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# RESEARCH ON OBTAINING *LIQUID* MYCELIA FROM *PLEUROTUS* SPP. STRAINS AND TESTING THEIR FRUITING POTENTIAL

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Current Trends in Natural Sciences

***Abstract***
*The paper focuses on the obtaining biotechnology and in vitro propagation of edible and/or medicinal macromycetes mycelia from the genus Pleurotus in submerged cultures in order to produce the biological "seeding" material - spawn. The quality and biological purity of the spawn is critical to the success of mushroom production and productivity. Starting from stock cultures from the collection of the RDIVFG Vidra, the mother cultures were obtained, the biological material being represented by two strains of Pleurotus ostreatus and one strain of Pleurotus citrinopileatus, each of them being cultivated in PDB + K2HPO4 (1,5g/l), pH 6.5 medium at different stirring speeds (0/100/150 rpm). The submerged growing biomass was used to inoculate wheat caryopsis, that evolved into spawn after complete colonization. The lignocellulosic substrate was obtained from wheat straws with the addition of nutritional supplements (wheat and maize bran and sunflower middlings) and distributed in the polypropylene bags. Their yielding capacity was verified in the fruiting chamber of the laboratory micro-mushroom farm test facility.*

***Keywords**: liquid inoculum, mycelium, Pleurotus citrinopileatus, Pleurotus ostreatus, submerged cultivation*

## 1. INTRODUCTION

The macromycetes species observed in this paper are lignivorous/xylophagous species from the genus *Pleurotus* which produce bundles of mushrooms of various colors ranging from pale violet to light/dark grey (*P. ostreatus*) and lemon-yellow (*P. citrinopileatus*). Their taste and smell are pleasant, mushrooms being highly valued for their nutritional, gastronomic and medicinal values, properties that give them the status of functional foods. The production of mycelium using submerged cultures leads to a wide range of economically significant applications including the production of nutritious biomasses for feeds, products or supplements containing biocompounds, the obtaining and isolating enzymes, nutraceuticals etc. (Gregori et al., 2007). The *in vitro* cultivation biotechnologies of macromycetes mycelium in submerged cultures have several advantages including: an increased rate of colonization both on cereal caryopsis and in the substrate, dispersal of metabolic products throughout the liquid, reducing their accumulations and more uniform characteristics of mycelial colonies. Submerged mycelium cultivation is an effective method for increasing biomass yields, especially when applied with naturally slow-growing species. Submerged mycelium cultivation is well-suited for rapid biomass production, especially when combined with stirred tank bioreactor technologies. This biotechnological technique allows for the rapid creation of therapeutic metabolites (Petre et al., 2021).


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## 2. MATERIALS AND METHODS

Biological material: 2 strains of *Pleurotus ostreatus*: PoM-77 and PoM-357 and one strain of *Pleurotus citrinopileatus*: PcM-95. Pure dikaryotic mycelium disks were aseptically obtained (cork borer Ø5mm) from PDA Petri dishes (90 mm) and inoculated into Erlenmeyer flasks containing 200ml of PDB (VWR Chemicals) + $K_2HPO_4$ (1.5 g/l), pH 6.5; each inoculated with 5 mycelium fragments. Incubation was performed in dark conditions on an orbital shaker at 3 different speeds (0/100/150 rpm) for 14 days and at a temperature of 24-26ºC.

For the preparation of the spawn, pre-cooked wheat caryopsis were homogenized with 2% $CaCO_3$ and 6% $CaSO_4$, distributed in Erlenmeyer flasks (250g each) and sterilized by autoclaving at 123ºC for 2h. The sterile grains were inoculated with a volume of approx. 65 ml liquid culture medium containing numerous mycelial fragments minced with a magnet, 5 replicates for each strain. Incubation was performed in the dark, at 26ºC for 10 days.

