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ORIGINAL RESEARCH PAPER

EVALUATION OF SOME BIOTECHNOLOGICAL PARAMETERS INFLUENCING THE *PLEUROTUS OSTREATUS* BIOMASS PRODUCTION BY SUBMERGED CULTIVATION

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The submerged culture of mushrooms represents a future for biotechnological processes at industrial level, in order to obtain biomass with economical value (food and ingredients, nutraceuticals and pharmaceuticals). *Pleurotus ostreatus* is well known worldwide for its culinary and medicinal value. The aim of the present study was to evaluate the most important biotechnological parameters that have influence on the biomass production of *P. ostreatus*, by cultivation in submerged conditions. Applying the Plackett-Burman experimental design, the significant parameters influencing the *P. ostreatus* biomass production were found to be the concentration of dextrose and yeast extract and time of cultivation. The best results in terms of maximising the biomass production (25.71 $g \cdot L^{-1}$) were obtained when the "+1" level of each independent variables was used in the Plackett-Burman experimental design. Analysis of variance (ANOVA) exhibited a high correlation coefficient (R^2) value of 0.9908, which certifies that the mathematical model was relevant for the biotechnological process.

Keywords: *Pleurotus ostreatus*, Plackett-Burman design, submerged cultivation, biomass production

Introduction

The medicinal properties and nutritional value of several mushrooms are widely known. However, only since the last decade of the 20th century has it been possible to isolate and partially characterize some biologically active compounds with antitumor effects (Kidd, 2000). For many years mushrooms have been consumed and appreciated by their nutritional value, and medicinal properties. The traditional mushroom cultivation takes too long and the macrofungi biotechnology has not been explored in its full potential yet (Smiderle *et al*., 2012).

P. ostreatus, also known as the oyster mushroom, is a *Basidiomycetes* belonging to the family *Pleurotaceae (Agaricales, Agaricomycetes)*. Interest for this species has

increased considerably in the last decade because of its gastronomic value and its nutraceutical properties (Barros *et al*., 2007). The medicinal beneficial effects of *P. ostreatus*, such as antioxidant, antitumor and cholesterol-lowering activities, have been investigated intensively (Gregori *et al*., 2007).

The aim of this research was to study how different biotechnological parameters affect the *Pleurotus ostreatus* biomass production by cultivation in submerged conditions in liquid medium with agitation. 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 (Horincar *et al*., 2014; Akinyele *et al*., 2012; Papaspyridi *et al*., 2012).

The Plackett-Burman factorial design is used to identify the most important factors early in the experimentation phase when complete knowledge about the fermentative medium is usually unavailable. That represents an efficient screening method to identify the active factors that influence a process (Pan *et al*., 2008).

Considering the great interest for mushrooms as a source of bioactive metabolites for the development of drugs and nutraceuticals, the aim of this research was to increase the yield of *P. ostreatus* biomass by optimizing some biotechnological growth parameters using the Plackett-Burman experimental design. In this study, by mathematical modelling and statistical analysis, seven biotechnological factors were analysed, i.e. carbon and nitrogen sources concentration (dextrose, yeast extract and peptone), inoculum concentration, time of cultivation, pH and agitation speed, in order to establish which factors have the greatest influence (positive and negative) on the *P. ostreatus* biomass production.

Materials and methods

Mushroom strains

The mushroom strain *P. ostreatus* was obtained from the Culture Collection of the Laboratory for research of fungi with role in the ecological reconstruction of heavy metal polluted soils – RECOSOL of "Alexandru Ioan Cuza" University of Iasi, Romania.

Inoculum preparation

The inoculum was obtained by stationary submerged cultivation in liquid medium for 4 days at 26°C. The medium composition for vegetative inoculum contains ($g \cdot L^{-1}$): glucose 40, yeast extract 5, peptone 5, pH = 5.5. The stock culture was preserved by cultivation on agar nutritive medium containing (g·L-1): 15 malt extract and 15 agar, in Petri dishes at 4°C.

Submerged cultivation

From pure culture grown on solid medium (consisted in ($g \cdot L^{-1}$): glucose 40, yeast extract 5, $KH_2PO_4 \cdot H_2O$ 0.5, $MgSO_4 \cdot 7H_2O$ 0.5 and agar 20) on the Petri dishes three square pieces of mycelium with 0.5 cm diameter were cut. These were used as start inoculum for 100 mL of fermentation medium. The fermentative 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 was realized in Erlenmeyer flasks placed in a rotary shaker SI-300R (Lab Companion, Korea) for 10 days at 26°C and 150 rpm.

