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BIOPROSPECTS OF MACROFUNGI

RECENT DEVELOPMENTS

Sunil Kumar Deshmukh,
Kandikere R. Sridhar and
Hesham Ali El Enshasy (eds.)

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Series: Progress in Mycological Research

BIOPROSPECTS OF MACROFUNGI

Recent Developments

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Preface

There are several gaps in our knowledge of health, nutrition, energy, agriculture and the environment. Such as the significance of plants and animals, the fifth kingdom, and the filamentous fungi possess the capacity to combat several current challenges. Mycology being a mega-science expanded our knowledge in several folds since the beginning of 21 century. Due to a wide variety of fungi and fungi-like organisms distributed in different ecological niches, several biotechnological applications have been considered to combat the challenges in value-added foodstuffs, sustainable agriculture, energy production, environmental cleaning and industrially valued products. Owing to fungal diversity, their varied lifestyles, substrate preference and versatile metabolites, they entered the applied science by drawing the attention of ecologists, geneticists, biochemists, biotechnologists, fermentation technologists, experimental biologists and bioengineers. Several contributions have emerged in the recent past about the biotechnological applications of fungi (e.g., Nevalainen 2020, Deshmukh et al. 2022, Meyer 2022).

Macrofungi are non-conventional resources that meet the requirements of nutrition, health, agriculture and industrial applications. A global conservative estimation of fungi ranges from 2.2−3.8 million and macrofungal diversity ranges between 0.14 and 1.25 million (Hawksworth 2019, Hawksworth and Lucking 2017). Evaluation of macrofungal reserves in different ecosystems increases our knowledge of their applications and future progress. In the choice of subjects, efforts have been made to pool together chapters that possess extensive applications worldwide. Thus, the present book projects various benefits mainly on their cultivation and products (metabolites, nutraceuticals, and biocomposites). Contributions of researchers from Algeria, Austria, Egypt, Korea, Malaysia, India, Japan, Indonesia, Italy, Mexico, Poland, Spain, South Africa, Turkey, the United Kingdom, and the United States of America offer 19 chapters in different applied areas. This volume possesses seven sections in macrofungal biotechnology (cultivation, bioactive compounds, biocomposites, nutraceuticals, association with fauna, biofertilizers and biocontrol).

We hope that the effort on the biotechnological potential of macrofungi offered in this book will be informative and valuable for a wide group of readers, beginners in the fields of mycology, biotechnology, and microbiology. This contribution mainly focused on various facets of applied mycology of macrofungi of different disciplines (e.g., metabolites, products, cultivation, nutraceuticals, biocomposites, biofertilizers and pest control). We are grateful to the kind gestures of the contributors and reviewers towards on-time submission and evaluation. CRC Press has offered their co-operation by honest official formalities to offer this book on time to the readers.

Pune, India — Sunil K. Deshmukh
Mangalore, India — Kandikere R. Sridhar
Johor Bahru, Malaysia — Hesham Ali El Enshasy

References

Deshmukh, S.K., M.V. Deshpande and K.R. Sridhar. 2022. Fungal Biopolymers and Biocomposites: Prospects and Avenues, Springer Nature Singapore Pte Ltd., p. 421.

Hawksworth, D.L. 2019. The macrofungal resource: Extant, current utilization, future prospects and challenges. pp. 1−9. *In*: Sridhar, K.R. and Deshmukh, S.K. (eds.). Advances in Macrofungi: Diversity, Ecology and Biotechnology. CRC Press, Boca Raton, USA.

Hawksworth, D.L. and R. Lücking. 2017. Fungal diversity revisited: 2.2 to 3.8 million species. Microbiology Spectrum 5: FUNK-0052-2016.

Meyer, V. 2022. Connecting materials sciences with fungal biology: A sea of possibilities. *Fungal Biol. Biotechnol*., 9: 5. 10.1186/s40694-022-00137-8.

Nevalainen, H. 2020. Grand Challenges in Fungal Biotechnology, Springer Nature Switzerland AG, p. 535.

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Advances in the Cultivation of Ectomycorrhizal Mushrooms in Japan

Takashi Yamanaka,[1,*] *Akiyoshi Yamada,*[2] *Hitoshi Furukawa,*[3] *Akihiko Kinoshita*[4] **and** *Keisuke Obase*[5]

1. Introduction

A variety of wild mushrooms have been harvested in Japan. People in Japan enjoy mushrooms prepared in various ways, including grilled, fried (tempura), flavored rice, and soup. Thus, mushrooms have been a common food ingredient in autumn. Among these edible mushrooms, shiitake (*Lentinula edodes*), nameko (*Pholiota microspora*), maitake (*Grifola frondosa*), enokitake (*Flammulina velutipes*), buna-shimeji (*Hypsizygus marmoreus*), etc., are saprotrophic species produced by log cultivation or bed cultivation. On the other hand, ectomycorrhizal (EM) species such as matsutake (*Tricholoma matsutake*), shoro (*Rhizopogon roseolus*), and amitake (*Suillus bovinus*) are difficult to cultivate because their growth and fruiting depend on living plant roots (Yamada et al. 2017). Consequently, these EM mushrooms

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are costly. While truffles (*Tuber* spp.), girolle (*Cantharellus cibarius* and several related species), and porcini (*Boletus edulis* and several related species) are also EM mushrooms that are expensive food ingredients in western cuisines (Guerin-Laguette 2021), only recently have these species been reported in Japan (Kinoshita et al. 2011, Endo et al. 2014, Ogawa et al. 2019a). The artificial cultivation technique of EM mushrooms would lead to the development of stable new markets, and numerous attempts have been made to cultivate them. Establishing symbiotic associations between EM species and trees by cultivating trees with EM species is the cultivation system for these EM mushrooms. However, a few EM mushrooms (*Lyophyllum shimeji*, *Boletus* spp.) could be cultivated in the same manner as saprotrophic mushrooms (Ohta 1994b, Yamanaka et al. 2000, Ohta and Fujiwara 2003). The following steps are necessary for establishing the cultivation of EM mushrooms: obtaining inoculums (spores and pure cultures of EM spp.), cultivating EM plants *in vitro* or *in vivo*, and acclimatizing the EM plants in the greenhouse and then in the field. In addition, field inoculation with spores or mycelia of EM species on mature trees is another technique for EM mushroom cultivation. The research conducted on EM mushroom species for cultivation in Japan is presented in Table 1. This article focuses on the EM mushrooms (*T. matsutake*, *T. bakamatsutake*, *L. shimeji,* and *Tuber* spp.) and describes recent efforts for their cultivation in Japan.

2. *Tricholoma matsutake* (Matsutake)

Tricholoma matsutake is an EM species that produces the coveted edible mushroom known as "matsutake" (Figure 1A). This mushroom has been revered in Japan as

Figure 1: Matsutakes harvested in Toyooka, Nagano, Japan (A); Bakamatsutakes developed after field inoculation with mycelia of this species on an oak plant in Nara, Japan (B); Honshimejis at Nosegawa in Nara, Japan (C); Japanese black truffles from Hyogo, Japan (D) and Japanese white truffles from Mie, Japan (E).

Table 1. Status of the research for the cultivation of Japanese EM mushrooms.

EM species	Pure culture	EM formation *in vitro*	Fruiting body formation				
						In the field	
			Pure culture	EM plant in pot	Plantation of EM sapling	Spore spray	Mycelial inoculum
Tricholoma matsutake	Kawai and Abe (1976), Shimazono (1979), Yamada et al. (2001c)	Yamada et al. (1999b, 2006)	Ogawa and Hamada (1975), Kawai and Ogawa (1976)		Kareki and Kawakami (1985), Ka et al. (2018)		
T. bakamatsutake	Shimazono (1979), Yamada et al. (2001c)	Yamanaka et al. (2014)			Kawai (2019)		
T. portensosum	Yamada et al. (2001a)	Yamada et al. (2001a, b)		Yamada et al. (2001a)			
T. saponaceum	Yamada et al. (2001a)	Yamada et al. (2001a, b)		Yamada et al. (2001a)			
T. flavovirens	Yamada et al. (2001a)	Yamada et al. (2001a, b)		Yamada et al. (2001a)			
T. terreum	Yamada et al. (2007)	Yamada et al. (2007)		Yamada et al. (2007)			
Lyophyllum shimeji	Ohta (1994a)	Yamada et al. (2001a, b)	Ohta (1994b)	Kawai (1997)	Kawai (1999)		Kawai (1999), Mizutani (2005)
L. semitale	Yamada et al. (2001a)	Yamada et al. (2001a, b)		Yamada et al. (2001a)			
Japanese *Tuber* spp	Obase et al. (2021), Nakano et al. (2020a, 2022a)						

Table 1 contd. ...

...Table 1 contd.

EM species	Pure culture	EM formation *in vitro*	Fruiting body formation				
						In the field	
			Pure culture	EM plant in pot	Plantation of EM sapling	Spore spray	Mycelial inoculum
Rhizopogon rubescens	Yamada et al. (2001a), Shimomura (2019)	Yamada et al. (2001a), Shimomura (2019)		Yamada et al. (2001a), Shimomura (2019)		Tomikawa (2006)	
Suillus bovinus	Yamada et al. (2001a)	Yamada et al. (2001a, b)					
S. luteus	Yamada et al. (2001a)	Yamada et al. (2001a, b)		Yamada et al. (2001a)			
S. granulatus	Yamada et al. (2001a)	Yamada et al. (2001a, b)		Yamada et al. (2001a)			
S. grevillei	Qu et al. (2003)	Qu et al. (2003)				Shibata (1989), Katagiri et al. (2021)	
Boletus edulis	Endo et al. (2014)	Endo et al. (2014)					
Amanita caesareoides	Endo et al. (2013)	Endo et al. (2013)					
Lactarius akahatsu	Yamada et al. (2001a)	Yamada et al. (2001a, b)		Yamada et al. (2001a)			
L. hatsutake	Yamada et al. (2001a)	Yamada et al. (2001a, b)		Yamada et al. (2001a)			
Japanese golden chantarella (*Cantharellus anzutake*)	Ogawa et al. (2019a)	Ogawa et al. (2019b)		Ogawa et al. (2019b)			

"a taste of autumn" for more than a millennium. In the early 1940s, the matsutake harvest was approximately 12,000 Mg but has since decreased to less than 100 Mg annually. In consequence, the price per kilogram of this mushroom rose from 370 JPY in 1952 to 67,000 JPY in 2017. Along with a decline in the domestic harvest, it is necessary to implement the artificial cultivation of these mushrooms (Yamanaka et al. 2020).

