Production of *Pleurotus albidus* mycelia in fermentation bottles

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ABSTRACT

The search for sustainable alternatives to animal-based protein sources has gained prominence in recent years. Fungi mycelium foams have emerged as a promising substitute for traditional meat, addressing environmental concerns and promoting consumer health. In this study, we developed a system for submerged fermentation of *Pleurotus albidus* mycelia. The experimental setup involved three lab bottles (1000mL each) equipped with silicone stoppers, air filters, stainless steel rods, and hoses and an adapted air sparger. A pre-inoculum was prepared through fermentation in Erlenmeyer flasks for seven days. The cultivation medium for the submerged fermentation in the system consisted of 20 g/L of glucose and 10 g/L of yeast extract and 50 mL/L of mineral medium. The experiment was repeated four times, with varying outcomes. Mycelium moisture was 94% and dry mass differed among the bottles between 3.3 to 4.3 g, possibly due to mycelium adherence to the flask walls and variations in aeration. Mycelium grew as a pellet with diverse dimensions (1.36 to 6.59 mm). Protein content achieved ranged from 29.2 to 34.8. Glucose concentration analysis revealed unexpected findings. The results acquired show the importance of more research concerning the usage of filamentous fungus *P. albidus* mycelium as a vegan protein source and the necessity of improving the submerged fermentation system in eventual future works about this type of cultivation.

Keywords: Mycelia. Fermentation. Bottle. Protein

1 INTRODUCTION

Lately, there has been a focus on the search for possibilities of alternatives to protein sources when referring to vegan food. Knowingly, the usage of animal originated products promotes serious negative impact in the environment, helping increase the climate changes that currently hurt our planet. Lots of vegan alternatives however face some difficulties such as availability and seasonality. Considering this, it has emerged, through lots of backed by companies of the field research, the idea of using biomass made from fungi mycelium as a substitute for traditional meat[1].

Also, it can be appointed that not only food produced this way can help diminish the ecological problems, but it can also be interesting in terms of product quality and consumer's health and safety. That because it is a fact that edible mushrooms are, for example, poor in fat and rich in essential amino acids. This could provide an increase in the general well-being of a person, as well as in the combat of cardiovascular diseases of those who consume it[2].

Therefore, this research aims to build a system composed by a lab bottle and other materials in order to produce mycelia from filamentous fungi *Pleurotus albidus* through submerged fermentation.

2 MATERIAL & METHODS

The materials used to build the fermentation system were: three lab bottles with 1000 mL of total volume, four silicone stoppers, five 0.2 µm air filters, two hose connectors, twelve stainless steel rods, 21 silicone hoses, three plastic taps, three metallic needles, and a Kitassato containing hydrophobic cotton. Each of the bottles were sealed with a perforated stopper, which contained the three stainless steel rods and the metallic needle.

One of the rods was meant for the entrance of air at the system, through a hose at the outside part of the bottle, and a hose full of tiny holes at the inside area. Collecting samples was possible through another one of the rods, that contained hoses connected in both far ends of it, but with the plastic tap at the handling location. The remaining rod had no hose coupled at the interior, for it had the proposal of taking away the air produced by the fungus in the fermentation. At last, the metallic needle was linked with a small diameter hose at the upper region in order to insert any necessary solutions or liquids in general. At Figure 1, it gets easier to visualize the proposed bioreactor system.

