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RESEARCH ARTICLE

STUDY OF PRELIMINARY BIOTECHNOLOGICAL CONDITIONS FOR *PLEUROTUS OSTREATUS* CULTIVATION ON SUBMERGED SYSTEM

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Abstract: The goal of this work was to identify the biotechnogical conditions (i.e. inoculum concentration, nutrional sources (carbon and nitrogen), pH and time of cultivation) for improving the yield of biomass production of *Pleurotus ostreatus* by cultivation on submerged culture conditions. The submerged cultivation was done in Erlenmeyer vessels in rotary shaker with controlled temperature, for 10 days at 26°C and 150 rpm. Four concentrations of inoculum 0.2%, 5.0%, 7.5% and 10.0%, and also three values of pH (5.0, 5.5 and 6.0) were tested. Also, it was tested the qualitative effect of some carbon and nitrogen sources for an optimal growth and multiplication of mushroom in liquid medium, in submerged conditions with agitation. The best *Pleurotus ostreatus* biomass yield (7.58 g dry biomass $\cdot L^{-1}$) was obtained with 0.2% inoculum concentration when dry biomass yields increased with 30%. By using dextrose in concentration of 40 g/L and peptone and yeast extract (1:1) in concentration of 5g/L have a positive influence of mycelium growth and biomass forming. These preliminary results will open new further studies on mushroom submerged cultivation.

Keywords: *Pleurotus ostreatus*, submerged cultivation, biomass yield.

Introduction

For many years, mushrooms had represented a rich source for pharmaceutical and nutraceutical products (Papaspyridi *et al.*, 2011). Submerged fermentation of the mushrooms promises an efficient production of fungal biomass, rich in bioactive compounds and also to obtain extracellular secondary metabolites with large practical applications. Commercial mushroom products are obtained from the fruiting bodies of field cultivated mushrooms, which represent an intensive process over a long period of time. The submerged cultivation offers the advantages of faster production for biomass and bioactive compounds in controlled condition, in reduced space and time and also with lesser chances for contamination (Tang *et al.*, 2007). The growing, of mushroom biomass in submerged culture has many advantages over the popular compost bed methods. There is a considerable reduction in time and expense in submerged cultures. Substantial quantities of biomass can be grown according to these techniques in several days while several weeks

are required for the conventional technique. Also the equipment required for the submerged process is much smaller than that required for the composting method. Another remarkable feature of this method is the cultivation of mushrooms with less contamination risk. The contamination of mushrooms cultures it's a serious problem because there have been accidents reported of consuming poisonous species leading to death or serious illness (Oei, 1996). Along with these methods the desired species will be cultivated in each batch.

Another important issue of this method of mushroom cultivation is that it will provide the same concentration of bioactive compounds in biomass because of the controlled biotechnological cultivation conditions. This is a very useful issue in terms of human food consumption because it represents an easy and time saver method for obtaining a pure source of bioactive compounds. Human population expands by 2% per year. Thus food production has to keep pace with population increase. Mushroom along with yeast are referred to as alternative source of food. Nutritionally, mushrooms are low in energy and fat but high in protein, carbohydrate, and dietary fibers, so, mushrooms can be considered as a potential new source of dietary fibers, since fungal cell walls are rich in non-starch polysaccharides, of which β-glucans are the most interesting functional components (Vetter, 2007). Moreover, mushrooms contain a variety of minerals and trace elements such as potassium, copper and vitamins such as riboflavin, niacin, and folates (Cheung, 2010). *Pleorutus ostreatus* is an edible mushroom occurring all over Europe. Currently, it is being cultivated as food on an industrial level (Vamanu, 2012). *Pleurotus* spp. having various biotechnological applications in medicine, food and drug industry is viewed promising as medicinal mushroom, exhibiting a wide range of biological activities (Gregori, 2007). Interest in this species has increased considerably in the last decade because of its gastronomic value and its nutraceutical properties (Barros, 2007). The beneficial effects to human health of *P. ostreatus*, such as their antioxidant activities, immunomodulatory effects, antitumor activities, anti-inflammatory and cholesterol-lowering activities, have been investigated intensively during the last years (Gregori, 2007).

In Romania, the study of mushrooms cultivation in submerged system is in its infancy, there aren't any protocols yet applied at industrial level. This study is appropriate for the setting preconditions for mushroom growing in the proposed system.

