Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 22 (2) : 2020 : 359-368

ISSN-0972-3005

EFFECT OF CULTURE PRESERVATION METHODS IN THE STABILITY AND NUTRITIONAL CHARACTERISTICS OF *PLEUROTUS OSTREATUS*

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(Received 16 January, 2020; accepted 3 March, 2020)

Key words: *Strain conservation, Pleurotus ostreatus, Productivity, Viability, Fruiting body.*

Abstract – The fungi of the genus *Pleurotus* correspond to the second group of macromycetes most commercialized worldwide. Its nutritional properties include a low caloric intake with a high content of protein, fiber, and carbohydrates, as well as the presence of essential amino acids. The yields of the *Pleurotus* production and its chemical composition can be affected by the substrates used for its production, as well as the strains conservation methods. The present investigation analyzed the influence of the methods of preserving *Pleurotus ostreatus* strains in sterile distilled water, sterile mineral oil, sterile saline solution and periodic subculture, the purity, and viability of the preserved strains, biological efficiency and rate of productivity in the crop, and the nutritional properties of the fruiting body of *Pleurotus ostreatus*. It was found that the conservation methods evaluated maintained the viability and purity of all preserved strains, maintaining their macro and microscopic characteristics after four months of storage at 4 °C without affecting the nutritional characteristics potential. Furthermore, the rate of productivity and biological efficiency of the cultivation was not affected by the preservation method evaluated for strain preservation of *Pleurotus ostreatus*.

INTRODUCTION

The fungi of the *Pleurotus* genus correspond to the second most commercialized group of macromycetes worldwide (Royse *et al*., 2017; Sekan, *et al*., 2019). They are characterized by their high nutritional, economic, ecological value, as well as their medicinal properties, exhibiting antitumor, antibiotic, antiviral anticholesterol, and immunomodulatory activity (Sarangi *et al*., 2006; Selegean *et al*., 2009). Its nutritional properties include a low caloric intake with a high content of protein, fiber, and carbohydrates, as well as the presence of essential amino acids such as arginine, glutamine, and glutamic acid (da Silva *et al*., 2012). *Pleurotus* fungi have enormous biotechnological potential given their ability to degrade lignocellulosic materials, bioaccumulate heavy metals, produce nutraceutical metabolites of interest, among others (Selegean *et al*., 2009; Dickson *et al*., 2020; Sekan *et al*., 2019).

The genus *Pleurotus* is made up of about 40 species of fungi (Kües and Liu, 2000), with *Pleurotus ostreatus* being the most economically important. This species is characterized by the presence of bioactive substances of medical interest such as polysaccharides, peptides, terpenoids, esters of fatty acids and polyphenols (Kumar, 2020), as well as their ability to grow on various lignocellulosic substrates producing violet oyster shaped black carpophores, whose size can vary between 5 and 12 cm in diameter depending on the age of the fungus and the growth conditions (Delmar *et al*., 2011). *Pleurotus* production yields and chemical composition may be affected by the substrates used for its production (Shashirekha *et al*., 2005).

However, the conservation of the strains plays a fundamental role in the viability, availability, and protection of the phenotypic, genotypic and industrial potential characteristics of microbial species (Ryan and Smith, 2004; Henao, *et al*., 2006; Morales *et al*., 2010; Montesinos Matías, *et al*., 2015).

There are various methods for the maintenance and conservation of microbial species such as lyophilization, cryopreservation, periodic transfer, conservation by suspension in sterile distilled water, conservation of mineral oil layer, drying on filter paper, drying on soil, sand, silica gel, among others (García and Uruburu, 2000; Ryan and Smith, 2004; Weng, *et al*., 2005; Gato, 2010). The choice and application of the method will depend directly on the expected shelf life and the type of microorganism (Rico *et al*., 2004). The purpose of this work is to evaluate the effectiveness of sterile mineral oil, 0.85% sterile saline solution, sterile distilled water, and periodic subculture as conservation methods of *Pleurotus ostreatus* and its impact on biological efficiency, crop productivity rate and the nutritional properties of the fruiting body.

