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Submerged Culture of Mushrooms in Bioreactors – Challenges, Current State-of-the-Art, and Future Prospects

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Summary

Medicinal mushrooms have profound health-promoting benefits. Recently, a number of substances of mushroom origin have been isolated, identified and shown to have physiological activities, such as antitumor, immunomodulating, cardiovascular, antihypercholesterolemia, antibacterial, antiviral, antiparasitic, hepatoprotective, and antidiabetic activities. Currently, commercial products from medicinal mushrooms are mostly obtained through the field-cultivation of the fruiting body. However, in this case it is difficult to control the quality of the final product. Submerged fermentation of the mycelial form of mushroom-producing fungi has received much attention as a promising alternative for efficient production of the biomass of medicinal mushrooms and their active metabolites. However, in order for the production to be successful at industrial scale, various technical problems need to be solved, including characterization of the variations that occur during the submerged cultivation of mushrooms in bioreactors and their effects on growth and product formation. This review outlines the major factors that affect the submerged cultivation of mushrooms in bioreactors, including oxygen supply, shear and mixing, morphology and rheology, as well as two-stage cultivation strategies and high-cell-density cultivation strategies such as fed-batch fermentation.

Key words: mushroom, macrofungi, submerged fermentation, bioreactor, bioactive metabolites, fed-batch fermentation, oxygen transfer, shear and mixing, morphology and rheology, two-stage cultivation process

Introduction

Mushrooms have long been regarded as effective medicines for the treatment of various human diseases. The medicinal properties are due to various cellular components and secondary metabolites, which have been isolated and identified from the fruiting-body, culture mycelium and culture broth of mushrooms and shown to have promising antitumor, immunomodulating, cardiovascular and hypercholesterolemia, antiviral, antibacterial, and antiparasitic effects (*1–3*). The number of mushroom species on Earth is estimated at 140 000, yet only 10 % (approximately 14 000 named species) are known (*4*). Few have been studied thoroughly from the standpoint of their commercial potential. Less than 25 species of mushrooms are accepted widely as food and even fewer have attained the status of items of commerce. This is unfortunate, because mushrooms comprise a vast and yet largely untapped source of potential new pharmaceutical products. In particular, and most importantly for modern medicine, they represent a source of new polysaccharides with antitumor and immunostimulating properties. Medicinal mushrooms are viewed as a rapidly developing area of biotechnology for cancer and other therapies (*3*).

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There is a growing market for the products derived from medicinal mushrooms. Currently, commercial mushroom products are mostly derived from the fruiting body of field-cultivated mushrooms. However, it usually takes several months to cultivate the fruiting body and it is difficult to control the quality of the final product, as it is subject to the vagaries of the weather and pests. Submerged culture of medicinal mushrooms is a promising alternative for efficient production of mycelium and metabolites and has received increasing attention around the world. However, in spite of several decades of efforts, the production of secondary metabolites by submerged culture of mushrooms is still facing many biological, physiological and engineering limitations. The lack of information on submerged cultivation of mushrooms in bioreactors is significant when compared to the relatively large body of information about the submerged culture of streptomycetes and microfungi.

This review focuses on the special challenges, current state-of-the-art, and future prospects of submerged cultivation of medicinal mushrooms to produce mycelium and bioactive metabolites. It highlights the need to overcome the difficulties in oxygen transfer and mixing that are caused by the changes in broth rheology that typically occur during the fermentation and also the use of fed-batch fermentation strategies to maximize product formation. However, first a brief overview of the products from mushrooms that have been produced by submerged culture will be given.

Bioactive Metabolites Produced by Submerged Cultivation of Mushrooms

A number of bioactive metabolites of higher basidiomycetes have been produced by submerged cultivation in bioreactors, as shown in Table 1 (*5–19*). The fol-

