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Solid-state fermentation for low-cost production of biosurfactant by promising *Mucor hiemalis* UCP 1309

Fermentação em estado sólido para produção de baixo custo de biossurfactante por *Mucor hiemalis* UCP 1309 promissor

Fermentación en estado sólido para la producción de bajo costo de biosurfactante por *Mucor hiemalis* UCP 1309 promisorio

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Abstract
Biosurfactants are amphipathic molecules with growing worldwide interest because of their low toxicity, high biodegradability and good ecological acceptability. They are produced by microorganisms and between them, filamentous fungi belonging Mucorales order have been reported as promising producers. However, most of these studies still use traditional submerged fermentation, a methodology with technical and economic problems. In this context, this work aimed the production of biosurfactant by *Mucor hiemalis* UCP 1309 using solid-state fermentation (SSF) as a low-cost alternative technology. Experiments were carried out using seven agro-industrial byproducts and wastes as substrates, supplemented with salt solution containing 5% waste soybean oil (WSO). Medium with the best result in the production of biosurfactant was used in a second fermentation, where a 2^2 full-factorial design (FFD) was performed to investigate the influence of inoculum size and concentration of WSO on surface tension. The results showed higher reduction of surface tension (to 28.1 mN/m) in fermentation using wheat bran. Also, the statistical analysis demonstrated significant effect of concentration of WSO on surface tension. Produced biosurfactant demonstrated excellent emulsifying properties with WSO, motor oil and burned motor oil, and the emulsions remained stable after 90 days of incubation. Therefore, this work demonstrated SSF as a suitable strategy for getting inexpensive and efficient biosurfactant and expediting its large-scale production. Moreover, we report, for the first time in the international literature, the production of biosurfactant using SSF by a Mucoralean fungus.
Keywords: Mucoralean fungus; Static fermentation; Surface tension; Emulsification index; Wheat bran.

Resumo
Biossurfactantes são moléculas anfipáticas com crescente interesse mundial devido à sua baixa toxicidade, alta biodegradabilidade e boa aceitação ecológica. São produzidos por micro-organismos e entre eles, fungos filamentosos pertencentes à ordem Mucorales têm sido relatados como produtores promissores. No entanto, a maioria desses estudos ainda utiliza a fermentação submersa tradicional, uma metodologia com problemas técnicos e econômicos.

Neste contexto, este trabalho teve como objetivo a produção de biossurfactante por *Mucor hiemalis* UCP 1309 utilizando fermentação em estado sólido (FES) como tecnologia alternativa de baixo custo. Os experimentos foram realizados usando sete subprodutos e resíduos agroindustriais como substratos, suplementados com solução salina contendo 5% de óleo de soja residual. O meio com melhor resultado na produção de biossurfactante foi utilizado em uma segunda fermentação, onde foi realizado um planejamento fatorial completo 2² para investigar a influência do tamanho do inóculo e da concentração de óleo de soja residual na tensão superficial. Os resultados mostraram maior redução da tensão superficial (para 28,1 mN/m) na fermentação usando farelo de trigo. Além disso, a análise estatística demonstrou efeito significativo da concentração de óleo de soja residual na tensão superficial. O biossurfactante produzido demonstrou excelentes propriedades emulsificantes com óleo de soja residual, óleo de motor e óleo de motor queimado, e as emulsões permaneceram estáveis após 90 dias de incubação. Portanto, este trabalho demonstrou que a FES é uma estratégia adequada para obter biossurfactante barato e eficiente e agilizar sua produção em larga escala. Além disso, relatamos pela primeira vez na literatura internacional a produção de biossurfactante usando FES por um fungo Mucorales.
Palavras-chave: Fungo Mucorales; Fermentação estática; Tensão superficial; Índice de emulsificação; Farelo de trigo.

