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SUBMERGED CULTIVATION OF SOME SPECIES OF EDIBLE BASIDIOMYCETES FOR THE SIMULTANEOUS BIOSYNTHESIS OF BIOMASS AND LACCASE ENZYME

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Abstract

Five species of edible basidiomycetes - Pleurotus ostreatus, Flammulina filiformis, Ganoderma lucidum, Lentinula edodes, and Polyporus squamosus - were isolated and cultivated as submerged mycelium for the biosynthesis of laccase enzymes and biomass production. The selected mushrooms were grown on different agar media to determine the average growth rate, tested on the Potato Dextrose Agar medium supplemented with guaiacol to determine the laccase enzyme index, and then grown submerged in a rotary incubator, in Erlenmeyer flasks. The development of these species was studied in a medium with sugarcane molasses and mineral salts. The dry biomass of the culture media was determined, and the laccase activity was analysed in the filtrate.

Key words*: basidiomycetes, laccase, biomass.*

INTRODUCTION

The quality, nutritional value and safety of food is an ever-present issue for the food industry and consumers. An important source of proteins and bioactive compounds is edible fungi (class Basidiomycetes), which can be cultivated as mycelium on the surface of solid substrates or submerged in liquid media, with aeration. Their medicinal and nutraceutical properties have been known for a long time, but they have not been used to their full value in human nutrition.
Unlike the traditional cultivation of edible mushrooms, which requires significant time, space and produces variable yields, mycelium cultivation is a current method, which significantly reduces growth time, external contamination and produces a biomass with valuable nutritional properties. In addition, some mushrooms have considerable therapeutic qualities, due to bioactive metabolites synthesized intra- and extracellularly: they have anti-inflammatory, antioxidant, anti-carcinogenic, hypocholesterolemic, anti-diabetic, immunomodulatory and other properties.
Also, some fungi can synthesize enzymes from the class of oxido-reductases (laccase with lignin degradation action) and hydrolases (amylases, cellulases, proteases and others), with a role in the decomposition of organic substrates. These enzymes can be recovered from liquid cultures of mycelial biomass production (Bakratsas et al, 2021; Dudekula et al., 2020; Sandargo et al., 2019; Lu et al., 2020; Zhang et al., 2019).
Laccases (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase) are a group of multi-copper containing enzymes, in the active site, which catalyse the oxidation reaction of various substrates such as phenolic and aromatic compounds (ortho- and para-diphenols, amino phenols, methoxy phenols, polyphenols), aliphatic amines and inorganic cations, resulting in water as a product of reduction of molecular oxygen (Ferdes et al., 2022).
The aim of this paper is to present an analysis of the capacities of five species of fungi belonging to the Basidiomycetes, to synthesize laccases, cellulases and other enzymes, and to produce biomass in liquid media.

MATERIALS AND METHODS

Biological material

Five species of edible mushrooms cultivated in the form of mycelium were used, namely:

Pleurotus ostreatus, Flammulina filiformis (commercially known as Enoki flower), *Ganoderma lucidum* (also known as Reishi), *Lentinula edodes* (Shiitake), and *Polyporus squamosus.*

Culture media

Potato Dextrose Agar (PDA) medium supplemented with 0.1% chloramphenicol was used to isolate fungal mycelium. The mycelium was cultured in tubes or Petri dishes on potato dextrose agar (PDA), Malt Extract Agar (MEA) and Czapek Dox Agar (CZA) media. Cultivation of the submerged mushroom species was carried out using the liquid medium with the following composition: sugar beet molasses 100 g/L, NH_4NO_3 3 g/L, $(NH_4)_3PO_4$ 1 g/L. Enzymes were detected on specific media that allowed the visualization of a modification reaction of the agar gel: laccase was detected on PDA medium supplemented with 0.02% guaiacol; for cellulases, a medium with carboxy methyl cellulose (CMC) 10 g/L, $(NH_4)_2SO_4$ 1.4 g/L, K_2HPO_4 2 g/L, $CaCl_2$ 0.3 g/L, $MgSO_4$ 0.3 g/L, yeast extract 0.25 g/L, peptones 0.75 g/L, agar 17 g/L was used; the medium is then colored with a Congo red solution, and the producing colonies become surrounded by a colorless zone. To evaluate the synthesis of proteolytic enzymes, the fungal colonies were cultivated on a medium with casein 2.5 g/L. Lipolytic enzymes were analysed on nutrient agar supplemented with $CaCl_2$ 1% and Tween 80 1%, and amylases on a medium with starch. The colony growth rate was determined on 3 culture media, namely: PDA, MEA and CZA.

Culture media were sterilized by autoclaving for 15 minutes at 121 ˚C.