Table 1. Current state of cultivation of mushrooms/macrofungi in bioreactors

Mushroom	Production	Scale	Results
Agaricus brasiliensis (synonym: *Agaricus blazei* ss. Heinem.) (*5*)	Polysaccharide	5-L agitated bioreactor with two six-bladed turbine impellers	(1.67±0.08) g/L
	Ergosterol		25 mg/g (DM)
Agrocybe cylindracea (synonym: *Agrocybe aegerita*) (*6*)	EPS	5-L stirred-tank reactor (KoBioTech Co., Seoul, Korea) working volume of 3 L	3.0 g/L
Antrodia camphorata (*7,8*)	EPS	5-L bioreactor (B. Braun, Germany)	148 mg/L
	Polysaccharide	500-L fermenting tank containing 350 L of cultural medium	23.2 % (yield)
	Total polyphenols		71 mg/g
	Crude triterpenoids		108 mg/g
Auricularia polytricha (*9*)	Exobiopolymer	5-L stirred-tank fermentor (KoBioTech Co., Seoul, Korea) with working volume of 3 L	3.1 g/L
Collybia maculate (*10*)	EPS	5-L stirred-tank reactor with working volume of 3 L	3.94 g/L
Cordyceps militaris (*11*)	Cordycepin	30-L centrifugal impeller bioreactor (CIB) with working volume of 21 L	188.3 mg/L
Cordyceps sinensis (*12*)	Cordycepin	5-L bioreactor (TopBio, Taiwan) with working volume of 3 L	1.9 μg/g
	Adenosine		2.7 μg/g
Grifola frondosa (*13,14*)	EPS	5-L stirred-tank bioreactor (KoBioTech Co., Incheon, Korea)	5.3 g/L
		5-L airlift bioreactor (Best Korea Co. Ltd., Daejeon, Korea)	4.53 g/L
	Exopolymer	15-L bioreactor (Biostat C10-3, B. Braun, Germany)	1.252 g/L
Inonotus obliquus (*15*)	Endopolysaccharide and EPS	300-L bioreactor (stirred type, KoBioTech Co., Seoul, Korea)	0.495 g/L
Phellinus (*P. baumii, P. gilvus* and *P. linteus*) (*16*)	EPS	5-L stirred-tank reactor (KoBioTech Co., Seoul, Korea) with working volume of 3 L	*P. baumii* 3.59 g/L *P. gilvus* 5.30 g/L *P. linteus* 2.43 g/L
Phellinus gilvus (*17*)	EPS	5-L stirred-tank reactor (KoBioTech Co., Seoul, Korea) with working volume of 3 L	5.3 g/L
Sarcodon aspratus (*18*)	EPS	5-L stirred-tank reactor (KoBioTech Co., Seoul, Korea) with working volume of 3 L	2.68 g/L
Schizophyllum commune (*19*)	L-Malic acid	12-L standard bubble column fermentor with working volume of 10 L	Yield<20 %
		8-L external-loop airlift column fermentor with working volume of 7.2 L	Yield>40 %
		5-L airlift reactor (Best Korea Co Ltd., Daejeon, Korea)	3.05 g/L

EPS – extracellular polysaccharide

lowing subsections briefly outline the key characteristics of each group of metabolites and their biological activities.

Polysaccharides

The fruiting bodies of many basidiomycetes, if not all, contain biologically active polysaccharides. Data on mushroom polysaccharides have been collected from 651 species and 7 infraspecific taxa from 182 genera of higher hetero- and homobasidiomycetes. In Japan, Russia, China, and the USA, several different polysaccharide antitumor agents have been developed from the fruiting body, cultured mycelia, and culture broth of various medicinal mushrooms including *Lentinus edodes, Ganoderma lucidum, Schizophyllum commune, Trametes versicolor, Inonotus obliquus,* and *Flammulina velutipes* (*2,20*).

These polysaccharides are of different chemical compositions, with most belonging to the group of β-glucans; these have β-(1→3) linkages in the main chain of the glucan and additional β-(1→6) branch points that are needed for their antitumor action. High molecular mass glucans appear to be more effective than those of low molecular mass. Most of the clinical evidence for antitumor activity comes from three commercial polysaccharides, namely lentinan produced by *Lentinus edodes*, PSK (polysaccharide krestin) produced by *Coriolus versicolor,* and schizophyllan produced by *Schizophyllum commune*. Their activity is especially beneficial in clinics when used in conjunction with chemotherapy. Mushroom polysaccharides prevent oncogenesis, show antitumor activity against various allogeneic and syngeneic tumors, and prevent tumor metastasis. They do not attack cancer cells directly, but produce their antitumor effects by activating different immune responses in the host. The antitumor action of polysaccharides requires an intact T cell component; their activity is mediated through a thymus-dependent immune mechanism (*2*).