This EM species primarily inhabits forests of Japanese red pine (*Pinus densiflora*), while *Picea glehnii*, *P. jezoensis*, *Pinus pumila*, *P. thunbergii*, *Tsuga diversifolia* and *T. sieboldii* have also hosted tree species (Imazeki and Hongo 1987). Asian, European, North American, and Central American specimens of matsutake and its close relatives have been documented.

Strains have been isolated from various geographic locations and utilized in phylogenetic studies based on genetic data, physiological properties, and EM formation on various host trees (Yamada et al. 2014, Narimatsu et al. 2019). Yamada et al. (1999a) described a typical EM formation by *T. matsutake* in the field. This was confirmed by inoculating pine seedlings *in vitro* with a strain of *T. matsutake* (Yamada et al. 1999b). Afterwards, many studies on EM formation using various combinations of strains and sterile seedlings of putative host species were conducted (Yamada et al. 2010, 2014, Nakano et al. 2020b). These studies demonstrated that was more likely to form EM with tree species *in vitro* than in the field, although the improved growth of tree seedlings may be affected by nutrient conditions, such as the soil materials used (Saito et al. 2018).

"Shiro", a mycelial aggregation of *T. matsutake*, forms under natural conditions in the field, in association with EM roots and soil particles, obtaining carbon sources from the tree roots with which it is associated. In autumn, matsutakes appear on the periphery of the shiro. Therefore, the cultivation of shiro in the field and/or in a greenhouse is required for the production of matsutake. Numerous field trials for developing shiros and fruiting matsutakes have been conducted, including inoculation with spores, mycelia, fragments of shiro, and planting pine saplings with EM of *T. matsutake* (Yamanaka et al. 2020). Among these trials, matsutake appeared in Hiroshima in 1983, five years after planting a pine sapling with EM of *T. matsutake* (Kareki and Kawakami 1985). In South Korea, it was reported that later matsutake occurred after pine saplings with EM were similarly planted in the field (Ka et al. 2018). However, it was not determined in either instance whether these matsutakes were genetically identical to the strains that were inoculated on the sapling.

Yamada et al. (2006) and Kobayashi et al. (2007) successfully produced shiros using EM seedlings in an *in vitro* system. These EM pine seedlings with shiros were planted in the field, but no shiros were subsequently developed (Kobayashi et al. 2015). There is no instance in which the development of shiro was positively identified after field planting.

Mycelia forming a shiro have long been believed to be genetically homogeneous, as a shiro develops from a dikaryotic mycelium after mating between two monokaryotic mycelia. However, Murata et al. (2005) reported that isolates from fruit bodies and spores from the same fruit bodies were genetically distinct, and

Lian et al. (2006) discovered that one to four genets were found within each shiro after analyzing fruiting bodies. These results demonstrated the natural occurrence of genetic mosaics in a shiro. Murata et al. (2005) suggested that the management of shiro should encourage colonization and fruit body production by maintaining a favorable mosaic status, which could be sustained by the dispersal and germination of basidiospores and their subsequent sexual interaction with shiro mycelia. This causes physiological variation among genets within a single shiro. These variations should be complementary between neighboring genet and linked to concurrent fruit body production within a shiro. Yamada et al. (2019) initially compared isolates established from the spores of a single fruiting body to determine the effect of mosaicism on the development of shiro and fruit body production. On media, the mycelial growth of these sibling isolates varied significantly. In addition, the isolates demonstrated commensal and amensal interactions with varying levels of nutrients. Then, sibling spore isolates were compared to ectmycorrhization ability (Horimai et al. 2020). The ratio of EM colonization varied significantly between isolates and was dependent on the nitrogen content of the soil. Mixed inoculations of three selected isolates with single pine seedlings revealed that paired inoculations of two of the three isolates and triple inoculations of these isolates resulted in significantly higher levels of ectomycorrhizal colonization than a single isolate. Moreover, Horimai et al. (2021) demonstrated that basidiospore germination in the EM system of a pine host produced new genets. These occurrences contribute to the comprehension of how maintained shiro structures, as well as the occurrence of alternating and newly produced genets. Besides, basidiospore inoculation in an already established EM system involving this fungus on a host of pine was beneficial for generating complex shiro structures that resemble the natural population structures.

Understanding the relationship between soil microorganisms and shiro (Vaario et al. 2011) is also essential for the establishment and growth of shiro. The active mycorrhizal zone of shiro possesses antimicrobial properties (Ohara and Hamada 1967). Lian et al. (2006) demonstrated that the EM fungal community underneath the active mycorrhizal zone is species-poor and that there are significant differences between those within and outside the shiros. Kataoka et al. (2012) demonstrated that certain bacteria, such as *Sphingomonas* and *Acidobacterium*, were present in the active mycorrhizal zone, although none were detected using the technique of dilution plating. These findings have been supported by a report by Ohara and Hamada (1967). In addition, in South Korea, fewer fungal communities were found beneath the fruiting zone of the shiro compared to the fruiting zone's interior and exterior (Kim et al. 2013, Oh et al. 2016). However, Kim et al. (2014) found no difference between the bacterial communities of the fruiting zone within and outside of the fruiting zone. Li et al. (2018) reported that oak roots colonized by *T. matsutake* changed the endophytic microbial community in the roots. According to Ohara and Hamada (1967), volatile monoterpenes like α- and β-pinene are antimicrobial metabolites. According to Nishino et al. (2016), the (oxalato) aluminate complex has antimicrobial properties. This is a product of the reaction of aluminum phosphate and oxalic acid secreted by EM fungi, and it releases soluble phosphorus that utilizes as

a nutrient to spread the shiro in the soil. Additionally, Vaario et al. (2015) reported that *T. matsutake* got soil minerals from rock fragments. Thus, shiro development has been chemically and biologically described but not induced artificially.

It has been believed that bed cultivation of matsutake is not possible because this EM species has a limited capacity to decompose organic matter (Kusuda et al. 2006, 2008, Shimokawa et al. 2017). Ogawa and Hamada (1975) and Kawai and Ogawa (1976) showed that produced fruit body primordia from mycelia grown in sterilized soil or vermiculite containing a nutrient solution. However, the fruiting bodies did not develop from the primordia. Recently, Murata et al. (2018, 2019) obtained a stable mutant of with enhanced amylose and cellulose degradation induced by heavy-ion beam exposure. Using barley-based substrate cultivation, a mutant of *T. matsutake* generated by irradiation of γ-rays, produced protuberances composed of non-aerial hyphal tissues (Murata et al. 2020). Although these protuberances did not develop into fruiting bodies, they were observed in three independent experiments in succession.

3. *Tricholoma bakamatsutake* (Bakamatsutake)

In Japan, three mushroom species have been reported as closely related to matsutake: bakamatsutake (*T. bakamatsutake*), nisematsutake (*T. fulvocastaneum*), matsutakemodoki (*T. robustum*) (Imazeki and Hongo 1987). Among them, bakamatsutake is anticipated to be an equally valuable edible mushroom with a strong matsutake aroma. *Tricholoma bakamatsutake* is a member of the Fagaceae-associated species and has been reported from China, North Korea, and New Guinea, in addition to Japan (Otani 1976, Gyong 2011, Yamanaka et al. 2011). In the field and *in vitro*, this species produced chlamydospores, thick-walled pigmented spores, at the terminal end of vegetative hyphae (Shimazono 1979, Terashima et al. 1993). The addition of amino acids *in vitro* enhanced the formation of chlamydospores (Yamanaka et al. 2019). *Tricholoma bakamatsutake* can form EMs with pine seedlings *in vitro*, indicating that it is *in vitro* host range is broader than in nature (Yamanaka et al. 2014). Multiple fruit bodies of this species have recently formed on the floor of a *Quercus serrata* forest following field inoculation with the planting of a *Q. phillyraeoides* sapling inoculated with the fungus mycelia (Figure 1B) (Kawai et al. 2019). In contrast, bakamatsutake strains demonstrated an enzymatic capacity for lignin decomposition (Oikawa et al. 2020), and a private company reported the formation of fruiting bodies in bed cultivation.

4. *Lyophyllum shimeji* (Honshimeji)

Honshimeji is *L. shimeji*'s fruit body (Figure 1C). Japanese people have a high regard for honshimeji, remarking, "matsutake has a good aroma, while shimeji has a good flavor." *Lyophyllum shimeji* is an EM species associated with *P. densiflora*, *Q. serrata*, *Q. crispula*, *Q. phillyraeoides*, etc. It is necessary to establish mycorrhizal associations between *L. shimeji* and its host plants for its cultivation in the field. Kawai (1997) inoculated *P. densiflora* saplings obtained by air-layering with spawns

of *L. shimeji* to produce EM in the pine saplings. Fruiting bodies were produced during the cultivation of these EM pine saplings in a pot with sterilized sandy soil. Moreover, after these EM pine saplings were planted in the pine forest, fruit bodies appeared on the forest floor (Kawai 1999).

This EM species is uniquely capable of decomposing organic matter and producing fruit bodies in bed culture. Ohta (1994a, b) reported that all strains of *L. shimeji* could grow on beech and pine sawdust and that some of these strains produced fruiting bodies on a medium containing barley, beech sawdust, and liquid synthetic nutrients. Then, four strains of *L. shimeji* were chosen from 60 wild strains for commercial honshimeji production (Ohta 1998).