Biomass yield

The biomass concentration during the experimental tests was determined by measuring the dry weight, expressed as $g \cdot L^{-1}$. It was measured by collecting wet biomass by vacuum filtering sample through reweighed cellulose acetate filter 0.45µ, Sartorius Stedim Biotech, GmbH, Germany. The filtered biomass was then washed twice with distilled water and filters were then placed in glass dishes and dried at 105°C at Drying Oven Sanyo, Japan until achieving a constant weight.

Identifying the significant variables using Plackett-Burman design

The Plackett-Burman experimental design is a valuable tool for the rapid evaluation of the effects of various parameters that influence the biotechnological process. It can pick up the main factors with the least number of experiments from a list of potential factors (Plackett and Burman, 1946).

For mathematical modelling a first-order polynomial model (Eq. 1) was used as follows:

$$Y = \beta_0 + \sum \beta_i \chi_i \qquad (1)$$

where Y is the predicted response (biomass yields, $g \cdot L^{-1}$), β_0 is the model intercept and β_i is the linear coefficient, and χ_i is the level of the independent variable (Plackett and Burman, 1946).

A total of 7 parameters, such as dextrose, yeast extract, peptone, pH, time, agitation speed and inoculums concentration, were studied in a matrix design containing 12 experiments (Table 1). The experimental design was established with the Placket-Burman tool generated by using the statistical software package Design Expert 6.0.8 (Stat-Ease, Minneapolis, MN, USA). All the experiments were carried out in 500 mL Erlenmeyer flasks by submerged cultivation. The response was filled as dry weight biomass.

Table 1. Levels of variation of independent variables in Plackett Burman design

Abbr.	Parameters	Levels of variation of independent variable	
		(-1)	(+1)
A	Dextrose, $g \cdot L^{-1}$	20.0	60.0
B	Yeast extract, $g \cdot L^{-1}$	2.5	7.5
C	Peptone, $g \cdot L^{-1}$	2.5	7.5
D	pH	5.0	6.0
E	Time, days	5	15
F	Agitation speed, rpm	100	200
G	Inoculum concentration, %	1.0	3.0

Results and discussion

Seven parameters (independent variables), including physical and nutritional factors such as concentration of some carbon and nitrogen sources (dextrose, yeast extract, peptone), inoculum concentration, pH value, agitation speed and the time of cultivation, were investigated in order to establish their effect on the *P. ostreatus* growth ability in a submerged system and the increase yield of biomass production.

The responses for biomass yield based on the variation of independent variables are presented in Table 2.

A large contrast mean, either positive or negative effects, indicates that each factor has a large impact on biomass production, while a mean close to zero means that the factor has little or no effect. This way, three factors were established that play an important role in biomass production: dextrose and yeast extract concentration and also the time of cultivation (Figure 1). The time of cultivation (*E*) seems to have the most significant effect on biomass production, followed by the yeast extract concentration (*B*). The dextrose concentration (*A*) could also have a positive influence on submerged mushroom multiplication also by correlation with the carbon and nitrogen ratio in fermentation medium composition. The other three biotechnological parameters – pH (D), agitation speed (F) and inoculum concentration (G)– have a negative influence on biomass growing and they were not considered for future study.

Table 2. The Placket-Burman factorial design of experiments and biotechnological responses

Run	Coded levels of variable							Yield of biomass
	A	**B**	**C**	**D**	**E**	**F**	**G**	**($g \cdot L^{-1}$)**
1	-1	1	1	1	-1	1	1	1.57
2	-1	1	1	-1	1	-1	-1	21.55
3	1	-1	-1	-1	1	1	1	9.57
4	-1	-1	-1	-1	-1	-1	-1	10.05
5	-1	-1	-1	1	1	1	-1	14.62
6	1	-1	1	1	-1	1	-1	9.72
7	1	1	1	-1	1	1	-1	25.71
8	-1	-1	1	1	1	-1	1	6.31
9	1	-1	1	-1	-1	-1	1	1.83
10	-1	1	-1	-1	-1	1	1	2.51
11	1	1	-1	1	-1	-1	-1	16.23
12	1	1	-1	1	1	-1	1	11.51

