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BIOTECHNOLOGY OF SUBMERGED FERMENTATION TO PRODUCE NUTRITIVE MYCELIAL BIOMASS THROUGH CONTROLLED CULTIVATION OF EDIBLE AND MEDICINAL MUSHROOMS

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Abstract

The main aim of this research work was focused on the establishment of the best food biotechnology in order to be applied in the leading and controlling of submerged fermentation by using three edible and medicinal mushroom species Ganoderma lucidum (Curt. Fr.) P. Karst and Lentinula edodes (Berkeley) Pegler that were grown on different substrata containing grain wastes as main constituents. The experiments were carried out by cultivating these mushroom species under controlled conditions inside the culture vessel of a modern laboratory-scale bioreactor designed at the highest food quality standards. The submerged fermentation was set up in the following conditions: temperature,25- 27° C; agitation speed, 100-120 rev. min $^{-1}$; pH level, 5.7 – 6.5 units; dissolved oxygen tension within the range of 30% - 70%. During the period of controlled submerged fermentation lasting from 120 to 170 h, the mycelial biomass of fungal pellets was developed inside the broth. At the end of the culture cycles, the fungal pellets were harvested by extracting them from the culture vessel of the bioreactor and separating them from the broth by slow vacuum filtration. Pellet size, the hairy length of pellets, and the free mycelia fraction in the total biomass were microscopically investigated and the chemical composition of fungal biomass was analysed to determine and compare the protein and reduced sugar contents.

Key words: *biotechnology, biomass, submerged cultivation, edible and medicinal mushrooms*

INTRODUCTION

Submerged cultivation in liquid media of mushroom mycelium is a promising method which can be used in novel biotechnological processes for obtaining pharmaceutical substances of antitumor, antiviral and immuno-modulating actions from fungal biomass and cultural liquids as well as for the production of liquid spawn [1, 2].

The researches made for getting nutritive supplements from the biomass of *Ganoderma lucidum* species (Reishi) have shown that the nutritive value of its mycelia is owned to the huge protein content, carbohydrates and mineral salts. *Lentinula edodes* species (Shiitake) is a good source of proteins, carbohydrates (especially polysaccharides) and mineral elements with beneficial effects on human nutrition [3-5].

The main purpose of this work consists in the application of biotechnology for continuous cultivation of medicinal mushrooms by submerged fermentation in agro-food industry which has a couple of effects by solving the ecological problems generated by the accumulation of plant wastes in agro-food industry through biological means to valorise them without pollutant effects as well as getting fungal biomass with high nutritive value which can be used to prepare functional food [5-7].

The continuous cultivation of medicinal mushrooms was applied using the submerged fermentation of different natural by-products of agro-food industry that provided a fast growth as well as high biomass productivity of the investigated strains [7].

MATERIAL AND METHOD

Ganoderma lucidum (Reishi) and *Lentinula edodes* (Shiitake) were used as pure mushroom strains. The stock cultures were maintained on malt-extract agar (MEA) slants, incubated at 25°C for 5-7 d and then stored at 4°C. The seed cultures were grown in 250-ml flasks containing 100 mL of MEA medium at 23°C on

rotary shaker incubators at 150 rev min^{-1} for 7 d [7-10].
The fungal cultures were achieved by inoculating 100 ml of culture medium using 3-5% (v/v) of the seed culture and then cultivated at 23-25°C in rotary shake flasks of 250 ml. The experiments were conducted under the following conditions: temperature, 25°C; agitation speed, 120-180 rev min^{-1}; initial pH, 4.5–5.5. After 10–12 d of incubation the fungal cultures were ready to be inoculated aseptically into the glass vessel of laboratory-scale bioreactor (Fig. 1).

Fig. 1 – Laboratoy-scale bioreactor for submerged cultivation of edible and medicinal mushrooms

For the fungal growing in this bioreactor special culture media were prepared by using liquid nutritive broth, having the following composition: 15% cellulose powder, 5% wheat bran, 3% malt extract, 0.5% yeast extract, 0.5% peptone, 0.3% powder of natural argillaceous materials.
After the steam sterilization at 121°C, 1.1 atm., for 15 min. this nutritive broth was transferred aseptically inside of the culture vessel of a laboratory scale bioreactor [12-14].
This culture medium was aseptically inoculated with activated spores of *Ganoderma lucidum* and *Lentinula edodes* species. After inoculation into the bioreactor vessel, a slow constant flow of nutritive liquid broth was maintained inside the nutritive culture medium by recycling it and adding from time to time a fresh new one.
The submerged fermentation was set up at the following parameters: constant temperature, 23°C; agitation speed, 80-100 rev. min^{-1}; pH level, 5.7–6.0 units; dissolved oxygen tension within the range of 30-70%.
After a period of submerged fermentation lasting up to 120 h, small fungal pellets were developed inside the broth [10-12].