The lignocellulosic substrate was made using 3-5cm wheat straws, brought to the required humidity by submersion in water. After 24h, the excess water was removed, leaving the substrate with a moisture content of 65-70%. After homogenization with 2% $CaCO_3$, 6% $CaSO_4$ and 3% nutritional supplements: wheat and maize bran along with sunflower middlings, it was distributed into polypropylene bags containing 1.5kg of substrate. Sterilization was done by autoclaving for 100 minutes at 121°C. The inoculation was performed under aseptic conditions, at a spawn rate of 8%, 120g spawn/1.5kg bag. Incubation was realized in no light conditions, at the temperatures of 26-28ºC for 3-4 weeks. The fruiting potential was observed when the bags were completely colonized by the spawn and transferred to the fruiting chamber.

## 3. RESULTS AND DISCUSSIONS

There were no fungal or bacterial infection detected in the samples. All mycelia showed vitality, exhibiting typical white, conforming cultural traits and similar tendencies as the macromycetes cultures developed. In static cultures, the PoM-77 was presented as globular masses (single/ few individual formations) with a tendency to conglomerate, hyphal fringes at the periphery of the colony, mostly submerged but with stroma at the surface of the liquid and a medium-raised value for the density of mycelial biomass. PoM-357 produced unique and spherical, almost entirely submerged cultures with very little stroma and an average mycelial density. PcM-95 strain developed completely submerged cultures, unique and globular, without stroma and a “fluffy” and airy appearance. Submerged mycelial cultures require agitation and aeration to facilitate the movement of nutrients and oxygen from and between the nutritive media and the mycelium. Figure 1 shows the submerged cultures developed in static submerged culture conditions.

At 100 rpm (Figure 2), the mycelium of the PoM-77 strain is displayed as a unitary, compacted biomass that tends to fill almost entirely the volume of the flasks, presenting a fibrous-fringed structure towards the periphery of the colony. PoM-357 developed as a unitary, globular mass, similar to the mycelium of the PoM-77 strain, with a smaller growth but larger than that of the PcM-95 strain. *P. citrinopileatus* produced unique, globular, loose, airy and fringed biomasses in smaller quantities than both *P. ostreatus* strains.

At a stirring speed of 150 rpm (Figure 3), it was found that all strains formed completely submerged mycelial biomasses, without stroma and developed as spherical bundles of different sizes. The PoM-77 strain shows globular mycelial masses that are much more intertwined with each other, with a tendency to form a unitary whole, as well as the largest of all three strains. The PoM-357 strain appearance of the mycelial spheres is similar to that of the other *P. ostreatus* strain, smaller


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and slightly better individualized. PcM-95 records the smallest dimensions of its better-defined colonies.

