²⁺ additions obviously promoted the release of extracellular products by increasing cell membrane permeability, resulting in enhanced EPS production.

To further enhance the accumulation of bioactive metabolite fermentation of the medicinal mushroom *G. lucidum*, these researchers devel-
oped a novel integrated strategy by simultaneously adopting multiple Cu²⁺ additions, three-stage light irradiation, and multi-pulse feeding of carbon and nitrogen sources. In the integrated strategy experiment, peptone (2.5 g/L) and yeast extract (2.5 g/L) were added on days 2 and 4. A total of 60 g/L lactose was fed into the G. lucidum fermentation process by four pulse feedings (15 g/L lactose on days 10, 14, 18, and 25). The maximal EPS production (2.08 g/L) obtained with the integrated strategy was 1.74-, 2.16-, and 1.19-fold higher than those achieved with multiple Cu²⁺ additions, three-stage light irradiation, and multi-pulse feeding. This finding indicated that lactose feeding in the multi-pulse feeding and integrated strategies might be an important factor for increasing EPS production. The maximal IPS production (3.35 g/L) obtained with the integrated strategy was higher by 2.28-, 1.08-, and 1.14-fold compared to those with multiple Cu²⁺ additions, three-stage light irradiation, and the multi-fed batch culture. The results showed that the total IPS accumulation might be enhanced by the synergistic effects of multiple pulse feedings and three-stage light irradiation. The highest ganoderic acid production (720.8 mg/L) obtained with the integrated strategy was enhanced by 2.07-, 1.55-, and 1.24-fold compared with the multiple Cu²⁺ additions, three-stage light irradiation, and the multiple pulse-fed batch culture, respectively, and is also the highest reported in a shaker-flask culture of G. lucidum. Finally, based on the integrated scale-up criterion, the fed-batch fermentation of G. lucidum was successfully scaled up from a 7.5- to a 200-L stirred-tank bioreactor.

**D. Other Strategies**

A cost-effective strategy for up-regulating the biosynthesis of bioactive metabolites (antioxidants), namely, a co-culture of Inonotus obliquus with Phellinus punctatus, has been described. In a monoculture, biomass accumulation showed an exponential increase beginning at day 3 and reached 7.6 g/L at day 11. Similar time courses of biomass accumulation were also seen in the co-culture, yet maximum biomass levels were reduced to 5.2 g/L. In contrast, metabolites accumulated in the co-culture were substantially higher than those found in the monoculture. In melanin production, for example, the maximum level in the monoculture reached only 1.23 g/L, but this increased to 3.61 g/L in the co-culture. Maximum total mycelial phenolic compound levels in the monoculture attained 29.89 mg/g, and increased to 43.91 mg/g in the co-culture. Total mycelial triterpenoids accumulation in the co-culture was also drastically raised in contrast to that in the monoculture.

**VI. OPTIMIZATION OF CULTURE CONDITIONS**

To achieve a higher yield in a submerged culture, it is a prerequisite to design an optimal production medium and optimal process operating conditions. Traditional methods of optimizing the culture medium involve changing one independent parameter while keeping the others constant. However, such one-dimensional methods are very laborious, time-consuming, and do not provide any information on interactions and correlations between parameters. Therefore, there are large numbers of reports on the optimization of culture media for mycelial growth and metabolites by statistical optimization techniques that permit the simultaneous optimization of many factors, thereby obtaining much quantitative information by only a few experimental trials. These techniques have been successfully applied to the improvement of culture media or the production of primary and secondary metabolites in the cultivation process. Thus, the optimization of submerged culture conditions and nutritional requirements of mycelial biomass and EPS production from Fomes fomentarius was studied using the orthogonal matrix method. Under optimal culture conditions, the maximum EPS concentration reached 3.64 g/L, which was more than four times higher than that in the initial basal medium.

Feng et al. employed statistical optimization to optimize the culture medium for maximum mycelial growth and EPS production in submerged cultivation of Lentinus edodes. The Plackett-Burman design was applied to determine significant factors, followed by the paths of steepest ascent to move to the general vicinity of the optimum and the Box-Behnken design to obtain the final optimum culture medium composition. Among eight factors tested, glucose, yeast powder, and pH were significant for fermentation. Accordingly, 15.4 g/L glucose and 5.32 g/L yeast powder as well as pH 4.61 were optimum for the biomass accumulation from 2.75 g/L to 6.88 g/L.
while 15.78 g/L glucose, 5.86 g/L yeast powder, and pH 4.48 were optimum for EPS production from 0.214 g/L to 0.751 g/L.