RESULTS AND DISCUSSIONS

Fermentation process was carried out by inoculating the growing medium volume (10,000 ml) with secondary mycelium inside the culture vessel of the laboratory-scale bioreactor (Fig. 1).
The whole process of growing lasts for a single cycle between 5-7 days in case of *L. edodes* and between 3 to 5 days for *G. lucidum*.
The strains of these fungal species were characterized by morphological stability, manifested by its ability to maintain the phenotypic and taxonomic identity.
The biomass collected after each one of these cultivation processes by submerged fermentation was dehydrated in order to be preserved for long time as mycelia powder. The biomass samples of *L. edodes* as well as *G. lucidum* are shown in figures 2, 3.

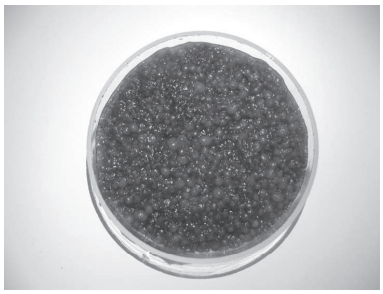

Fig. 2. Fungal pellets of *L. edodes* biomass

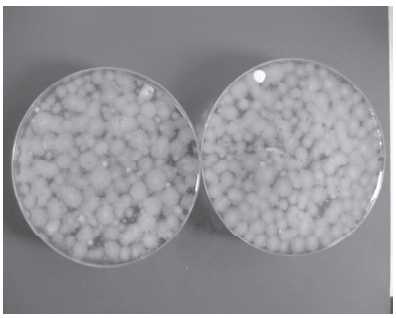

Fig. 3. Biomass of *G. lucidum* as fungal pellets

Observations on morphological and physiological characters of these two tested species of fungi were made after each culture cycle, highlighting the following aspects:

► sphere-shaped structure of fungal pellets, sometimes elongated, irregular with various sizes (from 2 to 5 mm in diameter), reddish-brown colour of *L. edodes* (Fig. 4).

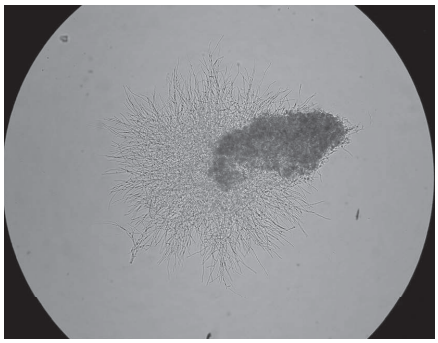

Fig. 4. Microscopical image of *L. edodes* fungal pellet (X100)

► globular structures of fungal pellets, irregular with diameters of 4 up to 7 mm or mycelia congestion, which have developed specific hyphae of *G. lucidum* (Fig. 5).

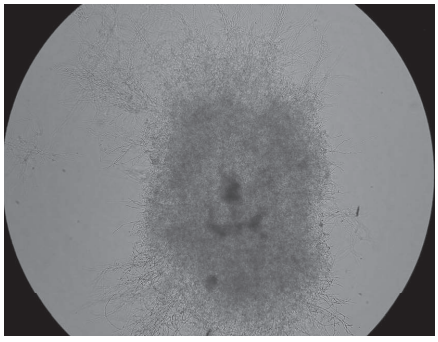

Fig. 5. Microscopical image of *L. edodes* fungal pellet (X100)

The experiments were carried out in three repetitions. Samples for analysis were collected at the end of the fermentation process, when pellets formed specific shapes and characteristic sizes.

For this purpose, fungal biomass was washed repeatedly with double distilled water in a sieve with 2 mm diameter eye, to remove the remained bran in each culture medium.

Biochemical analyses of fungal biomass samples obtained by submerged cultivation of edible and medicinal mushrooms were carried out separately for solid fraction and extract fluid remaining after the separation of fungal biomass by pressing and filtering [15-17].

The percentage distribution of solid substrate and liquid fraction in the preliminary samples of fungal biomass are shown in table 1, together with the percentage of substance remaining (which was measured for dry matter content) and the percentage level of liquid fraction (resulted after pressing the solid substrate).

Table 1. Percentage distribution of solid substrate and liquid fraction in the samples of fungal biomass

Mushroom species	Total liquid volume of per sample (ml)	Total biomass weight per sample (g)	Water content after separation (%)
L. edodes	83	5.81	83.35
L. edodes	105	7.83	82.50
L. edodes	95	7.75	82.15
L. edodes	80	5.70	79.55
G. lucidum	75	7.95	83.70
G. lucidum	115	6.70	82.95
G. lucidum	97	5.45	80.75
G. lucidum	110	6.30	77.70

Also, the most obvious sensory characteristics (color, odor, consistency) were evaluated and presented at this stage of biosynthesis taking into consideration that they are very important in the prospective view of fungal biomass using as raw matarials for nutraceuticals producing [18-23].