Resumen
Los biosurfactantes son moléculas anfipáticas con creciente interés a nivel mundial debido a su baja toxicidad, alta biodegradabilidad y buena aceptabilidad ecológica. Son producidos por microorganismos y entre ellos, los hongos filamentosos pertenecientes al orden Mucorales han sido reportados como productores promisorios. Sin embargo, la mayoría de estos estudios todavía utilizan la fermentación sumergida tradicional, una metodología con problemas técnicos y económicos. En este contexto, este trabajo tuvo como objetivo producir un biosurfactante por *Mucor hiemalis* UCP 1309 utilizando fermentación en estado sólido (FES) como una tecnología alternativa de bajo costo. Los experimentos se realizaron utilizando siete subproductos y residuos agroindustriales como sustratos, suplementados con solución salina con 5% de aceite de soya residual. El medio con mejor resultado en la producción de biosurfactante se utilizó en una segunda fermentación, donde se realizó un diseño factorial completo 2² para investigar la influencia del tamaño del inóculo y la concentración de aceite de soya residual en la tensión superficial. Los resultados mostraron una mayor reducción de la tensión superficial (a 28,1 mN/m) en la fermentación con salvado de trigo. Además, el análisis estadístico mostró un efecto significativo de la concentración de aceite de soja residual sobre la tensión superficial. El biosurfactante producido demostró excelentes propiedades emulsionantes con aceite de soja residual, aceite de motor y aceite de motor quemado, y las emulsiones se mantuvieron estables después de 90 días de incubación. Por lo tanto, este trabajo demostró que la FES es una estrategia adecuada para obtener biosurfactantes baratos y eficientes y acelerar su producción a gran escala. Además, reportamos por primera vez en la literatura internacional, la producción de biosurfactante usando FES por un hongo Mucorales.
Palabras clave: Hongo Mucorales; Fermentación estática; Tensión superficial; Índice de emulsificación; Salvado de trigo.

1. Introduction

Biosurfactants are amphiphilic compounds, capable of reducing surface and interfacial tension between immiscible fluids (Araújo, et al., 2019; Freitas, et al., 2022). The interest in these biomolecules has increased due to their advantages over their synthetic counterparts, including better environmental compatibility, production from renewable waste substrates, maintaining activity at harsh environmental conditions, lower or no environmental toxicity. These properties make feasible to utilize them for numerous environmental, food, pharmaceutical, cleaning and other industrial application purposes (Montero-Rodríguez, et al., 2015; Andrade, et al., 2018; Ferreira, et al., 2020).

Biosurfactants are produced by microorganisms, but most research has focused on those obtained by bacteria and yeast (Lima, et al., 2017; Rulli, et al., 2019). There are few studies on the production of these biocompounds by filamentous fungi, which have a high biotechnological potential for the production of tensoactives, due to their ability to adapt to different environmental conditions, as a result of variations in their physiological, biochemical and genetic activities (Andrade, et al, 2018; Silva, et al., 2018). Among them, there are representatives from the Mucorales order, such as *Cunninghamella echinulata*, *Mucor circinelloides*, *Rhizopus arrhizus* and *Absidia cylindrospora*, who have demonstrated excellent ability to produce surfactant compounds (Andrade, et al., 2018; Marques, et al., 2019; Mendonça, et al., 2021; Cândido, et al., 2022). Recently, Ferreira et al. (2020), reported the production of sustainable biosurfactant by *Mucor hiemalis* UCP 0039.

In other hand, most of the literature has reported the biosurfactant production using submerged fermentation (SmF). This method possesses technical and economic disadvantages, so solid-state fermentation (SSF) has gained significant attention in recent years for the development of industrial bioprocesses (Nalini & Parthasarathi, 2018; Banat, et al., 2021). SSF is a process that occurs in the absence or near absence of water and present benefits including lower energy requirement because of the reduced aeration and agitation demands, lesser risk of contamination due to lower water activity, higher product yields and lower effluent generation (Soccol, et al., 2017; Srivastava, et al., 2019).

In this context, several researches have focused on the production of biosurfactants by SSF (Brumano, et al., 2018; Rubio-Ribeaux, et al., 2020; dos Santos, et al., 2021); however, studies using filamentous fungi are still scarce (Castiglioni, et al., 2014; Velioglu & Urek, 2015; Lourenço, et al., 2018). In particular, the application of SSF for the production of biosurfactants by Mucorales fungi has not yet been reported in the international literature (Krieger, et al., 2010; Costa, et al., 2018; Banat, et al., 2021), which arouses interest in developing research aimed this topic. Therefore, this work focused on the biosurfactant production by a Mucoralean fungi, *M. hiemalis* UCP 1309, in SSF, in order to develop a low-cost process to obtain this useful biocompound.

2. Methodology

2.1 Microorganisms

M. hiemalis UCP 1309, isolated from Caatinga soil of the state of Pernambuco, Brazil, was kindly provided by the Culture Collection of Nucleus of Research in Environmental Sciences and Biotechnology (NPCIAMB), at Catholic University of Pernambuco (UNICAP), Recife-PE, Brazil, registered in the World Federation for Culture Collections (WFCC). The strain was maintained at 5°C on Sabouraud Dextrose Agar medium.