MATERIALS AND METHODS

Obtaining the biological material

The strain of the fungus *Pleurotus ostreatus* (PO2) was supplied by the National Coffee Research Center - CENICAFE. The strain was recovered in petri dishes with Potato dextrose agar (PDA, Oxoid) and was incubated at 28 °C for 15 days until the mycelium covered the entire surface of the petri dish (90 x15 mm).

Inoculation of the fungus on paper discs

In order to evaluate the different conservation methods, the fungus was inoculated in petri dishes with PDA agar (Oxoid) containing 5 mm diameter filter-sterilized paper discs (Whatman # 1). Incubation was performed at 28 °C in a Binder incubator (RedLine) for 15 days until the mycelium covered 100% of the surface of the petri dishes (90x15 mm).

Preservation of PO2 strain in distilled water, mineral oil and sterile saline solution

A 5 mm diameter filter paper disc inoculated with strain PO2 was added to cryovial tubes with 2 mL of sterile distilled water type III, with a pH of 7.5 and an electrical conductivity of 0.5 µS / cm (SI Analytics Handy Lab 680FK). The tubes were labeled and stored in refrigeration at 4 °C for four months. This procedure was further carried out for the evaluation of sterile mineral oil and sterile saline solution (0.85 % NaCl) as preservation methods. 50 replications were performed for each of the treatments.

Conservation of PO2 strain by periodic subculture (control)

Petri dishes were inoculated with PDA Agar (Oxoid) with a 5 mm diameter disc of filter paper covered with mycelium of the PO2 strain, subsequently were incubated at 28 °C for 7 days, until the mycelium cover the entire surface of the petri dishes, they were sealed with parafilm, labeled and stored in refrigeration at 4 °C for four months. 50 replicates were performed for this treatment.

Recovering of the strains conserved

The strains conserved were recovered monthly by inoculating the disks with the mycelium of the fungus in petri dishes of glass (90x15 mm) with agar PDA (Oxoid). The recovery had the objective to monitor the viability of the strains and their rate of growth. After culture, the petri dishes were incubated at 28°C for seven days.

Determination of the viability of the conserved PO2 strain

The fungus strain was considered viable when growth was observed. Macroscopic characteristics of the mycelium were evaluated, such as color, appearance, texture, surface, shape, and crop of the culture. Additionally, hyphal morphology was observed by direct microscopy before staining with lactophenol blue. The growth rate of the recovered strains was determined by the daily measurement of the mycelium diameter for 7 days. A period of conditioning to the culture medium of 3 days was established.

Spawn production for use in cultivation

Wheat hydrated up to 40% moisture was used to obtain the main spawn, which was packed in polypropylene bags of 500 g and subsequently autoclaved in three cycles at 121 °C for 15 min and 103 Kpa. The wheat was inoculated with the mycelium of the fungus from the petri dishes with PDA agar (Oxoid). The bags were incubated for a period of 1 to 2 weeks at 28 °C until the mycelium covered more than 90% of the wheat. For the

production of the main spawn, 10 replicates per treatment were randomly selected; each of them was used to inoculate two 500 g bags of wheat, for a total of 80 bags of spawn, 20 bags for each of the treatments. Subsequently, hydrated wheat was inoculated as previously performed with 3% *Pleurotus ostreatus* spawn and incubated for 2 weeks at 28 °C until the mycelium covering over 90% of wheat this will he called commercial spawn. A total of 132 bags of 500 g were obtained for mushroom cultivation, corresponding to 33 bags per treatment.

Preparation of the substrate for the growth of *Pleurotus ostreatus*

A formulation based on coffee pulp (40%), coffee husk (30%), oak sawdust (15%), coconut shell (13%), and calcium carbonate (2%) was made. The formulation was previously standardized in the educational plant of edible fungi of the SENA Regional Caldas (Manizales, Colombia). The substrate was packed in bags of polypropylene with a weight of 10 kg; later, they were then subjected to anaerobic fermentation (water immersion the substrate) for 8 days. Subsequently, the substrate water was drained for 12 h until reaching a final humidity of 75%. Finally, 1.8 kg of the substrate was packed in polypropylene bags. 40 substrate cakes, 10 units per treatment were produced.

