BIOSURFACTANT PRODUCTION AND PETROLEUM DEGRADATION USING SPENT MUSHROOM SUBSTRATE

Marília Santiago Carvalho[1], Carlos Godinho de Abreu[1], Leonardo de Figueiredo Vilela[2], Tatiana Cardoso e Bufalo[1], Eustáquio Souza Dias[1]*

[1]Federal University of Lavras, Brazil

[2]Faculdade de Ciências Médicas de Três Rios, Suprema - Três Rios/RJ

**Corresponding author: esdias@ufla.br*

Mushroom cultivation generates enormous amounts of post-cultivation substrate at the end of each production cycle, which is known as spent mushroom substrate (SMS). Once known as a waste product of mushroom production, SMS is actually a valuable co-product of mushroom production. There are countless possibilities for using this material and, considering the large volumes produced, it is interesting to continue looking for new alternatives for its use in different human activities. In this work, we evaluated the potential for biosurfactant production in SMS from different species of oyster mushrooms, in addition to evaluating the potential for oil degradation in the presence of SMS from Pleurotus ostreatus and Lentinula edodes. Even with the use of dry SMS, a decrease in several hydrocarbons was observed after 3 months of oil treatment. Furthermore, Pleurotus species showed high emulsification rates. Future studies will be necessary to characterize the molecules responsible for the bioemulsification activity, since both biosurfactants and bioemulsifiers can act in a similar way.

Keywords: spent mushroom substrate, SMS, mushrooms

DOI: 10.24263/EDSD-2024-6-5

Received 27.04.2024

Received in revised form 21.10.2024

Accepted 05.11.2024

Introduction

Mushroom cultivation is considered an ecologically friendly activity because it uses agricultural or animal waste (Ahlawat and Sagar, 2007). Furthermore, mushroom cultivation plays an important role in food security because it transforms waste into excellent quality food. On the other hand, mushroom production also results in a large volume of waste, which is called SMS (spent mushroom substrate), at a rate of 5kg of SMS for each kg of mushroom produced. Inadequate disposal of SMS can have worse consequences than agricultural waste itself, since this material has a higher concentration of salts than agricultural waste and, importantly, a high concentration of microorganisms, especially when the cultivation substrate is obtained by composting, as is the case with *Agaricus* mushrooms (*A. bisporus*, *A. bitorquis* and *A. subrufescens*).

As a result, there is a lot of work on the potential for using SMS in an environmentally friendly way. The most direct form of use is the application of SMS as an organic fertilizer (Ahlawat and Sagar, 2007). But there are many other applications for SMS, such as energy production, animal feed, enzyme production, substrate for the production of other materials, substrate composition for the production of other mushrooms, substrate for the production of plant seedlings, soil conditioning, production of nanoparticles, in addition to other applications (Grimm and Wosten, 2018; Moreira et al., 2020; Carmo et al., 2021; Zied et al., 2021; Alves et al., 2021; Alves et al., 2022; Alves et al., 2024).

This diversity of SMS applications is of great importance, since the increasing growth of mushroom cultivation around the world will also result in a significant increase in SMS, making it difficult to fully use it in production sites (Alves et al, 2021) . Therefore, it will always be very important to search for more alternatives for using SMS both for the production of bioactive compounds and for solving environmental problems.

In this context, the use of SMS both for the production of enzymes (Grimm and Wösten, 2018) and for solving environmental problems such as bioremediation (Leong et al, 2022) are examples of biotechnological applications that make SMS a high-value product rather than waste. All these examples demonstrate that SMS can have many other biotechnological applications that have not yet been explored, which make SMS not an environmental problem but a desirable product for several areas of human activity.

In this work, we began to study the SMS as a source of biosurfactants in function of the mushroom cultivation using substrates rich in lignocellulosic materials, as well as its potential for use in petroleum degradation.

