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Research article

Biosurfactant and bioemulsifier as promising molecules produced by *Mucor hiemalis* isolated from Caatinga soil

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ABSTRACT

Background: The present study describes the production of biosurfactant (BS) and emulsifier (BE) by the filamentous fungus *Mucor hiemalis* UCP 0039, as well as the characterization and stability of the both biomolecules for environmental or industrial applications.
Results: Biosurfactants and bioemulsifiers are amphiphilic compounds and are produced as extracellular molecules. The results showed that bioproduct obtained by shaker condition reduced the water surface tension of 72 to 32 mN/m and reached an emulsification index of 96%, while the static cultivation resulted in a biomolecule with a surface tension of 40 mN/m and an emulsification index of 96%, suggesting the production of a biosurfactant and bioemulsifier, respectively. The compounds showed glycolipid nature but the biosurfactant presented cationic charge, while the bioemulsifier, anionic charge. Thus, the results confirmed that *M. hiemalis* produced two distinct biomolecules under different parameters and in the same culture medium.
Conclusions: It is the first time that biosurfactant and emulsifier production has been described in the same medium and under different physical conditions by *Mucor hiemalis*. Both biomolecules showed thermal stability, as well as have significant effect on the viscosity of hydrophobic compounds, indicating the excellent potential for environmental safety or industrial applications to improve the efficiency of sustainable and economic technologies.
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1. Introduction

Surfactants are soluble compounds that reduce surface tension and/or interfacial tension between two immiscible liquids [1,2]. While an emulsifier does not necessarily reduce surface tension to significant values, it nevertheless assists in the dispersion of droplets from one immiscible liquid into another and prevents coalescence [3,4].

Methods for detecting biological surfactants in culture media are often based on surface tension measurement [5]. Other methods include drop collapse, oil displacement, hemolysis tests and the use of the emulsification index (EI_{24}) [6]. Normally, bioemulsifiers are best known for emulsifying liquids without significant changes in the surface tension of their growth medium [7]. Moreover, experimental reports have shown that surface tension measurements and emulsification index screening methods do not correlate [8,9,10]. These methods often results in the elimination of bioemulsifier as they do not exhibit significant changes in surface/interfacial tension and may yield negative results during screening tests [11].

The chemical composition of surface-active biomolecule agents and bioemulsifiers is different and this fact may contribute to their specific roles in nature and biotechnological applications. At this point, it is important to note that the ability to reduce surface and interfacial tension stands out as the distinct contrast between surface-active biomolecule and bioemulsifiers, an especially important feature for accurate screening and identification procedures for microbial broths [12].

Surfactants and chemical emulsifiers can be used in different industrial segments such as cosmetic, petroleum, textile, agriculture,

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medicine, and food [13]. However, these chemicals cause negative impacts on the environment, thus the availability of a minor or non-toxic alternative such as bioemulsifiers and biosurfactants is desirable [14,15,16].

Research on the applications of the surface-active biomolecule agents and bioemulsifiers in reducing environmental pollution began some years ago, but it is relevant to emphasize the relationship with the relevant sustainability theme. Makkar and Cameotra [17] highlighted the importance of the concept of "reducing, reusing and recycling" for waste management due to concerns about the hazardous and non-hazardous waste generation rate and the inherent cost of treating and disposing of it. The authors emphasized the need for cost-effective biomolecule production to address these growing concerns. Several inexpensive waste materials are explored as substrates for the production of surface-active biomolecules, thus bringing an effective cost reduction strategy together with the need for waste management [18]. The use of industrial waste for the production of valuable compounds has become important in recent times, not only in the economics of any commercial production process but also in establishing a sustainable effort to effectively manage unprecedented waste [19].

Mucor hiemalis is a filamentous, dimorphic fungus widely highlighted in the literature for its biotechnological potential, especially with the production of enzymes and bioethanol. However, there are still few studies relating the microorganism to the production of surface-active biomolecule and bioemulsifier.