***Pleurotus ostreatus* fungus production**

The substrate was inoculated with 3% commercial spawn produced from each of the conservation methods evaluated. Incubation was performed at 25 °C in dark conditions for 30 days until the mycelium completely covered the substrate. Crosscuts were made to the colonized substrate using a sterile blade in order to favor fruiting. The entry of light, fresh air re-circulation was allowed, and the relative humidity was increased up to 90% by water irrigation and an average temperature of 25 °C. Once fruiting began, daily monitoring of the production of fruiting bodies was carried out in each of the 10 experimental units per treatment. The mushrooms produced were harvested, weighed, and stored in refrigeration for later physicochemical characterization.

Determination of biological efficiency and crop productivity rate

The biological efficiency of the strain conserved by each of the methods was determined by the relationship between the mass of fresh mushrooms obtained and the dry substrate at planting, as proposed by Miles and Chang (2004) (Equation 1).

$$BE = \frac{\text{Fresh mushroom mass (kg)}}{\text{Dried substrate (kg)}} * 100$$

(Equation 1)

The productivity rate corresponds to the relationship between biological efficiency and the time between inoculation and harvest, as proposed by Sánchez and Royse (2001)(Equation 2).

$$PR = \frac{\text{Biological efficiency}}{\text{Time elapsed between the inoculation and harvest}} * 100$$

(Equation 2)

Nutritional characterization of the fruiting body

The moisture of the mushroom was determined by the gravimetric method according to the technical standard AOAC, 925.45 Mod. Ed. 16. The total protein of the carpophores was measured using the Kjeldahl method according to the technical standard AOAC, 988.05 Ed. 15. The fat and total ash content were determined using the Soxhlet and AOAC methods 923.03 Ed. 16 respectively. The carbohydrate content was obtained by the difference between 100 and the sum of the variables previously calculated. The physicochemical analyses were performed in the Basic Sciences laboratory of the SENA Regional Caldas (Manizales, Colombia).

Statistic analysis

The conservation method with four levels, sterile distilled water, sterile mineral oil, sterile saline solution and positive control (periodic subculture) were taken as experimental factors for the evaluation of their possible incidence on the strain length response; and finally, the storage time of the strains with five levels: 24 hours of storage and 1, 2, 3 and 4 months of storage. Since additionally, the response was followed for 7 consecutive days for each of the treatments corresponding to the combination of storage time and conservation method, and knowing that this new variable (days) would generate an impact on the response, for the statistical analysis of the results this variable was subjected to the blocking principle. Therefore an experimental design of bifactorial randomized blocks was used, where the effects of treatments correspond to the conservation method and storage time factors, while the block effects are due to the variable day measurement. For the statistical

analysis of the information, the XLSTAT demo version software was used.

RESULTS AND DISCUSSION

Viability of conserved strains

The totality of the replicates per treatment remained viable during the four months of the experiment, with a viability percentage higher than 80%. There were no morphological changes, contamination by bacteria or other fungi (Figures 1, 2, 3, and 4). These results are equivalent to those reported for the conservation of basidiomycete fungi using the method of Castellani (Burdsall and Dorworth, 1994; Richter, *et al.*, 2010; Palacio, *et al.*, 2014; García-García, *et al.*, 2014). The conservation of basidiomycete fungi in sterile distilled water is economical and practical, allowing strains to be stored without requiring periodic transfers, which reduces the costs associated with culture media, laboratory space, and labor costs. The pH and electrical conductivity of distilled water do not affect the biological function of membrane proteins, nor the enzymatic activity present in the cell wall (Sánchez and Royse, 2001).