Materials and methods

Mushroom strain

Pleurotus ostreatus strain was obtained from *Culture Collection* of *Laboratory for research of fungi with role in the ecological reconstruction of heavy metals polluted soils-RECOSOL* of Alexandru Ioan Cuza University of Iasi.

Inoculum preparation

Two different types of inoculum were used. First of them was pellet sized, obtained on stirred submerged cultivation in liquid medium for 4 days on 26°C and the last one obtained by stationary cultivation also for 4 days at 26°C. The media composition for pellet inoculum was ($g \cdot L^{-1}$): glucose 5, yeast extract 5, malt extract 5 and ammonium sulfate 1, pH = 5.5, while for the stationary inoculum, was used a medium which contains ($g \cdot L^{-1}$): glucose 40, yeast extract 5, peptone 5, pH = 5.5. Stock culture was preserved by cultivation on agar nutritive medium containing 15 $g \cdot L^{-1}$ malt extract and 15 $g \cdot L^{-1}$ agar, in Petri dishes with conservation at 4°C.

Submerged cultivation

From pure culture on the Petri dishes was cut three square pieces of mycelium with 0.5 cm in diameter. These were used as start inoculum. The fermentative basal medium used for submerged mushroom cultivation consisted in ($g \cdot L^{-1}$): glucose 40, peptone 3, yeast extract 5, $KH_2PO_4 \cdot H_2O$ 0.5, $MgSO_4 \cdot 7H_2O$ 0.5. The cultivation took place in Erlenmeyer vessels placed in a rotary shaker (Lab Companion, Korea) with controlled temperature, for 10 days at 26°C and 150 rpm. Three concentrations of pellet inoculum were tested 5.0%, 7.5% and 10.0%. From the inoculum obtained on stationary conditions was tested only one concentration of 0.2%, to be compared with classic pellet sized inoculum technique. Also were tested three values of pH of cultivation medium, pH 5.0, 5.5 and 6.0, respectively.

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Testing nutritive effect of different carbon and nitrogen sources

The carbon sources in the medium play an important role for mushrooms growth and ligninolytic enzyme production (Mansur, 2007). The qualitative influence of different carbon sources were tested in order to determinate which has more impact on biomass yields. Thus, were tested five carbon sources: glucose, dextrose, lactose, celulose and xylan, all with laboratory purity grade. The concentration of these five carbon sources was kept constant at 40 g/L. The basal medium composition was the same like in the above experiment, but only the carbon source was changed.

To better understand the behavior of oyster mushrooms in submerged cultivation, were tried five different nitrogen sources to determine which one have a better influence on *Pleurotus ostreatus* biomass yield. In order to determine their influence on biomass yield was tested the following nitrogen sources: complex fertilizer, urea, peptone, yeast extract and peptone and yeast extract (1:1) all with laboratory purity grade. Because the nitrogen source is an important factor that can highly influence the biomass yields it were tested three concentrations 1.0, 3.0 and 5.0 g/L.

Statistical analysis

All the experiments were done in triplicate and the data presented here represents the mean of these replicates. Data related to the dry biomass yield were subjected to analysis of variance (one way ANOVA) in Duncan multiple range test using SPSS (version 10) statistical software. The differences with $p < 0.05$ were considered significant.

Results and discussion

It seems that *P. ostreatus* grows better in culture media inoculated with a stationary inoculum with 0.2 % concentration. In this case the production of dry biomass is grater with 27% than the best sample inoculated with pellet inoculum, used in concentration of 10% (Table 1).

Table 1. *P. ostreatus dry biomass production on submerged cultivation*

Sample	Inoculum	Dry biomass (g/L)	pH
1		2.9±0.14	5.0
2	5%	2.25±0.11	5.5
3		1.93±0.09	6.0
4		4.98±0.25	5.0
5	7.50%	4.85±0.24	5.5
6		4.19±0.21	6.0
7		4.84±0.14	5.0
8	10%	5.53±0.26	5.5
9		5.23±0.25	6.0
10		7.09±0.32	5.0
11	0.20%	7.58±0.34	5.5
12		0.84±0.04	6.0

The best results in terms of dry biomass yield were obtained by the sample inoculated with 0.2% inoculum, from stationary liquid cultivation used for submerged cultivation at pH 5.5. After 10 days, 7.58 $g \cdot L^{-1}$ dry biomass of *P. ostreatus* was produced. This represents an important factor for further work in practice because the quantity used of stationary inoculum is 50 times less than pellet inoculum. These results are according with the studies of Germano S. et al, in 2003, on *Pleurotus* spp.

biomass production. After a 7 day incubation it was obtained a superior yield (16.8 g dry biomass /L). Also a good dry biomass production was obtained at pH 5.0 in samples inoculated with 7.5% and 10% pellet inoculum.