The present study aims to evaluate the surfactant characteristics of a surface-active biomolecule and bioemulsifier produced by *Mucor hiemalis* UCP 0039 using post-frying soybean oil as carbon source, as well as to determine their respective stability against different environmental factors, generating efficient products, economically viable for future industrial application.

2. Materials and methods

2.1. Microorganism

The filamentous fungi *Mucor hiemalis* UCP 0039 was isolated from Caatinga soil, Pernambuco, Brazil. The strain was kindly provided by the Culture Collection of the Catholic University of Pernambuco, registered in the World Federation for Culture Collections (WFCC) under the number 927.

2.2. Renewable substrate

The renewable substrate used for biomolecule production was waste soybean oil (WSO), obtained from a local food trade.

2.3. Preparation of inoculum

The inoculum was prepared from young culture of *M. hiemalis* grown in solid medium Sabouraud dextrose agar during four days at 28°C, until sporulation. Young spores of *M. hiemalis* were transferred to Erlenmeyer flasks containing sterile water. The spores were then counted until 10^7 spores/mL were obtained and 5% of the spore suspension was used as inoculum in the culture media.

2.4. Detection of hemolytic activity

The ability of *M. hiemalis* to produce BS and BE agents was preliminarily detected by the hemolytic activity test according to Mahjoubi et al. [20]. Spores of the microorganism were inoculated in the center of a plate containing blood agar medium and incubated at 28°C for 96 h. The experiment was monitored every 24 h until the hemolysis halo formation.

2.5. Production of BS and BE

BS and BE production was investigated in the same culture medium consisting of a salt solution (1 g/L $NH_4 \cdot NO_3$, 0.2 g/L KH_2PO_4 and 0.2 g/L $MgSO_4 \cdot 7H_2O$) supplemented with 1% sodium glutamate (nitrogen source) and 5% post-frying soybean oil (carbon source), according to production media proposed by Pele et al. [21]. However, changes in inoculum size and agitation were performed as described in Table 1. The growth of *M. hiemalis* occurred during 96 h, 150 rpm at 28°C. After this period of fermentative processes, the metabolic liquid free cells were obtained by filtration, followed by centrifugation (10,000 × *g* for 15 min).

2.6. Determination of surface tension (ST)

After cultivation, surface tensions of cell-free metabolic liquids were determined using the Du Nouy ring method, in automatic tensiometer (model Sigma 70 KSV Ltd., Finland). Measurements were performed in triplicate according to Kuyukina et al. [22].

2.7. Determination of emulsification index (EI)

The ability to form emulsions was evaluated by emulsification index (IE) after 24 h, following the method of Cooper and Goldenberg [23]. The hydrophobic substrates used were motor oil and motor oil burned in 1:1 ratio (oil/metabolic liquid). Measurements were performed in triplicate.

2.8. Determination of the type of emulsion formed

The emulsion was characterized using light field microscopy and a digital camera was used to capture the images. After homogenization of the samples, an aliquot of the emulsion was transferred to the slide with the aid of a Pasteur pipette and visualized with an increase of 10×. Characteristics such as state of aggregation and emulsion type were analyzed.

2.9. Oil displacement area (ODA) test

The ability of biomolecules to disperse burned motor oil in water was evaluated. For this, 40 mL of distilled water, 1 mL of burned motor oil and 0.5 mL of cell-free metabolic liquids were added to the Petri dish. The oil displacement area (ODA) was obtained according to the [Equation 1] [24]:

$$ODA = 3.14 \times r^2 \quad [1]$$

2.10. Influence on viscosity of hydrophobic compounds

The influence of the action of BS and BE produced by *M. hiemalis* UCP 0039 on the viscosity of petroleum derivative (burned motor oil) and vegetable oil (soybean oil) was investigated. Results were expressed as centipoise (Cp) and percentage (%). For this purpose, a fixed volume of 6 mL of the burned motor oil was inserted into graduated tubes and 2 mL of the cell-free metabolic liquid containing BS/BE. The viscosity readings were performed at 25°C using standard viscometer

Table 1
Parameters selected for the production of biosurfactant and bioemulsifier by *Mucor hiemalis*.