Terpenoids

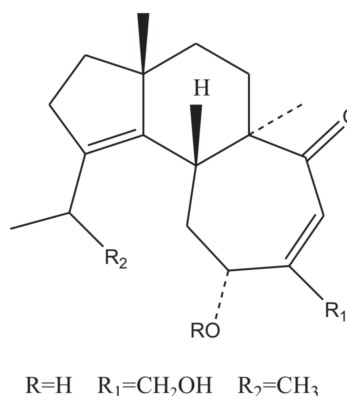
Terpenoids have been produced from *G. lucidum*, although other species are being increasingly investigated for the production of triterpenoids and related steroids. Terpenoids are comprised of four groups: (*i*) volatile mono- and sesquiterpenes (essential oils) (C10 and C15), (*ii*) less volatile diterpenes (C20), (*iii*) non-volatile triterpenoids and sterols (C30), and (*iv*) carotenoid pigments (C40) (*21,22*). Ziegenbein *et al.* (*21*) and Zhou *et al.* (*22*) have summarized the structure and bioactivity of terpenoids in *G. lucidum*. Tri-, di- and monoterpenoids are discussed separately in the following subsections.

Triterpenoids

The physicochemical properties of over 130 lanostane-type triterpenoids have been described since ganoderic acid A and B were first described (*22*). The biological activities of triterpenes from *G. lucidum* have received recent attention, with three different types of activity being demonstrated. Firstly, a triterpene fraction extracted from the mycelium inhibited growth of human hepatoma cells (*23*). Secondly, the triterpene fraction of mycelial extracts, which contained ganoderic acids A, B, C and D, lucidenic acid B and ganodermanotriol, showed antioxidant activities against pyrogallol-induced oxidation of erythrocyte membrane and Fe(II)-ascorbic-acid-induced lipid peroxidation in liver mitochondria (*24*). Thirdly, triterpenes isolated from fruiting bodies, such as ganoderic acid, ganoderiol B and ganodermanontriol, showed significant anti-HIV-1 and anti-HIV-1 protease activity (*25*).

Diterpenoids

Diterpenoids have one of four types of carbon skeletons: (*i*) cyathane-type (Fig. 1); In 1965, a new bird's nest fungus of the genus *Cyathus* was discovered in Canada and named *Cyathus helenae*. Ayer and Browne (*26*) proved that the bioactive component of *Cyathus helenae* belonged to a new class of diterpenoids, which have been named the cyathins. Since the 1980s, a series of new compounds with cyathane-type carbon skeletons have been found in the fruiting bodies of *Sarcodon scabrosum* and the culture broths of *Hericium erinaceus* and *Hericium ramosum*. In 2000, a new cyathane-xyloside, erinacine P, was isolated from the mycelium of the basidiomycete *Hericium erinaceum*. Since its aglycon closely resembles typical cyathane diterpenoids, this glycoside seems to be an important metabolite in the biosynthesis of erinacines and striatins; (*ii*) trichoaurantiane-type (Fig. 2); This type of carbon skeleton was first reported in 1995, and has only been found in a few basidiomycetous fungi, including *Tricholoma aurantium* and *Lepista sordida* (*27*); (*iii*) sphae-



R=H R_1=CH_2OH R_2=CH_3

Fig. 1. The structure of cyathin A_3 (a typical diterpenoid with a cyathane–type carbon skeleton)

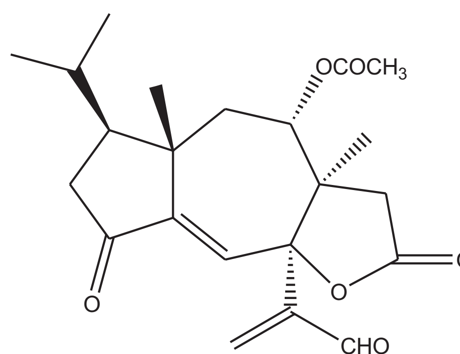


Fig. 2. The structure of trichoaurantianolide A (a typical diterpenoid with a trichoaurantiane–type carbon skeleton)

roane-type (Fig. 3); This type of carbon skeleton has only been found in *Sphaerococcus coronopifolius* and algae. In 1998, a new compound, tintinnadiol, was found in the fruiting body of *Mycena tintinnabulum*, and was shown to have a sphaeroane-type carbon skeleton (*28*); (*iv*) other styles; In the 1950s, some other compounds with new skeleton types were extracted from the basidiomycete *Pleurotus mutilis* and from *Drosophila subatrata*, and were shown to have antimicrobial activity (*29*).

