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Production of biosurfactants by Mucoralean fungi isolated from Caatinga bioma soil using industrial waste as renewable substrates

Produção de biossurfactantes por fungos Mucorales isolados do solo do bioma Caatinga usando resíduos industriais como substratos renováveis

Producción de biosurfactantes por hongos Mucorales aislados del suelo del bioma Caatinga utilizando residuos industriales como sustratos renovables

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Abstract
In this work it was investigated the potential of Mucorales fungi isolated from the Caatinga of Pernambuco state for production of biosurfactants using renewable substrates. The strains (*Mucor circinelloides* UCP 0005, *M. circinelloides* UCP 0006 and *Rhizopus arrhizus* UCP 1609) were cultivated in alternative culture media consisting of instant noodle waste (INW), corn steep liquor (CSL) and post-frying soybean oil (PFSO), according to conditions established by a 2^3 full-factorial design (FFD). The production of biosurfactants was evaluated by determining surface tension and emulsification index (EI_{24}) and statistical analysis was performed using Pareto diagram. The presence of the main sources of carbon and nitrogen in production medium was confirmed by FTIR spectroscopy. According to the results, the three fungi evaluated were able of produce biosurfactant in media containing renewable sources. However, the strain that showed the greatest reduction in surface tension (72 to 27 mN/m) was *M. circinelloides* UCP 0006 in condition 3 of the FFD (1% INW and 4% CSL, in absence of PFSO). The infrared analysis of the INW showed the presence of carbohydrates, fatty acids and proteins, proving that this is a suitable substrate for the cultivation of fungi. The biosurfactants produced by *M. circinelloides* UCP 0005 and *M. circinelloides* UCP 0006 were able to form water-in-oil emulsions and the biosurfactant from *R. arrhizus* UCP 1609 formed oil-in-water emulsions. The present study demonstrated that the three Mucorales fungi tested were able to produce biosurfactants from renewable sources, with emphasis on *M. circinelloides* UCP 0006.
Keywords: Biomolecule; Fungi; Surface tension; Emulsification; Sustainability.

Resumo
Neste trabalho foi investigado o potencial de fungos Mucorales isolados da Caatinga do estado de Pernambuco para produção de biossurfactantes utilizando substratos renováveis. As cepas (*Mucor circinelloides* UCP 0005, *M. circinelloides* UCP 0006 e *Rhizopus arrhizus* UCP 1609) foram cultivadas em meios de cultivo alternativos constituídos por resíduo de macarrão instantâneo, milhocina e óleo de soja pós-fritura, de acordo com um planejamento fatorial completo 2^3. A produção de biossurfactantes foi avaliada pela determinação da tensão superficial e índice de emulsificação (IE_{24}) e a análise estatística foi realizada por meio do diagrama de Pareto. A presença das principais fontes de carbono e nitrogênio no meio de produção foi confirmada por espectroscopia FTIR. De acordo com os resultados, os três fungos avaliados foram capazes de produzir biossurfactante em meios contendo as fontes renováveis. No entanto, a cepa que apresentou maior redução na tensão superficial (72 a 27 mN/m) foi *M. circinelloides* UCP 0006 na condição 3 do planejamento (1% resíduo de macarrão instantâneo, 4% de milhocina, na ausência do óleo de soja pós-fritura). A análise no infravermelho do resíduo de macarrão instantâneo mostrou a presença de carboidratos, ácidos graxos e proteínas, comprovando que este é um substrato adequado para o cultivo de fungos. Os biossurfactantes produzidos por *M. circinelloides* UCP 0005 e *M. circinelloides* UCP 0006 foram capazes de formar emulsões do tipo água em óleo e o biossurfactante de *R. arrhizus* UCP 1609 formou emulsões do tipo óleo em água. O presente estudo demonstrou que os três fungos Mucorales testados foram capazes de produzir biossurfactantes a partir de fontes renováveis, com destaque para *M. circinelloides* UCP 0006.
Palavras-chave: Biomolécula; Fungos; Tensão superficial; Emulsificação; Sustentabilidade.