Inoculum (%)	Agitation (rpm)	References
5	150	Pele et al. [21]
1	0	Pele et al. [21] modified

Brookfield TC 500 (Middleboro, MA, USA), with spinder no. 42 at 50 rpm. Samples were vortexed for 1 min and aliquots of emulsion evaluated by viscometer [25].

2.11. Extraction of BS and BE

The biomolecules produced by *M. hiemalis* were isolated from cell-free metabolic liquids using the 1:1 (v/v) acetone precipitation method according to Paraszkiewicz et al. [26]. The precipitates were left to stand for 24 h at 4°C, and then centrifuged at 4000 rpm for 15 min at 5°C.

2.12. Determination of ionic charge and functional groups

The identification of ionic charge was investigated by zeta potential with Zeta-Meter system 3.0 + ZM3-DG Direct Imaging (Zeta Meter, Inc., USA), while the functional groups were identified by the infrared spectroscopy technique. Infrared spectra were recorded on a Mattson 1000 FT-England FTIR system within the range of 500–4000 cm^{-1}.

2.13. Determination of biochemical composition

Biochemical composition of biomolecules was investigated by determination of total protein, total carbohydrate and total lipid content. To quantify the total protein content, the Diagnostic S.A. Brazil kit was used. The kit has the biuret reagent used for detecting the presence of peptide bonds. In the presence of peptides, a copper (II) ion forms mauve-colored coordination complexes in an alkaline solution, and used albumin as standard. Total protein concentration was obtained according to the [Equation 2]: Total protein = (Test Absorbance / Standard Absorbance) × (4 $g \cdot dL^{-1}$). The total carbohydrates were determined according to the methodology described by Dubois et al. [27], using phenol and sulfuric acid as reagents. D-Glucose was used as standard. The total amount of lipids was determined using the method proposed by Manocha et al. [28]. The total lipids were extracted with chloroform: methanol in different proportions (1:1, 1:2, and 2:1 by v/v). The organic extracts were gathered then evaporated under vacuum and the total lipid content was determined by gravimetry.

2.14. Stability of dispersing and ability action of stability

In order to evaluate the stability of dispersing ability of biomolecules, the metabolic liquids were subjected to different value of pH (2, 4, 6, 8, 10 and 12), temperature (5, 10, 20, 40, 80 and 100°C) and NaCl concentration (0, 5, 10, 15 and 20%). Subsequently, samples containing BS and BE were submitted to the oil displacement area (ODA) test.

2.15. Stability of emulsifying capacity

Stability of emulsifying capacity was assessed by the emulsification index test using the BE. The samples were submitted to different value of pH (2, 4, 6, 8, 10 and 12), temperature (5, 10, 20, 40, 80 and 100°C) and NaCl concentration (0, 5, 10, 15 and 20%) and then, the samples were subjected to determination of the emulsification index (IE).

3. Results and discussion

3.1. Hemolytic activity of Mucor hiemalis

The potential of *M. hiemalis* UCP 0039 in the production of surfactant biomolecules was preliminarily detected by hemolytic activity. Thus, the result demonstrated the appearance of the clear zone (25 mm) around the colony, in the first 24 h (Fig. 1A), and the increase of the halo to 69 mm at 96 h (Fig. 1B). Pele et al. [21] identified species of *Rhizopus* genus isolated from Caatinga soil for biosurfactant production after detecting halo formation of 40 mm in diameter. Therefore, the response obtained in hemolysis test in this work is significant because hemolytic activity is a qualitative preliminary test described by many studies that determined the ability of microorganisms in the production of BSs [29,30,31,32].