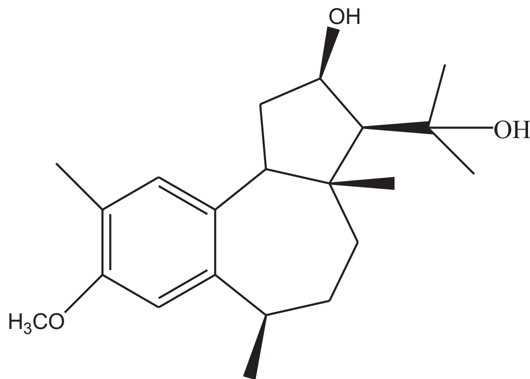


Fig. 3. The structure of tintinnadiol (a typical diterpenoid with a sphaeroane–type carbon skeleton)

Many papers have shown that diterpenoids can be inducers of nerve growth factor and have antimicrobial and antitumor activity. They can also inhibit the synthesis of squalene synthase and act as agonists of the opioid receptor (*29*).

Sesquiterpenoids and monoterpenoids

There is relatively little research into monoterpenes in mushrooms in comparison with other terpenes. A few papers have demonstrated that sesquiterpenoids can promote the synthesis of nerve growth factor (*30*) and have insecticidal activity (*31*). A new lactarane sesquiterpene was isolated from a mushroom of the *Russulaceae* family, *Lactarius vellereus*, and found to promote radicle elongation in lettuce seedlings (*32*). Five monoterpene alcohols have been isolated from the mushroom *Dictyophora indusiata* (*33*). Finally, a new monoterpene isolated from the basidiomycete *Cheimonophyilum candidissimum* showed nematicidal activity to *Caenorhabditis elegans* (*34*).

Cordycepin

Cordycepin (3'-deoxyadenosine), which comes from the Chinese medicinal fungus *Cordyceps militaris* (Linn.) Link, was extracted from the culture broth of this fungus by Cunningham *et al.* in 1951 (*35*). It is the first bacteriophage nucleotide separated from fungi and it has been recognized as a kind of cytotoxin (*36*). Cordycepin has antitumor, antibacterial, antifungal, antileukemic and antiviral activities and inhibits the activity of protein kinase (*36,37*). With respect to its antitumor activity, cordycepin restrains the growth of the mouse Ehrlich ascites tumor cell, HeLa human cervical carcinoma cell and Lewis lung cancer (*38,39*).

Ergosterol

Ergosterol is an important chemical in the pharmaceutical and chemical industry. It is the precursor of vitamin D; under ultraviolet radiation it can be converted into cortisone and progestin. Ergosterol is an important part of the fungal cell membrane, playing a role in maintaining membrane integrity, the activity of membrane-associated enzymes, and the fluidity of the membrane and the generation of cellular energy. Fruiting bodies of the medicinal mushroom *Agaricus brasiliensis* are relatively rich in ergosterol (*5*), which has also been found in *Cordyceps militaris*. However, commercial production of ergosterol still involves either yeast fermentation or extraction from the waste mycelium resulting from penicillin production.

Ceramide

Ceramide is a functional element that can be added to foods and cosmetics. It has effects such as improving immunity, preventing cancer and combating existing cancers. Its function as an effective moisturizing agent gives it the potential to be an important ingredient in cosmetics. Ceramide-containing products can dramatically increase the skin's hydration level, repair the cutaneous barrier, prevent vital moisture loss, and contribute to reducing dry flaky skin and aged appearance (*40*).

Four new phytosphingosine-type ceramides have been isolated from the fruiting bodies of *Grifola frondosa* (*41*). In 2001, a new C18-phytosphingosine ceramide containing a non-hydroxy fatty acid, armillaramide, was isolated from the fruiting bodies of the basidiomycete *Armillaria mellea* (*42*).

Submerged Cultivation of Mushrooms in Bioreactors

The bioactive metabolites of mushrooms can potentially be produced industrially by submerged cultivation, but success on a commercial scale depends on the cost compared with existing technology and whether industry sees any economic advantages. Expansion of the use of this technology will be facilitated by increases in product yields and the development of novel production systems.