5. *Tuber himalayense* and *T. japonicum* (Japanese Truffle)

"True truffles" are the hypogynous sporocarps of *Tuber* spp. (Ascomycota, Pezizales). Several truffle species, including the Périgord black truffle (*T. melanosporum*), the Italian white truffle (*T. magnatum*), the summer truffle (*T. aestivum*), and the Bianchetto truffle (*T. borchii*), are highly valued in European cultures due to their distinctive aroma (Hall et al. 2007). All known species are EM, allowing them to coexist with a variety of temperate forest tree species. Recently, Kinoshita et al. (2011) demonstrated that Japan is home to at least 20 species of Japanese truffles, the majority of which are distinct from European and North American species. Some of them were subsequently reported as new species (Kinoshita et al. 2016, 2018a, 2021).

Several experiments have been conducted to determine the optimal management of truffle cultivation in fields. Several truffle species are cultivated artificially using EM plants inoculated with these species (Hall et al. 2007, Iotti et al. 2016, Bach et al. 2021). In Japan, research is being conducted on the artificial cultivation of these truffles. Initially, two species of Japanese truffles (Figure 1D and IE) (*T. himalayense* and *T. japonicum*) were chosen for artificial cultivation because these species have been observed in many regions of Japan and their fruit bodies are of an appropriate size for human consumption. *Tuber japonicum* was collected from Miyagi to Okayama (Kinoshita et al. 2011, 2016), and *T. himalayense* from Hokkaido to Kyushu (Kinoshita et al. 2011, 2018a). Moreover, the analysis of odor-active volatile compounds revealed that 1-octen-3-ol and 3-methyl-2, 4-dithiapentane contributed significantly to the odor of *T. japonicum*, whereas 2,4-dithiapentane is the most important odorant of *T. magnatum* (Shimokawa et al. 2020). The chemical components of *T. japonicum* were identical to those of *T. magnatum* and *T. melanosporum*, both of which are edible. Acute oral toxicity tests revealed no abnormalities, with an LD_{50} of over 2,000 mg/kg under experimental conditions. These results indicated that *T. japonicum* may have a potentially high market value.

Based on information gathered from the collection of these truffles in their natural habitat, the vegetation and soil properties of their habitat were studied. These truffles are typically grown in oak forests, but birch and pine can also serve as hosts. *Tuber himalayense* inhabits neutral to slightly alkaline (pH 6.4–8.0) soils, whereas *T. japonicum* inhabits slightly acid (pH 5.6–6.0) soils (Furusawa et al.

2020). The pure cultures of these truffles have been obtained and utilized to elucidate their physiological properties. At pH 5.0 and 6.0, *T. japonicum* grew well and *T. himalayense* thrived at pH 7.0 (Nakano et al. 2020a). These findings suggest that the optimal pH for mycelial growth differs among species. The growth data collected in these studies could be used to determine the optimal pH conditions for the artificial cultivation of these truffles. For truffle mycelial growth, EM formation, and in situ fruit body production, soil pH is one of the most vital factors.

Strains of *T. japonicum* grew optimally on malt extract and modified Melin–Norkrans medium (20°C or 25°C) (Nakano et al. 2022a). This fungus utilized both inorganic (NH_4^+ and NO_3^-) and organic sources of nitrogen (casamino acids, glutamine, peptone, urea, and yeast extract). Moreover, this fungus utilizes numerous carbon sources, including monosaccharides (arabinose, fructose, galactose, glucose, and mannose), disaccharides (maltose, sucrose, and trehalose), polysaccharides (dextrin and soluble starch), and sugar alcohol (mannitol). However, growth-promoting nutrient sources and their effects varied considerably between strains. Interestingly, *T. japonicum* formed mitospores on the vegetative hyphae mycelium (Nakano et al. 2022b). Among 25 strains, 20 strains developed mitospores on modified Melin–Norkrans agar, demonstrating that mitospore formation is likely a trait shared by many *T. japonicum* strains. This *in vitro* nature of *T. japonicum* will be useful in the future for understanding the functions of mitospores in the genus under controlled environmental conditions.

Nakamura et al. (2020) investigated the local genotypic status of *T. himalayense* fruiting bodies utilizing 15 newly developed and 4 existing simple sequence repeat markers. The results revealed low genetic diversity in the truffle grounds, but that these truffle grounds remained productive over the sampling years, signifying that low genetic diversity does not necessarily hurt truffle production, at least not over an extended period. These data should significantly contribute to the continuous reproduction of Asian truffle species for which fundamental data on fine-scale genetic structure are lacking.

6. Conclusion

Many studies on the artificial fruit body formation of EM species have been conducted in Japan. In order to cultivate EM mushrooms in the field, it is necessary to create favorable conditions for the growth of EM species, based on the data on the ecology, physiology, and genetics of both EM fungi and their host plants. Most edible EM mushrooms are only found in the wild or with established techniques for their cultivation, the majority of which are only preliminary from a commercial perspective. Thus, EM mushrooms obtained from wild-collected samples may lead to the depletion of resources for cultivation research. The preservation of natural habitats where these fungi grow and perpetuate is a pressing concern. Strategies for the conservation of edible fungi's habitat provide information useful for the field cultivation of these species. However, it is not possible to implement cultivation practices with the expectation of immediate success. There is a need for more long-term research projects on the cultivation of EM mushrooms.

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References

Bach, C., P. Beacco, P. Cammaletti, Z. Babel-Chen, E. Levesque, F. Todesco, C. Cotton, B. Robin and C. Murat. 2021. First production of Italian white truffle (*Tuber magnatum* Pico) ascocarps in an orchard outside its natural range distribution in France. *Mycorrhiza*, 31: 383–388.

Endo, N., S. Gisusi, M. Fukuda and A. Yamada. 2013. *In vitro* mycorrhization and acclimatization of *Amanita caesareoides* and its relatives on *Pinus densiflora*. *Mycorrhiza*, 23: 303–315.

Endo, N., F. Kawamura, R. Kitahara, D. Sakuma, M. Fukuda and A. Yamada. 2014. Synthesis of Japanese *Boletus edulis* ectomycorrhizae with Japanese red pine. *Mycoscience*, 55: 405–416.

Furusawa, H., T. Yamanaka, A. Kinoshita, S. Nakano, K. Noguchi and K. Obase. 2020. Soil properties in *Tuber himalayense* and *Tuber japonicum* habitats in Japan. *Bull. FFPRI*, 19: 55–67. (In Japanese)

Guerin-Laguette, A. 2021. Successes and challenges in the sustainable cultivation of edible mycorrhizal fungi – furthering the dream. *Mycoscience*, 62: 10–28.

Gyong, J.S. 2011. Study on distribution characteristics of *Tricholoma matsutake* and its allied species and food culture. *Edible Fungi China*, 30 (Supp.): 110–111.

Hall, I.R., G.T. Brown and A. Zambonelli. 2007. Taming the Truffle. The History, Lore, and Science of the Ultimate Mushroom. Timber Press, Portland, pp. 304.

Horimai, Y., H. Misawa, K. Suzuki, M. Fukuda, H. Furukawa, K. Masuno, T. Yamanaka and A. Yamada. 2020. Sibling spore isolates of *Tricholoma matsutake* vary significantly in their ectomycorrhizal colonization abilities on pine hosts *in vitro* and form multiple intimate associations in single ectomycorrhizal roots. *Fungal Ecol*., 43: 100874.

Horimai, Y., H. Misawa, K. Suzuki, Y. Tateishi, H. Furukawa, T. Yamanaka, S. Yamashita, T. Takayama, F. Fukuda and A. Yamada. 2021. Spore germination and ectomycorrhizae formation of *Tricholoma matsutake* on pine root systems with previously established ectomycorrhizae from a dikaryotic mycelial isolate of *T. matsutake*. *Mycorrhiza*, 31: 335–347.

Imazeki, R. and T. Hongo. 1987. Colored illustrations of mushrooms of Japan (vol. I). Osaka: Hoikusha, Osaka, pp. 325. (In Japanese)

Iotti, M., F. Piattoni, P. Leonardi, I.R. Hall and A. Zambonelli. 2016. First evidence for truffle production from plants inoculated with mycelial pure cultures. *Mycorrhiza*, 26: 793–798.

Ka, K.H., H.S. Kim, T.C. Hur, H. Park, S.M. Jeon, R. Ryoo and Y. Jang. 2018. Analysis of environment and production of *Tricholoma matsutake* in matsutake-infected pine trees. *Korean J. Mycol*., 46: 34–42. (In Korean)

Kareki, K. and Y. Kawakami. 1985. Artificial formation of Shiro (fungus colony) by planting the pine saplings infected with *Tricholoma matsutake* (Ito et Imai) Sing. *Bull. Hiroshima Pref. For. Exp. Stat.*, 20: 13–23. (In Japanese)

Katagiri, K., K. Kato and K. Masuno. 2021. Effect of rainfall and soil temperature on the production of *Suillus grevillei*. *Bull. Nagano Pref. For. Res. Cent.*, 35: 83–92. (In Japanese)

Kataoka, R., Z.A. Siddiqui, J. Kikuchi, M. Ando, R. Sriwati, A. Nozaki and K. Futai. 2012. Detecting nonculturable bacteria in the active mycorrhizal zone of the pine mushroom *Tricholoma matsutake*. *J. Microbiol*., 50: 199–206.

Kawai, M. 1997. Artificial ectomycorrhizal formation on roots of air-layered *Pinus densiflora* saplings by inoculation with *Lyophyllum shimeji*. *Mycologia*, 89: 228–232.