Methods

Average hourly growth rate estimation

The fungal species were grown in tubes and kept at 4˚C in the refrigerator until use.

The average hourly growth rate was determined on the previously mentioned culture media, at temperatures of 20˚C and 30˚C, using the formula:

$$V = \frac{Colony\ diameter\ (mm)}{time\ (hours)}$$

Qualitative method for enzymatic activity

To test the production of enzymes, the diameters of the modified zones around the fungal colonies cultivated in Petri dishes on the media mentioned above were measured.

The enzyme indices were calculated according to the formula:

$$I_E = \frac{Zone\ diameter}{Colony\ diameter}$$

Submerged cultivation

The submerged cultivation of fungi was carried out in liquid medium with molasses, in Erlenmeyer flasks (200 mL/flask), on a rotary incubator, at 150 rpm and 20˚C, for 7 days. Dry biomass, pH and dry matter (in the filtrate) were measured in the collected samples. The obtained biomass was separated by filtering and washing with distilled water, dry matter was analyzed with a thermobalance at a temperature of 103˚C.

Guaiacol assay method for laccase activity

The method is based on the oxidation reaction of guaiacol in the presence of laccase, with the formation of a reddish brown coloration.

Guaiacol can be used as a hydrogen donor (substrate) in the assay of laccase. Upon oxidation, it forms tetraguaiacol with a molar extinction coefficient $\varepsilon = 26.6\ mM^{-1}cm^{-1}$.

A reaction mixture consisting of 1) Guaiacol (2 mM) 1 ml; 2) Sodium acetate buffer (10 mM) 3 ml; 3) Enzyme 1 ml was prepared. A control is prepared, which contains distilled water instead of the enzyme. The reaction mixture was incubated at 30˚C for 15 minutes and then the absorbance was read at 450 nm in a UV-Vis spectrophotometer. 1 enzymatic unit (U) was defined as the amount of enzyme required to oxidize 1 µmol of guaiacol per minute. Enzyme activity was calculated according to the formula (Isik et al., 2023):

$$\text{Laccase activity (LA)} = \frac{Abs \times Vol \times DF}{\varepsilon \times t \times v}$$

Where:

LA = Laccase activity

A = Absorbance 450 nm

V =Total mixture volume (ml)

v = enzyme volume (ml)

t = incubation time

ε = Guaiacol [12100 M^{-1} cm^{-1}] molar absorption coefficient.

DF=dilution factor.

RESULTS AND DISCUSSIONS

Mushroom cultures developed in tubes or Petri dishes on the mentioned media showed white color, fluffy appearance and characteristic flavor (Figure 1).

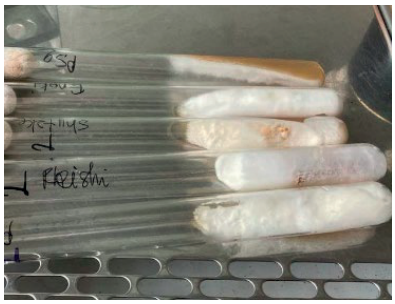


Figure 1. Appearance of cultures in tubes on PDA medium

The average hourly growth rates on PDA, MEA and CZA culture media at temperatures of 20˚C and 30˚C are shown in Figures 2 and 3.

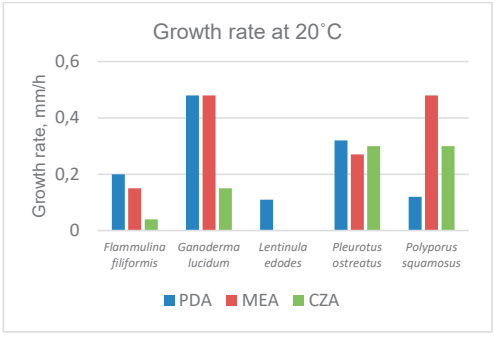


Figure 2. Average hourly growth rates of colonies on PDA, MEA and CZA at 20˚C

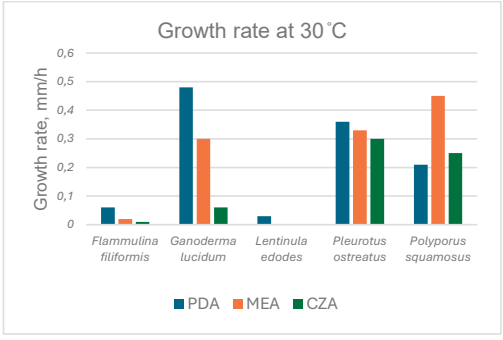


Figure 3. Average hourly growth rates of colonies on PDA, MEA and CZA at 30˚C

Colony growth of the 5 species of basidiomycetes was strongly influenced by the composition of the culture medium. In general, the highest growth rates were recorded for the PDA and MEA media, while for the CZA medium the growth was minimal for the species of *Flammulina filiformis* and *Lentinula edodes*. For the following studies this was important for choosing the optimal parameters for biomass accumulation and enzymes production. The temperature had a less important influence on the growth of the studied mushroom colonies. *Flammulina filiformis, Ganoderma lucidum* and *Lentinula edodes* species seem to grow better at 20˚C, while *Pleurotus ostreatus* and *Polyporus squamosus* have comparable growth rates at 20˚C and 30˚C.