Resumen
En este trabajo se investigó el potencial de hongos Mucorales aislados de la Caatinga do estado de Pernambuco para la producción de biosurfactantes utilizando sustratos renovables. Las cepas (*Mucor circinelloides* UCP 0005, *M. circinelloides* UCP 0006 y *Rhizopus arrhizus* UCP 1609) se cultivaron en medios de cultivo compuestos por residuo de fideos instantáneos, agua residual de maíz y aceite de soya post fritura, de acuerdo con un diseño factorial completo 2^3. La producción de biosurfactante se evaluó por la determinación de la tensión superficial y el índice de emulsificación (IE_{24}) y el análisis estadístico se realizó mediante el diagrama de Pareto. La presencia de las principales fuentes de carbono y nitrógeno en el medio de producción fue confirmada por espectroscopía FTIR. De acuerdo con los resultados, los tres hongos evaluados fueron capaces de producir biosurfactantes en medios que contenían fuentes renovables. Sin embargo, la cepa que mostró la mayor reducción de la tensión superficial (72 a 27 mN/m) fue *M. circinelloides* UCP 0006 en la condición 3 del diseño factorial (1% residuo de fideos instantáneos, 4% agua residual de maíz, en ausencia de aceite de soya post fritura). El análisis infrarrojo del residuo de fideos instantáneos mostró la presencia de carbohidratos, ácidos grasos y proteínas, comprobando que este es un sustrato adecuado para el cultivo de hongos. Los biosurfactantes producidos por *M. circinelloides* UCP 0005 y *M. circinelloides* UCP 0006 formaron emulsiones de tipo agua en aceite y el biossurfactante de *R. arrhizus* UCP 1609 formó emulsiones de tipo aceite en agua. El presente estudio demostró que los tres hongos Mucorales evaluados produjeron biosurfactantes a partir de fuentes renovables, destacándose *M. circinelloides* UCP 0006.
Palabras clave: Biomolécula; Hongos; Tensión superficial; Emulsificación; Sostenibilidad.

1. Introduction

The Caatinga is biome unique of Brazil that has a wide variety of species and is the target of growing interest of industries for therapeutic purposes (Sá filho et al, 2021). Occupies about 10% of the national territory, located between the Atlantic forest and the savannah, it is present in 9 northeastern states. With the characteristic semi-arid climate, it has a highly diverse resident microbiota that presents peculiar characteristics, being able to produce bioproducts with innovative properties of high added value and industrial interest (Santos et al, 2021).

There are few records in literature with microorganisms isolated from the Caatinga bioma for the production of biosurfactants. Among the microorganisms, fungi have biotechnological potential due their extensive reproduction capacity, fast and easy adaptation (Riordon et al, 2019).

Biosurfactants are secondary metabolites produced by several microorganisms such as filamentous fungi, yeasts and bacteria (Araújo et al, 2019). Structurally, the molecule has amphipathic characteristics, that is it non-polar (hydrophobic portion) and polar (hydrophilic portion soluble in water) in same molecule (Uzoigwe, 2015). The main properties of potent biosurfactants are emulsifying and solubilizing capacity, reduction of surface tension and interfacial activity. These properties are already widely applied in industrial area as wetting, solubilizing and foaming substances, among others (Antunes et al,

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2013).

The advantage of using biosurfactants in relation to the chemical surfactants is the low toxicity, biodegradability and synthesis from renewable and low-cost substrates (Pacwa-Plociniczak et al., 2011). In this context, the ability of microorganisms in bioconvert industrial residues for the production of biosurfactants is a sustainable alternative, as it meets the environmental demand by reuse of industrial residues reducing the process costs, making the process attractive and easy to industrial employ (Rivera et al, 2019; Oliveira et al, 2020).

In this context, the present study aims to evaluate the capacity of different isolates of Mucorales fungi to production of biosurfactants of high industrial interest using industrial waste as alternative substrates.

2. Methodology

2.1 Microorganisms

In this study, three isolates of Mucorales fungi (*Mucor circinelloides* UCP 0005, *Mucor circinelloides* UCP 0006 and *Rhizopus arrhizus* UCP 1609) were used. The strains are maintained in Culture Bank of the Catholic University of Pernambuco (UNICAP), registered with the World Federation of Culture Collections (WFCC) under the number 927.

