

NUTRITIVE MUSHROOM BIOMASS PRODUCING THROUGH SUBMERGED FERMENTATION OF AGRICULTURAL ORGANIC WASTES

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ABSTRACT. The submerged cultivation of edible and medicinal mushrooms is a novel biotechnological process to get nutritive mushroom biomass that can be used for food supplement production.

The main aim of this research was focused on the establishment of the best biotechnology of leading and controlling the submerged fermentation organic agricultural wastes by using three edible and medicinal mushroom species *Ganoderma lucidum* (Curt. Fr.) P. Karst, *Lentinula edodes* (Berkeley) Pegler and *Pleurotus ostreatus* (Jacquin ex Fries) Kummer. These mushrooms were cultivated on liquid substrata containing as main constituents different sorts of grain by-products as well as winery wastes.

The experiments were carried out by cultivating these mushrooms under controlled conditions inside the culture vessel of a modern laboratory-scale bioreactor designed at the highest biotechnological 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%-50%. 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, agricultural wastes*

INTRODUCTION

The submerged cultivation of mushroom mycelium is a promising method which can be used in novel biotechnological processes for obtaining pharmaceutical substances of anticancer, antiviral, immuno-modulating, and anti-sclerotic action

from fungal biomass and cultural liquids and also for the production of liquid spawn (Breene, 1990). The researches that were carried out to get 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 (Wasser and Weis, 1994; Mizuno et al., 1995). It is well known the anti-tumor activity of polysaccharide fractions extracted from mycelia of *Pleurotus ostreatus*, known on its popular name as Oyster Mushroom (Mizuno et al., 1995; Hobbs, 1996).

The main purpose of this work consists in the application of biotechnology for continuous cultivation of edible and 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 (Carlile and Watkinson, 1996; Moser, 1994).

The continuous cultivation of medicinal mushrooms was applied using the submerged fermentation of natural wastes of agro-food industry, such as different sorts of grain by-products as well as winery wastes that provided a fast growth as well as high biomass productivity of the investigated strains (Petre and Teodorescu, 2011).

MATERIALS AND METHODS

Ganoderma lucidum Curt. Fr.) P. Karst, *Lentinula edodes* (Berkeley) Pegler and *Pleurotus ostreatus* (Jacquin ex Fries) Kummer were used as pure 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 (20% malt extract, 2% yeast extract, 20% agar-agar) at 23°C on rotary shaker incubator at 100 rev.min⁻¹ for 7 d (Petre and Petre, 2008). The fungal cultures were grown 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 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 a laboratory-scale bioreactor (Fig. 1).

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. The culture medium was aseptically inoculated with activated spores belonging to *G. lucidum*, *L. edodes* and *P. ostreatus* 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.



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

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. The experimental model of biotechnological installation, represented by the laboratory scale bioreactor (Fig. 1), was designed to be used in submerged cultivation of the mentioned mushroom species that were grown on substrata made of wastes resulted from the industrial processing of cereals and grapes (Petre et al., 2005; Stamets, 1993).

Table 1. The composition of compost variants used in mushroom cultures

| Variants of culture substrata | Composition |
|-------------------------------|---|
| S1 | Mixture of winery wastes and wheat bran 2.5% |
| S2 | Mixture of winery wastes and barley bran 2.5% |
| S3 | Mixture of winery wastes and rye bran 2.5% |
| Control | Pure cellulose |

RESULTS AND DISCUSSION

The whole process of mushroom mycelia growing lasts for a single cycle between 5-7 days in case of *L. edodes* and between 3 to 5 days for *G. lucidum* and *P. ostreatus*. All experiments regarding the fermentation process were carried out by inoculating the growing medium volume (15 L) with secondary mycelium inside the culture vessel of the laboratory-scale bioreactor (see Fig. 1).

The strains of these fungal species were characterized by morphological stability, manifested by its ability to maintain the phenotypic and taxonomic identity. 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 7 to 12 mm in diameter), reddish-brown colour—*G. lucidum* specific culture (Fig. 2a);



Fig. 2a. *Fungal pellets of G. lucidum*

- ▶ globular structures of fungal pellets, irregular with diameters of 5 up to 10 mm or mycelia congestion, which have developed specific hyphae of *L. edodes* (Fig. 2b);



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

► round-shaped pellets with diameter measuring between 5 and 15 mm, having a white-cream colour and showing compact structures of *P. ostreatus* mycelia (Fig. 2c).



Fig. 2c. Fungal pellets of *P. ostreatus*

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 the solid fraction and extract fluid remaining after the separation of fungal biomass by pressing and filtering. 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 materials for nutraceuticals producing. 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 the protein content ranging from 0.5 to 20 mg/100 mg sample (Bae et al., 2000; Lamar et al., 1992). 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 table 2 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 *G. lucidum*, *L. edodes* and *P. ostreatus* mycelia grown by submerged fermentation

| Mushroom species | Culture variants | Fresh biomass (g) | Dry biomass (%) | Total proteins (g % d.w.) |
|---------------------|------------------|-------------------|-----------------|---------------------------|
| <i>G. lucidum</i> | I | 25.94 | 9.03 | 0.67 |
| <i>G. lucidum</i> | II | 22.45 | 10.70 | 0.55 |
| <i>G. lucidum</i> | III | 23.47 | 9.95 | 0.73 |
| | Control | 5.9 | 0.7 | 0.3 |
| <i>L. edodes</i> | I | 20.30 | 5.23 | 0.55 |
| <i>L. edodes</i> | II | 23.55 | 6.10 | 0.53 |
| <i>L. edodes</i> | III | 22.27 | 4.53 | 0.73 |
| | Control | 4.5 | 0.5 | 0.2 |
| <i>P. ostreatus</i> | I | 21.50 | 5.73 | 0.63 |
| <i>P. ostreatus</i> | II | 23.95 | 7.45 | 0.55 |
| <i>P. ostreatus</i> | III | 23.25 | 4.79 | 0.75 |
| | Control | 4.7 | 0.5 | 0.3 |