The results of determining the enzyme indices according to the mentioned formula are shown in Table 1.

Table 1. Enzyme index values of fungal colonies

Enzymatic indices					
Species	ILc	IC	IA	IP	ILi
Flammulina filiformis	1,4	0,25	0	6	0
Ganoderma lucidum	2,7	2	0	2	5
Lentinula edodes	2,2	2	0	0	3
Pleurotus ostreatus	4,6	1,1	0	3	4,6
Polyporus squamosus	1	1,5	0	1,1	0

Note: ILc=laccase index; IC=cellulase index; IA=amylase index; IP=protease index; ILi=lipase index.

The aspect of the modified areas around the producing colonies is shown in the Figures 4-7. Laccase was highlighted in the case of *Pleurotus ostreatus, Ganoderma lucidum* and *Lentinula edodes* species, known for their growth on lignocellulosic materials. *Polyporus squamosus* had the lowest laccase production, the reverse side of the colony being slightly coloured in reddish brown. Laccase biosynthesis started after the first 3 days of colony development.

Cellulolytic enzymes could be visualized on the medium with CMC also after the first 3-5 days of development. By staining the culture medium with 1% Congo red solution and washing with a 1M NaCl solution, the colorless hydrolysis zone could be visualized around the colonies of *Ganoderma lucidum, Lentinula edodes, Polyporus squamosus* and *Pleurotus ostreatus*.

Some species of Basidiomycetes have synthesized proteases and lipases. *Flammulina filiformis* and *Pleurotus ostreatus* had the highest proteolytic indices, of 6 and 3, respectively. *Ganoderma lucidum, Lentinula edodes* and *Pleurotus ostreatus* synthesized lipases, highlighted by the appearance of an opaque area around the colonies.
No fungal colony synthesized amylolytic enzymes.

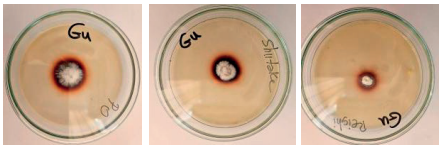


Figure 4. Appearance of basidiomycetes colonies on the medium for laccases: *Pleurotus ostreatus, Lentinula edodes, Ganoderma lucidum*

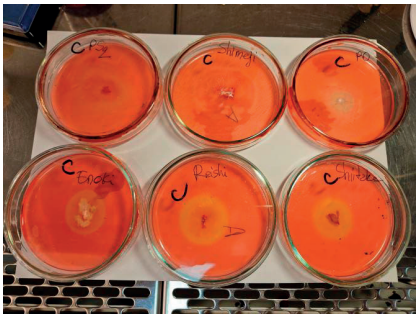


Figure 5. Appearance of basidiomycete cultures on the medium for *cellulolytic* enzymes

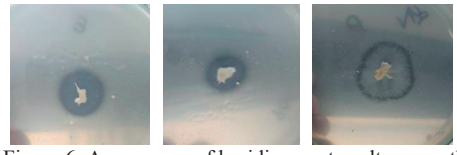


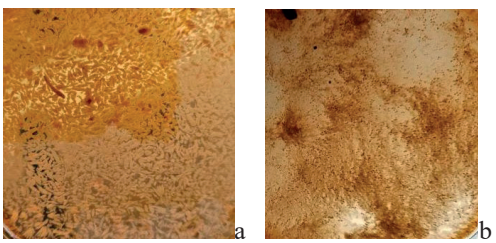
Figure 6. Appearance of basidiomycete cultures on the medium for proteolytic enzymes: *Flammulina filiformis, Pleurotus ostreatus, Polyporus squamous*

Figure 7. Appearance of basidiomycete cultures on the medium for lipolytic enzymes: *Pleurotus ostreatus, Ganoderma lucidum, Lentinula edodes*

The accumulation of biomass in liquid media
The mushroom species were cultivated in the liquid medium with sugar cane molasses and mineral salts, on the rotary incubator, at 150 rpm, for 7 days. Dry biomass, pH and dry matter% values in the filtrate were analysed. The results presented in the Table 2 demonstrated that the largest amount of biomass was obtained in the cultures of *Pleurotus ostreatus* and *Lentinula edodes*, of 3.49 and 3.24 g/L, respectively. The mycelium developed in the form of filaments, sometimes forming spheres with a diameter of several millimetres (Figure 8 and Figure 9). The accumulation of biomass was correlated with the decrease of dry matter in the filtrates, and the pH values in the culture liquid varied between 4.7 and 7.1 units, depending on the species.