Kawai, M. 1999. Artificial infection and fruit body occurrence by inoculation of *Lyophyllum shimeji* mycelial culture in *Pinus densiflora* forest. *Bull. Nara For. Exp. Stat*., 29: 1–7. (In Japanese)

Kawai, M. 2019. Researches on the cultivation of ectomycorrhizal mushrooms in Nara prefecture. *Shinrin Kagaku* 86: 30–33. (In Japanese)

Kawai, M. and S. Abe. 1976. Studies on the artificial reproduction of *Tricholoma matsutake* (S. Ito et Imai) Sing. I. Effects of carbon and nitrogen sources in media on the vegetative growth of *T. matsutake*. *Trans. Mycol. Soc. Jpn*., 17: 159–167. (In Japanese)

Kawai, M. and M. Ogawa. 1976. Studies on the artificial reproduction of *Tricholoma matsutake* (S. Ito et Imai) Sing. IV. Studies on a seed culture and a trial for the cultivation on solid media. *Trans. Mycol. Soc. Jpn*., 17: 499–505. (In Japanese)

Kim, M., H. Yoon, Y.H. You, Y.E. Kim, J.R. Woo, Y. Seo, G.M. Lee, Y.J. Kim, W.S. Kong and J.G. Kim. 2013. Metagenomic analysis of fungal communities inhabiting the fairy ring zone of *Tricholoma matsutake*. *J. Microbiol. Biotechnol*., 23: 1347–1356.

Kim, M., H. Yoon, Y.E. Kim, Y.J. Kim, W.S. Kong and J.G. Kim. 2014. Comparative analysis of bacterial diversity and communities inhabiting the fairy ring of *Tricholoma matsutake* by barcoded pyrosequencing. *J. Appl. Microbiol*., 117: 699–710.

Kinoshita, A., H. Sasaki and K. Nara. 2011. Phylogeny and diversity of Japanese truffles (*Tuber* spp.) inferred from sequences of four nuclear loci. *Mycologia*, 103: 779–794.

Kinoshita, A., H. Sasaki and K. Nara. 2016. Two new truffle species, *Tuber japonicum* and *Tuber flavidosporum* spp. nov. found from Japan. *Mycoscience*, 57: 366–373.

Kinoshita, A., K. Nara, H. Sasaki, B. Feng, K. Obase, Z.L. Yang and T. Yamanaka. 2018a. Using mating-type loci to improve taxonomy of the *Tuber indicum* complex, and discovery of a new species, *T. longispinosum*. *PLoS ONE*, 13(3): e0193745.

Kinoshita, A., K. Obase and T. Yamanaka. 2018b. Ectomycorrhizae formed by three Japanese truffle species (*Tuber japonicum*, *T. longispinosum*, and *T. himalayense*) on indigenous oak and pine species. *Mycorrhiza*, 28: 679–690.

Kinoshita, A., H. Sasaki, T. Orihara, M. Nakajima and K. Nara. 2021. *Tuber iryudaense* and *T. tomentosum*: Two new truffles encased in tomentose mycelium from Japan. *Mycologia*, 113: 653–663.

Kobayashi, H., T. Watahiki, M. Kuramochi, S. Onose and A.Yamada. 2007. Production of pine seedlings with the shiro-like structure of the Matsutake mushroom (*Tricholoma matsutake* (S. Ito et Imai) Sing.) in a large culture bottle. *Mushroom Sci. Biotechnol*., 15: 151–155. (In Japanese)

Kobayashi, H., M. Terasaki and A. Yamada. 2015. Two-year survival of *Tricholoma matsutake* ectomycorrhizas on *Pinus densiflora* seedlings after outplanting to a pine forest. *Mushroom Sci. Biotechnol*., 23: 108–113.

Kusuda, M., M. Ueda, M. Nakazawa, K. Miyatake and K. Yamanaka. 2006. Detection of β-glucosidase as saprotrophic ability from an ectomycorrhizal mushroom, *Tricholoma matsutake*. *Mycoscience*, 47: 184–189.

Kusuda, M., M. Ueda, K. Miyatake and T. Terashita. 2008. Characterization of the carbohydrase productions of an ectomycorrhizal fungus, *Tricholoma matsutake*. *Mycoscience*, 49: 291–297.

Li, Q., C. Xiong, X. Li, X. Jin and W. Huang. 2018. Ectomycorrhization of *Tricholoma matsutake* with *Quercus aquifolioides* affects the endophytic microbial community of host plant. *J. Basic Microbiol*., 58: 238–246.

Lian, C., M. Narimatsu, K. Nara and T. Hogetsu. 2006. *Tricholoma matsutake* in a natural *Pinus densiflora* forest: correspondence between above- and below-ground genets, association with multiple host trees and alteration of existing ectomycorrhizal communities. *New Phytol*., 171: 825–836.

Mizutani, K. 2005. Fruit body occurrence during 5 years by inoculation of *Lyophyllum shimeji* mycelial culture in *Pinus densiflora* forest. *Bull. Gifu Pref. For. Sci. Res. Inst*., 34: 1–6. (In Japanese)

Murata, H., A. Ohta, A. Yamada, M. Narimatsu and N. Futamura. 2005. Genetic mosaics in the massive persisting rhizosphere colony "shiro" of the ectomycorrhizal basidiomycete *Tricholoma matsutake*. *Mycorrhiza*, 15: 505–512.

Murata, H., T. Abe, H. Ichida, Y. Hayashi, T. Yamanaka, T. Shimokawa and K. Tahara. 2018. Heavy-ion beam mutagenesis of the ectomycorrhizal agaricomycete *Tricholoma matsutake* that produces the prized mushroom "matsutake" in conifer forests. *Mycorrhiza*, 28: 171–177.

Murata, H., S. Nakano, T. Yamanaka, T. Shimokawa, T. Abe, H. Ichida, Y. Hayashi, K. Tahara and A. Ohta. 2019. Conversion from mutualism to parasitism: A mutant of the ectomycorrhizal agaricomycete *Tricholoma matsutake* that induces stunting, wilting and root degeneration in seedlings of its symbiotic partner *Pinus densiflora in vitro*. *Botany*, 97: 463–474.

Murata, H., T. Yamanaka, T. Shimokawa and A. Ohta. 2020. Morphological changes in a γ-ray irradiation-induced mutant of the ectomycorrhizal agaricomycete *Tricholoma matsutake* during *in vitro* spawning on barley-based substrates. *Bull. FFPRI*, 19: 153–157.

Nakamura, N., J.P. Abe, H. Shibata, A. Kinoshita, K. Obase, J.R.P. Worth, Y. Ota, S. Nakano and T. Yamanaka. 2020. Genotypic diversity of the Asiatic black truffle, *Tuber himalayense*, collected in spontaneous and highly-productive truffle grounds. *Mycol. Prog.*, 19: 1511–1523.

Nakano, S., A. Kinoshta, K. Obase, N. Nakamura, H. Furusawa, K. Noguchi and T. Yamanaka. 2020a. Influence of pH on *in vitro* mycelial growth in three Japanese truffle species: *Tuber japonicum*, *T. himalayense*, and *T. longispinosum*. *Mycoscience*, 61: 58–61.

Nakano, S., T. Yamanaka and H. Kawagishi. 2020b. Effects of 2-azahypoxanthine and imidazole-4-carboxamide on the growth and ectomycorrhizal colonization of *Pinus densiflora* seedlings inoculated with *Tricholoma matsutake*. *Mycoscience*, 61: 259–263.

Nakano, S., A. Kinoshita, K. Obase, N. Nakamura, H. Furusawa, K. Noguchi and T. Yamanaka. 2022a. Physiological characteristics of pure cultures of a white-colored truffle *Tuber japonicum*. *Mycoscience*, 63: 53–57.

Nakano, S., K. Obase, N. Nakamura, A. Kinoshita, K. Kuroda and T. Yamanaka. 2022b. Mitospore formation on pure cultures of *Tuber japonicum* (Tuberaceae, Pezizales) *in vitro*. *Mycorrhiza*, 32: 353–360.

Narimatsu, M., M. Yamaguchi, T. Yamanaka, S. Gisusi, T. Azuma, Y. Tamai, T. Fujita and M. Kawai. 2019. Comparison of mycelial growth of different *Tricholoma matsutake* strains in soil medium at varying temperatures. *Asian J. Biotechnol. Bioresour. Technol*., 5: 1–8.

Nishino, K., M. Shiro, R. Okura, K. Oizumi, T. Fujita, T. Sasamori, N. Tokitoh, A. Yamada, C. Tanaka, M. Yamaguchi, S. Hiradate and N. Hirai. 2016. The (oxalato) aluminate complex as an antimicrobial substance protecting the "shiro" of *Tricholoma matsutake* from soil micro-organisms. *Biosci. Biotechnol. Biochem*., 81: 102–111.

Obase, K., S. Yamanaka, A. Kinoshita, Y. Tamai and T. Yamanaka. 2021. Phylogenetic placements and cultural characteristics of *Tuber* species isolated from ectomycorrhizas. *Mycoscience*, 62: 124–131.

Ogawa, M. and M. Hamada. 1975. Primordia formation of *Tricholoma matsutake* (Ito et Imai) Sing. in pure culture. *Trans. Mycol. Soc. Jpn*., 16: 406–415. (In Japanese)

Ogawa, W., N. Endo, Y. Takeda, M. Kodaira, M. Fukuda and A. Yamada. 2019a. Efficient establishment of pure cultures of the yellow chanterelle, *Cantharellus anzutake*, from ectomycorrhizal root tips, and the morphological characteristics of ectomycorrhizae and cultured mycelium. *Mycoscience*, 60: 45–53.

Ogawa, W., Y. Takeda, N. Endo, S. Yamashita, T. Takayama, M. Fukuda and A. Yamada. 2019b. Repeated fruiting of Japanese golden chanterelle in pot culture with host seedlings. *Mycorrhiza*, 29: 519–530.

Oh, S.Y., J.J. Fong, M.S. Park and Y.W. Lim. 2016. Distinctive feature of microbial communities and bacterial functional profiles in *Tricholoma matsutake* dominant soil. *PLoS ONE*, 11: e0168573.

Ohara, H. and M. Hamada. 1967. Disappearance of bacteria from the zone of active mycorrhizas in *Tricholoma matsutake* (S. Ito et Imai) singer. *Nature*, 213: 528–529.

Ohta, A. 1994a. Some cultural characteristics of mycelia of a mycorrhizal fungus, *Lyophyllum shimeji*. *Mycoscience*, 35: 83–87.