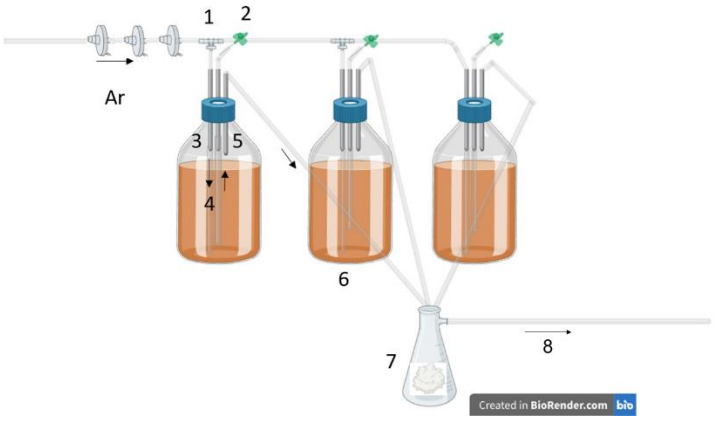

Figure 1 Schematic representation of the fermentation system, made in Biorender. 1- Filters and connectors; 2 - Sample collection; 3 - Air entrance; 4 - Sample suction; 5 - Air exit; 6 - Air conduction to Kitassato; 7 - Cotton; 8 - Air flowing to environment

The fungus *Pleurotus albidus* was provided by the Enzyme and Biomass Laboratory at the Institute of Biotechnology, University of Caxias do Sul and then transported to the laboratory Lieb at the Federal University of Santa Catarina. In order to keep the research going on, the strain was cultivated through an allocation of a 5 mm diameter disc of the microorganism mycelia on Petri dishes previously filled with PDA medium, followed by an incubation at 28ºC in the vacancy of light for 14 days.

Later, these Petri dishes were put in a refrigerator at 4ºC. The pre-inoculum was prepared in a way that three 250 mL Erlenmeyer flasks were filled each with a 66 mL culture medium that had this composition: 10 g/L of glucose, 5 g/L of yeast extract, as well as 50 mL/L of a mineral medium adjusted to pH 5.3, that was constituted of 14 g/L of $(NH_4)SO_4$, 3 g/L of Urea, 20 g/L of KH_2PO_4, 3 g/L of $MgSO_4.7H_2O$, 1.2 g/L of $CaCl_2$, 0.05 g/L of $FeSO_4.7H_2O$, 0.014 g/L of $ZnCl_2$, 0.0156 g/L of $MnSO_4.H_2O$, e 0.02 g/L of $CoCl_2.6H_2O$. After that, one disc of *P.albidus* mycelia that was stored was transferred to each of the flasks, previously sterilized, and then these were put in a shaker at 28 ºC and in a rotation of 150 rpm where it fermented for 7 days[3].

At the seventh day, the Erlenmeyer flasks were taken into a flow, where the entire volume of pre-inoculum contained in them was transferred to the bottles, which had previously been filled with 600 mL of medium and sterilized, making the final volume in each had a volume ratio of 10% v/v between pre-inoculum and inoculum. This medium directed to fermentation in the bottles was prepared from 20 g/L of glucose, 10 g/L of yeast extract, and 50 mL/L of mineral medium.

Then, 100 µL of Ampicillin was added to each bottle for bactericidal effects, as well as 0.5 mL of anti-foaming agent. The next step was to connect all the hoses and assemble the system while still in the flow, in order to avoid the possibility of contamination as much as possible. With this part completed, the set was taken to an incubator at 28°C where it was connected and the air flow was adjusted. The fungi were fermented for seven days, with samples of the medium being collected every 24 h, counting the first day (sample zero). These were used to make an analysis of the glucose concentration, with the application of the high-performance liquid chromatography (HPLC) method.

Regarding the biomass collected, in order to quantify this result of the experiment, the mycelium of each recipient was filtered separately using a vacuum pump. The wet mass was then collected in 50 mL Falcon flasks, whose weights were previously determined, and then weighted each at an analytical balance. A part of the mass from test 4 was then put in a Petri disc filled with water in order to make a pellet size evaluation. Size data were obtained using the software Image J. Later those Falcons were lyophilized and again weighted. To determine the protein percentage, the micro-Kjeldahl method was utilized. [3]

3 RESULTS & DISCUSSION

The experiment was repeated four times. At the first two runs no visible growth of the microorganism was observed, which was caused both by a failure in the continuous aeration system and by contamination from some other biological agent. At the third one, the system functionality was a success in general and no problems regarding contamination were perceived, however the results related to the glucose consumption by the microorganisms were at odds with what was expected. This way, fermentation was carried out for the fourth time, where the sampling system was improved so that an analysis of sugars in the process could be obtained, and this time one of the bottles happened to get its cultive medium compromised by the presence of another microorganism, at the sixth day of fermentation. The biomass constituted of *P.albidus* mycelium had a satisfactory growth after seven days of fermentation under this conditions.