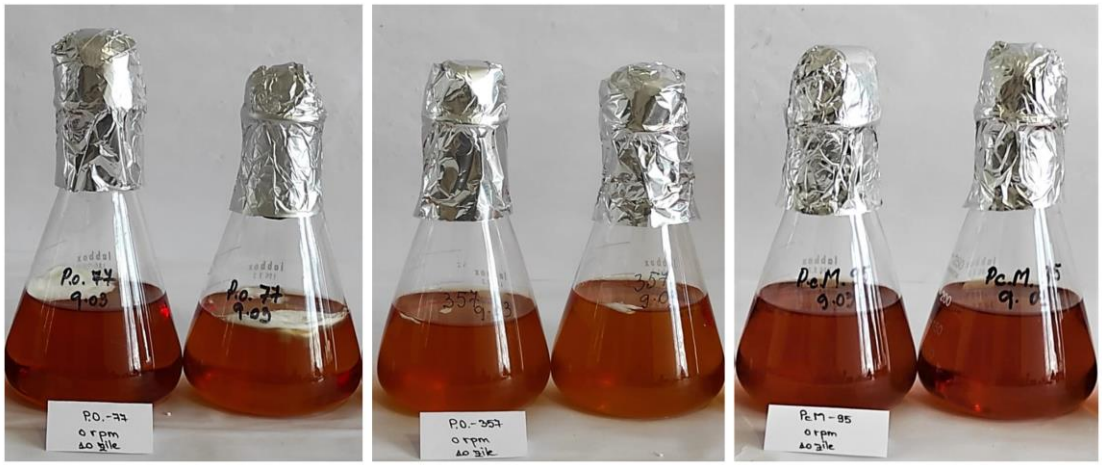

***Figure 1. Mycelial biomass grown submerged at 0 rpm (static cultures)***

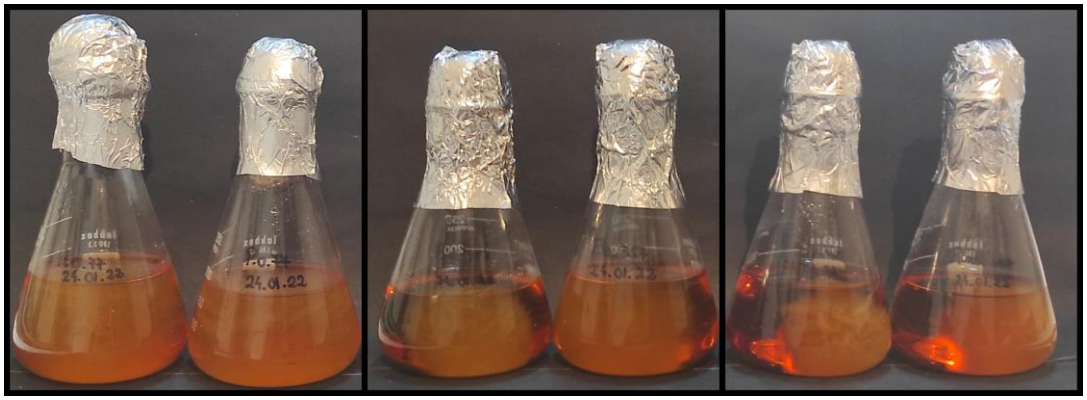

***Figure 2. Mycelial biomass grown submerged at 100 rpm (L-R: PoM-77, PoM-357 and PcM-95)***

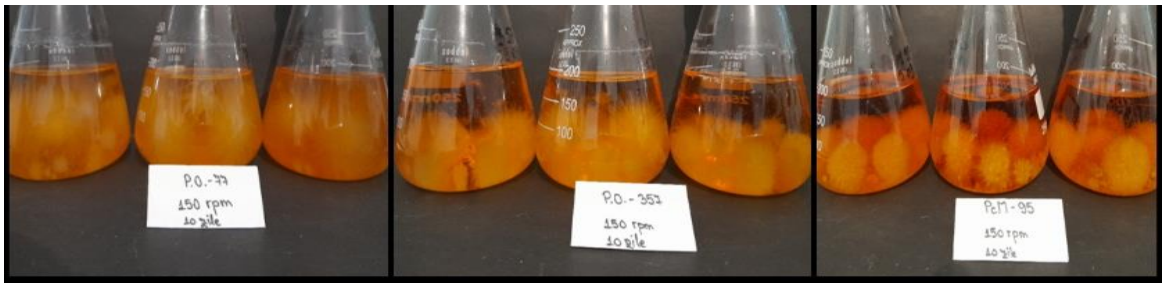

***Figure 3. Mycelial biomass grown submerged at 150 rpm***

The targeted species, nutrient composition, temperature, value of pH, $O_2$ and $CO_2$ concentration, light conditions, inoculum volume, agitation speed and the presence/ absence of particles into the liquid media are all closely related to the qualitative and quantitative attributes of submerged evolution of the mycelial biomass. Stirring speed is an important physical factor for the cultures


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cultivated in the liquid culture media because it facilitates the transport of nutrients and oxygen from the medium towards the mycelium, the agitation regime influencing the quantity and quality of fungal colonies.

To obtain and evaluate the resulting biomass at the end of incubation, they were separated from the medium, washed, filtered and dried in an oven for 72 hours at 40°C, the weight of the biomass being measured before and after drying (Table 1).