100% of the strains preserved using sterile mineral oil remained viable, pure, and morphologically stable (Figure 2). These results are consistent with the reported by Panizo, Reviákina, Montes, and González (2005) and Pinzón Gutiérrez, Bustamante, and Buitrago (2009); mineral oil suppresses the morphological changes of the strains while maintaining their stability, limits the availability of oxygen, and reduces mite infestation

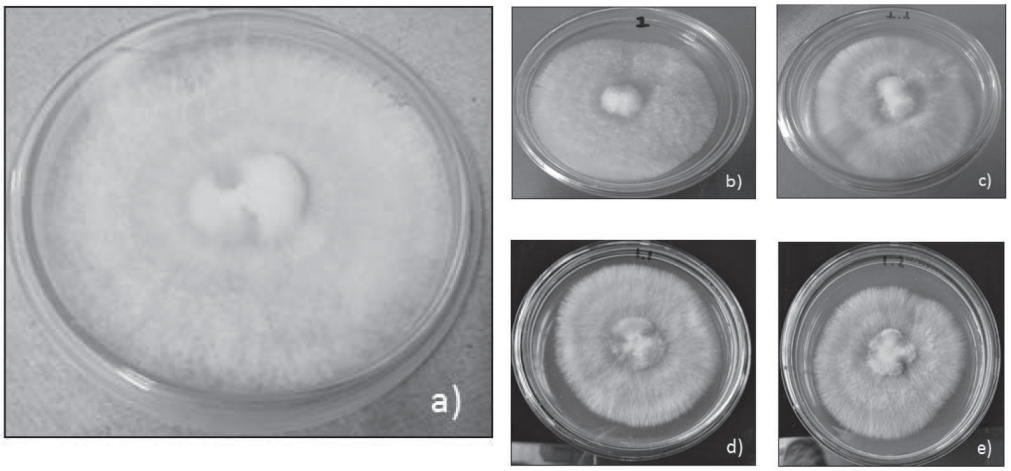

Fig. 1. Growth of the PO2 strain after preservation with sterile distilled water. a) 24 hours of conservation, b) One month of conservation, c) Two months of conservation, d) Three months of conservation, e) Four months of conservation.

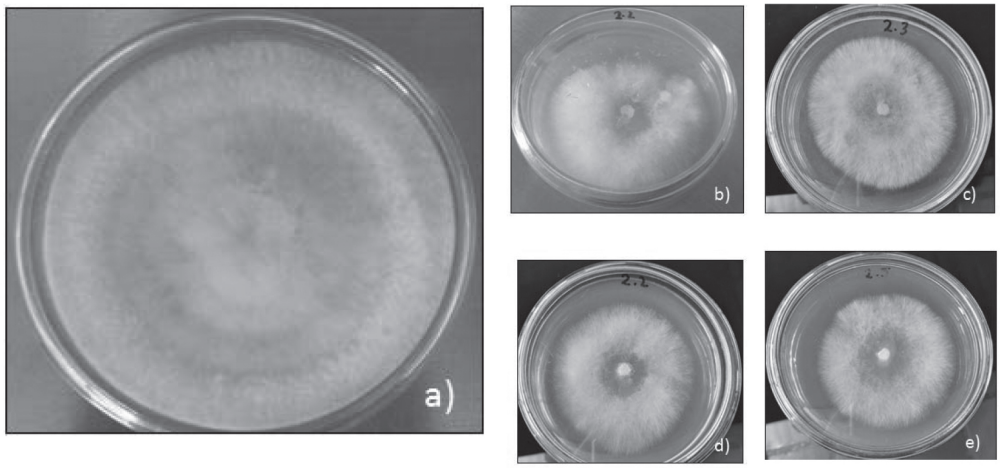

Fig. 2. Growth of the PO2 strain after preservation with sterile mineral oil. a) 24 hours of conservation, b) One month of conservation, c) Two months of conservation, d) Three months of conservation, e) Four months of conservation.