For sample 12, inoculated with 0.2% stationary inoculum, it was observed that at pH 6.0 the *P. ostreatus* didn't grow. It hasn't the same behavior as in case of inoculation with pellet inoculum where in all different ranges of concentration the biomass of *P. ostreatus* was produced, indeed less than at pH values between 5.0 and 5.5.

Because of the highest biomass yield obtained in samples inoculated with 0.2% inoculum at pH 5.5, this method will be chosen for further studies because of the small inoculum quantity used, wich represents an economical factor in industry.

The screening on the influence of carbon sources on biomass yield has shown that in the culture medium with dextrose and glucose was recorded the higher values 16.62 respectively 12.66 g dry biomass/L medium (Table 2). For the further studies, dextrose will be chosen as the main carbon source because it was demonstrated that it has the higher influence on biomass yield.

Table 2. *Dry biomass forming by P. ostreatus on submerged system cultivation on liquid media with different carbon sources (submerged cultivation 10 days, at 25°C and 150 rpm)*

Carbon source	Glucose	Dextrose	Lactose	Celulose	Xylan
Yield of dry biomass (g/L)	12.66±0.63	16.62±0.81	2.79±0.13	0	0

Celulose and lactose hasn't any influence on P. *ostreatus* biomass yield. They both haven't shown any growth of mycelium. At the final cultivation time (10 days) the culture medium was clearly as in the initial phase with no traces of any mushroom growth. This reason therefore reinforces the idea of no longer consider them the further studies.

In terms of what nitrogen source is the best for using in submerged cultivation of *P. ostreatus*, the growth of oyster mushroom biomass was reached when complex fertilizer, yeast extract and peptone and yeast extract (1:1) were used as nitrogen sources. The best *P. ostreatus* biomass yield was obtained in sample supplemented with 5 g/L peptone and yeast extract (1:1). In this case 47.04 g dry biomass/L was obtained (Table 3).

Similarly study on different carbon and nitrogen sources has reported comparable biomass yields (Papaspyridi, 2010). There was obtained a maximum yield of 39.2 g dry biomass/L.

This certifies that the method used can be chosen for future studies. Also for the lowest concentrations of 1 and 3g/L respectively, the values of biomass growth was higher than the others nitrogen sources.

Table 3. *Dry biomass growth of P. ostreatus on submerged system cultivation on liquid media with different nitrogen sources (submerged cultivation 10 days, at 25°C and 150 rpm)*

Type and concentration of nitrogen source (g/L)	1.0	3.0	5.0
Complex fertilizer	11.36±0.56	8.68±0.43	4.18±0.21
Ureea	-	-	-
Peptone	-	-	-
Yeast extract	1.61±0.08	1.72±0.08	1.65±0.08
Peptone and yeast extract (1:1)	5.67±0.28	32.08±1.61	47.04±2.35

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When urea and peptone were used, the *P. ostreatus* biomass hasn't shown any growth. The lowest biomass growth was determined when yeast extract was used as nitrogen source when was recorded between 1.61 and 1.72 g dry biomass/L.

4. Conclusions

The liquid medium composition, the pH and also the type of used inoculum have a strong influence on yield of biomass production during *Pleurotus ostreatus* cultivation in submerged system. Dry biomass yields increased with 30% were obtained by inoculation with 0.2% inoculum obtained from stationary culture. It was observed that the optimum pH for *Pleurotus ostreatus* submerged cultivation was pH 5.5. When dextrose in concentration of 40 g/L was used in terms of the best carbon sources, it was obtained the best biomass yield (16.62 g/L). The higher biomass growth concentration was achieved when peptone and yeast extract (1:1) was used as nitrogen sources. At a concentration of 5g/L peptone and yeast extract (1:1), and also 40 g/L dextrose a yield of 47.04 g dry biomass/L was obtained.

For future studies these results will be a springboard, because they set the initial conditions of increasing *P. ostreatus* biomass. Continuing this step it can be establish the basic conditions to obtain a maximum yield of biomass when mushrooms are cultivated in submerged system on liquid medium. This will be also the main results that will open new further studies to make this process more economically.

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