***Table 1. Results of weighing fresh and dry biomasses***

| Strain/ replicate | 0 rpm | | 100 rpm | | 150 rpm | |
|---|---|---|---|---|---|---|
| | Fresh biomass (g) | Dried biomass (g) | Fresh biomass (g) | Dried biomass (g) | Fresh biomass (g) | Dried biomass (g) |
| PoM-77/1 | 2.446 | 0.078 | 10.802 | 0.604 | 6.367 | 0.704 |
| PoM-77/2 | 1.201 | 0.068 | 13.211 | 0.695 | 4.246 | 0.506 |
| PoM-77/3 | 0.808 | 0.189 | 10.056 | 0.581 | 6.528 | 0.558 |
| PoM-357/1 | 0.342 | 0.089 | 7.712 | 0.449 | 4.169 | 0.256 |
| PoM-357/2 | 0.690 | 0.004 | 8.201 | 0.411 | 8.738 | 0.492 |
| PoM-357/3 | 1.728 | 0.067 | 7.921 | 0.437 | 3.933 | 0.370 |
| PcM-95/1 | 0.430 | 0.090 | 5.311 | 0.440 | 5.119 | 0.588 |
| PcM-95/2 | 0.607 | 0.010 | 3.535 | 0.379 | 6.920 | 0.579 |
| PcM-95/3 | 0.726 | 0.138 | 4.032 | 0.435 | 7.967 | 0.486 |

***Table 2. Influence of strain and agitation speed on amount of fresh biomass***

| Strain/ speed | Average fresh biomass (g) | Relative fresh biomass (%) | Difference ± d (g) | Significance of difference |
|---|---|---|---|---|
| PoM-77/st | 1.485 | 161.4 | +0.565 | - |
| PoM -77/100 rpm | 11.356 | 1234.3 | +10.436 | *** |
| PoM /150 rpm | 5.714 | 621 | +4.794 | *** |
| PoM-.357/st (Mt) | 0.920 | 100 | - | - |
| PoM-.357/100 rpm | 7.945 | 863 | +7.025 | *** |
| PoM-.357/150 rpm | 5.613 | 610.1 | +4.963 | *** |
| PcM-95/ st | 0.588 | 63.9 | -0.332 | - |
| PcM-95/100 rpm | 4.293 | 466.6 | +3.373 | ** |
| PcM-95/150 rpm | 6.669 | 724.8 | +5.749 | *** |

DL 5 % = 2.391
DL 1 % = 3.294
DL 0,1 % = 4.535


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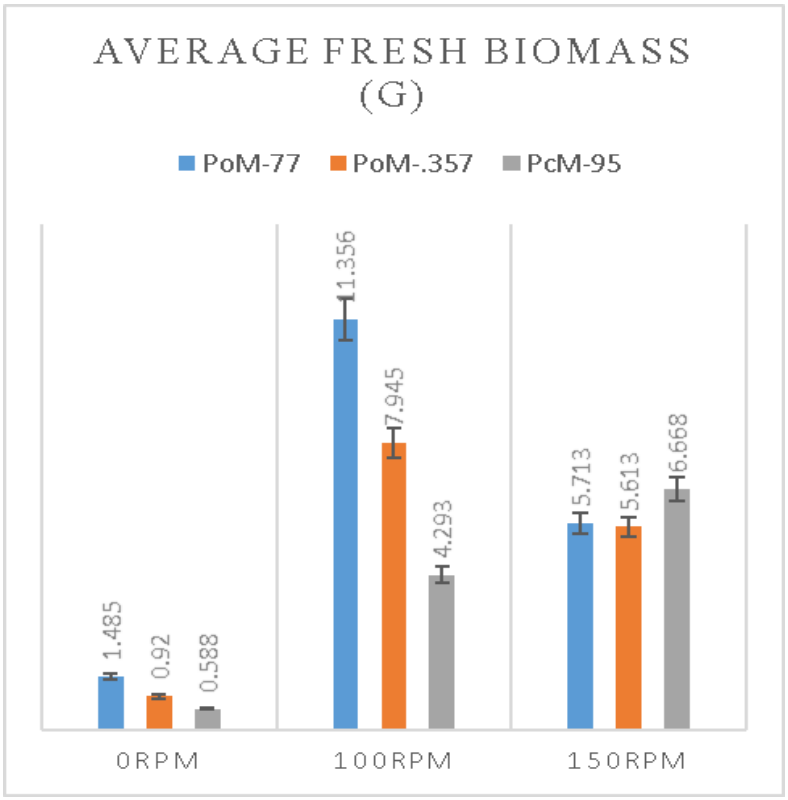