In each experimental variant the amount of fresh biomass mycelia was determined. Percentage amount of dry biomass was determined by dehydration obtained at a temperature of 70° C, until constant weight.

The total protein content was determined by biuret method, whose principle is similar to the Lowry method, this method being recommended for protein content ranging from 0.5 to 20 mg/100 mg on each sample [15-17].

In addition, this method requires only one sample incubation period (20 min) and using them is eliminated interference with various chemical agents (ammonium salts, for example).

The principle method is based on reaction that takes place between copper salts and compounds with two or more peptides in the composition in alkali, which results in a red-purple complex, whose absorbance is read in a

spectrophotometer in the visible domain (λ - 550 nm).
In tables 2 and 3 are presented the amounts of fresh and dry biomass as well as the protein contents for each fungal species and variants of culture media.

Table 2. Fresh and dry biomass and protein content of *L. edodes* grown by submerged fermentation

Mushroom culture variants	Fresh biomass (g)	Dry biomass (%)	Total proteins (g % d.w.)
I	20.30	5.23	0.55
II	23.95	6.10	0.53
III	22.27	4.79	0.73
IV	20.10	4.21	0.49
Control	4.7	0.5	0.2

Table 3. Fresh and dry biomass and protein content of *G. lucidum* grown by submerged fermentation

Mushroom culture variants	Fresh biomass (g)	Dry biomass (%)	Total proteins (g % d.w.)
I	25.94	9.03	0.67
II	22.45	10.70	0.55
III	23.47	9.95	0.73
IV	21.97	9.15	0.51
Control	5.9	0.7	0.3

According to registered data, using wheat bran strains the growth of *G. lucidum* biomass was favoured, while the barley bran led to increased growth of *L. edodes* mycelium and *G. lucidum* as well.
In contrast, dry matter content is significantly higher when using barley bran for both species used. Protein accumulation is more intense when using barley bran compared with those of wheat and rye, at both species of mushrooms.
The sugar content of dried mushroom pellets collected after the biotechnological experiments was determined by using Dubois method.
The mushroom extracts were prepared by immersion of dried pellets inside a solution of NaOH pH 9, in the ratio 1:5.
All dispersed solutions containing the dried pellets were maintained 24 h at a precise temperature of 25^{o}C, in full darkness, with continuous homogenization to avoid the oxidation reactions.
After the removal of solid residues by filtration the samples were analyzed by the previous mention method [15-17.
The nitrogen content of mushroom pellets was analyzed by Kjeldahl method.
All registered results are related to dry weight of mushroom pellets that were collected at the end of each biotechnological culture cycle (Table 4).

Table 4. The sugar and total nitrogen contents of dried mushroom pellets

Mushroom species	Culture variant	Sugar content (mg/ml)	Kjeldahl nitrogen (%)
L. edodes	I	5.15	6.30
L. edodes	II	4.93	5.35
L. edodes	III	4.50	5.70
L. edodes	IV	4.35	5.75
	Control	0.55	0.30
G. lucidum	I	4.95	5.95
G. lucidum	II	5.05	6.15
G. lucidum	III	5.55	6.53
G. lucidum	IV	4.70	5.05
	Control	0.45	0.35

Comparing all registered data, it could be noticed that the correlation between the dry weight of mushroom pellets and their sugar and nitrogen contents is kept at a balanced ratio for each tested mushroom species.
From these mushroom species that were tested in biotechnological experiments *G. lucidum* – culture variant III showed the best values concerning the sugar and total nitrogen content. In order, on the very next places, *L. edodes* – culture variant I and *G. lucidum* - culture variant II could be mentioned from these points of view.
These registered results concerning the sugar and total nitrogen contents had higher values than those obtained by other researchers [15-18].
The nitrogen content in fungal biomass is a key factor for assessing its nutraceutical potential, but the assessing of differential protein nitrogen compounds requires additional investigations.

CONCLUSIONS

1. The grain by-products used as substrata for growing the fungal species *L. edodes* and *G. lucidum* by controlled submerged fermentation showed optimal effects on the mycelia development in order to get high nutritive biomass.
2. The dry matter content of fungal biomass produced by submerged fermentation of barley bran was higher for both tested species.
3. The protein accumulation is more intense when using barley bran compared with those of wheat and rye, at both fungal species.

4. The correlation between the dry weight of mushroom pellets and their sugar and nitrogen contents is kept at a balanced ratio for each tested mushroom species.
5. *G. lucidum* - culture variant III showed the best values of sugar and total nitrogen contents, being followed by *L. edodes* – culture variant I.

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