2.2 Agro-industrial substrates

Seven agro-industrial substrates were used in formulation of production media: sugarcane bagasse (SCB), wheat bran (WB), corn bran (CB), instant noodle waste (INW), pineapple peels (PAP), pineapple crown (PAC) and tangerine peels (TP). SCB was kindly donated by Usina Japungu, Santa Rita (Paraíba, Brazil), INW was kindly provided by the instant noodle industry, and WB, CB, pineapples and tangerines were bought at a local market in city of Recife (Pernambuco, Brazil). WB, CB and INW did not receive any kind of pre-treatment. SCB was maintained at -4°C until its use, then it was thawed at room temperature, oven-dried at 70°C for 24 h and ground in a blender. Pineapples and tangerines were washed and the wastes were separated from the edible pulps, oven dried at 70°C for 72 h and ground in a blender. Then, all substrates were sieved and the fraction used was either that retained between 16 and 32 mesh sieves (opening of 1.0 and 0.5 mm, respectively) (dos Santos, et al., 2021).

In addition, it was used waste soybean oil (WSO), kindly supplied by a local restaurant in the city of Recife (Pernambuco, Brazil).

2.3 Screening of substrates for biosurfactant production in SSF

Each 250 ml Erlenmeyer flasks containing 5 g of dry solid substrate were autoclaved at 121°C for 15 min. Then, corresponding amount of impregnating solution inoculated with 10^7 spores/ml of *M. hiemalis* and 3% WSO was mixed into solid substrates. The amount of impregnation solution for each dry substrate was defined as described by Camilios-Neto et al. (2011). The impregnation solution itself contained KH_2PO_4 (2 g/L), MgSO4 (1 g/L) and 1 ml of trace solution: $FeSO_4.7H_2O$ (0.63 mg/L), $MnSO_4$ (0.01 mg/L) and $ZnSO_4$ (0.62 mg/L) (Pele, et al., 2018). The inoculated flasks were incubated for 120 h at 28°C under static conditions.

2.4 Obtaining biosurfactant-containing extract from SSF

After the fermentation period, 100 ml of distilled water was added to each Erlenmeyer flask and the contents were agitated for 1 h at 200 rpm and 30°C on an orbital shaker. Then, the suspensions were filtered using cheesecloths and the liquid excess was squeezed out manually (Nalini & Parthasarathi, 2014). This procedure was done three times. The extracts were centrifuged for 20 min at 10000 *g* and the cell-free metabolic liquids were used to determination of surface tension.

2.5 Measurement of surface tension

Surface tension was determined on metabolic cell-free liquid obtained by centrifugation and subsequent filtration of cultures, using a tensiometer model Sigma 70 (KSV Instruments Ltd., Finland) by the Noüy ring method at room temperature (± 28°C). Measurements of surface tension from distilled water were used as control (Kuyukina, et al., 2001).

2.6 Full-factorial design (FFD)

After the selection of substrate with the best result in initial screening, a second SSF was carried out using 5 g of this substrate. A 2^2 full-factorial design (FFD) was applied in order to investigate the effects of each independent variables (inoculum size and concentration of WSO in the impregnation solution), as well as the interactions between them, on surface tension as response variable. Table 1 shows the levels studied and the decoded matrix of FFD. A set of seven assays with three replicates at the central point was performed.

Table 1: Levels of the variables studied in a 2^2 full-factorial design for the production of biosurfactant by *M. hiemalis* UCP 1309 in solid-state fermentation.

Variables	Levels		
	Low (-1)	**Central (0)**	**High (+1)**
Inoculum size (spores/ml)	10^4	10^7	10^{10}
Waste soybean oil (%, *v/v*)	1	3	5

Source: Authors.

The inoculated flasks were incubated at 28°C for 120 h in static conditions. After fermentation, the Erlenmeyer flasks were processed and surface tension was measured as previously described. The data obtained from the experiments were subjected to statistical analysis by Statistica® software, version 10.0 (StatSoft Inc., USA) and the significance of the results was tested at $p < 0.05$ level.