3.2. Biosurfactant and bioemulsifier production by Mucor hiemalis using waste soybean oil in submerged cultivation

M. hiemalis UCP 0039 was able to metabolize the components of the production medium (1% sodium glutamate and 5% WSO) proposed by Pele et al. [21], resulting in the extracellular production of two biomolecules with different surfactant characteristics as shown in Table 2.

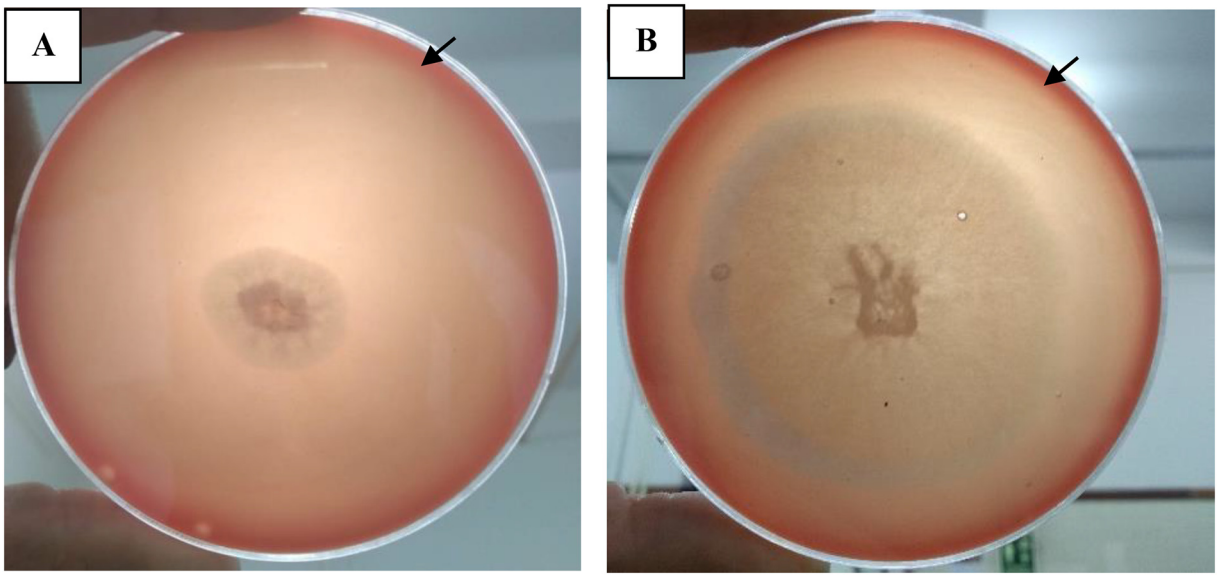

Fig. 1. Detection of hemolytic activity of the biosurfactant produced by *Mucor hiemalis* UCP 0039: (A) Hemolysis after 24 h and (B) hemolysis after 96 h.

Table 2
Production of biosurfactant and bioemulsifier by *Mucor hiemalis*.

Conditions		Results			
Inoculum (%)	Agitation (rpm)	Surface tension (mN/m)	Emulsification index (EI_{24}%)	Yield (g/L)	Produced biomolecule
5	150	32	96	7.73	BS
1	0	40	98	1.17	BE

Table 3
Surface tension and emulsification index of biosurfactants produced by Mucoralean fungi using waste soybean oil as a carbon source, described in the literature and compared to present study.