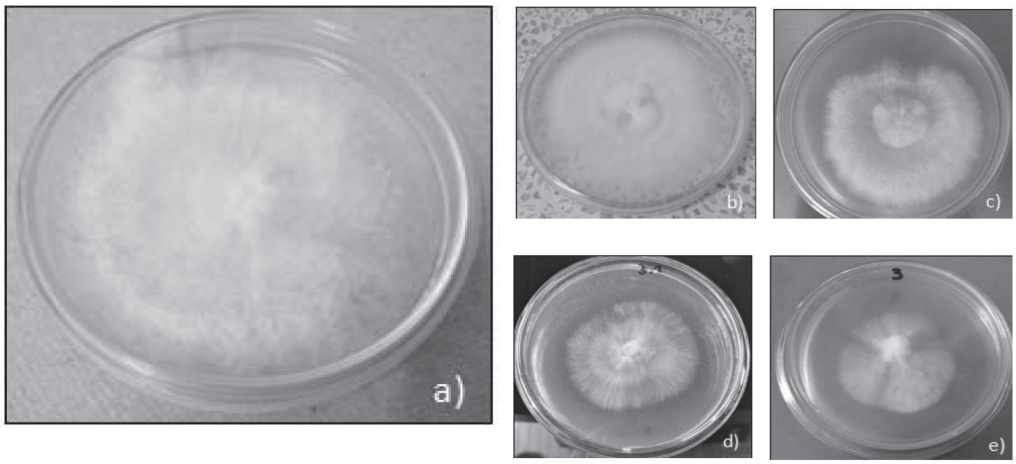

Fig. 3. Growth of the PO2 strain after preservation with saline solution. a) 24 hours of conservation, b) One month of conservation, c) Two months of conservation, d) Three months of conservation, e) Four months of conservation.

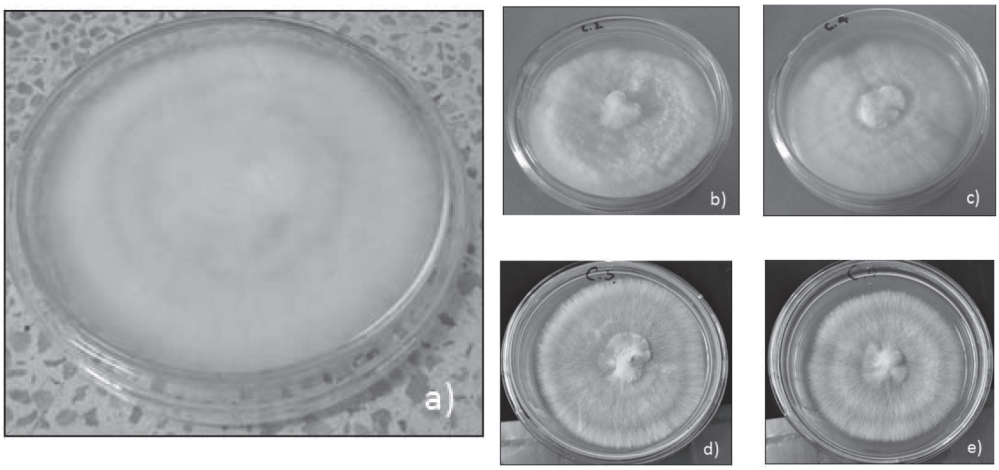

Fig. 4. Growth of the PO2 strain after preservation by periodic subculture. a) 24 hours of conservation, b) One month of conservation, c) Two months of conservation, d) Three months of conservation, e) Four months of conservation.

during storage, guaranteeing the proper preservation of fungi for extended periods.

The sterile saline solution evaluated at 0.85% maintains the water balance of the conserved cells avoiding cytoplasmic damage due to the destabilization of the osmotic processes. The results obtained for this treatment are consistent with those reported by Henao *et al.* (2006) and Sarmiento Rangel, *et al.* (2013), who in their studies on fungal conservation, obtained viability percentages higher than 90%, without presenting contamination or morphological changes of stored strains (Figure 3).