2.7 Emulsification index and stability

The emulsification index was determined according to the methodology described by Cooper & Goldenberg (1987) using the metabolic liquid from the condition with lowest value of surface tension. Vegetable oils (soybean, corn, canola, castor, sunflower and waste soybean oil) and petroderivatives (motor and burned motor oil) were used as hydrophobic substrates. The stability of emulsions was verified after 24 h, 7, 30, 60 and 90 days.

3. Results and Discussion

3.1 Solid-state fermentation for biosurfactant production

A microorganism is considered viable to produce biosurfactant when its metabolic liquid is able to reduce surface tension of distilled water from 72 to 40 mN/m or at least achieve a reduction of ≥ 20 mN/m (Rahman, et al., 2018). According to the literature, bacterial biosurfactants are more effective in reducing surface tension to values around 27-28 mN/m (Varjani, & Upasani, 2016; Liu, et al., 2018; Zhao, et al., 2020). However, in recent decades filamentous fungi have been explored in this sense, such as *Aspergillus niger*, *Cunninghamella echinulata* and *Mucor circinelloides* (Silva, et al., 2018; Sperb, et al., 2018; Marques, et al., 2019).

In addition, high energy demand and water consumption, as well as the high-cost downstream step, are the main disadvantages of conventional SmF, which hinder the commercialization of biosurfactants (Montero-Rodríguez, et al., 2018; Rubio-Ribeaux, et al., 2020). In this context, we investigated the biotechnological potential of *M. hiemalis* UCP 1309 for biosurfactant production in SSF, as low-cost alternative to SmF. Table 2 shows the surface tension values obtained in media containing different agro-industrial substrates. The reduction in surface tension to 28.1 mN/m not only demonstrates WB as the most promising substrate, but also confirms that SSF is a technology as effective as conventional SmF for the production of microbial surfactants (Marcelino, et al., 2020). In addition, to our knowledge, this is the first report regarding a Mucoralean fungus that produces biosurfactant by SSF.

Table 2: Surface tension values obtained by *M. hiemalis* UCP 1309 using agro-industrial byproducts and wastes as substrates in SSF, during 120 h at 28°C.

Substrates	Surface tension (mN/m)
Sugarcane bagasse	33.6
Wheat bran	28.1
Corn bran	30.7
Instant noodle waste	31.9
Pineapple peels	38.9
Pineapple crown	38.7
Tangerine peels	38.8

Source: Authors.

3.2 Full-factorial design (FFD)

Besides the choice of the appropriate substrate for biosurfactant production, it is necessary to investigate the factors that influence the process. Thus, the effects of inoculum size and concentration of WSO on the reduction of surface tension, were investigated here using a 2^2 FFD. The results presented in Table 3 showed that the lowest value of surface tension (28.4 mN/m) was obtained in condition 4 of the FFD, at the highest values of inoculum size (10^7 spores/ml) and concentration of WSO (5%) contained in the impregnation solution.

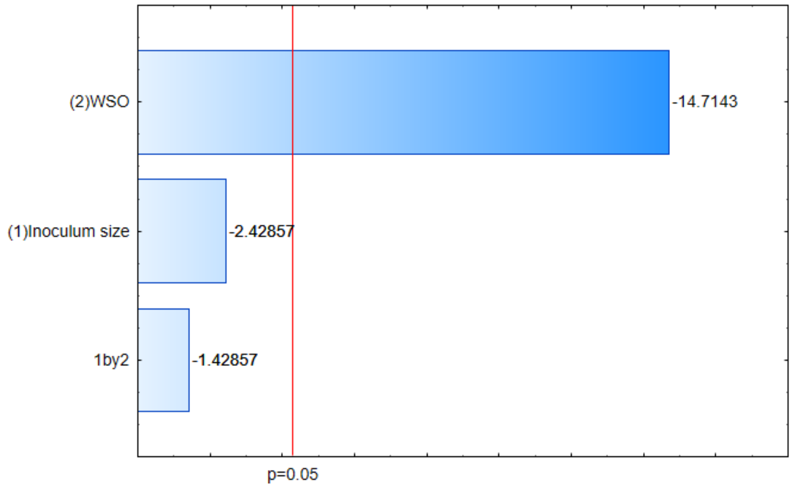
Table 3: Factorial design applied to production of biosurfactant by *M. hiemalis* UCP 1309 using wheat bran in solid-state fermentation.

Assays	Inoculum size	Waste soybean oil	Surface tension (mN/m)
1	-1	-1	40.4
2	+1	-1	39.7
3	-1	+1	31.1
4	+1	+1	28.4
5	0	0	40.8
6	0	0	40.6
7	0	0	39.5

Source: Authors.