Materials and Methods

Microorganisms and cultivation

For mushroom cultivation trials, the species *Pleurotus ostreatus*, *P. djamor*, *P. eryngii* and *Lentinula edodes* were used. All fungi were grown in Petri dishes with PDA culture medium. The plates were inoculated with mycelial discs measuring approximately 6 mm. The plates were incubated at 25ºC until complete colonization.

To prepare the spawn, husk rice enriched with 1% gypsum and 2% limestone was used. To do this, the rice was cooked for 30 minutes and, shortly after, supplemented with gypsum and limestone. The mixture was packaged in glass flasks (containing approximately 350 g each). The flasks were autoclaved at 121ºC for 2 hours twice, after an interval of 24 hours. After cooling, each flask was inoculated with 3 fragments of approximately 1 cm^2 of the colonized PDA plates. The flasks were incubated at 25ºC until complete colonization of the grains (15 to 20 days).

Pleurotus ostreatus and *P. djamor* were cultivated in sawdust (48%), coffee husk (48%) and limestone (4%) substrate. *P. eryngii* was grown in sawdust (60%), wheat bran (15%), corn flour (20%) and limestone (4%) substrate. *L. edodes* was grown in sawdust (76%), wheat bran (4%) and limestone (4%) substrate. All substrates were placed in high-density polyethylene bags with a gas exchange window (2kg/bag) and autoclaved at 121ºC/2h. The process was repeated under the same conditions after an interval of 24 hours. Each bag received 40g of Spawn, which was distributed across the top and sides of the bag. The blocks were incubated at room temperature (25±2ºC) until complete colonization. For *L. edodes*, after complete colonization of the substrate, it was necessary to complete 3 months of incubation to allow the browning process, which is essential for shiitake fruiting. For mushroom fruiting, *P. ostreatus* and *P. djamor* were transferred to a room at 25ºC and relative humidity above 90%. For *P. eryngii* and *L. edodes*, fruiting was carried out in a room at 19ºC and relative humidity above 90%.

Obtaining the SMS

After growing the mushrooms, the substrate was removed from each bag and dried under the sun for 2 days. After that, it was crushed to obtain fragments with a maximum length of 1cm. This SMS was used for oil degradation tests. For the biosurfactant production tests, SMS was used with the natural humidity of the substrate after finishing the mushroom cultivation.

Oil degradation test

For the oil degradation tests, the SMS from two species of mushrooms were used: *P. ostreatus* and *L. edodes*. For each test, 10g of dry SMS were used. The SMS was rehydrated with sterile distilled water in order to reach 65% moisture. After that, crude oil was added at a rate of 10%. The entire mixture was made in glass flasks. After mixing well, the flasks were protected with aluminum foil and incubated at room temperature for up to 3 months. The moisture was maintained at around 65% by adding water every week. The assays were carried out in triplicate.

Biodegradability analyzes of hydrocarbons

Chromatographic analyzes were carried out on a gas chromatograph coupled to a mass spectrometer, aiming to quantify some hydrocarbons presente in the crude oil.

The SMS samples with petroleum were macerated with liquid nitrogen for headspace extraction. Two grams of sample were placed in hermetically sealed 15 ml vials and heated for 15 minutes at 60ºC to reach equilibrium in the sample headspace. The adsorption of volatile compounds on the fiber was carried out by inserting the SPME into the top of the flask for 30 minutes at 60ºC. To desorb the compound, the fiber was placed in the injection port of the chromatograph heated to 230°C for 2 minutes. The SPME fiber used was 75mm carboxen/poly (dimethylsiloxane) (DVB/CAR/PDMS) (Supelco Co., Bellefonte, PA, USA).