Microorganisms	Waste soybean oil as carbon source (%)	Surface tension (mN/m)	Emulsification index (EI_{24}%) (burned motor oil)	References
Mucor hiemalis	5	32.0	96	Present study
Cunninghamella echinulata	2	31.7	98.7	De Souza et al. [40]
C. echinulata	0.5	32.4	81.4	Andrade Silva et al. [24]
Rhizopus arrhizus UCP 1607	5	31.8	79.4	Pele et al.[41]
R. arrhizus var. arrhizus UCP 1295	5	35.0	69.0	Pele et al. [21]
R. microsporus var. chinensis UCP 1296	5	33.3	91.7	Pele et al. [21]
C. echinulata	3	36.0	80.0	De Souza et al. [40]
Synthetic surfactant	petroderivative	36.0	64.0	Christofi and Ivshina [42]

Medium with 5% inoculum and cultivation at 150 rpm, produced 7.73 g/L of a compound with significant surfactant properties, since it showed surface tension of 32 mN/m and an emulsification index of 96%. In this condition, the biomass yield was 2.18 g/L. According to the literature, surface tension values below 35mN/m indicate that the microorganism is an efficient biosurfactant producer [33,34,35,36]. Moreover, these results demonstrate that the BS produced has significant emulsifying properties, since Willumsen and Karlson [37] consider significant values above 50% emulsification.

On the other hand, with 1% inoculum and in static condition, *M. hiemalis* produced 1.17 g/L of a BE with potential to reduce surface tension to 40 mN/m and emulsification index of 98%. The biomass yield was 2.10 g/L. These results are in agreement with Rahman et al. [38] stating that BEs are not capable of causing significant changes in the reduction of surface tension between liquids, besides presenting high emulsifying activity.

It is also noteworthy that in this work, BS and BE production was carried out in medium containing WSO as the sole carbon source. Therefore, the results obtained in this study were compared to those recently published using this residue and noted that the BSs cited are capable of forming stable emulsions after 24 h. In addition, it is possible to state that the surface tension values cited in the literature corroborate the result obtained in present study, with values around 31–36 mN/m (Table 3).

3.3. Characterization of emulsions formed by BS and BE produced by M. hiemalis

The emulsions formed by the BS and BE produced by *M. hiemalis* were visualized in a light field microscope, characterized by the droplet aggregation state and the emulsion type formed after the use of the burned motor oil as hydrophobic substrate (Fig. 2).

According to the results obtained, the BS favored the formation of oil-in-water (O/W) emulsions with thermodynamically stable and heterogeneous globular droplets (Fig. 2A). The same characteristics were identified in the BE, with a difference in the presence of a larger number of (O/W) droplets when compared to the BS emulsions (Fig. 2B). These data are in agreement with Souza et al. [39] which characterizes emulsions as O/W type when the oil droplets (dispersed phase) are suspended in the aqueous (continuous) phase.

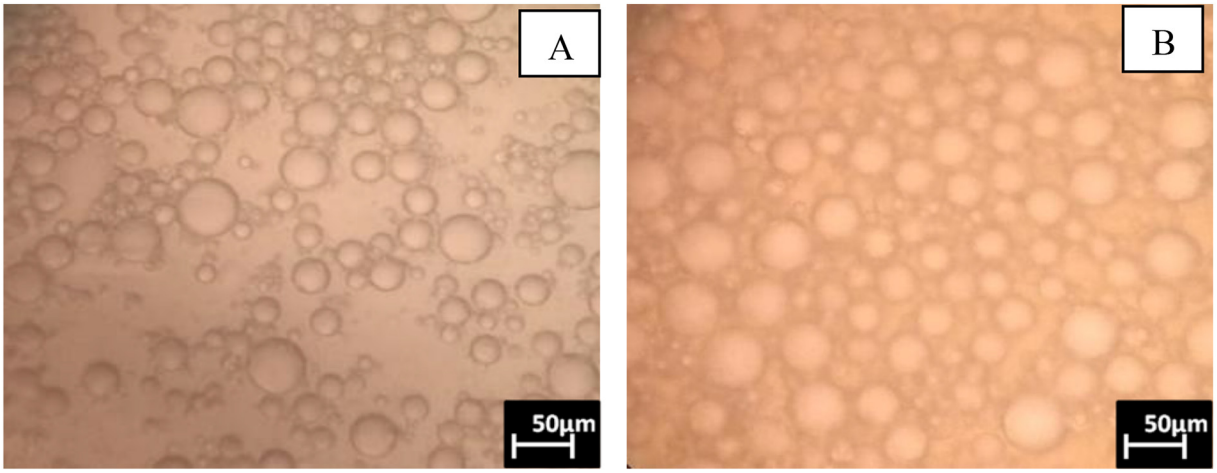

Fig. 2. Microscopic observation (40×) of the emulsion droplets formed by BS (A) and BE (B) of *M. hiemalis*.