2.2 Renewable substrates

The renewable substrates used in this study were previously established by Andrade et al., (2018): instant noodle waste (INW), kindly provided by local industry; corn steep liquor (CSL) obtained of corn processing industry (Corn Products, Brazil) located in the municipality of Cabo de Santo Agostinho-PE, and post-frying soybean oil (PFSO), kindly supplied by a local food trade in the city of Recife-PE, Brazil. INW was macerated, and then the particles were uniformed in a 32 mesh (500 µm) sieve. The composition of the selected substrates is shown in Table 1.

Table 1 - Composition of instant noodle waste, corn steep liquor and post-frying soybean oil used as carbon and nitrogen sources in the culture media formulated for production of biosurfactants by species of Mucorales fungi (*Mucor circinelloides* and *Rhizopus arrhizus*)

Instant noodle waste Components	**Amount (mg)**
Carbohydrates (carbon source)	51.000
Proteins (nitrogen source)	9.400
Total fats (carbon source)	16.000
Sodium	1.357
Thiamine	0.84
Riboflavin	0.91
Niacin	11
Pyridoxine	0.91
Corn steep liquor Main component	**Amount (%)**
Amino Acids (nitrogen source)	62.49
Post-frying soybean oil Components in fatty acids	**Amount (%)**
Saturated (carbon source)	21.06
Monounsaturated (carbon source)	29.78
Polyunsaturated (carbon source)	55.97

Source: Andrade et al (2018) adapted.

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2.3 Inoculum preparation

To prepare the inoculum, 100 mL sterile water were added to the Erlenmeyer flasks and young spores of different fungal isolates were added to the Erlenmeyer flasks. Then, it was performed the count in Neubauer chamber until 10^7 spores/mL. 5% this suspension was used as inoculum in the production media.

2.4 Biosurfactant production

The production was carried out in 250 mL-Erlenmeyers flasks containing 100 mL of the production media, consisting of renewable substrates (INW, CSL and PFSO), at concentrations established by a 2^3 full-factorial design (FFD). The pH of the media was adjusted to 5.5, and then, they were sterilized in autoclave and inoculated with 5% spore solution. Fermentations were carried out under orbital shaking at 150 rpm and 28°C, for 96 h. After this period, the cultures were subjected to filtration and centrifugation, in order to separate the biomass from the metabolic liquids. Cell-free metabolic liquids were used to determination of surface tension and emulsification index.

2.5 Factorial design

A 2^3 FFD was carried out in order to investigate the influence of concentration of each low-cost substrate (INW, CSL and PFSO), as well as the interaction between them, on surface tension as response variable. Table 2 shows the levels studied for each independent variable of FFD. A set of eight assays with four replicates at the central point was performed. The data obtained from the experiments were subjected to statistical analysis by Statistica® software, version 12.0 (StatSoft Inc., USA) and the significance of the results was tested at $p < 0.05$ level.

Table 2 - Levels and variables of the 2^3 full-factorial design for biosurfactant production by different Mucorales fungi.

Variables	**Levels**		
	-1	**0**	**+1**
Instant noodle waste (%)	1	2	3
Corn steep liquor (%)	0	2	4
Post-frying soybean oil (%)	0	0.5	1

Source: Authors.

2.6 Determination of surface tension

Surface tension was measured in triplicate on cell-free metabolic liquids using Du Noüy ring method in an automatic tensiometer model Sigma 70 (KSV Instruments Ltd., Finland), at temperature of 28°C (Kuyukina et al, 2001). The measurement of surface tension on distilled water was used as control (surface tension of water = 72 mN/m).

2.7 Determination of emulsification index (EI_{24})

The ability of the biosurfactant in form emulsions was verified after 24 h of homogenization, according to Cooper and Goldenberg (1986). The hydrophobic substrate used was burnt engine oil burned in ratio of 1:1 and in triplicate. The emulsification index (EI_{24}) was evaluated according with following equation:

$$EI_{24} (\%) = \text{Emulsion height (EH)} / \text{Total height (TA)} \times 100 \qquad \text{(Eq. 1)}$$

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2.8 Microscopic analysis of emulsions

The type of emulsion (water in oil/oil in water) was determined after the formation of emulsion by homogenization of the metabolic liquid (containing the biosurfactant) and the hydrophobic substrate (burnt engine oil). Then, a drop of the emulsion was transferred with a Pasteur pipette to slide and visualized in optical microscope with increase of 40x. From the image, the emulsion formed was classified according to the type and formation of bubbles.