***Figure 4. Average fresh biomass production***

***Table 3. Influence of strain and agitation speed on the amount of dry biomass***

| Strain/ speed | Average dried biomass (g) | Relative of dried biomass (%) | Difference ± d (g) | Significance of difference |
|---|---|---|---|---|
| PoM-77/st | 0.112 | 211.3 | +0.059 | - |
| PoM -77/100 rpm | 0.627 | 118.3 | +0.574 | *** |
| PoM /150 rpm | 0.589 | 111.1 | +0.536 | *** |
| PoM-.357/st (Mt) | 0.053 | 100 | - | - |
| PoM-.357/100 rpm | 0.451 | 850.9 | +0.398 | *** |
| PoM-.357/150 rpm | 0.373 | 703.8 | +0.320 | *** |
| PcM-95/ st | 0.079 | 149.1 | +0.026 | - |
| PcM-95/100 rpm | 0.418 | 788.7 | +0.365 | *** |
| PcM-95/150 rpm | 0.551 | 104 | +0.498 | *** |

DL 5 % = 0.123
DL 1 % = 0.169
DL 0,1 % = 0.233


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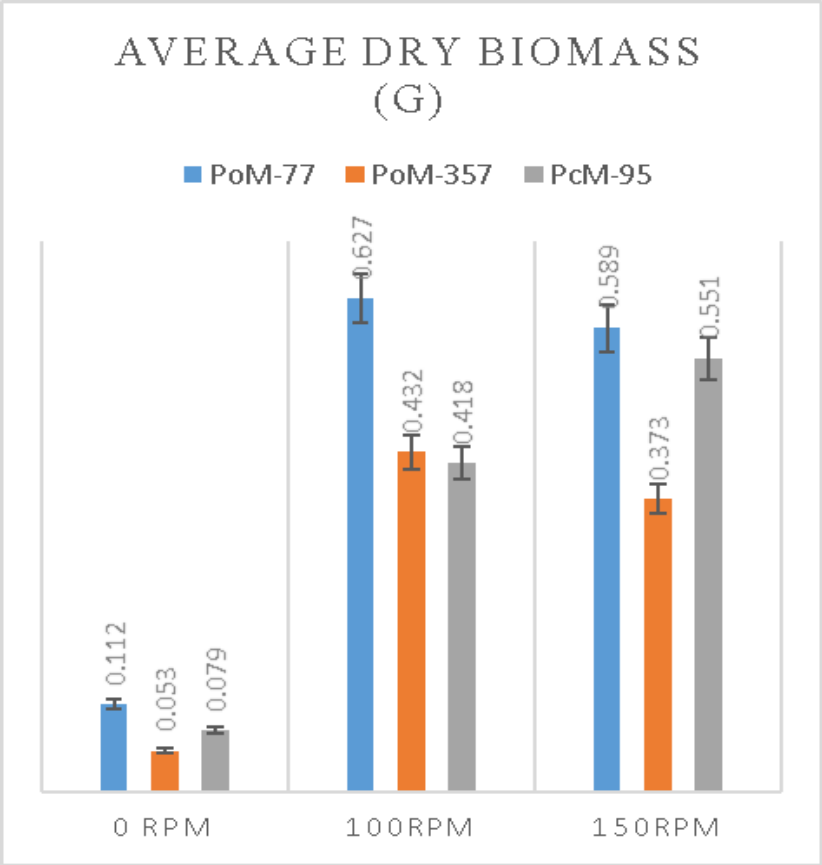


