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Biotechnology of Mushroom Pellets Producing by Controlled Submerged Fermentation

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Abstract

The main aim of this work was focused on testing a new biotechnological procedure to optimise the efficiency of mushrooms cultivation by controlled submerged fermentation, in order to enhance the cellular activity to synthesize mushroom pellets that contain bioactive compounds, such as polysaccharides, proteins, phenols and flavones. Specific culture media for mushroom growing were prepared by using liquid nutritive media prepared from cereal wastes, like wheat bran, grain powder etc. After the steam sterilization of nutritive broths inside of the culture vessel of a 15 l laboratory scale bioreactor all the culture media were aseptically inoculated by using a suspension of activated spores of some pure strains of Ganoderma lucidum, Lentinula edodes and Pleurotus ostreatus. 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%. During a certain period of controlled submerged fermentation lasting up to 120 h, small mushroom pellets were developed inside the broth. On the base of registered results the optimal in vitro culture conditions were evaluated and the best values of all physical and chemical factors which could influence the evolution of biotechnological processes for fungal biomass synthesis were established.

Keywords: biotechnology, controlled cultivation, mushroom pellets, edible and medicinal mushrooms, submerged fermentation

Introduction

The submerged cultivation of edible and medicinal mushrooms 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 [1]. 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 own to the huge protein content, carbohydrates and mineral salts [2, 3].
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 [4, 5, 6]. *Lentinula edodes* species (Shiitake) is a good source of proteins, carbohydrates (especially polysaccharides) and mineral elements with beneficial effects on human nutrition [5, 6].
The main purpose of this work consists in the application of biotechnology for controlled cultivation of medicinal mushrooms by submerged fermentation in order to get fungal biomass with high nutritive value in the shape of mushroom pellets which can be used to prepare functional food [3, 7]. As a result of the experiments that we have accomplished to

produce mushroom pellets by submerged fermentation, the biotechnology of controlled cultivation of edible and medicinal mushrooms was tested in different physical and chemical conditions that could influence the fast growth as well as high biomass productivity of the investigated strains [7].

Materials and methods

Micro-organisms and culture media

According to the main purpose of this work, three fungal species from Basidiomycetes, namely *Ganoderma lucidum* (Curt.:Fr.) P. Karst, *Lentinula edodes* (Berkeley) Pegler and *Pleurotus ostreatus* (Jacquin ex Fries) Kummer were used as pure cultures in experiments. The stock cultures were maintained on malt-extract agar (MEA) slants. Slants were incubated at 25°C for 5-7 d and then stored at 4°C [7, 8, 9].
The fungal cultures were grown in 250-ml flasks containing 100 ml of MEYE medium (20% malt extract, 2% yeast extract) at 23°C on rotary shaker incubators at 110 rev min $^{-1}$ for 5 - 7 d [10, 11].

Methods used in experiments

The fungal cultures were prepared by aseptically inoculating 100 ml of culture medium by using 3-5% (v/v) of the seed culture and then cultivated at 23-25°C in 250 ml rotary shake flasks. The biotechnological 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 [7, 12].
For the mushroom growing inside the culture vessel of a laboratory-scale bioreactor, special culture media were prepared by using different liquid nutritive broths. After the steam sterilization at 121^{0}C, 1.1 atm., for 15 min. each nutritive broth was transferred aseptically inside of the culture vessel of a laboratory scale bioreactor. This kind of culture medium was aseptically inoculated with the previously activated spores of *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.
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% [13]. After a period of submerged fermentation lasting up to 120 h, small mushroom pellets were developed inside the nutritive broth. The experimental model of biotechnological installation, represented by the laboratory scale bioreactor, was designed to be used for controlled submerged cultivation of the mentioned medicinal mushrooms on substrata made of wastes resulted from the industrial processing of cereal grains [14, 15].

Results and discussion

During the experiments of controlled submerged fermentation achieved by using the previous mentioned mushroom species the effects of carbon, nitrogen and mineral sources as well as pH level on mycelia growing and especially on fungal pellets formation were investigated. In order to find a suitable carbon source for the mycelia growth and consequently for fungal biomass synthesis, the pure cultures of *G. lucidum*, *L. edodes* and *P. ostreatus* were cultivated in different nutritive media containing various carbon sources, and each carbon source was added to the basal medium at a concentration level of 1.5% (w/v) for 7 d. When the cells were grown in the maltose medium, the fungal biomass production was

the highest among those tested (Table 1). What is very important to be noticed is that the maltose has a significant effect upon the increasing of mycelia growth and fungal biomass synthesis [16, 17].