The feasibility tests performed showed that the four conservation methods evaluated are applicable for the preservation of the *Pleurotus ostreatus* strain PO2. The conservation in sterile distilled water or mineral oil allows maintaining high percentages of the viability of the preserved strains without presenting changes in their morphology and purity, even after being stored for up to 32 years (Nakasone, *et al.*, 2004; Homolka, 2014).

The macroscopic morphological characteristics of the conserved strains were not affected by the conservation methods employed. A filamentous white mycelium of dry appearance was obtained, with round topography of high margin for all conservation methods and periods of growth (Figures 1, 2, 3, and 4). No morphological changes were observed in hyphae depending on the conservation strategy (Figure 5). The techniques evaluated do not affect the viability, morphology, and stability of the strains; therefore, they are suitable for the conservation of microbial strains (García and Uruburu, 2000; Arencibia, *et al.*, 2008;

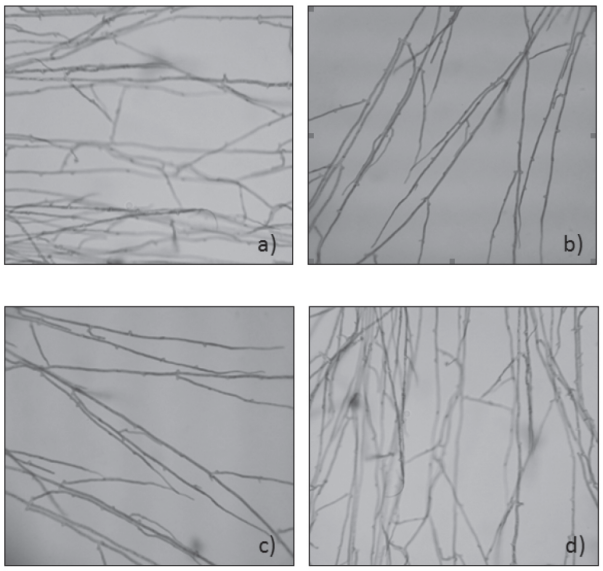

Fig. 5. Microscopic characterization of *Pleurotus ostreatus* using staining with lactophenol blue, a) Distilled water, b) Mineral oil, c) Saline solution 0.85%, d) Periodic subculture.

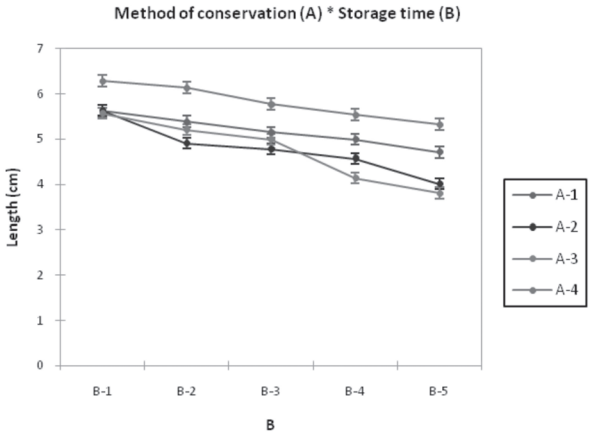

Fig. 6. Interaction between the analyzed factors. A-1 (Sterile distilled water), A-2 (Sterile mineral oil), A-3 (0.85% sterile saline), A-4 (Periodic subculture) and storage time B -1 (24 hours), B-2 (1 month), B-3 (2 months), B-4 (3 months), B5 (4 months), concerning the growth length of the *Pleurotus ostreatus* strains.

Morales *et al.*, 2010).

The growth rate of conserved strains

The analysis of the growth rate of the strains conserved in sterile distilled water, sterile mineral oil and saline solution, allowed to observe an inversely proportional relationship between storage time and the speed at which the organism reaches its maximum growth (total colonization of the petri dish) The relationship between time and the speed of growth is directly affected by the preservation method used, being the methods of periodic subculture and storage in sterile distilled water who have higher growth rates. The conservation methods of microbial strains can generate stress conditions that affect the speed of growth (García and Uruburu, 2000; Arencibia *et al.*, 2008). Data were analyzed following a balanced experimental block design, the assumptions of normality, homoscedasticity, and independence to a significance of 5% were checked. A three-way analysis of type I variance was performed for the design of blocks with 95% confidence, obtaining a relationship between the conservation method and the conservation time (Figure 6), which together influences the growth of the hyphae ($p<0.0001$).