The analysis of volatile compounds in the samples was carried out using a gas chromatograph (GC) coupled to a mass spectrometer model QP-2010 SE and a silica capillary column HP-5MS (30. m / 0.25 mm / 0. 25mm). The temperature program started with 5 min at 60 °C, followed by a gradual increase of 10 °C/min up to 200 °C. The temperature was then maintained at 200 °C for 30 min. Injector and detector temperatures were maintained at 230 °C. Carrier gas (He) was used at a flow rate of 1.2 mL/min. Volatile compounds were identified by comparing mass spectra and alkane retention index and with the Shimadzu database (Rodriguez-Campos et al., 2011).

Emulsification tests to evaluate the production of biosurfactants

For each species or strain of mushroom, the crude extract of wet SMS was obtained, using distilled water in a proportion of 1 liter of water for 1 kg of wet SMS. After mixing well, the substrate was pressed through a 200 mesh sieve to release the crude extract. For the emulsification tests, 2 mL of the crude extract was mixed with 2 mL of kerosene, using a 10 mL test tube. The mixture was vortexed for 2 min and then left to rest for 24 h (Martins et al., 2021). The emulsification index was calculated according to the formula EI= [(He/Ht) x100], where: EI: emulsifier index (%), He: emulsion height, Ht: total height.

Results and Discussion

Crude oil biodegradation

The results of quantification of volatile hydrocarbons are shown in Table 1. These tests were carried out in small volumes with the aim of validating the methodology for future tests on larger volumes of substrate. The results demonstrated that the 3-month period was sufficient to see the degradation of part of the hydrocarbons, especially those that were in higher concentration in the uncultivated substrate, such as heptadecane, pentadecane and tetradecane. For several hydrocarbons, no degradation was observed in the time evaluated for any of the cultivated fungal species. For one compound (Pentadecane, 2,6,10-trimethyl), a drastic reduction in the SMS of *L. edodes* was observed. Similarly, it was observed that nonadecane completely disappeared in *the P. ostreatus* SMS, but not in the *L. edodes* SMS. For one hydrocarbon (Tridecane, 2-methyl), a complete absence was observed in the SMS of both mushroom species.

Table 1. Quantification of volatile hydrocarbons in SMS treatments contaminated with oil after 3 months of treatment. UCS: uncultivated substrate; Le: Lentinula edodes; Po: Pleurotus ostreatus

Hydrocarbons	UCS + crude oil	Le SMS + crude oil	Po SMS + crude oil
Dodecane	0.049	0.097	0.090
Dodecane, 2.7.10-trimethyl-	0.047	0.076	0.058
Hexadecane	0.182	0.125	0.202
Heptadecane	0.384	0.217	0.150
Hexadecane, 2.6.10.14- tetramethyl-	0.068	0.082	0.114
Nonadecane	0.038	0.046	0.000
Octadecane	0.108	0.157	0.172
Pentadecane	0.680	0.404	0.288
Pentadecane, 2.6.10-trimethyl-	0.196	0.017	0.118
Tetradecane	0.509	0.398	0.340
Tridecane	0.163	0.201	0.198
Tridecane, 2-methyl-	0.045	0.000	0.000

The use of basidiomycetes for oil degradation studies is already well documented (Isikhuemhen et al., 2003), including in consortium with bacteria (Acevedo-Sandoval et al., 2018). However, microbial succession probably occurs during the period of oil degradation. During the tests, the fruiting of a different species of basidiomycete was even observed at the end of the process. Therefore, the oil degradation activity, probably is not directly associated with the cultivated mushroom species, but with other microbial species that were installed either during the cultivation of the mushrooms or during the biodegradation tests, both of bacteria and fungi. The study of this microbiota could be very important in future studies.

Emulsification index of extracts obtained from wet SMS of oyster mushrooms

SMS from different species and strains of *Pleurotus* showed high rates of emulsification, as shown in Table 2. Initially, there was interest in cultivating these mushrooms in a substrate containing a high proportion of coffee husks, due to its high lignin content. However, *P. eryngii* did not colonize this type of substrate well. *P. ostreatus* and *P. djamor* colonized the substrate very well, however, mushroom production was not good (data not shown). Despite this, the SMS obtained provided extracts with a high emulsification index, especially *P. ostreatus* MB and *P. djamor*, which presented rates close to 100%. In the case of *P. eryngii*, as this fungus did not grow well in the substrate with coffee husks, it was necessary to grow it in a conventional substrate, based on eucalyptus sawdust and wheat bran. The emulsification index was not that high but, despite this, it was above 90%, demonstrating that the fungus is also quite efficient in this aspect, as well as the substrate containing sawdust.