At the same time, to investigate the effect of nitrogen sources on mycelia growth and fungal biomass production, the pure cultures of these fungal species were cultivated in media containing various nitrogen sources, where each nitrogen source was added to the basal medium at a concentration level of 10 g/l. As it could be noticed, malt extract was one of the best nitrogen sources for a high mycelia growth. Peptone and yeast extract are also known as efficient nitrogen sources for fungal biomass production by using the pure cultures of such fungal species [17]. In comparison to organic nitrogen sources, inorganic nitrogen sources gave rise to relatively lower mycelia growth and fungal biomass production [18]. From all five nitrogen sources examined, wheat bran was the most efficient for mycelia growth and fungal pellets production.

The influence of various mineral sources upon fungal biomass production was examined at a standard concentration level of 1% (w/w). Among the various mineral sources examined ($CaCO_3$, $CaSO_4$, K_2HPO_4 and KH_2PO_4), $CaCO_3$ yielded the best mycelia growing as well as fungal biomass production at 28-32 g% and for this reason it was registered as the most favourable mineral source. . All the experiments were carried out for 12 days at 25 °C with the initial pH 5.5. Data are the means ± S.D. of triple determinations (Table 1).

Table 1. The effects of carbon, nitrogen and mineral sources upon *G. lucidum*, *L. edodes* and *P. ostreatus* mycelia growth and fungal pellets formation

Carbon Source (1.5% w/v)	**Fresh Fungal Biomass Weight (g/l)**			**pH Level**		
	G. lucidum	***L. edodes***	***P. ostreatus***	***G. l***	***L. e***	***P. o***
Glucose	27±0.14	41±0.05	43±0.03	5.5	5.3	5.1
Maltose	27±0.15	45±0.12	49±0.05	5.8	5.4	5.3
Sucrose	25±0.23	35±0.03	37±0.09	5.1	5.2	5.7
Xylose	26±0.07	38±0.07	35±0.07	5.3	5.5	5.9
Nitrogen Source (1%, w/v)	**Fresh Fungal Biomass Weight (g/l)**			**pH Level**		
	G. lucidum	***L. edodes***	***P. ostreatus***	***G. l***	***L. e***	***P. o***
Wheat bran	37±0.10	57±0.05	73±0.23	5.7	5.6	5.8
Malt extract	36±0.12	55±0.03	69±0.20	5.3	5.2	5.7
Peptone	32±0.03	43±0.12	57±0.15	5.1	5.0	5.3
Yeast extract	35±0.21	30±0.01	61±0.14	5.0	5.1	5.2
Mineral Source (5 mg)	**Fresh Fungal Biomass Weight (g/l)**			**pH Level**		
	G. lucidum	*L. edodes*	***P. ostreatus***	*G. l*	*L. e*	*P. o*
$CaCO_3$	45±0.07	57±0.05	59±0.07	5.1	5.1	5.7
KH_2PO_4	37±0.15	45±0.07	53±0.12	5.5	5.3	5.9
K_2HPO_4	35±0.25	55±0.09	63±0.28	5.6	5.4	6.1
$CaSO_4$	38±0.35	56±0.12	57±0.48	5.7	5.5	5.8

Similar observations were made also by other researchers during the experiments concerning the cultivation of the same species but using the solid state fermentation [16, 17, 19]. Also, as one of the tested mineral sources, $CaSO_4$ have shown the most favourable influence upon the fungal pellets growing [19, 20]. At the same time, the mineral sources K_2HPO_4 and KH_2PO_4 as essential phosphates could improve the pH level through their buffering action, but they were less favourable for mycelia growing in submerged as well as in surface cultures of mushrooms. All the experiments were carried out for 12 days at 25°C with the initial pH 5.5. Data are the means ± S.D. of triple determinations.

In order to characterize the chemical composition of the collected mushroom pellets the sugar as well the nitrogen content were investigated. Firstly, the dry weight of the fungal biomass was established for each mushroom species used in experiments.

Secondly, the sugar content of dried mushroom pellets collected after the biotechnological experiments was determined by using Dubois method [16, 19]. The mushroom extracts were prepared by immersion of dried pellets inside a solution of NaOH pH 9, in the ratio 1:5. All these dispersed solutions containing the dried pellets were maintained 24 h at a precise temperature of 25^0C, in full darkness, with continuous homogenisation to avoid the oxidation reactions. After the removal of solid residues by filtration the samples were analysed by the previously mentioned method. The nitrogen content of the same samples of mushroom pellets was analysed 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 2).

Comparing all registered data, it could be noticed that the balance between the dry weight of mushroom pellets and their sugar and nitrogen contents was kept at almost the same ratio for each tested mushroom species. Among all twelve mushroom samples that were tested in biotechnological experiments *G. lucidum* G-3 has shown the best values of their composition in sugars and total nitrogen content. Also, the mushroom samples *L. edodes* L-1 and *P. ostreatus* P-1 could be highlighted as good results from the point of view of their sugar and nitrogen contents (Table 2).