A Tukey multiple comparisons (DHS) analysis was performed with 95% confidence to assess the differences in the rate of mycelium generation between the different treatments. It was obtained that the method of periodic subculture with storage between 24 h and one month showed higher growth rates with hyphae lengths of up to 6.25 cm. There were no significant differences between storage for four months in saline or mineral oil; these combined conditions generated growths below 4.13 cm. With the results obtained in the evaluation of the growth rate, it is possible to affirm that the method of conservation by distilled water could be used as an alternative to replacing the technique of periodic subculture, which has traditionally been used to preserve *Pleurotus ostreatus* strains. This technique does not significantly affect the speed of growth and behavior of the subsequent recovery strains on artificial culture media, presenting a radial growth of up to 1.10 cm daily. This result is consistent with that reported by Bermúdez Savón, García Oduardo, and Mourlot López (2007), who reported growth values between 0.76 - 1.12 cm per day for *Pleurotus* sp.

Determination of biological efficiency and productivity rate in the culture of *Pleurotus ostreatus*

Biological efficiency and productivity rate were determined using equations 1 and 2 for each of the experimental units per treatment. It was observed that fungi obtained by conservation in mineral oil had a slightly higher biological efficiency than the other conservation strategies (Table 1). However, it was determined by an Anova test that there are no

Table 1. Average biological efficiency and productivity rates for the cultivation of *Pleurotus ostreatus*, according to the method of conservation of the strains

Crop indicators	Treatment			
	Distilled sterile water	Sterile mineral oil	Saline sterile solution	Periodic subculture
Average biological efficiency (%)	89.33 ± 0.98	89.93 ± 0.65	89.78 ± 0.54	90.67 ± 0.60
Average productivity rate (%)	1.24 ± 0.01	1.25 ± 0.01	1.25 ± 0.01	1.25 ± 0.01

significant differences in biological efficiency between treatments (p> 0.05). These results indicate that the conservation methods evaluated do not affect the ability of the fungus to degrade complex polymers such as lignin, cellulose, and hemicellulose; therefore, they allow to conserve all their industrial potential at lower costs than other techniques such as cryopreservation in liquid nitrogen or lyophilization, which can affect the productivity of the strain (Lara *et al.,* 1998).

The biological efficiency achieved during the pilot test is consistent with that reported using oak sawdust as a substrate (López-Rodríguez, *et al.,* 2008). Rodríguez and Jaramillo (2004) reported values in the biological efficiency of *Pleurotus ostreatus* of 88.90% in a mixture of coffee pulp and sawdust from the stem, as well as 62.50% and 76.70% from mixtures of coffee pulp and coffee parchment in different proportions. A substrate is considered to be profitable at the productive level if the biological efficiency reaches minimum values between 40 and 50% (Sánchez and Royse, 2001; Rodríguez and Jaramillo, 2004 ; Bermúdez Savón *et al.,* 2007). In the present study, average productivity values above 89% were obtained, indicating that both the conservation methods evaluated and the substrate used for the cultivation of *Pleurotus ostreatus* are optimal for the production process of this type of fungi. In the same way, it can be affirmed that the results regarding biological efficiency are because the formulation of the substrate used for the crop provided an adequate relationship between all the nutritional components necessary for the growth and development of the carpophores. Conservation in mineral oil had the highest productivity rate with an average of 1.25%, followed by conservation in saline solution, periodic subculture, and sterile distilled water, with, productivity rates of 1.25%, 1.25% and 1.24% respectively.