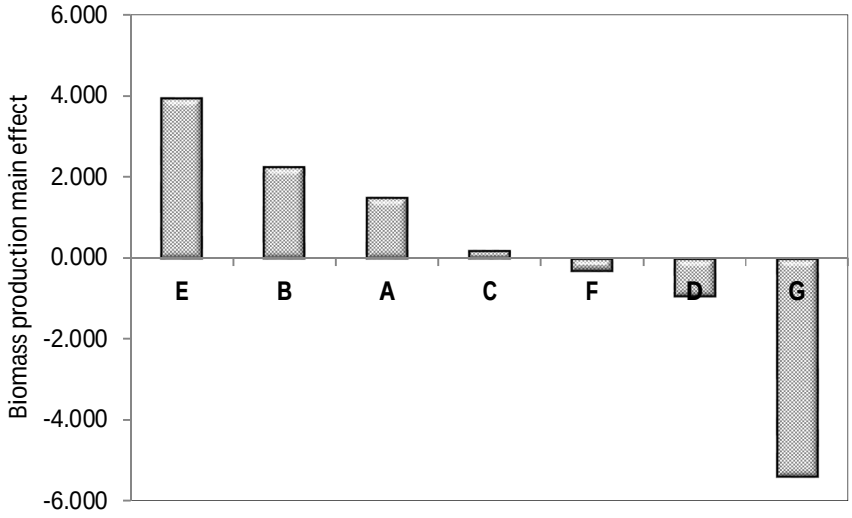

Figure 1. Effect of most important independent variables that influence *P. ostreatus* biomass production by cultivation in submerged conditions

After applying the ANOVA statistical test, the polynomial model equation was established to describe the *P. ostreatus* biomass production (Eq.2):

$$Y= 10.93 +1.50\,A +2.25B +0.18C -0.94D +3.95E -0.32\ \ F -5.38G \qquad (2)$$

where *Y* was the predicted *P. ostreatus* biomass production ($g \cdot L^{-1}$), *A* the concentration of dextrose ($g \cdot L^{-1}$), *B* the concentration of yeast extract ($g \cdot L^{-1}$), *C* the concentration of peptone ($g \cdot L^{-1}$), *D* the pH, E the inoculum concentration (%), *F* the agitation speed (rpm) and *G* the time of cultivation (days).

Table 3. Statistical analysis of biotechnological process for *P. ostreatus* biomass production by the independent variables variation

Source	Sum of Squares	DF	MeanSquare	F Value	Prob > F
Model	634.16	1	790.59	61.82	0.0007
A	26.88	1	26.88	18.34	0.0128
B	60.66	1	60.66	41.39	0.0030
C	0.40	1	0.40	0.28	0.6276
D	10.57	1	10.57	7.21	0.0549
E	186.91	1	186.91	127.55	0.0004
F	1.19	1	1.19	0.81	0.4183
G	347.55	1	347.55	237.16	0.0001
Residual	5.86	4	1.47		
Cor Total	640.02	11			

C.V.% =11.07; R^2=0.9908; adjusted R^2=0.9748

According to the data in Table 3, the Model F-value of 61.82 shows that the model was significant. There is only a 0.07% chance that a "Model F-Value" this large could occur due to noise. The R^2 values (multiple correlation coefficients) closer to 1 denoted high agreement between the experimental and the predicted responses and indicate that the mathematical model is very reliable in the present study. The coefficient of variation (CV) indicated the degree of precision with which the experiments were compared. A lower reliability of the experiment is usually indicated by a high value of CV; in the present case the low value of CV (11.07) showed that the experiments conducted were precise and reliable.

The parity plot (Figure 2) showed a satisfactory correlation between the experimental and the predicted values (obtained from Eq.2) of the *P. ostreatus* yield of biomass production in submerged condition cultivation, where the points around the diagonal line which indicated an optimal fit of the model, since the deviation between the experimental and predicted values was minimal.