2.9 Identification of functional groups of industrial waste

INW used in production medium was subjected to Fourier-transform infrared (FTIR) spectroscopy, in order to identify the functional groups in its composition. The functional groups of CSL and PFSO were identified according with Naumann et al, (2000) and Forato et al (2013), respectively.

3. Results and Discussion

3.1 Production of biosurfactant by Mucorales fungi

The three Mucorales fungi used in this study were able of metabolize agro-industrial residues for production of biosurfactants, as shown in Table 3. The strain that showed the greatest reduction in surface tension (72 to 27 mN/m) was *M. circinelloides* UCP 0006 in condition 3 of the FFD (1% INW and 4% CSL, in absence of PFSO). Significant values of surface tension were also obtained by *M. circinelloides* UCP 0005 (29 mN/m) and *R. arrhizus* UCP 1609 (31 mN/m) in condition 7 (1% INW, 4% CSL and 1% PFSO).

In this context, *M. circinelloides* UCP 0006 was selected by its promising potential when compared to the biosurfactant-producing bacteria which reduce the surface tension to values between 25-28 mN/m. Previously, several researchers reported similar results to those obtained in the present study, with biosurfactants produced by *Serratia marcescens* (25.92 mN/m) (Araújo et al, 2019), *Bacillus stratospshericus* (28 mN/m) (Hentati et al, 2019), *Streptomyces* sp. (28 mN/m) (Santos et al, 2019) and *Pseudomonas cepacia* (29 mN/m) (Soares et al, 2018).

Table 3 - Surface tension results obtained by *Mucor circinelloides* UCP 0005, *Mucor circinelloides* UCP 0006 and *Rhizopus arrhizus* UCP 1609 in 2^3 full-factorial design.

Conditions	Renewable substrates			Surface tension (mN/m)		
	Instant noodle waste (%)	Corn steep liquor (%)	Post-frying soybean oil (%)	*Mucor circinelloides* UCP 0005	*Mucor circinelloides* UCP 0006	*Rhizopus arrhizus* UCP 1609
1	1	0	0	40	39	43
2	3	0	0	37	36	38
3	1	4	0	41	27	34
4	3	4	0	34	29	37
5	1	0	1	33	34	40
6	3	0	1	34	32	37
7	1	4	1	29	28	31
8	3	4	1	31	28	32
9	2	2	0.5	31	30	35
10	2	2	0.5	32	29	36
11	2	2	0.5	31	28	36
12	2	2	0.5	30	29	37

Source: Authors.

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Table 4 shows the surface tension results obtained in this study compared to the literature in the last five years. The literature search was carried out from works that also used industrial substrates of renewable origin as raw material in the culture media for the production of biosurfactants by filamentous fungi. It worth highlighting that in the study carried out by Andrade et al. (2018) with *Cunninghamella echinulata* UCP 1299, using the same industrial waste (2% INW, 0.5% PFSO and 2% CSL), the surface tension was 34 mN/m. In addition, the isolate *M. circinelloides* UCP 0006 showed to be a promising microorganism for the production of a biosurfactant capable of competing with the chemical surfactant sodium dodecyl sulfate (SDS), which reduce the surface tension to 37 mN/m. Oliveira et al (2020) obtained a similar result in their study with *Penicillium sclerotiorum*, also using renewable substrates. It corroborates the ability of filamentous fungi to use different sources of carbon and nitrogen to produce biosurfactants, justifying the importance and necessity of investing in the researches with these microorganisms.

Table 4 - Surface tension obtained in this study compared with the literature in last five years involving culture of filamentous fungi in media containing substrates of renewable origin.