***Figure 5. Average dry biomass production***

Mycelial growth was accelerated in stirred flasks compared to the non-stirred flasks. The highest quantity of mycelial biomass was obtained at a stirring speed of 100rpm, with PoM-77 having the highest growth among the strains with an average of 11.35g fresh biomass, respectively 0.62g dried biomass, followed by the PoM-357 strain with 7.94g fresh biomass and 0.43g dried biomass respectively and finally the PcM-95 strain with 4.29g fresh biomass and 0.41g dried biomass. The stirring has a significant effect on biomass production, other studies mentioning better results in terms of obtaining submerged biomasses in the range of this stirring speed (Yang and Liau, 1998; Wu, 2008, Vamanu et al., 2011; Kirsch et al., 2016).

The *liquid* inoculum is prolific in the colonization of the wheat grains due to the multitude of inoculation points that led to a rapid and complete colonization of the caryopsis and the obtaining of the spawn. The PoM-77 and PoM-357 strains had the fastest and most vigorous growth, with the tendency to compact the cereal grains, a more pronounced aspect in the case of the PoM-77 strain. The PcM-95 strain colonized caryopsis at a slightly lower rate than both *Pleurotus ostreatus* strains, presenting a looser mycelium with its typical sweet fragrance.

The inoculated bags did not show bacterial or fungal infections and at the same time no exudate drops were observed. Although all the 3 strains have similar tendencies and characteristics as mycelium grows and develops with its tendency to compact the lignocellulosic substrate, both *P. ostreatus* strains had the most vigorous growth compared with the PcM-95 strain, PoM-77 having the fastest growth.


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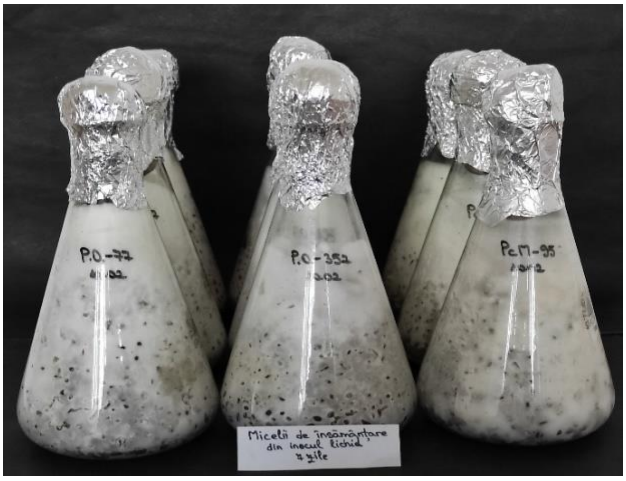


***Figure 6. Spawn produced from liquid inoculum – Day 7 of incubation***

***Figure 7. Mycelial colonization into substrate***

For an exact measurement of the mycelial growth, 24 glass tubes (24x4.5x0.5 cm) were filled with 50g of 2 lignocellulosic substrate variants:

- V0: wheat straws + 2% $CaCO_3$ + 6% $CaSO_4$;
- V1: wheat straws + 2% $CaCO_3$ + 6% $CaSO_4$ + 3% nutritional supplements (wheat bran, maize bran and sunflower middlings), 10% corn cobs

The sterilization of tubes was made by autoclaving for 90' at 121°C. After cooling, the inoculation has been aseptically performed using 7-10g of spawn at the surface of the substrate, thus being able to measure the development front of the mycelium. Incubation was made in the absence of light at 24-26°C for 2 weeks.