However, the Pareto diagram (Figure 1) demonstrated that only WSO had a significant and negative influence, from the statistical point of view, in the obtained surface tension values. This means that an increase in WSO concentration led to lower surface tension, suggesting the production of biosurfactant in the culture medium. Previously, several studies demonstrated the effectiveness of hydrophobic substrates such as vegetable and waste frying oils as inductors for microbial biosurfactant production (Montero-Rodríguez, et al., 2015; Sperb, et al., 2018; Araújo, et al., 2019).

Figure 1: Pareto diagram showing the effects of independent variables: inoculum size (1) and waste soybean oil (WSO) (2) on surface tension of containing wheat bran, after SSF by *M. hiemalis* UCP 1309.



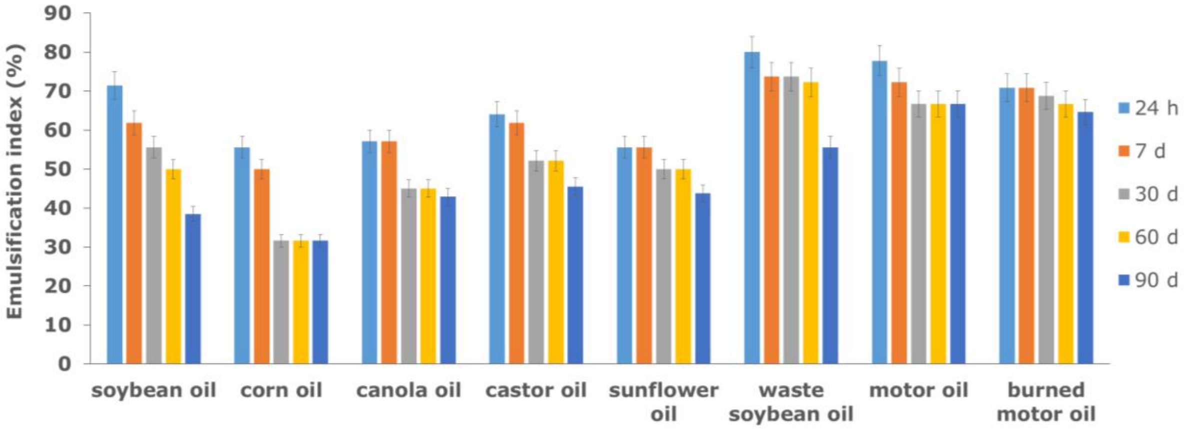
Source: Authors.

3.3 Emulsification index and stability

Determination of emulsification index has often been used as a suitable method for identifying biosurfactants which are markedly characterized by their excellent ability of emulsion stabilizing (Souza, et al., 2016; Pele, et al., 2018; Ferreira, et al., 2020). In this sense, the emulsification index was determined using the metabolic liquid of condition 4 of the FFD and the results are shown in Figure 2. The property of emulsion stabilization can be evaluated by the ability to maintain at least 50% of the original emulsion volume after 24 hours of formation (Lima, et al., 2017). Thus, the biosurfactant produced by *M. hiemalis* showed good emulsifying properties with all hydrophobic compounds tested, mainly WSO (80%), motor oil (77.8%), soybean oil (71.4%) and burned motor oil (70.8%).

In addition, emulsion stability was monitored up to 90 days at room temperature, and it was found that emulsions with motor oil, burned motor oil and WSO showed the highest stability, with emulsification index higher than 50% after three months (Figure 2). The ability to form stable emulsions is one of the most important characteristics to be considered in a biological surface-active compound, and suggest its application in formulation of detergents, cosmetics and food products (Montero-Rodríguez, et al., 2015; Vecino, et al., 2017; Rulli, et al., 2019).

Figure 2: Stability of emulsions formed by biosurfactant of *M. hiemalis* UCP 1309 in condition 4 of FFD.



Source: Authors.

4. Conclusion

This study reports for the first time in the literature the production of biosurfactant by a Mucoralean fungus using SSF. The biosurfactant exhibited excellent potential for environmental applications due to its ability to form emulsions, that remained stable after three months of incubation. In addition, the present study confirmed the suitability of SSF to convert cheap and under-utilized agro-industrial by-products into industrially relevant biosurfactant, making attractive this bioprocess for the industry.

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