The submerged cultivation of mushrooms is characterized by an increase in broth viscosity with time, which can be due to one or more factors, including the increase in cell concentration within the bioreactor, changes in growth morphology, or the production of extracellular products that alter the rheological character of the culture fluid. Such viscosity increases are usually viewed by the process operator as an undesirable but inevitable phenomenon (*43–46*), since they cause difficulties in the supply of oxygen, in the removal of carbon dioxide, and in the provision of adequate agitation. In the following sections we discuss strategies that have been developed for the cultivation of mushrooms in order to overcome the difficulties caused by viscosity increases and to maximize the formation of the desired metabolites.

Within these sections, special attention is given to *Ganoderma lucidum* (Fr.) Krast (*Polyporaceae*), one of the most famous traditional Chinese medicinal mushrooms,

known for more than 2000 years and used as a popular folk medicine to treat many diseases, such as hepatitis, hypertension, hypercholesterolemia and gastric cancer (*24,47,48*). The polysaccharides isolated from the fruiting body and culture mycelium of *G. lucidum* also have antitumor activities (*48*). Recently, ganoderic acids have been isolated from the fruiting bodies and cultured mycelium of *G. lucidum* and biological functions such as anti-HIV-1 and anti-HIV-1 protease have been reported (*25,49*). As a result of this biotechnological potential, there has been a significant interest in developing processes for the culture of *G. lucidum* in bioreactors.

Fed-batch fermentation in stirred-tank bioreactors

Tang and Zhong (*50*) developed an efficient fed-batch fermentation process for simultaneous production of ganoderic acid (GA) and polysaccharides by *G. lucidum*. The initial studies focused on the effects of carbon source and initial sugar concentration on the process. Sucrose was a suitable carbon source for the production of extracellular polysaccharides (EPS), although the cells did not grow well. Lactose promoted good cell growth and production of GA and intracellular polysaccharides (IPS). When the initial lactose concentration exceeded 35 g/L, the GA accumulation was decreased; GA production was remarkably improved by using a low level of initial lactose and feeding lactose in pulses in such a manner as to maintain its concentration in the culture broth between 5 and 10 g/L. The fed-batch fermentation process in shake flasks was successfully reproduced in stirred bioreactors (STR) with dissolved oxygen (DO) controlled to be between 20 and 35 % of air saturation during fermentation. The maximum cell density reached 22 and 21 g/L, EPS reached 0.87 and 0.75 g/L, while IPS reached 2.5 and 2.4 g/L, in the STR and flask, respectively. GA reached 367 and 298 mg/L in the STR and flask, respectively, with the corresponding productivities being 30 and 14 mg/(L·day). Not only the cell density but also the content, production and productivity of GA and IPS obtained in this work are the highest values reported in agitated cultures of *G. lucidum*. This appears to be due to the fact that the low lactose concentration maintained during the fermentation avoided the inhibitory effects of high lactose levels on the production of GA.

Similar processes have been developed for other organisms. A fed-batch fermentation of *Agaricus brasiliensis* enhanced growth and the production of EPS and ergosterol (*5*). In the case of *Ganoderma resinaceum* (*51*), the maximum concentrations of mycelial biomass (42 g/L) and EPS (4.6 g/L) were obtained when 50 g/L of glucose were fed on day 6. By optimizing the time and the amount of NH_4^+ feeding to the fed-batch, Mao and Zhong (*52*) increased the production of cordycepin by the medicinal mushroom *Cordyceps militaris* from 209 mg/L in batch culture to 346 mg/L in fed-batch culture.

Oxygen supply

Tang and Zhong (*53*) studied the effect of oxygen supply on growth and the production of polysaccharides and ganoderic acid (GA) by *Ganoderma lucidum*, over a range of initial values of the volumetric oxygen transfer coefficient (K_La) from 16.4 to 96.0 h^{-1}. In terms of biomass and intracellular polysaccharide (IPS) production, the best initial K_La was 78.2 h^{-1}, giving a biomass concentration of 15.6 g/L, an IPS yield of 2.19 g/L and an IPS productivity of 217 mg/(L·day). An increase of initial K_La to 96.0 h^{-1} led to the formation of larger mycelial aggregates. This increase in the initial K_La also maximized the yield and productivity of GA, both of these being 1.8-fold greater than the values obtained with an initial K_La of 16.4 h^{-1}. The effects of K_La on the performance of the cultivation process are mediated by their effects on the dissolved oxygen (DO) tension. This parameter was studied more directly by maintaining the DO constant during the cultivation. The growth of *G. lucidum* was quite poor when the DO was controlled to be at 10 % of air saturation, which appears to be due to the limited oxygen availability in the interior of the mycelial aggregates. Despite the poor growth, the production of extracellular polysaccharides (EPS) and the contents of IPS and GA within the biomass at a DO of 10 % were higher than those obtained at DO of 25 %. However, the overall yields and productivities of IPS and GA at lower DO were lower than those at the higher DO.