Malinowska et al.\(^{95}\) optimized cultivation conditions of *Hericium erinaceus* in a submerged culture using the one-factor-at-a-time method and a central composite rotatable design (CCRD) experiment. Of the various factors examined, including carbon and nitrogen sources, vitamins, mineral elements, and initial pH, those that proved to have a significant effect were then tested using a 24 CCRD. Under the optimal culture conditions, the maximal yield of biomass reached 14.24 g/L and was 1.85-fold higher than in the basal medium. On the basis of the results obtained from the CCRD experiment, *H. erinaceum* was cultivated in a fermenter filled with optimized media. The maximal biomass growth was equal to 15.3 g/L on day 8 of cultivation. During the first days of cultivation, a major increase in the EPS yield was observed, which reached 2.75 g/L after 8 days of cultivation and then gradually declined together with the biomass yield.

Defined medium composition was optimized using the orthogonal matrix method for mycelial growth and EPS production by submerged culture of *Phellinus igniarius*.\(^{96}\) An optimal combination of culture medium ingredients included 40 g/L glucose, 4 g/L glutamic acid, and 4 g/L \((\text{NH}_4)_2\text{SO}_4\), with an initial pH at 6.0. Based on the optimal chemically defined medium, the maximal mycelial biomass and EPS production in shake flasks was 12.33 and 1.21 g/L at 192 h, respectively. The fermentation process was further scaled up in a 3-L fermenter. In this case, the maximal mycelial biomass and EPS production reached 13.86 and 1.92 g/L, respectively.

Luo et al.\(^{97}\) optimized the medium composition for the production of EPS from *Phellinus baumii* in submerged culture using a \(2^{7-3}\) fractional factorial design to select glucose, yeast extract, and diammonium oxalate monohydrate, medium components having significant effects on EPS production. Subsequently, the concentrations of the three factors were optimized using a central composite design in response surface methodology. As a result, a quadratic model was found to fit EPS production, and the optimal medium composition was determined, providing an accumulation of 2.36 g/L EPS.

VII. CONCLUSIONS AND FUTURE PERSPECTIVES

The information presented in this review clearly indicates that higher Basidiomycetes are metabolically versatile fungi that could be used in various biotechnological applications. Some of these applications are traditional and practiced throughout the world, while some need further research and development. Undoubtedly, basidiomycetes are an immensely rich resource of a wide range of useful, easily accessible, natural products that could promote human well-being. However, it is a resource that has barely been tapped because only a fraction of the world’s fungal species are known to science, whereas the majority of already known pharmacologically important mushrooms are still far from being thoroughly explored.

The available literature data prove that the capability to synthesize BAM is widespread among the higher Basidiomycetes, and the spectrum of detected pharmacological activities of mushrooms is very broad. Moreover, their biosynthetic potential is species and strain dependent, and varies, to a large extent, according to the composition of the media, cultivation conditions, and techniques. However, the practical application of BAM obtained from medicinal mushrooms depends not only on their unique properties but also on availability of industrially relevant competitive technologies. As pointed out in this review, the submerged culture of mushrooms has significant industrial potential, but its success on a commercial scale depends on costs compared to the existing field-cultivation technology and whether industry sees an economic advantage.

Till now researchers paid scant attention either to the regulatory aspects of BAM synthesis and secretion, or to the large-scale cultivation of fungi and downstream processing. The challenge is to discover the physiological and biochemical mechanisms regulating and enhancing BAM synthesis and secretion. All published studies have focused only on describing the effects of physical or chemical factors, and no work has been carried out in a way that might provide an explanation of the cause of the effects observed. Surprisingly, no data exist on the fundamental factors affecting the synthesis of antioxidants and lectins in submerged and solid-state fermentation of lignocellulose. This represents a large field for future research, which
must be addressed if optimal fermentation strategies are to be developed in a rational manner.

Greater attention is needed in mushroom cultivation to various fermenters. Production of BAM could be significantly enhanced by fed-batch cultivation or application of two-stage and multi-stage cultivation strategies when important environmental factors are well controlled to achieve a highly productive process on a large scale. Increasing our knowledge of how mushrooms behave in varying environments would permit the design of processes and bioreactors around the capabilities of fungi.

Finally, with the increasing demands for cheap bioactive compounds, developing cost-effective technologies based on biomass feedstock is challenging. Higher Basidiomycetes are one of the most efficient decomposers of lignocelluloses because they are capable of synthesizing all relevant extracellular enzymes required to degrade the major components of the substrate into low-molecular-weight compounds that can be assimilated for fungi nutrition. Therefore, they may be cultivated on a large number of locally available substrates. Food industry residues are extremely important and most appropriate as growth substrates for mushroom cultivation because such residues are rich in sugars and other useful compounds, which are easily metabolized by mushrooms.

Undoubtedly, gaining knowledge in physiology, biochemistry, and molecular biology of mushrooms as well as improving screening methods (high-throughput screening, genomics, and proteomics) will help increase the application of mushrooms for medicinal purposes.

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