Microorganisms	Substrates	Surface tension (mN/m)	References
Mucor circinelloides UCP 0006	INW and CSL	27	This study
M. circinelloides UCP 0005	INW, CSL and PFSO	29	This study
Rhizopus arrhizus UCP 1609	INW, CSL and PFSO	31	This study
M. circinelloides UCP 0005	Jatobá (*Hymenaea stilbocarpa*) husks and CSL	34	Santiago et al (2021)
M. hiemalis UCP 0039	PFSO	40	Ferreira et al (2020)
Penicillium sclerotiorum UCP 1361	Whey and barley	27	Oliveira et al (2020)
Absidia cylindrospora UCP 1301	Crude glycerol, CSL and whey	30	Mendonça et al (2020)
Cunninghamella echinulata UCP 1299	INW, CSL and PFSO	32	Andrade et al (2018)

Source: Authors.

3.2 Influence of carbon and nitrogen sources for biosurfactant production by *Mucor circinelloides* and *Rhizopus arrhizus*

From the results it was possible to identify that the isolates of *M. circinelloides* (UCP 0005 and UCP 0006) and *R. arrhizus* (UCP 1609) used the carbohydrate present in INW (starch) as the first carbon source, as it is a hydrophilic source of easy assimilation. The nitrogen source in the culture medium also plays a fundamental role in the metabolism of microorganisms to obtain biosurfactants, which may have a limiting effect on the bioprocess by altering the pH of the medium

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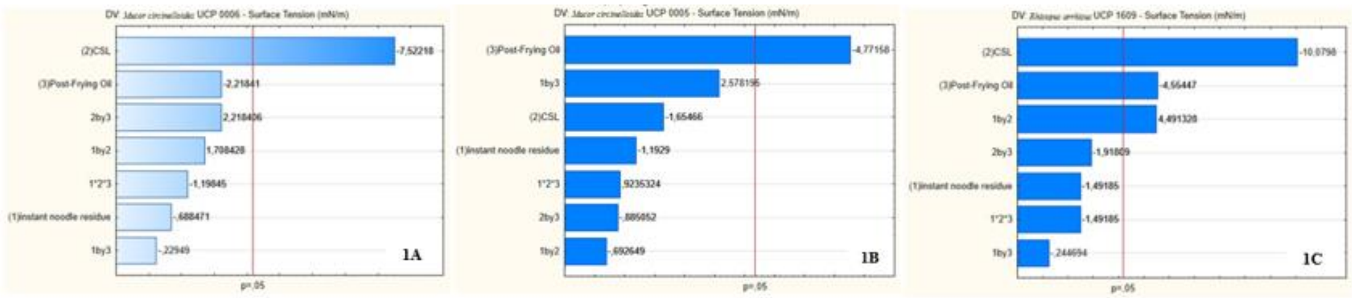
by releasing the chemical amino group, making it acidic (Marcelino et al. al, 2020). Thus, in the present study, the production of the biosurfactant by the Mucorales fungi was favored by the amino acids present in CSL.

The influence of concentrations of carbon sources (INW and PFSO) and nitrogen source (CSL), as well as their interactions in production of biosurfactants by *M. circinelloides* UCP 0005, *M. circinelloides* UCP 0006 and *R. arrhizus* UCP 1609, were statistically evaluated by Pareto diagram (Figure 1).

Figure 1 – Pareto diagrams obtained from the statistical analysis of the 2^3 full-factorial design applied to the production of biosurfactants by *Mucor circinelloides* UCP 0006 (A), *Mucor circinelloides* UCP 0005 (B) and *Rhizopus arrhizus* UCP 1609 (C). The point at which the effect estimates were statistically significant (p = 0.05) is indicated by dashed lines



Source: Authors.

Figure 1A demonstrates that for production by *M. circinelloides* UCP 0006 only CSL has significant influence on the reduction of surface tension. This isolate showed the greatest reduction in surface tension (72 to 27 mN/m), and it is possible to confirm that this nitrogen source favored the production of the biomolecule. For the isolate *M. circinelloides* UCP 0005 (Figure 1B), PFSO was the most important variable to reduce surface tension in condition 7 (1% INW, 4% CSL and 1% PFSO), being the preferred carbon source used by this isolate. CSL and INW may have greater statistical significance in the results within a new design including other concentrations of these substrates.