According to registered data, using a mixture of wheat bran 2.5% and winery wastes the growth of *G. lucidum* biomass was stimulated, 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 2.5% mixed with winery wastes for both species used. Protein accumulation is more intense when using barley bran compared with those of wheat bran and rye bran, at both mushroom species. 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°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 (Wasser and Weis, 1994).

The nitrogen content of mushroom pellets was analyzed by Kjeldahl method. All the registered results are related to the dry weight of mushroom pellets that were collected at the end of each biotechnological culture cycle (Table 3).

Comparing all the 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* (variant III) showed the best values concerning the sugar and total nitrogen content. On the very next places, *L. edodes* (variant I) and *G. lucidum* (variant II) could be mentioned from these points of view. These registered results concerning the sugar and total nitrogen contents have higher values than those obtained by other researchers (Bae et al., 2000; Moo-Young, 1993).

Table 3. The sugar and total nitrogen contents of dried mushroom pellets

| Mushroom species | Culture variants | Mushroom pellets d. w. (%) | Sugar content of dried pellets (mg/ml) | Kjeldahl nitrogen of dried pellets (%) |
|---------------------|------------------|----------------------------|--|--|
| <i>G. lucidum</i> | I | 17.64 | 4.93 | 5.15 |
| <i>G. lucidum</i> | II | 14.51 | 3.70 | 5.35 |
| <i>G. lucidum</i> | III | 20.16 | 5.23 | 6.28 |
| | Control | 0.7 | 0.45 | 0.30 |
| <i>L. edodes</i> | I | 19.67 | 4.35 | 6.34 |
| <i>L. edodes</i> | II | 17.43 | 3.40 | 5.03 |
| <i>L. edodes</i> | III | 15.55 | 4.75 | 6.05 |
| | Control | 0.5 | 0.45 | 0.35 |
| <i>P. ostreatus</i> | I | 19.70 | 5.15 | 6.43 |
| <i>P. ostreatus</i> | II | 14.93 | 4.93 | 6.25 |
| <i>P. ostreatus</i> | III | 15.63 | 5.10 | 5.83 |
| | Control | 0.55 | 0.50 | 0.35 |

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 cereal by-products and winery wastes used as substrata for growing the fungal species *G. lucidum*, *L. edodes* and *P. ostreatus* 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* (variant III) registered the best values of sugar and total nitrogen contents, being followed by *L. edodes* (variant I)

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REFERENCES

- Bae, J.T., Sinha, J., Park, J.P., Song, C.H., Yun, J.W., 2000. Optimization of submerged culture conditions for exo-biopolymer production by *Paecilomyces japonica*. *J. Microbiol. Biotechnol.*, **10**:482-487.
- Breene, W.M., 1990. Nutritional and medicinal values of mushrooms. *J. Food Prot.*, **53**:833-894.
- Carlile, M.J. and Watkinson, S.C., 1996. *Fungi and biotechnology*. In: Carlile MJ, Watkinson SC, editors, *The Fungi*. London: Academic Press, 423-450.
- Hobbs, C., 1996. *Medicinal mushrooms*. Santa Cruz, Botanika Press, 251-270.
- Lamar, R.T., Glaser, J.A., Kirk, T.K., 1992. White rot fungi in the treatment of hazardous chemicals and wastes. In: Leatham, G.F. (ed.), *Frontiers in industrial mycology*, Chapman & Hall, New York, 223-245.
- Mizuno, T., Saito, H., Nischitoba, T., Kawagishi, H., 1995. Antitumor active substances from mushrooms. *Food Reviews International*, **11**:23-61.
- Moser, A., 1994. Sustainable biotechnology development: from high-tech to eco-tech. *Acta Biotechnologia*, **12**:2-6.
- Moo-Young, M., 1993. Fermentation of cellulose materials to mycoprotein foods, *Biotechnol. Adv.*, **11**(3): 469-482.
- Petre, M., 2009. *Biotehnologia protectiei mediului*, vol. 2, Ed. CD Press, Bucuresti, 23-50.
- Petre, M. and Petre, V., 2008. Environmental biotechnology to produce edible mushrooms by recycling the winery and vineyard wastes. *Journal of Environmental Protection and Ecology*, **9**(1):87-97.
- Petre, M., Teodorescu, A., 2011. Recycling of Vineyard and Winery Wastes as Nutritive Composts for Edible Mushroom Cultivation. *Proceedings of the International Conference on Advances in Materials and Processing Technologies AMPT 2010*, American Institute of Physics, 1539-1545.
- Stamets, P., 1993. *Growing gourmet and medicinal mushrooms*. Ten Speed Press, 554 -560.
- Wasser, S.P. and Weis, A.L., 1994. Medicinal properties of substances occurring in higher Basidiomycetes mushrooms: current perspectives. *Int. J. of Medicinal Mushrooms*, **1**(1):31-62.
- Wainwright, M., 1992. *An Introduction to Fungal Biotechnology*. Wiley, Chichester.