It was determined by an analysis of variance and a comparative test of Tukey that there were no statistically significant differences between the evaluated treatments (p> 0.05). That allows affirming that the rate of productivity of the crop is not affected by the method of conservation of the strain. However, it will depend directly on the biological efficiency, the nutritional quality of the substrates, and the total time required for the production cycle (Bermúdez Savón *et al.,* 2007). The productivity values obtained for the substrate used are in the range reported for other formulations (Bermúdez Savón *et al.*, 2007; Romero *et al.*, 2010; Castañeda Diaz and Carvajal, 2014).

Nutritional quality of harvested mushrooms

The nutritional quality analysis for harvested mushrooms allowed to observe that the strains conserved by periodic subculture have higher humidity than those conserved by distilled water, saline solution, and mineral oil (Table 2). However, for all treatments, the humidity remained around 90%; this value is within the optimal ranges reported for *Pleurotus ostreatus* (Miles and Chang, 2004; Cheung, 2008; and Alam *et al.,* 2008). The results obtained indicate that the humidity of the fruiting bodies is not affected by the conservation methods evaluated.

Fungi preserved by all the evaluated methods presented ashes percentages higher than 6.87% (Table 2). It was determined by a Tukey test that there are no significant differences for the ash content by different methods. This variable is directly related to the bromatological composition of the substrates used in mushroom production, being directly proportional to the ash content of the substrate (Nieto and Chegwin, 2010).

Nutritional properties such as protein, lipid, and carbohydrate content did not show differences associated with the conservation method (Table 2). The protein content present in the *Pleurotus ostreatus* carpophores ranges from 10 to 40%, depending on the substrate used for its production (Rodríguez and Jaramillo, 2004; Bernardi, *et al.,* 2009; Nieto and Chegwin, 2010). In the present investigation, protein percentages obtained were close to 6%, which is

Table 2. Nutritional properties of fungi harvested from *Pleurotus ostreatus*, according to the method of conservation of the strains

Percentage (%)	Treatment			
	Distilled sterile water	Sterile mineral oil	Sterile saline solution	Periodic subculture
Humidity	91.16 ± 0.83	89.78 ± 1.50	89.96 ± 1.30	91.79 ± 0.56
Ashes	6.87 ± 0.74	6.98 ± 1.33	7.27 ± 0.99	7.00 ± 0.61
Protein	5.52 ± 0.79	5.73 ± 0.81	5.55 ± 0.85	5.74 ± 0.45
Lipids	2.80 ± 0.26	2.82 ± 0.41	2.98 ± 0.39	2.99 ± 0.26
Carbohydrates	84.82 ± 1.06	84.48 ± 1.18	84.20 ± 1.53	84.28 ± 0.43

directly related to the nutritional composition of the substrates used for the cultivation in a pilot plant, in which coffee pulp is a limiting nutrient for growth and fruiting since it provides the highest nitrogen content (Rodríguez and Jaramillo, 2005). The percentage of lipids and carbohydrates obtained for harvested mushrooms is within the ranges established for this species of macromycete. The present investigation allows affirming that the conservation methods evaluated do not directly affect properties such as the moisture of the carphores, the percentage of ashes, or the concentration of lipids, proteins or carbohydrates. The formulation used for the generation of the substrate affects the production of carbohydrate-rich mushrooms with low protein concentrations.

CONCLUSION

Preservation methods evaluated in this study, managed to maintain the viability and purity of all strains preserved, maintaining their macro and microscopic characteristics after four months of storage at 4 °C. These methods are economically viable alternatives for the preservation of fungi basidiomycetes as the *Pleurotus ostreatus*.

Microbial conservation methods affected the growth rate of *Pleurotus ostreatus* (PO2) strains after their recovery, with methods of periodic subculture and storage in sterile distilled water higher growth rates.

The strain conservation methods evaluated (sterile distilled water, mineral oil, saline solution, and periodic subculture) did not affect the nutritional properties such as moisture, ash, lipids, proteins, or carbohydrates of *Pleurotus ostreatus*, nor the biological efficiency and productivity rate of the fungus crop.

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