Mao and Zhong (*11*) investigated the effect of oxygen supply on cordycepin production in submerged cultivation of *Cordyceps militaris* in a 5-litre turbine-agitated bioreactor. Initial K_La values influenced cordycepin production significantly over the range from 11.5 to 113.8 h^{-1}. The highest cordycepin concentration of 168 mg/L was obtained at an initial K_La value of 54.5 h^{-1}.

Shear force and mixing

In submerged cultures of mushroom, it is necessary to agitate the culture broth in order to obtain good mixing and thereby promote heat and mass transfer. However, agitation also creates shear force. This shear force can cause damage to hyphae and provoke morphological changes in the mycelium, and these changes can cause variations in growth rate and product formation (*54*).

Gong and Zhong (*55*) studied the influence of shear force on growth and production of ganoderic acid and polysaccharides by *G. lucidum* in stirred tank bioreactors. The parameter used to characterize the shear force was the tip speed of the impeller. The tip speed significantly affected the maximum biomass concentration obtained over the range from 0.51 to 1.53 m/s in a 5-litre stirred bioreactor: with a tip speed of 0.51 m/s, the maximum biomass concentration obtained after 9 days of cultivation was 13.8 g/L, whereas at a tip speed of 1.53 m/s it was only 10.6 g/L. The mycelial morphology of *G. lucidum* was also affected by the tip speed. During the first 3 days of cultivation, the mean projected area of dispersed hypha increased much more at a tip speed of 0.51 m/s than at higher tip speeds. The maximum mean projected areas of dispersed hypha obtained were $3.7\cdot10^4$, $2.5\cdot10^4$ and $2.1\cdot10^4$ μm^2 with impeller tip speeds of 0.51, 1.02, and 1.53 m/s, respectively. The pellet size at the lowest tip speed (0.51 m/s) increased more rapidly than it did at the higher tip speeds (1.02 or 1.53 m/s). In other words, smaller pellets and shorter hyphae were formed under conditions of higher shear stress. A lower shear environment also favored IPS accumulation. On

the other hand, EPS accumulation was not affected much by the tip speed. Finally, the optimum production of GA occurred at the intermediate tip speed, 1.02 m/s.

In fact, there has been relatively little research into culture broth mixing and rheology in the cultivation of mushrooms. These issues have received more attention in the case of other microorganims (*56*), with studies focusing on: (*i*) the effects of impeller design and operation on the development of rheology and texture; (*ii*) empirical measurement of rheology during mixing from mixer torque or power consumption; (*iii*) effect of rheology on mixing patterns and performance; and (*iv*) simulation and prediction of mixing flow deformation patterns as functions of impeller geometry and rheology.

Morphology and rheology

Cell morphology and broth rheology affect the metabolism of mushroom during submerged fermentations in bioreactors (*57*). Changes in mycelial morphology and the consequent changes in broth rheology affect several phenomena, such as mass and heat transfer, oxygen uptake and carbon dioxide evolution, shear stress and mixing efficiency. Unfortunately, the relationship between growth morphology and metabolite production by mushrooms is not well understood (*58*). The multicellular structure of the mycelium, which allows morphological and physiological heterogeneity to occur along the length of hyphae during the culture, makes it difficult to construct mathematical models to predict culture performance and to guide control strategies (*59*).

One or both of two growth forms occur during the cultivation of most mushroom in submerged culture: the filamentous and the pellet form (*60*). Often the morphology of the filamentous element is simply characterized by the total hyphal length and the number of actively growing tips. The morphology of pellets is characterized by the pellet shape (degree of circularity), the pellet diameter, the area covered by the pellet, and the hairiness and roughness of the pellet.

Fang *et al.* (*61*) investigated the impact of pellet size on the biosynthesis of intracellular polysaccharides (IPS) and ganoderic acid (GA) by *G. lucidum*. The contents of IPS in pellets decreased as the pellet diameter increased, being 85, 69 and 55 mg/g (dry mass basis) for diameters smaller than 1.2 mm, from 1.2 to 1.6 mm, and larger than 1.6 mm, respectively. In contrast, the content of GA increased with pellet size, being 10, 13 and 16 mg/g (dry mass basis), respectively, for the same three pellet sizes listed above. Mycelial pellet size therefore has different effects on formation of polysaccharide and ganoderic acid in *G. lucidum* cultures (*61*).