Table 2. Emulsification index of the extract obtained from different strains or species of Pleurotus after the mushroom cultivation cycle

Species /strain	Mushroom substrate	Emulsification index (%)
P. ostreatus MB	Serragem + casca de café	99.5
P. ostreatus Luigi	Serragem + casca de café	94.7
P. djamor	Serragem + casca de café	98.0
P. eryngii	Serragem + farelo de trigo	93.6

Biosurfactants have several potential applications, including cleaning oil tanks (Banat et al., 1991) and improving oil biodegradation and soil decontamination (Shekhar et al., 2015). Therefore, the production of enzymes by the microbiota present in SMS as well as the biosurfactants produced by cultivated basidiomycetes can be an excellent combination for this purpose as well. Future studies should address different sources of lignin to optimize not only mushroom production but also the production of biosurfactants in extracts obtained from SMS. Furthermore, analyzes will be necessary to characterize the compounds produced to differentiate biosurfactants from bioemulsifiers, since both may have the same emulsification capacity.

Conclusions

In this work, we evaluated the potential for biosurfactant production in spent mushroom substrate from different species of oyster mushrooms, in addition to evaluating the potential for oil degradation in the presence of spent mushroom substrate from Pleurotus ostreatus and Lentinula edodes. Even with the use of dry spent mushroom substrate, a decrease in several hydrocarbons was observed after 3 months of oil treatment. Furthermore, Pleurotus species showed high emulsification rates. Future studies will be necessary to characterize the molecules responsible for the bioemulsification activity, since both biosurfactants and bioemulsifiers can act in a similar way.

Acknowledgement

The authors acknowledge FAPEMIG, FINEP, CNPq, CAPES for financial support.

Conflict of interest

The authors state no conflict of interest.

References

Acevedo-Sandoval, O., Gutierrez-Alcantara, E.J., Perez-Balan, R., Rodriguez-Vazquez, G., Zamorategui-Molina, A., & Tirado-Torres, D. (2018). Degradation of polycyclic aromatic hydrocarbons using bacterial isolate from the contaminated soil and white rot fungus Pleurotus ostreatus. Applied Ecology and Environmental Research, *16*(4), 3815-3829.

Ahlawat, O.P. and Sagar, M.P. (2007). Management of spent mushroom substrate. National Research Centre for Mushroom (Indian Council of Agricultural Research). Chambaghat, Solan, India.. 48p.

Alves, L.S., Moreira, B.R.A., Viana, R.S., Pardo Giménez, A, D, Noble, R., Zied, D.C. (2021). Recycling spent mushroom substrate into fuel pellets for low-emission bioenergy producing systems. Journal of Cleaner Production, 313, 127875.

Alves, L.S., Caitano, C.E., Cardoso, S.F, Vieira Júnior, W.G., Heinrichs, R., Moreira, B.R.A., Pardo-Giménez, A., Zied, D.C. (2022). Application of Spent Sun Mushroom Substrate in Substitution of Synthetic Fertilizers at Maize Topdressing. Agronomy-Basel, 12, 2884.

Alves, L.S., Moreira, B.R.A., Viana, R.S.; Dias, E.S., Rinker, D.L., Pardo Giménez, A., Zied, D.C. (2022). Spent mushroom substrate is capable of physisorption-chemisorption of CO_2. Environmental Research, 204, 111945.

