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

Produção de biossuintantes 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

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$_{30}$) 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.
Resumen

En este trabajo se investigó el potencial de hongos Mucorales aislados de la Caatinga del 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 2³. La producción de biosurfactante se evaluó por la determinación de la tensión superficial y el índice de emulsificación (IE₉₂) 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 biosurfactante en medios conteniendo las fuentes renovables. Sin embargo, la cepa que mostró mayor reducción de la tensión superficial (72 a 27 mN/m) fue M. circinelloides UCP 0006 y M. circinelloides UCP 0006 fueron capaces de formar emulsiones de tipo agua en aceite y el biosurfactante de R. arrhizus UCP 1609 formó emulsiones de tipo aceite en agua. El presente estudio demostró que los tres hongos Mucorales evaluados son capaces de producir biosurfactantes a partir de fuentes renovables, destacándose M. circinelloides UCP 0006.

Palabras clave: Biomolécula; Hongos; Tensión superficial; Emulsificación; Sustentabilidad.

1. Introducción

La Caatinga es un ecosistema único de Brasil que tiene una variedad de especies y es el objetivo de un creciente interés de industrias para fines terapéuticos (Sá filho et al., 2021). Ocupa un 10% del territorio nacional, ubicado entre el Parque Nacional de la Caatinga y la sabana. Es present en 9 estados del noreste. Con su clima semiaride, es capaz de producir bioproductos con propiedades innovadoras de alta valoración y interés industrial (Santos et al., 2021).

Existen pocos registros en la literatura sobre microorganismos aislados de la Caatinga para la producción de biosurfactantes. Entre los microorganismos, los hongos tienen un potencial biotecnológico debido a su capacidad de reproducción extensa, rápido y fácil (Riordon et al., 2019).

Biosurfactantes son metabolitos secundarios producidos por varios microorganismos, como hongos, levaduras y bacterias (Araújo et al., 2019). Structurally, la molécula tiene características amphipáticas, que es no-polar (tipo hidrofóbico) y polar (tipo hidrofílico soluble en agua) en la misma molécula (Uzoigwe, 2015). Las propiedades principales de los biosurfactantes son su capacidad emulsionante y solubilizante, la reducción de la tensión superficial y la actividad interfacial. Estas propiedades son ampliamente utilizadas en la industria como agente de frota, solubilizante y espumante, entre otros (Antunes et al., 2015).
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 | 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 | Amino Acids (nitrogen source) | 62.49  |
| Post-frying soybean oil Components in fatty acids | Saturated (carbon source) | 21.06  |
|                                 | Monounsaturated (carbon source) | 29.78  |
|                                 | Polyunsaturated (carbon source) | 55.97  |

Source: Andrade et al (2018) adapted.
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:

\[ EI_{24} (%) = \frac{\text{Emulsion height (EH)}}{\text{Total height (TA)}} \times 100 \] (Eq. 1)
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 stratosphericus (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 2³ 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.
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*  | INW and CSL            | 27                     | This study                  |
| UCP 0006                |                        |                        |                             |
| *M. circinelloides*     | INW, CSL and PFSO      | 29                     | This study                  |
| UCP 0005                |                        |                        |                             |
| *Rhizopus arrhizus*     | INW, CSL and PFSO      | 31                     | This study                  |
| UCP 1609                |                        |                        |                             |
| *M. circinelloides*     | Jatobá (Hymenaea       | 34                     | Santiago et al (2021)       |
| UCP 0005                | stilbocarpa) husks and |                        |                             |
|                         | CSL                    |                        |                             |
| *M. hiemalis*           | PFSO                   | 40                     | Ferreira et al (2020)       |
| UCP 0039                |                        |                        |                             |
| *Penicillium sclerotiorum* | Whey and barley     | 27                     | Oliveira et al (2020)       |
| UCP 1361                |                        |                        |                             |
| *Absidia cylindrospora* | Crude glycerol, CSL and | 30                     | Mendonça et al (2020)       |
| UCP 1301                | whey                   |                        |                             |
| *Cunninghamella echinulata* | INW, CSL and PFSO     | 32                     | Andrade et al (2018)        |
| UCP 1299                |                        |                        |                             |

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.
by releasing the chemical amino group, making it acidic (Marcelino et 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 2³ 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

![Pareto diagrams](image)

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 4% 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).
Figure 2 - Infrared spectrum (FTIR) of the structure of the INW used in production medium of biosurfactants by Mucorales fungi.

![Infrared spectrum](image)

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.

![Emulsification Index](image)
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 tensioactive 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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