Table 2. The results of biomass accumulation in the culture medium with molasses

Species	Dry biomass, g/L	pH in filtrate	Dry matter in filtrate, %	Laccase activity, U/mL
Control	-	5,7	9,8	0
Flammulina filiformis	2,0	6,54	9,2	0
Ganoderma lucidum	6,8	4,92	8,1	6
Lentinula edodes	16,2	4,73	3,7	4
Pleurotus ostreatus	17,5	5,77	3,5	10
Polyporus squamosus	13,1	7,16	2,7	0



a b

Figure 8. The *mycelium* of a) *Pleurotus ostreatus*, b) Polyporus squamosus in liquid medium

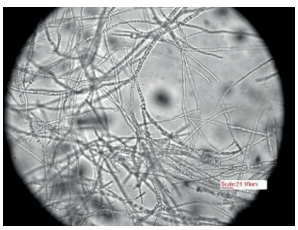


Figure 9. Hyphae of *Polyporus squamosus* on microscope camera

After 7 days of thermostating in the rotary incubator, the quantitative analysis of the

laccase activity by the guaiacol method showed that only three strains synthesized the enzyme in the culture medium: *Pleurotus ostreatus, Ganoderma lucidum*, and *Lentinula edodes*.
Comparable results were obtained for the same reaction conditions by other authors, namely by Suleman et al., 2020, who determined a maximum activity between 10 and 26 U/mL for different fungal isolates; Kumar et al., 2016. found that the laccase activity for an *Aspergillus flavus* strain was 26 U/mL, and Isik et al., 2023, obtained an enzyme activity of approximately 300 U/mg from *Trametes versicolor*. Using other substrates such as ABTS, syringaldazine, or 2,6-dimethoxyphenol, in different test conditions, the values of laccase enzymatic activities are different.
Similar studies were carried out using different species of basidiomycetes, cultivated in culture media with diverse composition (Krupodorova et al., 2021). *Agaricus bisporus* (Ma et al., 2014; Salmones et al., 2018), *Ganoderma adspersum* (Badalyan et al., 2019), *Lentinula edodes* (Krupodorova et al., 2020) *Pleurotus* spp. (Phadke et al., 2020) were cultivated in different conditions.
Although mushrooms contain valuable nutrients for food, a relatively small number of publications is dedicated to the study of the influence of cultivation parameters on their growth. The presence and nature of carbon and nitrogen sources, mineral salts and growth factors greatly influence both the yield of biomass production, as well as the growth time and the duration of the lag phase.
The various researches on the growth of the fungal mycelium have shown that there is no single commonly accepted standard media for the optimal fungal growth, because the influence of the type of nutrients on the mycelial growth can vary depending on fungi species and their strains. Media such as PDA, CZA and MEA were found to be the best for the growth of many basidiomycetes.
In addition, the various agro-industrial by-products, such as molasses, have a complex nature and variable composition over time, making it difficult to determine some physiological characteristics such as the rate of substrate uptake.

CONCLUSIONS

Five species of basidiomycetes (*Pleurotus ostreatus, Flammulina filiformis*, *Ganoderma lucidum*, *Lentinula edodes*, and *Polyporus squamosus)* were tested in terms of growth speed on different agar media, enzyme production and biomass production in liquid media.
Mushrooms are becoming more and more important for the human diet due to their nutritional, organoleptic and pharmacological characteristics. Considering the optimal concentration of minerals, the low value of lipids and the caloric content, the nutritional value of mushrooms was re-evaluated. In addition, it seems that some mushrooms can prevent some diseases such as forms of cancer, hypercholesterolemia and hypertension.
Colony growth of the 5 species of basidiomycetes on PDA, MEA and CZA was strongly influenced by the composition of the culture medium, while the temperature had a less significant influence.
The best producers of laccase and cellulase were *Pleurotus ostreatus* and *Ganoderma lucidum*, which had values of 3 and 4 respectively for laccase enzyme indices.
The maximum biomass values in the culture medium with molasses were recorded for the *Pleurotus ostreatus, Lentinula edodes*, and *Polyporus squamosus* species, being 17.5, 16.2, and 13.1 g/L respectively.
Only three species of basidiomycetes, namely *Pleurotus ostreatus, Ganoderma lucidum* and *Lentinula edodes*, synthesized laccase in the liquid medium used in this study.

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