Many operating variables affect the cell morphology and broth rheology during submerged cultivation of mushrooms in bioreactors, including the inoculation density, culture pH, temperature and nutrient supply. Although these effects have not been extensively studied, some observations have been made and are presented in the following paragraphs.

In the case of *G. lucidum*, the study of Fang *et al.* (*61*) showed a decrease in pellet size as inoculation density increased. With an inoculum size of 670 mg/L (dry mass basis), 68 % of the pellets had diameters smaller than 1.2 mm; however, at an inoculum size of 70 mg/L (dry mass basis), 91 % of the pellets had diameters larger than 1.6 mm.

During the submerged fermentation of *G. lucidum* in an airlift bioreactor without pH control, Lee *et al.* (*62*) observed a change in mycelial morphology from an initial pellet-like form to a filamentous form. On the other hand, when the pH was controlled at 6, the mycelial morphology was pellet-like throughout the whole culture period.

Pellet size and hairy mycelial length in a submerged fermentation of *G. lucidum* were significantly affected by mechanical forces. In a novel two-stage cultivation process of *G. lucidum* developed by Fang and Zhong (*63*), and Tang and Zhong (*64*), flasks were shaken for the first four days and then left static. An interesting phenomenon was that a white mycelium layer appeared on the surface of culture broth and that this layer produced a higher yield of ganoderic acid than was obtained in the conventional submerged fermentation (*63,64*).

The aeration rate can affect the morphology significantly. When *Cordyceps militaris* was grown under different aeration conditions, it always grew in pellet form, but the morphology of the pellets varied notably. The mean diameter and compactness of the pellets were higher at 2 vvm, a condition that also favored exobiopolymer biosynthesis. In cultures grown at 0.5 vvm, the core of the pellets autolyzed during the later stages of the fermentation, whereas in cultures grown at 4 vvm the outer hairy region of the pellet was sheared off (*65*).

In their studies on the effect of K_La on the rate on the growth and production formation by *G. lucidum* described above, Tang and Zhong (*53*) also showed that K_La affected pellet morphology. In these studies the variations in the initial K_La were obtained by adjusting the aeration rate and keeping the agitation rate constant. The mycelium from cultures grown with the highest initial K_La value was more aggregated than the mycelium grown with lower initial K_La values. An increase of initial K_La from 16.4 to 78.2 h^{-1} increased the percentage of mycelial aggregates with diameters larger than 0.5 mm and also those with diameters between 0.25 and 0.5 mm, while the percentage of mycelial aggregates with diameters below 0.25 mm decreased. When the initial K_La was increased further to 96.0 h^{-1}, there was a big increase in the percentage of mycelial aggregates over 0.50 mm in diameter.

The rheological characteristics of the broth during the culture of mushrooms are strongly influenced by the yield of mycelial biomass, the morphological form of this biomass and the production of extracellular polymers (*16*). High biomass and extracellular polymer yields lead to large increases in the viscosity of the culture broth during the fermentation. As a result, it can be difficult to achieve good nutrient and oxygen transfer outside the zone swept by the impeller. With respect to the morphological form, cultures in which the mushroom grows as pellets tend to be less viscous than those in which it grows as dispersed filaments. When growth occurs in the pellet form, the culture broth typically only becomes viscous and deviates from Newtonian behavior at high biomass concentrations (*66*).

Two-stage culture process

It is not uncommon for the optimal conditions for cell growth to be quite different from those for product biosynthesis. Directed shifting of process parameters can be used to optimize cell and product yields. However, a successful application of process parameter shifts requires a good understanding of the physiological changes that are provoked within the cells.

Lee *et al.* (*62*) proposed a bistage pH control process for improving EPS production by *G. lucidum* in an airlift bioreactor. Starting with a pH of 3 and changing this to 6 at the beginning of the exponential growth phase allowed an EPS yield of 20 g/L, compared to the yield of 4.1 g/L obtained in the culture without pH control (*62*). This strategy avoided the adverse effects on growth that occurred when the pH was maintained constant at 6 throughout the whole culture period.