Alves, L.S., Tolardo, G., Caitano, C.E., Vieira Júnior, W.G., Gomes Freitas, P.N., Zied, D.C. (2024). Use of spent mushroom substrate for cherry tomato seedlings; a potential alternative to peat in horticulture. Biological Agriculture & Horticulture, 40, 1-14.

Banat, I. M., Samarah, N., Murad, M., Horne, R., & Banerjee, S. (1991). Biosurfactant production and use in oil tank clean-up. World Journal of Microbiology and Biotechnology, *7*, 80-88.

Carmo, C.O., Rodrigues, M.S., Silva, F., Irineu, T.G.M., Soares, A.C.F. (2021). Spent mushroom substrate of *Pleurotus ostreatus* Kummer increases basil biomass and essential oil yield. Rev. Caatinga, 34(3), 548-558. https://doi.org/10.1590/1983-21252021v34n306rc

Chibuzo, U., Burgess, J.G., Ennis, C.J., Rahman, P.K.S.M. (2015). Bioemulsifiers are not biosurfactants and require different screening approaches. Frontiers in Microbiology, v. 6. DOI=10.3389/fmicb.2015.00245

Grimm, D., Wösten, H.A.B. (2018). Mushroom cultivation in the circular economy. *Appl* Microbiol Biotechnol 102, 7795–7803. https://doi.org/10.1007/s00253-018-9226-8

Isikhuemhen, O.S., Anoliefo, G.O. & Oghale, O.I. (2003). Bioremediation of crude oil polluted soil by the white rot fungus, *Pleurotus tuberregium* (Fr.) Sing. *Environ Sci & Pollut Res* **10**, 108–112 https://doi.org/10.1065/espr2002.04.114

Martins, R.C., Bravo, C.E.C, Vilela, L.F., Costa, L.M.A.S., Dias, E.S. (2021). Multivariate analysis of nutritional parameters required for the submerged cultivation of three coprinaceous fungi for biosurfactant production. Ciência e Agrotecnologia, 45, e022220.

Moreira, B.R.A., Viana, R.S., Magalhaes, A.C., Caraschi, J.C., Zied, D.C, Dias, E.S., Rinker, D.L. (2020). Production of *Pleurotus ostreatus* var. florida on briquettes and recycling its spent substrate as briquettes for fuel grade biosolids. Journal of Cleaner Production, 1, 123919.

Rodriguez-Campos, J., Escalona-Buendía, H.B., Orozco-Avila, I.; Lugo-Cervantes, E., Jaramillo-Flores, M.E. (2011). Dynamics of volatile and non-volatile compounds in cocoa (*Theobroma cocoa* L.) during fermentation and drying process using principal components analysis. Food Res. Int. 44, 250–258.

Shekhar, S., Sundaramanickam, A., & Balasubramanian, T. (2015). Biosurfactant Producing Microbes and their Potential Applications: A Review. Critical Reviews in Environmental *Science and Technology*, 45(14), 1522–1554. https://doi.org/10.1080/10643389.2014.955631

Wang, S., Xu, F., Li, Z., Zhao, S., Song, S., Rong, C., Geng, X., Liu, Y. (2015). The spent mushroom substrates of *Hypsizigus marmoreus* can be an effective component for growing the oyster mushroom Pleurotus ostreatus. Scientia Horticulturae, 186, 217-222, https://doi.org/10.1016/j.scienta.2015.02.028.

Yoong Kit Leong, Te-Wei Ma, Jo-Shu Chang, Fan-Chiang Yang. (2022). Recent advances and future directions on the valorization of spent mushroom substrate (SMS): A review. Bioresource Technology, 344, Part A, 126157. https://doi.org/10.1016/j.biortech.2021.126157.

Zied, D.C., Abreu, C.G., Alves, l.S., Prado, E.P., Pardo Giménez, A, Melo, P.C., Dias, E.S. (2021). Influence of the production environment on the cultivation of lettuce and arugula with spent mushroom substrate. Journal of Environmental Management, 281, 111799-6.