The development of mycelium was achieved at very close values on both substrate variants, slightly higher on the V0 type, taking into account the biological nature of the material and its response, the spatial development in a tube because the seeding mycelium is normally homogenized throughout the mass of the substrate for a fast and multidirectional colonization compared to the colonization started from the substrate surface, combined with the more difficult to digest composition of the V1 variant, the $O_2/CO_2$ differences from the substrate surface compared to the levels inside the tubes and knowing the fact that a slightly slower mycelium growth in a recipient does not necessarily imply a lower yield of fruiting.


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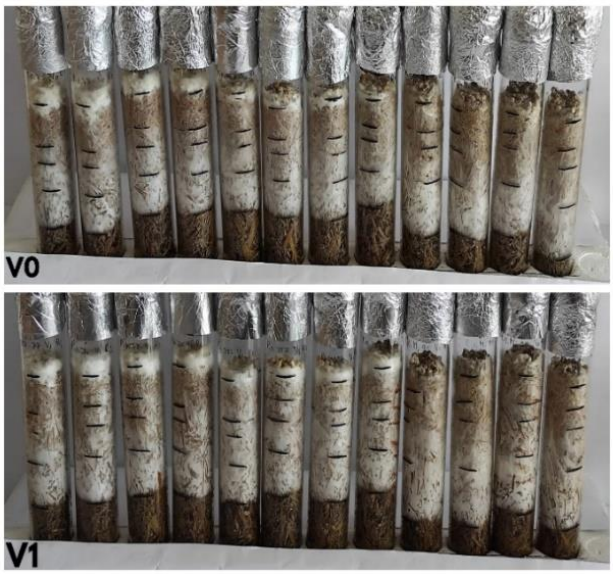


***Figure 8. Mycelial growth into substrate tubes (L-R: PoM-77, PoM-357 and PcM-95)***

After the spawn produced from the *liquid* inoculum had colonized the lignocellulosic substrate, all the bags were transferred to the fruiting chamber to observe their mushroom production. The onset of fruiting was marked by the appearance of the primordia, and the microclimate conditions provided in the fruiting chamber aimed a temperature of 16-18$^{o}$C, a relative humidity in the air of at least 90-95% at the appearance of the primordia, decreasing to 80-85% with the development of basidiofruits, a light regime of 8 hours/day, a maximum $CO_2$ concentration of 1200 ppm and adequate ventilation to ensure air recirculation, the introduction of fresh air and at the same time a relatively slow movement of air masses very beneficial to *Pleurotus* mushrooms. Mushroom will reach harvest maturity in 6-8 days from the onset of primordia.

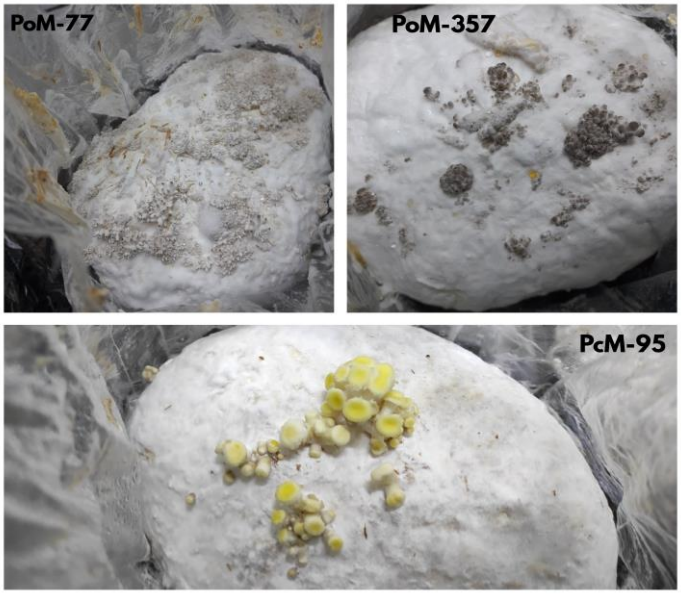


***Figure 9. Fruiting onset and primordia appearance***


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***Figure 10. Firsh flush***