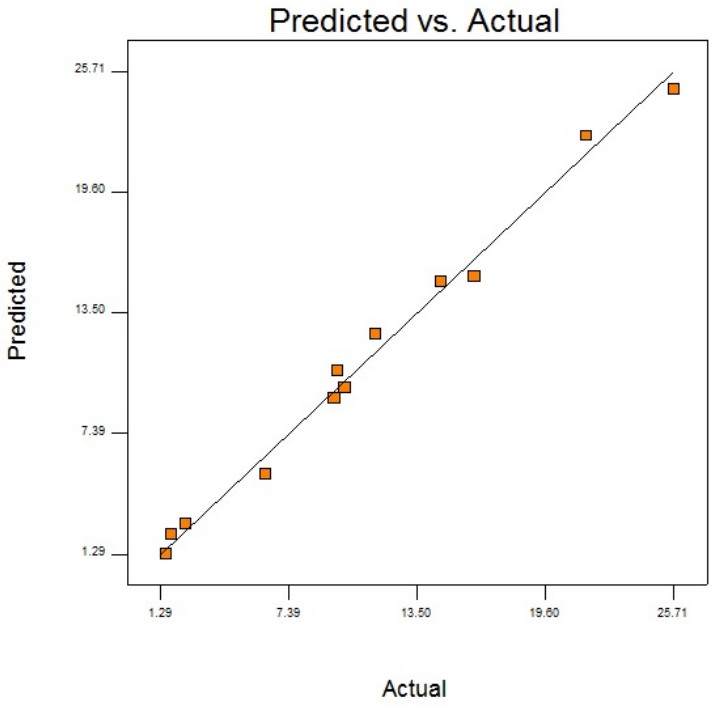

Figure 2. Parity plot presenting the distribution of experimental vs. predicted values of *P. ostreatus* biomass production

The literature describes several studies regarding the culture media optimization for *P. ostreatus* growing in submerged conditions. Gern *et al.* (2008) have used CSL (corn steep liquor) medium for obtaining the best biomass yield 29.64 $g \cdot L^{-1}$.

Comparable results were also obtained in our study where maximum biomass yields of 25.71 $g \cdot L^{-1}$ were achieved. The concentration of carbon source is important for *P. ostreatus* growing and this was also seen in other studies where an increase in the glucose concentration from 20 to 40 $g \cdot L^{-1}$ had a positive significant effect on the concentration of biomass, the maximum biomass productivity and the maximum specific growth rate (Gern *et al*., 2008). The carbon source was also investigated by Papaspyridi *et al*. (2010) where the effects of different carbon sources on the mycelia growth of *P. ostreatus* were investigated in shake flask cultures. The maximum biomass of 23.7±0.5, 21.1±0.5, and 20.5±0.8 $g \cdot L^{-1}$ was obtained when xylose, glucose, and trehalose were used, respectively. In other previous reports (Gbolagade *et al*., 2006; Gern *et al*., 2008) glucose was used as carbon source. In the present study dextrose indicated the best yields and was used as the primary carbon source for *P. ostreatus* submerged cultivation.

Papaspyridi *et al*. (2010) have demonstrated that biomass production was generally higher with organic nitrogen sources. This is consistent with the results of previous studies (Mikiashvili*et al*., 2006; Xu*et al*., 2008) that most basidiomycetes prefer complex organic nitrogen sources, because certain essential amino acid(s) may not be synthesized from inorganic nitrogen sources in the submerged culture of higher fungi. There are some researchers that have used organic nitrogen source for the production of bioactive compounds from basidiomycetes (Gern *et al*., 2008). In the present study the maximum *P. ostreatus* biomass yield of 25.71 $g \cdot L^{-1}$ was obtained using yeast extract as nitrogen source. Similar results, such as 23.4 and 21.5 $g \cdot L^{-1}$, were obtained by other researchers using CSL and soy-bean meal as nitrogen source (Papaspyridi *et al*., 2010).

Conclusions

The results offer information about the submerged cultivation conditions of *P. ostreatus* mushrooms in order to increase the yield of biomass. This plays a key role in adjusting the principal biotechnological parameters in order to increase the efficiency of the biotechnological process.

These preliminary data are important to establish the biotechnological conditions for mushroom cultivation in liquid medium with agitation and aeration. By applying the Plackett-Burman design of experiments and statistical analysis, it was possible to identify the most important three parameters with positive influence on biomass yield for seven independent variables. It was found that dextrose concentration, yeast extract concentration and cultivation time have the highest influence on biomass production in tested biotechnological conditions. The inoculum concentration and also the pH of the fermentation medium showed an indirect influence on mushroom multiplication. Based on the obtained results, it will be possible to optimize the biotechnological conditions by studying the correlative effect of the three variables with positive effect and applying the central composite design of experiments and the response surface methodology.

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