Table 2. The sugar and total nitrogen contents of dried mushroom pellets

Sample number	Mushroom species	Mushroom pellets d. w. (%)	Sugar content of dried pellets (mg/ml)	Kjeldahl nitrogen of dried pellets (%)
G-1	*G. lucidum*	17.64	4.93	5.15
G-2	*G. lucidum*	14.51	3.70	5.35
G-3	*G. lucidum*	20.16	5.23	6.28
L-1	*L. edodes*	19.67	4.35	6.34
L-2	*L. edodes*	17,43	3.40	5.03
L-3	*L. edodes*	15.55	4.75	6.05
P-1	*P. ostreatus*	19.70	5.15	6.43
P-2	*P. ostreatus*	14.93	4.93	6.25
P-3	*P. ostreatus*	15.63	5.10	5.83

In this stage, 70-80% of the former fungal pellets were separated by collecting them from the culture vessel of the bioreactor and separating from the broth by slow vacuum filtration. On the base of registered results the optimal *in vitro* culture conditions were evaluated and the best values of all physical and chemical factors which could influence the evolution of biotechnological processes for fungal biomass synthesis were established.

Taking into consideration all these registered results it was established the biotechnology of mushroom pellets producing by controlled submerged fermentation including the most important stages (Fig. 1).

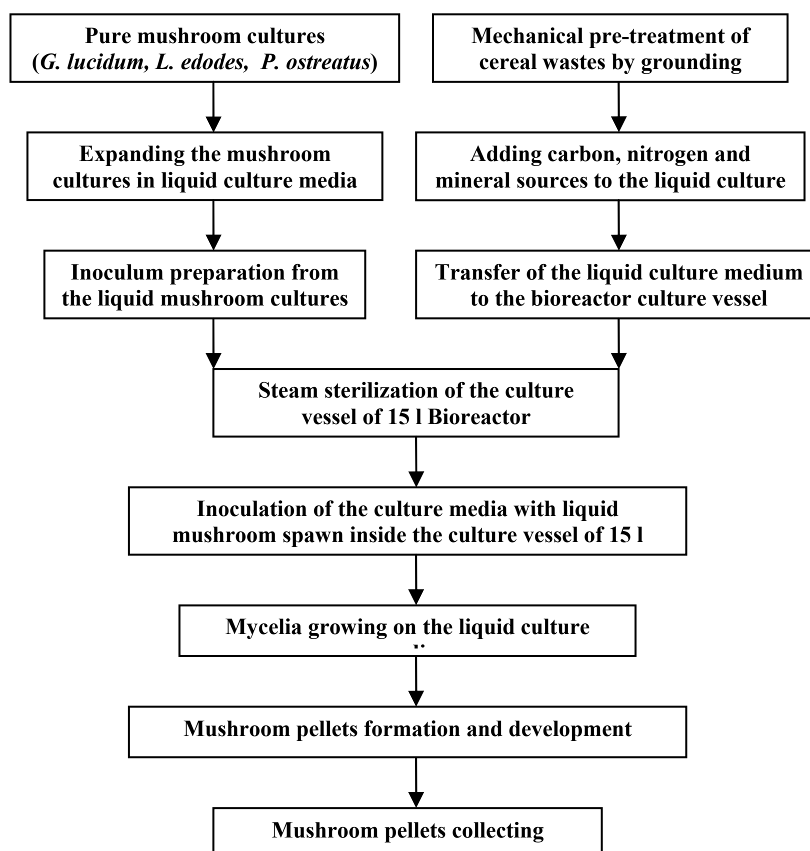

Fig. 1. Schematic flow of the biotechnology for mushroom pellets producing by submerged fermentation

Conclusions

Taking into consideration the main registered results of the accomplished experiments that were made to test the biotechnology of mushroom pellets producing by controlled submerged fermentation, the following conclusions could be mentioned:

1) The edible and medicinal mushrooms of *G. lucidum, L. edodes* and *P. ostreatus* species were used in a biotechnological process of controlled cultivation by submerged fermentation to increase the mushroom metabolism in a laboratory-scale bioreactor.
2) The optimal *in vitro* culture conditions were tested and compared in order to select the best values of all physical and chemical factors which could influence the evolution of biotechnological processes for mushroom pellets synthesis.
3) When the cells were grown in the maltose medium, the fungal biomass production was the highest among those tested.
4) From all five nitrogen sources examined, wheat bran was the most efficient for mycelia growth and fungal pellets production.
5) As one of the tested mineral sources, $CaSO_4$ have shown the most favourable influence upon the fungal pellets growing.
6) Among all twelve mushroom samples that were tested in biotechnological experiments *G. lucidum* G-3 has shown the best values of their composition in sugars and total nitrogen content.
7) The collected mushroom pellets having high nutritional value were produced by applying the biotechnology of controlled submerged fermentation in order to be used in functional food producing.

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