Figure 1C demonstrates that for production of biomolecule by *R. arrhizus* UCP 1609, PFSO and CSL were the components of the production medium that most contributed with the reduction of surface tension. Therefore, INW, in concentration used, did not statistically influence the reduction of surface tension, requiring the increase of this concentration.

3.3 Chemical characterization of waste used for production of biosurfactants by Mucorales fungi.

The chemical composition (functional groups) of the residues used was identified by infrared spectroscopy. According with results obtained by Forato et al (2013), CSL has the presence of different amino acids (protein source) in its composition (peaks between 1700 and 1000 cm^{-1}), confirming its function in production medium as the main nitrogen source. Moreover, CSL also shows the versatility of its composition with the presence of fatty acids (peaks between 3100 and 2800 cm^{-1}) and oligo and polysaccharides (peaks between 1200 and 1000 cm^{-1}) (Naumann et al, 2000, Forato et al 2013), also proving to be an excellent source of carbon for microorganisms. On the other hand, INW (Figure 2) demonstrates a more intense spectral band in 1014 cm^{-1} (Figure 3), evidencing the presence of oligo and polysaccharides (1151 cm^{-1} and 1078 cm^{-1}) confirming the presence of carbohydrates. In addition to the presence of carbohydrates in composition of INW, it was also evidenced the presence of fatty acids (3296 to 2800 cm^{-1}) and proteins (1742 cm^{-1}) in its composition (Naumann et al, 2000).

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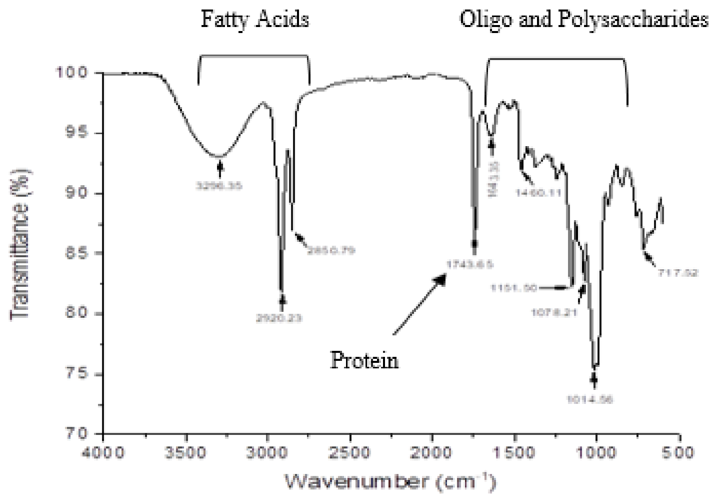
Figure 2 - Infrared spectrum (FTIR) of the structure of the INW used in production medium of biosurfactants by Mucorales fungi.

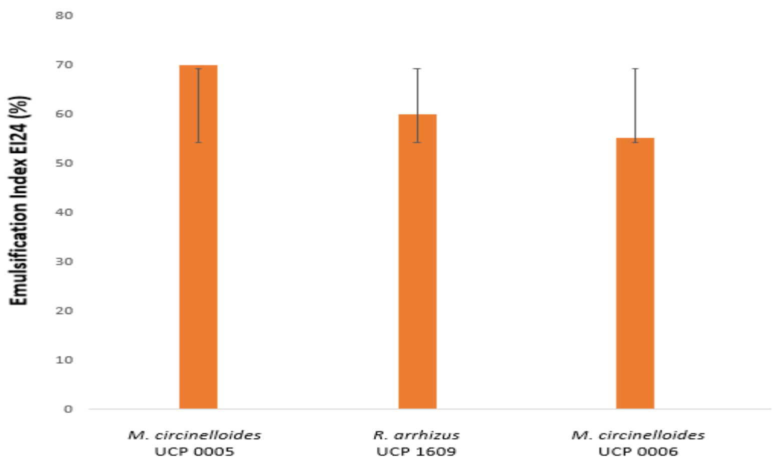


Source: Authors.