Based on an analysis of the relationship between the specific rate of formation of cordycepin and DO in submerged cultivation of *Cordyceps militaris*, Mao and Zhong (*11*) developed a two-stage DO control strategy to maintain a high specific rate of cordycepin formation. The DO was controlled at 60 % of air saturation in the initial stages of the cultivation and then shifted to 30 % of air saturation when the specific rate of cordycepin formation started to decrease. With this strategy they were able to obtain a high cordycepin yield of 201 mg/L and a high productivity of 15.5 mg/(L·day), these values being 15 and 30 % higher, respectively, than the values obtained in conventional experiments in which the DO was maintained constant. The proposed DO control strategy was also applied to a 5-litre centrifugal impeller bioreactor, with the cordycepin yield and productivity being 188.3 mg/L and 14.5 mg/(L·day), respectively. The process was also successfully scaled up from the 5-litre bioreactor to a 30-litre bioreactor of the same type.

In our own work, a two-stage fermentation process of *G. lucidum* was developed for enhanced GA production by combining the first stage comprised of a conventional shake-flask culture with the second stage comprised of static culture. 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 the static culture process, glucose was consumed at a slower rate and converted to biomass more efficiently. The highest cell density, 21 g/L (dry mass basis), was achieved through a 4-day shake-flask fermentation followed by a 12-day static culture. The GA production in this two-stage process was considerably enhanced, with its content in the biomass increasing from 14 (shaken control) to 32 mg/g, the latter result being much higher than previously obtained (*63,64*). A thick layer of white mycelia formed on the liquid surface during the static culture.

Scale-up of the static second stage was studied in order to improve GA production. The initial volumetric oxygen transfer coefficient (K_La) and the area of liquid surface per liquid volume (A_s) were identified as key factors affecting the process. A multilayer static bioreactor was designed on the basis of these parameters. At a low initial K_La level of 2.1 h^{-1}, a thick layer of white mycelia formed on the liquid surface, and an optimal total yield of GA was obtained, taking into account the GA produced both in the liquid and in the white mycelial layer. In the case of A_s, both the formation of white mycelia and production of GA in the mycelial layer at the liquid surface increased as A_s increased from 0.24 to 1.53 cm^2/mL. On the other hand, the total yield of GA was optimal at an A_s value of 0.90 cm^2/mL. The process was scaled up successfully from a 20-mL static T-flask to a 7.5-litre three-layer static bioreactor, by maintaining the initial K_La constant. The maximum biomass (21 g/L, dry basis), GA content (50 mg/g, dry basis), and total GA production (1.0 g/L) were attained in static bioreactors.

A simple unstructured kinetic model was constructed to describe this novel static culture process (*67*). Parameters in the model were determined by fitting the model to the experimental data. The model was able to describe the dynamics of cell growth, substrate consumption and GA accumulation by *G. lucidum* cells. A parameter sensitivity analysis indicated that the model predictions were sensitive to changes in the maximum specific growth rate, the saturation constant, the biomass yield on lactose and the growth-associated product formation constant. The model was able to predict the static culture process of *G. lucidum* in various laboratory-scale multilayer static bioreactors with working volumes from 20 mL to 7.5 L. The model might be useful in guiding the scale-up of the process to industrial scale.

Conclusions and Perspectives

The study and manufacture of products by submerged fermentation of mushrooms confronts us with numerous difficulties. In recent years, considerable endeavor has been made in monitoring and control of the cultures of mushrooms, but relatively little headway has been made in this area, despite widespread acknowledgment of the limitation of existing bioreactors.

Another area of comparative neglect has been the study of the behavior of mushrooms in bioreactors. Gradients in the concentrations of substrate and other chemical species are especially pronounced in submerged cultures of mushrooms, yet our knowledge of how mushrooms behave when exposed to changes in their environment is scanty. Fortunately, our ability to carry out such studies is improving, due to the increasingly sophisticated techniques that are becoming available to study the internal processes of cells. A better knowledge of how mushrooms behave in varying environments would permit the design of processes and bioreactors around the capabilities of the fungus. Such knowledge of mushrooms does not obviate the need for fundamental studies related to control of bioreactor performance; in fact, the need for close cooperation between the chemical engineer and the microbial physiologist has never been greater.

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. Increases in productivity through the optimization of cul-

ture conditions and bioreactor operating strategies will help to realize the commercialization of these processes.

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