Ohta, A. 1994b. Production of fruit-bodies of a mycorrhizal fungus, *Lyophyllum shimeji*, in pure culture. *Mycoscience*, 35: 147–151.

Ohta, A. 1998. Culture conditions for commercial production of *Lyophyllum shimeji*. *Jpn. J. Mycol*., 39: 13–20. (In Japanese)

Ohta, A. and N. Fujiwara. 2003. Fruit-body production of an ectomycorrhizal fungus in genus *Boletus* in pure culture. *Mycoscience*, 44: 295–300.

Oikawa, Y., Y. Yoneda, S. Kawai, T. Yamanaka and M. Kawai. 2020. Selection of *Tricholoma bakamatsutake* stains with laccase-like enzyme-secreting and decomposition of phenolic lignin model compounds by these strains. Abstracts presented at the 70th *Annual Meeting Jpn. Wood Res. Soc*., L17-P2-11. (In Japanese)

Otani, Y. 1976. *Tricholoma bakamatsutake* Hongo collected in New Guinea (In Japanese). *Trans. Mycol. Soc. Jpn*., 17: 363–365. (In Japanese)

Qu, L., A.M. Quoreshi, K. Iwase, Y. Tamai, R. Funada and T. Koike. 2003. *In vitro* ectomycorrhiza formation on two larch species of seedlings with six different fungal species. *Eurasian J. For. Res*., 6: 65–73.

Saito, C., W. Ogawa, H. Kobayashi, T. Yamanaka, M. Fukuda and A.Yamada. 2018. *In vitro* ectomycorrhization of *Tricholoma matsutake* strains is differentially affected by soil type. *Mycoscience*, 58: 89–97.

Shibata, H. 1989. Cultivation of *Suillus grevillei* in *Larix leptolepis* forests. *Bull. Yamanashi Pref. For. Res. Exp. Inst*., 17: 16–23. (In Japanese)

Shimazono, H. 1979. Comparative studies on morphological characteristics of the colonies of *Tricholoma matsutake*, *T. fulvocastaneum* and *T. bakamatsutake* on agar media. *Trans. Mycol. Soc. Jpn.*, 20: 176–184. (In Japanese)

Shimokawa, T., M. Yamaguchi and H. Murata. 2017. Agar plate assays using dye-linked substrates differentiate members of *Tricholoma* sect. *caligata*, ectomycorrhizal symbionts represented by *Tricholoma matsutake*. *Mycoscience*, 58: 432–437.

Shimokawa, T., A. Kinoshita, N. Kusumoto, S. Nakano, N. Nakamura and T. Yamanaka. 2020. Component features, order-active volatiles, and acute oral toxicity of novel white-colored truffle *Tuber japonicum* native to Japan. *Food Sci. Nutr*., 8: 410–418.

Shimomura, N. 2019. Study of the artificial cultivation of the ectomycorrhizal mushroom, *Rhizopogon roseolus*. *Mushroom Sci. Biotechnol*., 26: 148–155. (In Japanese)

Terashima, Y., K. Tomiya, M. Takahashi and H. Iwai. 1993. Distribution and characteristics of shiros of *Tricholoma bakamatsutake* in a mixed forest of *Pasania edulis* and *Castanopsis cuspidata* var. *sieboldii*. *Trans. Mycol. Soc. Jpn.*, 34: 229–238. (In Japanese)

Tomikawa, Y. 2006. Effects of inoculation with fruit bodies suspension on cultivation of *Rhizopogon rubescens* in nursery of *Pinus thunbergii*. *Bull. Shimane Mt. Reg. Res. Cent*., 2: 43–49. (In Japanese)

Vaario, L.M., H. Fritze, P. Spetz, J. Heinonsalo, P. Hanajík and T. Pennanen. 2011. *Tricholoma matsutake* dominates diverse microbial communities in different forest soils. *Appl. Environm. Microbiol*., 77: 8523–8531.

Vaario, L.M., T. Pennanen, J. Lu, J. Palmén, J. Stenman, J. Leveinen, P. Kilpeläinen and V. Kitunen. 2015. *Tricholoma matsutake* can absorb and accumulate trace elements directly from rock fragments in the shiro. *Mycorrhiza*, 25: 325–334.

Yamada, A., S. Kanekawa and M. Ohmasa. 1999a. Ectomycorrhiza formation of *Tricholoma matsutake* on *Pinus densiflora*. *Mycoscience*, 40: 193–198.

Yamada, A., K. Maeda and M. Ohmasa. 1999b. Ectomycorrhiza formation of *Tricholoma matsutake* isolates on seedlings of *Pinus densiflora in vitro*. *Mycoscience*, 40: 455–463.

Yamada, A., T. Ogura and M. Ohmasa. 2001a. Cultivation of mushrooms of edible ectomycorrhizal fungi associated with *Pinus densiflora* by *in vitro* mycorrhizal synthesis. I. Primordium and basidiocarp formation in open-pot culture. *Mycorrhiza*, 11: 59–66.

Yamada, A., T. Ogura and M. Ohmasa. 2001b. Cultivation of mushrooms of edible ectomycorrhizal fungi associated with *Pinus densiflora* by *in vitro* mycorrhizal synthesis. II. Morphology of mycorrhizas in open pot soil. *Mycorrhiza*, 11: 67–81.

Yamada, A., T. Ogura, Y. Degawa and M. Ohmasa. 2001c. Isolation of *Tricholoma matsutake* and *T. bakamatsutake* cultures from field-collected ectomycorrhizas. *Mycoscience*, 42: 43–50.

Yamada, A., K. Maeda, H. Kobayashi and H. Murata. 2006. Ectomycorrhizal symbiosis *in vitro* between *Tricholoma matsutake* and *Pinus densiflora* seedlings that resembles naturally occurring ‘shiro’. *Mycorrhiza*, 16: 111–116.

Yamada, A., H. Kobayashi, T. Ogura and M. Fukuda. 2007. Sustainable fruit-body formation of edible mycorrhizal *Tricholoma* species for 3 years in open pot culture with pine seedling hosts. *Mycoscience*, 48: 104–108.

Yamada, A., H. Kobayashi, H. Murata, E. Kalmiş, F. Kalyoncu and M. Fukuda. 2010. *In vitro* ectomycorrhizal specificity between the Asian red pine *Pinus densiflora* and *Tricholoma matsutake* and allied species from worldwide Pinaceae and Fagaceae forests. *Mycorrhiza*, 20: 333–339.

Yamada, A., N. Endo, H. Murata, A. Ohta and M. Fukuda. 2014. *Tricholoma matsutake* Y1 strain associated with *Pinus densiflora* shows a gradient of *in vitro* ectomycorrhizal specificity with Pinaceae and oak hosts. *Mycoscience*, 55: 27–34.

Yamada, A., H. Furukawa and T. Yamanaka. 2017. Cultivation of edible ectomycorrhizal mushrooms in Japan. *Revista Fitotecnia Mexicana*, 40: 379–389.

Yamada, A., N. Hayakawa, C. Saito, Y. Horimai, H. Misawa, T. Yamanaka and M. Fukuda. 2019. Physiological variation among *Tricholoma matsutake* isolates generated from basidiospores obtained from one basidioma. *Mycoscience*, 60: 102–109.

Yamanaka, K., K. Namba and A. Tajiri. 2000. Fruit body formation of *Boletus reticulatus* in pure culture. *Mycoscience*, 41: 189–191.

Yamanaka, K., T. Aimi, J. Wan, H. Cao and M. Chen. 2011. Species of host trees associated with *Tricholoma matsutake* and close allies in Asia. *Mushroom Sci. Biotechnol.*, 19: 79–87. (In Japanese)

Yamanaka, T., Y. Ota, M. Konno, M. Kawai, A. Ohta, H. Neda, Y. Terashima and A. Yamada. 2014. The host ranges of conifer-associated *Tricholoma matsutake*, Fagaceae-associated *T. bakamatsutake* and *T. fulvocastaneum* are wider *in vitro* than in nature. *Mycologia*, 106: 397–406.

Yamanaka, T., M. Konno, M. Kawai, Y. Ota, N. Nakamura and A. Ohta. 2019. Improved chlamydospore formation in *Tricholoma bakamatsutake* with the addition of amino acids *in vitro*. *Mycoscience*, 60: 319–322.

Yamanaka, T., A. Yamada and H. Furukawa. 2020. Advances in the cultivation of the highly-prized ectomycorrhizal mushroom *Tricholoma matsutake*. *Mycoscience*, 61: 49–57.

2

Mushroom Cultivation in Submerged Culture for Bioactive Compounds Production

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1. Introduction

For centuries, mushrooms have been recognized as one of the most important bioresources for food and medicine in different ancient cultures. They have been recognized as part of the treatment plans of different folk medicine practices in the treatment of different diseases (El Enshasy et al. 2013). This is based on their high content of bioactive molecules of functional properties as antioxidants, antimicrobial, anti-diabetic, immunomodulatory, hepatoprotective, anticancer, and many others (El Enshasy 2010, El Enshasy and Hatti-Kaul 2013, Bains et al. 2021, Yadav and Negi 2021). This is based on the presence of a wide range of important chemical substances belonging to different chemical classes as phenolics, terpenoids, proteins, glycoproteins, and polysaccharides (Kalač 2012, Kumar et al. 2021, Campestrini and Salles-Campos 2021).

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Mushrooms grow naturally in different habitats in almost all parts of the world. However, due to the increased demand for mushrooms in both food and medicine, people start to cultivate mushrooms to make them available in large quantities throughout the year. At first, tree logs were been used as a traditional approach for mushroom cultivation. This is based on the capacity of mushrooms to produce a large number of hydrolytic enzymes which are able to degrade wood lignocellulosic materials to produce consumable carbohydrates for mushroom growth. Further development in cultivation came with the introduction of the greenhouse concept and the growth of mushrooms in a solid substrate composed of different balanced nutrients to support the growth and production of bioactive metabolites. Greenhouse cultivation is the most common type of mushroom large-scale production in many countries. This method is characterized by its ease and cheap approach with higher yield compared to the log method. In addition, the design of medium ingredients in the greenhouse cultivation system not only led to significant improvement in mushroom yield but also make it able to use many agro-industrial wastes as a nutrient source which help in both reducing pollution and improving the overall mushroom production cost. More recently, submerged culture has been introduced as an alternative cultivation system for sustainable mushroom production of mushrooms (Tang et al. 2007, Bakratsas et al. 2021). This is based on the fact that mushrooms as higher fungi and cultivation in submerged culture is possible like many other fungal species. The submerged cultivation systems exhibited many advantages compared to conventional solid-state cultivation in the greenhouse.