3.4 Potential of the biosurfactant obtained from Mucorales fungi for formation of stable emulsions

The emulsifying capacity of a biomolecule is an important parameter that expresses the versatility of its application in different areas (Zargar et al, 2022). The literature indicates that a biosurfactant with the potential to form stable emulsions is one that has an emulsification index (EI_{24}) above 50% (Ferreira et al, 2020). Thus, according to Figure 3, the metabolic liquids obtained from culture of three Mucorales fungi were able to present excellent EI_{24}, showing the bifunctional potential of these filamentous fungi to reduce surface tension and form stable emulsions. However, *M. circinelloides* UCP 0005 showed the best emulsification potential at the central point of FFD (70% in condition 11), followed by *R. arrhizus* UCP 1609 (60% in condition 8) and *M. circinelloides* UCP 0006 (55.2% in condition 3), the latter being the same condition of lower surface tension. A similar emulsification result (EI_{24} of 79.17%) was obtained by another Mucorales fungus, *Absidia* sp. UCP 1144, after growth in medium containing CSL (3%), glycerol (3%) and whey (4%) (Mendonça et al, 2019).

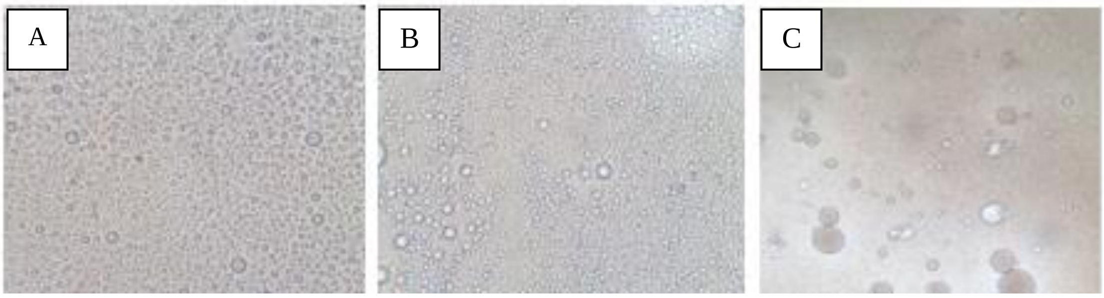
Figure 3 – Potential of the biosurfactant produced by *Mucor circinelloides* UCP 0006, *Mucor circinelloides* UCP 0005 and *Rhizopus arrhizus* UCP 1609 in formation of emulsions using burnt engine oil.



Source: Authors.

Figure 4 shows the optical microscopy of emulsions formed by biosurfactant from *M. circinelloides* UCP 0005 (Figure 2A), *M. circinelloides* UCP 0006 (Figure 2B) and *R. arrhizus* UCP 1609 (Figure 2C). Figure 2A and Figure 2B show the formation of oil-in-water emulsions by the biosurfactant of *M. circinelloides* UCP 0005 and *M. circinelloides* UCP 0006, respectively. With a greater number of globular and homogeneous droplets between the phases, there are fewer empty spaces between the bubbles, characteristic of oil drops from a dispersed phase suspended in a continuous or aqueous phase (Souza et al, 2016). On the other hand, Figure 2C shows the formation of water-in-oil emulsions formed by the biosurfactant of *R. arrhizus* UCP 1609.

Figure 4 – Microscopic observation (40x increase) of the emulsions formed by biosurfactant of *Mucor circinelloides* UCP 0005 (A), *Mucor circinelloides* 0006 (B) and *Rhizopus arrhizus* UCP 1609 (C).



Source: Authors.

4. Conclusion

In this study, the maximum efficiency in production of biosurfactant from renewable sources was evidenced by *M. circinelloides* UCP 0006. However, the isolates *M. circinelloides* UCP 0005 and *R. arrhizus* UCP 1609 also showed potential to produce biomolecule with tensoactive and emulsification properties of high industrial interest. The production of biosurfactant by Mucorales fungi was influenced by favorable constitution of alternative production medium rich in hydrophilic carbon source (starch present on INW) and hydrophobic carbon source (PFSO), as well as is rich in source of nitrogen (amino acids present in CSL). Future studies can be carried out using a new factorial design with alteration of the amounts of residues used, considering the possibility of contribution of INW on biosurfactant production by *M. circinelloides* UCP 0005.

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