| | |
|---|---|
| PoM-77 has a shell-shaped cap in a variety of hues ranging from purple to light and dark gray. The gills are decurrent till they reach the stipes and pileus insertion point. The spores are purplish in color. The stipes is white, short and inserted laterally. They grow in bouquets and has a pleasant odor and flavor. |  |
| PoM-357 features a shell-shaped cap that ranges in color from light and dark gray to brownish. The gills are decurrent until they reach the stipes and pileus insertion site. White stipes are short and inserted laterally. They grow in bouquets and have a great aroma and taste. |  |
| PcM – 95 has a lemonish yellow cornet-shaped hat. The gills reach all the way to the bottom of the stipes (lengthy and firmly bent). The bouquets are used to create the appearance. The mushrooms have a typical pleasant scent. Basidiofruits appear in bouquets with a common insertion point and will achieve commercial maturity in 4-6 days from the primordial occurence, which corresponds to the closed cone phase with flat sides. |  |


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The three strains produced bundles of robust and high-quality mushrooms exhibiting *Pleurotus*-specific traits as well as strain-specific characteristics, such as pileus color ranging from light to dark gray, pale violet, brownish and lemon yellow (*P. citrinopileatus*) with delicate stripes of various colours. The average amount of mushrooms harvested in the 1st fruiting flush was: PoM-77: 226.85g; PoM-357: 175.15g and PcM-95: 60g.

## 4. CONCLUSIONS

The PoM-77 strain had the fastest growth and was the most vigorous of all the three strains, giving the best results in most of the experiments and mushrooms production yield. Stirring speed is essential for mycelial cultures, among with other psychological factors for submerged cultivation. The stirring influenced the quantity and quality of the mycelial cultures. The highest quantity of biomass was obtained at a stirring speed of 100 rpm. At the same time, the stirring speed had a significant impact on the formation of mycelial colonies, static cultures producing less biomass, being more airy, loose and having a lower hyphal density, whereas stirring rates of 100/150 rpm resulted in higher biomass quantities and volumes and a stronger compaction tendency. The expansion rate of the macromycetes mycelium cultures in the glass tubes gives valuable information on the vitality of a strain, the rate of growth and development being one of many quality factors for the characterization of the macromycetes mycelia. The obtaining of submerged mycelium biomass has several advantages including the increased growth rate over the cereal grains due to the multiple inoculation points, assured biological purity because of an easier detection of contaminations, the ease of handling of the Erlenmeyer flasks because the inoculation was performed in a single pouring movement of liquid inoculum, a fast and efficient technique that lowers the risk of infections.

## 5. ACKNOWLEDGEMENTS

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## 6. REFERENCES

Gregori, A., Švagelj, M., Pohleven, J. (2007). Cultivation techniques and medicinal properties of *Pleurotus* spp. *Food Technology and Biotechnology*, *45*(3), 238-249.

Kirsch, L.D.S. et al. (2016). Production of mycelial biomass by the Amazonian edible mushroom *Pleurotus albidus*, *Brazilian journal of microbiology*, *47*, 658-664.

Petre, A., Ene, M., Vamanu, E. (2021). Submerged Cultivation of Inonotus obliquus Mycelium Using Statistical Design of Experiments and Mathematical Modeling to Increase Biomass Yield. *Appl. Sci.*, *11*, 4104.

Vamanu, E. et al. (2011). Antioxidant and antibacterial properties of the extracts from *Pleurotus ostreatus* EVFB1 and EVFB4, *Romanian Biotechnological Letters*, *16 (1)*, 40-46.

Wu, C.Y. (2008). The influence of cultivation conditions on mycelial growth and exopolysaccharide production of culinary-medicinal mushroom *Pleurotus citrinopileatus* Singer (*Agaricomycetideae*), *International Journal of Medicinal Mushrooms*, *10* (3).

Yang, F.C., Liau, C.B. (1998). Effects of cultivating conditions on the mycelial growth of *Ganoderma lucidum* in submerged flask cultures, *Bioprocess Engineering*, *19 (3)*, 233-236.


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