2. Solid State Cultivation vs. Submerged Cultivation

Mushroom cultivation using a solid-state cultivation system can be carried out in a greenhouse (under controlled temperature and humidity conditions) or in a small-scale cultivation system (glass vessels) or solid-state bioreactor. However, a greenhouse is the most commonly used for mushroom cultivation, especially when used as a source of food. In the greenhouse cultivation practice, the cultivation medium is usually composed of many lignocellulosic materials as cheap agriculture waste. This is based on the high capacity of mushrooms to produce a wide range of hydrolases enzymes such as lignocellulases and laccases enzymes which are capable of the cells to hydrolyze the complex organic materials to fermentable sugar which is consumed for fungal growth. This makes it possible for the mushroom to be cultivated using many cheap agro-industrial wastes organic substrates such as sawdust, rice husk, corncob, bagasse, coffee husk and many other solid waste plant residues (Hoa et al. 2015, Chai et al. 2021). However, using complex lignocellulosic materials as the main substrate prolong the cultivation time and also exhausts cells which need to produce a wide range of hydrolytic enzymes before the production of biomass and bioactive metabolites.

As shown, the submerged cultivation system is characterized by many advantages over the traditional greenhouse cultivation system. These advantages make it more suitable for the cultivation of rare mushrooms with a high growth rate, ease to extract and purify bioactive mushroom compounds, and full compliance to

Table 1: Comparison between greenhouse and submerged cultivation systems.

	Green house cultivation	**Submerged cultivation**
Advantages	- Easy cultivation system with low investment - Simple Control System (Temperature/humidity) - Scalable process	- Fully controlled production process (controllable/optimizable process). - Short cultivation time (15–25 days cultivation) Scalable Process - Cultivation under complete sterile condition (fully compliable to cGMP) - Less purification step especially if the targeted product produced extracellularly - Easy for process validation - More suitable for cultivation of poisonous mushrooms
Disadvantages/ Limitations	- Many wild types mushroom cannot cultivated in green house - Not suitable for cultivation of poisonous mushrooms. - Different purification steps are required to obtain the final bioactive material in pure form - Not fully sterile cultivation system. Not fulfil the requirements of cGMP for sterile manufacturing	- Large capital investment - Higher production cost - Need more skilled workers

Good Manufacturing Practice (cGMP) which is needed for bioactive compounds production in pharmaceutical industries. In addition, submerged cultivation is a closed cultivation system with complete control and thus can be used for the safe cultivation of poisonous mushrooms for bioactive metabolite production (Garcia et al. 2020, Lee et al. 2021). All these together make submerged cultivation a more attractive system for the production of mushroom bioactive compounds of high medicinal value. In summary, the greenhouse cultivation system is more suitable for mushroom production for the food and nutraceutical market, whereas, submerged cultivation is a more attractive alternative system for bioactive compounds for medical industries.

3. Mushroom Submerged Cultivation

In general, submerged cultivation of mushrooms is mostly needed for the production of high-value bioactive compounds for nutraceutical and medical applications. Medium is usually composed of soluble and easily consumable substrates compared to solid-state cultivation which uses complex substrates which need a mushroom enzymatic system for degradation before utilization. Therefore, this system is not exhausting the mushroom cells by undergoing two steps cultivation system (degradation of the substrate followed by the production of bioactive as in solid-state cultivation system). Thus, the easily consumed substrates can be utilized directly for biomass production and bioactive metabolites biosynthesis. Therefore, the submerged cultivation system is widely used for the production of mushrooms' bioactive compounds of medicinal

values such as polysaccharides, terpenoids, lectins, fungal immunomodulatory proteins (FIP), enzymes and many other metabolites (Elisashvili 2012, Dudekula et al. 2020, Bakratsas et al. 2021).

Different types of submerged cultivation vessels have been used for mushroom cultivation on different scales. These include shake flasks, stirred tank bioreactors, air-lift bioreactors and many other specially designed vessels of lower costs. In addition, the cultivation in shake flask has been also carried out using static mode (which favour some metabolites production) or shake mode (which supports more biomass production), or a combination thereof to increase biomass and bioactive metabolites production at the same time (Tang et al. 2015).

3.1 Factors Affecting Mushroom Cultivation in Submerged Cultivation System

Like other submerged cultivation systems of fungal strains, mushroom growth and production of bioactive metabolites are governed by many factors. These factors can be classified under three main groups:

1. Strain-dependent factors: Type of strain, inoculum size and type, inoculum concentration, growth rate, and growth morphology.
2. Medium dependent factor: Carbon and nitrogen sources, C: N ratio, complex organic substrate, phosphate concentration, minerals and trace elements, and precursors.
3. Cultivation condition-dependent factors: Temperature, pH, agitation rate, aeration rate, type and design of cultivation vessel, and shear stress.

All these factors together affect the overall cultivation system. Therefore, it is important to understand the key factors and the weight of individual factors and their effect on biomass growth, desired metabolite production, and by-product formation to optimize the cultivation strategy for the achievement of maximal yield. In addition, these factors also affect growth morphology which is considered one of the key bottlenecks which are important to control to achieve maximal production.

3.2 Mushroom Morphology in Submerged Culture System

Mushrooms exhibited the same associated cultivation challenges related to growth morphology in the submerged culture systems. Like other fungi, mushrooms can grow either in filamentous or in bio-pellet form. In addition, many mushrooms have high adhesion capacity to bioreactor surfaces which causes wall growth and also grow on bioreactor sensors such as (pH, DO, foam, level, and temperature sensors) which lead to false readings of essential bioprocessing parameters. Therefore, for many cultivations, growth in micro-pellet is the preferred morphology in submerged culture. Mushroom growth morphology and bio-pellet production are governed by many factors as those related to other fungi which include: strains-dependent factors, medium-dependent factors, and bioprocess engineering-dependent factors as shown in Figure 1 (El Enshasy 2022). However, for each cultivation process,

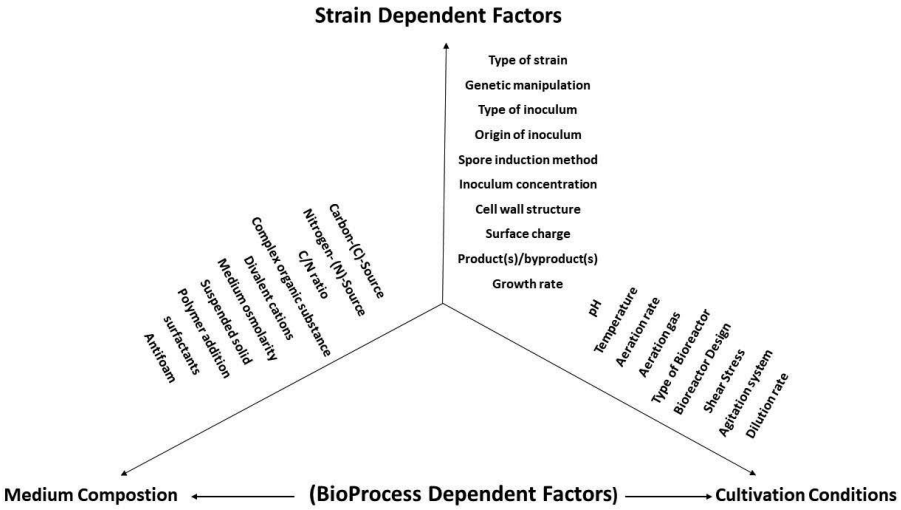

Figure 1: Effect of different strain-dependent factors and bioprocess-dependent factors on the fungal morphology during cultivation in submerged culture (El Enshasy 2022).

it is important to determine the most significant factor for each particular strain to control growth morphology. As reported by many authors, growth in form of a small pellet non-compact pellet structure is more desired as it helps to achieve the maximal productive biomass during the aerobic cultivation process with minimal oxygen transfer limitation in the bio- pellet structure (El Enshasy et al. 2006). In most cases, the type/size/preparation method of inoculum, agitation, and aeration is considered the key factors during scaling up for mushroom cultivation to achieve the desired morphology and maximal productivity as well.

4. Bioactive Metabolites Production in Submerged Culture

For many years, different types of mushrooms have been successfully cultivated in submerged culture for the production of a wide range of bioactive productions of different classes including polysaccharides, lipids, glycoproteins, proteins, lipids, enzymes, terpenoids, phenolics, and many other compounds. However, the production of polysaccharides, ganoderic acid, and cordycepin is the most well-studied metabolites in submerged culture. This is based on their high therapeutic values and increased demand in the medicinal bioactive compound market. During submerged cultivation, a single mushroom can produce more than bioactive compounds such as *Cordyceps* sp. which has the capacity to produce polysaccharides and cordycepin concurrently. *Ganoderma* sp. is also another attractive example that can produce ganoderic acid and polysaccharides concomitantly in the same culture. However, the ratio between the bioactive compounds produced is highly dependent on medium composition and the cultivation parameters. In other cases, a combination between two cultivation modes (static and shake, change from uncontrolled to controlled pH, or from uncontrolled DO to controlled DO) has been used to optimize the cultivation process to increase the production yield.