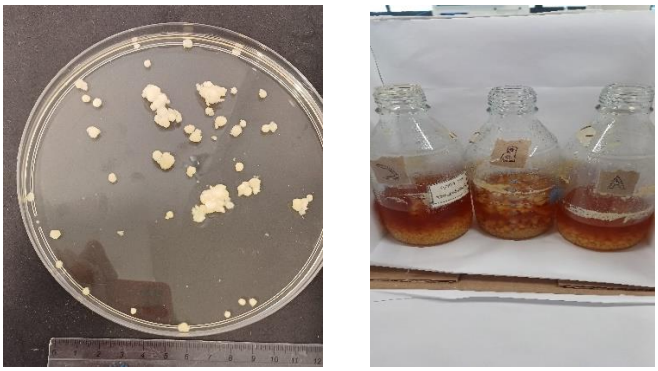

Figure 2 Picture of generated pellets on the fourth run (left), and the bottles from the third run after the fermentation was finished (right).

Considering the calculated moisture content, the values were respectively: 94.5% ± 1.0% for run III; 94.2% ± 0.3% for run IV (Table 1). The total dry mass was 3.3 g ± 0,04 g for run III; 4.0 g ± 0.4 g for run IV revealing reproducibility between the bottles, but not between the runs. It can be explained because part of the fungus biomass got taken to the bottle's wall, where it got stuck. Also, another reason could be that some of the mycelium actually grew over the aeration hose, the one containing small holes, what probably caused a difference in the bottles air flux, and consequently the mass growth. Concerning the dimensions of the generated pellets in the fourth test, the values obtained were 2.86 ± 1.33 mm for bottle B and 4.75 ± 1.11 mm for bottle C. It could be concluded through a Tukey test with a 5% significance level that there was a significant difference between the pellet size values. When visualizing the inside of the glass, there could be seen junctions of cells that had various shapes and mostly big sizes comparatively. Also, the measurements ranged from 1.36 mm to 6.59 mm. The glucose concentration for the fourth test, however, showed some unexpected results. It was expected that initial amount of glucose (20 g/L) would be consumed throughout the days, but at the end of 7 days the concentration increased to 33.7 g/L. One explication could be that during the fermentation a considerable part of the water evaporated, what led to a possible concentration of the growth medium. Concerning the protein percentage for the collected biomass, the results are the following: 34.8% ± 1,2% for III; and 29.2% ± 1.0% for IV. These percentages were superior if compared to the 20.4% reported by other study of *P. albidus*[2].

Table 1- Summary of the main results of the runs performed with the build fermentation system using *P. albidus*.

Experimental Run	Moisture (%)	Dry Mass (g)	Protein (%)	Pellet size range (mm)
III	94.5 ± 1.0	3.3 ± 0,04	34.8 ± 1,2	-
IV	94.2 ± 0.3	4.0 ± 0.4	29.2 ± 1.0	1.36 to 6.59

4 CONCLUSIONS

In conclusion, the results acquired show the importance of more research concerning the usage of filamentous fungus *P. albidus* mycelium as a vegan protein source and the necessity of improving the submerged fermentation system in eventual future works about this type of cultivation. A suggestion of improvement would be the implementation of a condensation system at each bottle, in order to avoid medium concentration due to evaporation. Another suggestion would be the reallocation of the air filters to the bottles' aeration entrance, being that it would potentially lower the chances of contamination of the system. The data regarding protein percentage presented small difference between the flasks per experiment, and were in accordance to the expected, as well as the dry weight and moisture calculated.

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