4.1 Polysaccharides

Mushroom polysaccharides are one of the major targeted biotherapeutic compounds based on their bioactivity as immunomodulators and anticancer agents (El-Deeb et al. 2019). Therefore, most submerged culture studies were focused on polysaccharides especially β-glucans in submerged culture. Different types of mushrooms have been reported for their capacity to produce functional polysaccharides in submerged cultures. Among these polysaccharides, Pleuran (from *Pleurotus ostreatus*), Lentinan (from *Lentinus edodes*), Different types of cultivation vessels have been successfully used in this process and showed high system productivity for both intracellular and extracellular polysaccharides. Table 2 shows us some examples of polysaccharides produced in a submerged cultivation system and the production of the corresponding polysaccharides yield. The quality and biotherapeutic efficiency of the produced polysaccharides were almost the same as those polysaccharides produced in fruiting bodies and mycelium in the greenhouse. However, the production of polysaccharides in submerged cultivation systems is not only carried out in a shorter production time with a high yield but also reduces the downstream steps for extraction and purification significantly especially when polysaccharides as an extracellular product. Figure 2 shows a comparative production platform for pleuran production by *P. ostreatus* between submerged cultivation in a large-scale stirred tank bioreactor (Process A) and greenhouse cultivation (Process B) (Modified from El Enshasy and Hatti-Kaul 2013).

Table 2: Production of different bioactive polysaccharides in submerged cultivation systems using different mushroom biofactories.

Mushroom	**Polysaccharides type**	**Maximal product yield**	**Cultivation vessel**	**Reference**
Agaricus bisporus	IPS	3.43	SF	Argyropoulos et al. (2022)
Agaricus blazei	IPS	0.72 g/L	SF, STR	Liu and Wang (2009)
Auricularia polytricha	EPS	9.42 g/L	SF	Yang et al. (2022)
Cordyceps militaris	EPS	7.30 g/L	SF	Kim et al. (2003)
Ganoderma lucidum	EPS	0.79 g/L	SF	Montoya et al. (2013)
Ganoderma lucidum	EPS	5.00 g/L	SF, STR	Abd Alsaheb et al. (2020)
Lentinula edodes	EPS	1.33 g/L	SF	Liu and Zhang (2019)
Lentinula edodes	EPS	0.94 g/L	SF	Garcia-Cruz et al. (2020)
Phellinus baumii	EPS	150 mg/L	SF, STR	Lung and Deng (2022)
Phellinus gilvus	EPS	5.30 g/L	STR	Hwang et al. (2004)
Pleurotus eryngii	IPS	3.82 g/L	SF	Argyropoulos et al. (2022)
Pleurotus ostreatus	EPS	2.10 g/L	STR	El Enshasy et al. (2010)
Pleurotus ostreatus	EPS	1.98 g/L	STR	Maftoun et al. (2013)
Trametes trogii	EPS	1.86 g/L	STR	Xu et al. (2013)
Tuber sinese	EPS	5.86 g/L	SF	Tang et al. (2008)

Note: EPS: Exopolysaccharides; IPS: Indopolysaccharides; SF: Shake Flask; STR: Stirred Tank reactor.

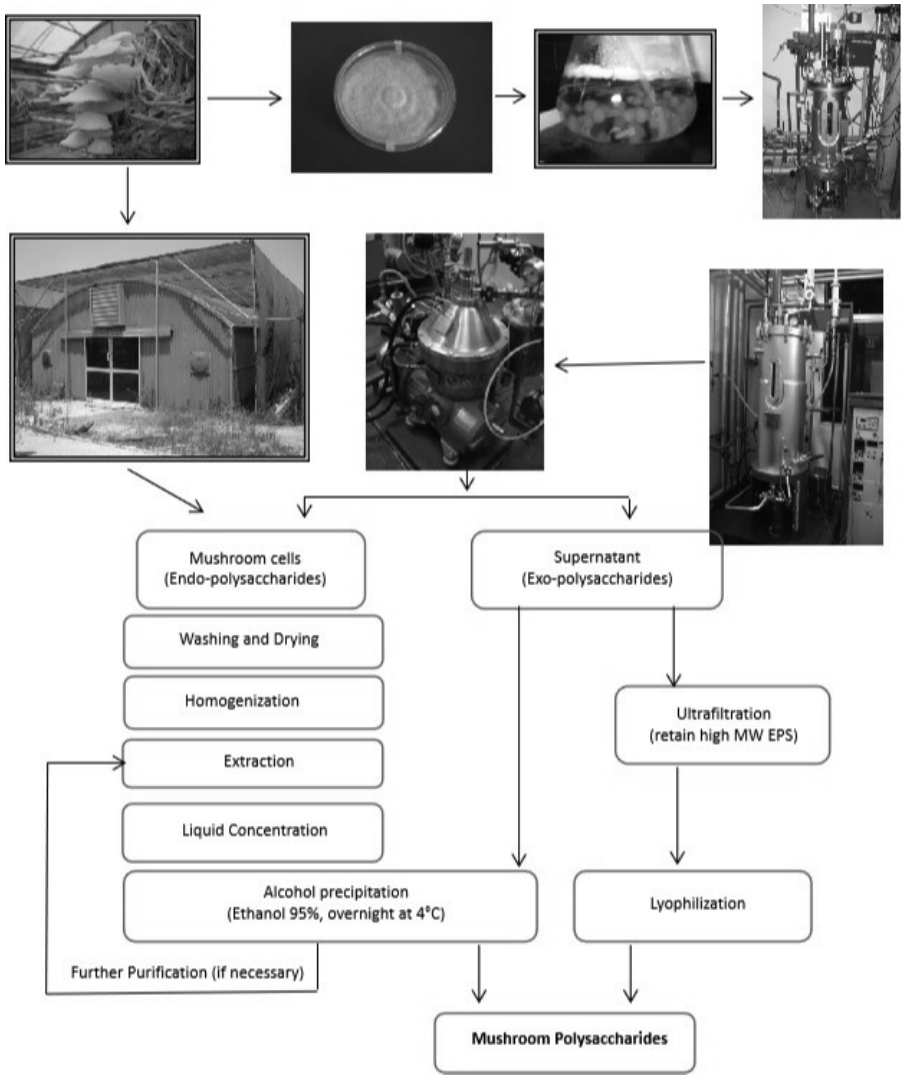

Figure 2: The summarized schematic diagram for bioprocess of mushroom β-glucan polysaccharides production using *Pleurotus ostreatus* for both submerged cultivation in stirred tank bioreactor (Process A) and greenhouse cultivation (Process B) (Modified from El Enshasy and Hatti-Kaul 2013).

In general, the bioactivity of the produced polysaccharides in submerged culture is largely influenced by molecular weight, degree of branching, monosaccharides composition, and degree of glycosylation "in case of glycoprotein" (Bae et al. 2013, Wang et al. 2017, Zhang et al. 2022). Most of the mushroom polysaccharides belong to branched β-glucans. For example, most of the immunomodulator bioactive polysaccharides have the backbone of 1–3, -β-D-glucan with short branched side chains of 1-6, β-linkage. The degree of branching also affects the bioactivity as well which is generally between 20% to 33% for most active polysaccharides. However,

it is worth noting, that the immunomodulatory and antitumor activity of lentinan is not linked to the presence of branching in the polysaccharides molecule as reported by Ren et al. (2012). Therefore, in addition to considering the bioprocessing and morphological factors, factors affecting the polysaccharides' molecular activity need to be also considered during the cultivation process to ensure that the produced molecules are effective with full functional properties.

4.2 Ganoderic Acid

This tri-terpenoid high molecular weight functional acid of molecular formula ($C_{30}H_{44}O_7$; CAS No. 81907-62-2). Two forms of ganoderic acids GA-A and GA-B were first isolated from the fruiting bodies, mycelium and spores of *G. lucidum* by Japanese scientists (Kubota and Asaka 1982). Ganoderic acid (GA) has been widely used as part of traditional Chinese medicine in Asia. So far, this compound exists in fruiting bodies of mushrooms belonging to *Ganoderma* sp. such as *G. lucidum* and *G. sinese*. This compound exhibited many proven therapeutic activities as a hepatoprotective, antitumor, antimicrobial, and anti-hyperlipidemic agent (Bryant et al. 2017, Lixin et al. 2019, Guo et al. 2020, Lv et al. 2022). However, in addition to ganoderic acid, more than 430 secondary metabolites have been isolated and identified from different species of *Ganoderma* (Baby et al. 2015).

Production of GA using a greenhouse is a very long process takes 6 months to produce fruiting bodies. This long-term cultivation, makes it sensitive to contamination as it is difficult to control sterility in open cultivation system. With the increased market demand, GA has been produced by *Ganoderma lucidum* in submerged cultivation system in both static and shake cultures. This process is highly governed by medium composition, pH, and oxygen. An early study reported that the maximal production of biomass of 17.3 g/L with 207.9 mg/L was achieved in shake culture at initial pH of 6.5. Lowering the pH from 6.5 to 3.5 to increase the production of the polysaccharide (intracellular- and extracellular-polysaccharides) concomitant with the decrease of GA production (Fang and Zhong 2002a). The same researchers also reported that the best medium composition for biomass and GA production of 16.7 g/L and 212.3 mg/L, respectively, can be achieved in a medium composed of mixed complex nitrogen sources composed of yeast extract and peptone in the concentration of 5 g/L each and glucose as a carbon source of 50 g/L (Fang and Zhong 2002b). Further improvement in GA production was achieved in liquid static culture by oxygen enrichment in the gaseous oxygen level. A maximal biomass of 29.8 g/L and GA production of 1427 mg/L were obtained after 16 days by aeration with an 80% oxygen-enriched gas phase (Zhang et al. 2010). In addition, nitrogen plays a regulatory role in GA production and the production can be enhanced under nitrogen-limiting conditions (Zhao et al. 2011). Other interesting study was reported on the significant influence of light irradiation on GA production in *G. lucidum*. A light shift cultivation strategy in three stages: 2 days of dark cultivation, followed by 6 days with white light irradiation of 0.94 W/m^2, increased up to 4.70 W/m^2 for the other four days increasing the production of GA by 548% compared to dark cultivation and reached 466.4 mg/L (Zhang and Tang 2008). On the other hand,

using mixed carbon sources of glucose and sucrose enhanced the production of GA production. A fed-batch cultivation strategy was developed starting with a glucose/sucrose mixture in the batch phase, followed by sucrose feeding after 5 days resulting in a significant increase of GA production in a 10-L bioreactor up to 636 mg/L (Wei et al. 2016). It has been also reported that the production of GA by a high-producer isolated strain *G. lucidum* RCKB-2010 can reach up to 2755 mg/L in liquid static culture compared to only 373 mg/L in shaking culture after 25 days (Upadhyay et al. 2014).

On the other hand, other research also reported on the potential enhancement of GA biosynthetic gene expression using different precursors such as phenobarbital (Liang et al. 2010). A recent study reported also the possible control of metabolic flux to affect the distribution in the production of GA-R, GA-S, and GA-T by exogenous addition of oleic acid (Yan et al. 2022).

Recent studies on the production of GA in submerged culture have been carried out using genetic manipulation either using homologous or heterologous approaches. Fei et al. (2019) reported on the overexpression of homologous farnesyl-diphosphate synthase genes to increase GA production in *G. lucidum.* The developed recombinant strain had the capacity to increase the production of all GAs (GA-Me, GA-S, and GA-T) by 2.80-, 2.62-, and 2.27-folds, respectively. To overcome the problems related to fungal growth (growth morphology and low growth rate), and the limitation of the biosynthetic capacity of the mushroom strains, a recombinant *Saccharomyces cerevisiae* has been used for GA production in a submerged culture system. The GA was found to be highly influenced by oxygen supply and the maximal production was only 8.79 mg/L after only 120 h cultivation (Liu and Zhong 2021). However, having GA expressed in a unicellular eukaryotic host with a high growth rate will ease large-scale production even though more studies are needed to increase the production yield.

4.3 Cordycepin

Cordycepin or (9-(3)-Deoxy-β-D-ribofuranosyl adenine) is an adenine-like compound with a molecular formula of $C_{10}H_{13}N_5O_3$ and CAS No. 73-03-0. This potent bioactive compound is produced by the fruiting bodies and in submerged culture of some rare mushrooms belonging to *Cordyceps* sp. such as *C. militaris*, *C. kyushuensis*, and *C. siniensis* (Leung and Wu 2007, Ling et al. 2009). This low molecular weight bioactive molecule exhibited potent biological activities as an anticancer, immunomodulator, anti-inflammatory and hypoglycemic agent (Soltani et al. 2018, Ashraf et al. 2020). Based on the difficulties of growing mushrooms related to *Cordyceps* sp. in the greenhouse, a high level of interest has been paid for the production of bioactive compounds in liquid static culture and submerged culture systems. Much research has been carried out related to the optimization of fermentation medium and cultivation conditions in different scales (Jiapeng et al. 2014, Kang et al. 2014). In one of the early studies, it has been reported that the biosynthesis of cordycepin in submerged culture is highly regulated by carbon and nitrogen sources during the cultivation of *C. militaris* (Mao et al. 2005). Glucose was

found to be the most suitable carbon source and the maximal production of 345.5 g/L was achieved when using a medium containing 42 g/L glucose and 15.8 g/L peptone. However, further studies reported also that the production of the surface culture of *C. militaris* can yield up to 640 mg/l in a medium composed of glucose, yeast extract, and peptone (Masuda et al. 2006). Another interesting research studied the effect of amino acids supplementation to a chemically defined medium on cordycepin production. A maximal production of 2.5 g/L was achieved in a medium supplemented with 16 g/L glycine and 1 g/L adenine. The volumetric production of cordycepin in this medium was almost 4 times higher compared to the initial basal medium (Masuda et al. 2007). Another study showed also that medium supplementation with 1 g/L ferrous sulphate can enhance cordycepin production by 70% reaching 596 mg/L (Fan et al. 2012). More recent research also reported that a maximal cordycepin production of 846 mg/L was achieved in submerged culture after 24 days of cultivation at 25°C, and pH of 5.5 in a medium composed of glucose, yeast extract, potassium phosphate monobasic/and dibasic, sodium chloride, and magnesium sulphate when using inoculum size of 8% (Tuli et al. 2014). Another study reported that the optimal medium composition for cordycepin production in submerged culture is composed of (g/L): sucrose, 20.7; peptone, 20.0; $K_2HPO_4 \cdot 3H_2O$, 1.11; $MgSO_4 \cdot 7H_2O$, 0.90, Alanine 12.23; hypoxathine, 5.34 with the addition of VB_1 in the concentration of 10 mg/L. A maximal production of about 2 g/L of cordycepin was achieved in liquid static culture after 35 days without pH control (Kang et al. 2014). Another bit of research was carried out to investigate the effect of the two-step cultivation strategy of the shake stage followed by the static stage. The shake stage promotes more cell growth, while the static stage supports more cordycepin production (Tang et al. 2015). Through this strategy, the production of cordycepin can reach up to 2.62 g/L compared to the maximal production of 1.03 g/L and 0.51 g/L in the case of static culture and shake culture, respectively.

Recent research was focused on using agro-industrial wastes and lignocellulosic biomass as potential substrates for cordycepin production. The potential use of casein hydrolysate as a cheap substrate for cordycepin production in submerged cultivation using a mutant strain of *C. militaris* KYL05 has been studied by Lee et al. (2019). After 6 days of cultivation in the shake flask culture at 150 rpm, 25°C, and pH 6.0, a maximal production of 445 mg/L was achieved (this was almost 3 times improvement in production compared to the initial conventional medium with wild-type strain). Another study by Ha et al. (2021) also reported on the potential use of alkaline-treated pine sawdust for efficient short-time production of cordycepin production in the submerged cultivation system of *C. militaris*. A maximal cordycepin production of 922 mg/L was achieved in after only 72 hours.

5. Conclusion

Submerged cultivation systems not only shorten the cultivation time but also decrease the steps needed for bioactive compound isolation and purification in downstream processes. These all together, decrease the production cost and thus improve the overall economy of the process. In addition, as a closed cultivation system and

running under completely sterile conditions, submerged cultivation is suitable for the production of mushroom bioactive compounds according to cGMP. Thus, the produced molecules can be used in the pharmaceutical and biopharmaceutical industries. However, further research is needed to improve the cultivation system and cultivation strategy to improve the yield of production. Scaling up is also one of the other challenges due to the high accumulative behaviour of fungal biomass which usually leads to intense hairy growth and makes it difficult to control culture during cultivation on a large scale. Therefore, controlling growth morphology using different bioprocess engineering approaches is needed to make this process more applied in the mushroom cultivation industry. Further research is also needed to investigate in depth the link between bioprocessing parameters, growth morphology, and polysaccharides (production, glycosylation, helix structure, and molecular weight distribution) to design more efficient production of bioactive molecules. In addition, further research on the design of two modes strategy of cultivation as a combination of static and shaking cultures to develop high cell density culture with maximal desired metabolites production is needed to be further investigated to design the most efficient strategy for the production of particular metabolites.

References

Abd Alsaheb, R., K.Z. Zjeh, R. Abd Malek, J.K. Abdullah, A. El Baz, N. El Deeb, D. Dailin, S.Z. Hanapi, D. Sukmawati and H. El Enshasy. 2020. Bioprocess optimization for exopolysaccharides production by *Ganoderma lucidum* in semi-industrial scale. *Rec. Pat. Food Nutr. Agric*., 11: 211–218.

Argyropoulos, D., C. Psallida, P. Sitareniou, E. Flemetakis and P. Diamantopulou. 2022. Biochemical evaluation of *Agaricus* and *Pleurotus* strains in batch cultures for production optimization of valuable metabolites. *Microorganisms*, 10: 964.

Ashraf, S.A., A.E.O. Elkhalifa, A.J. Siddiqui, M. Patel, A.M. Awadelkareem, M. Snoussi, M.S. Ashraf, M. Adnan and S. Hadi. 2020. Cordycepin for health and wellbeing: A potent bioactive metabolite of an entomopathogenic medicinal fungus *Cordyceps* with its nutraceutical and therapeutic potential. *Molecules*, 25: 2735.

Baby, S., A.J. Johnson and B. Govindan. 2015. Secondary metabolites from *Ganoderma*. *Phytochem*., 114: 66–101.

Bae, I.Y., H.W. Kim, H.J. Yoo, E.S. Kim, S. Lee, D.Y. Park and H.G. Lee. 2013. Correlation of branching structure of mushroom β-glucan with its physiological activities. *Food Res. Int*., 51: 195–200.

Bains, A., P. Chawla, S. Kaur, A. Najda, M. Fogarasi and S. Fogarasi. 2021. Bioactives from mushroom: Health attributes and food industry applications. *Materials*, 14: 7640.

Bakratsas, G., A. Polydera, P. Katapodis and H. Stamatis. 2021. Recent trends in submerged cultivation of mushrooms and their application as a source of nutraceuticals and food additives. *Future Foods*, 4: 100086.

Bryant, J.M., M. Bouchard and A. Haque. 2017. Anticancer activity of Ganoderic acid DM: Current status and future perspective. *J. Clin. Cell. Immunol*., 8: 535.

Campestrini, L.H. and C. Salles-Campos. 2021. Aspects of mushroom cultivation to obtain polysaccharides in submerged cultivation. *Afr. J. Biotechnol*., 20: 100–107.

Chai, W.Y., U.G. Krishnan, V. Sabaratnam and J.H.L. Tan. 2021. Assessment of coffee waste in formulation of substrate for oyster mushrooms *Pleurotus ostreatus* and *Pleurotus floridanus. Future Foods*., 4: 100075.

Dudekula, U.T., K. Doriya and S.K. Devarai. 2020. A critical review on submerged production of mushroom and their bioactive metabolites. *3 Biotech*., 10: 337.

El-Enshasy, H., J. Kleine and U. Rinas. 2006. Agitation effects on morphology and protein productive fractions of filamentous and pelleted growth forms of recombinant *Aspergillus nige*. *Process Biochem*., 41(10): 2103–2112.