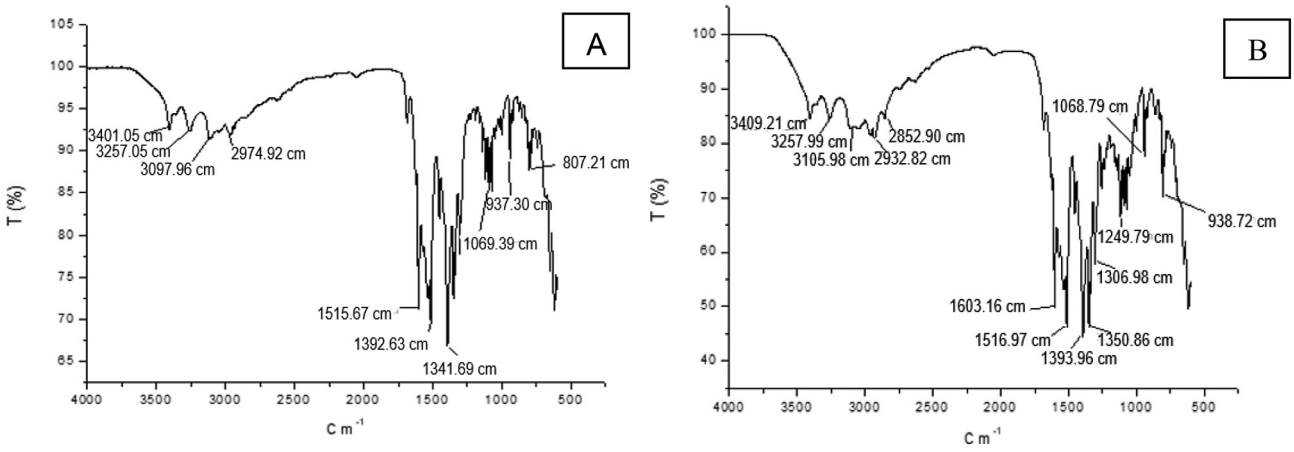

Fig. 3. Identification of functional groups of biosurfactant A) and bioemulsifier B) produced by *M. hiemalis* analyzed by infrared spectroscopy.

3.4. Ionic charge, chemical composition and functional groups

BS produced by *M. hiemalis* showed positive charge in the hydrophilic region after Zeta potential analysis, with +28.6 ZPmV, 1778 μS/cm at 23.9°C, indicating that it is a cationic surfactant. However, the BE showed negative charge, with -56.6 ZPmV, 438.2 μS/cm at 23.4°C, indicating and anionic profile. On the other hand, the results of the biochemical composition indicated that BS contained 56% lipids, 30% carbohydrates and 6% proteins, while the BE is composed by 48% lipids, 38% carbohydrates and 7.2% proteins., demonstrating that both belong to the glycolipid class.

The identification of the functional groups present in BS (Fig. 3A) are similar to those of the BE (Fig. 3B) as shown by the FT-IR spectra. Both biomolecules exhibited similarity with peaks between 3401–2974 cm^{-1}, indicating the presence of CH stretch vibrations of CH_3 and $=CH_2$ functional groups, usually of fatty acids, which confirms the presence of lipids in the hydrophobic region. Functional groups of the hydrophilic region were identified by the presence of 1000–1200 cm^{-1}

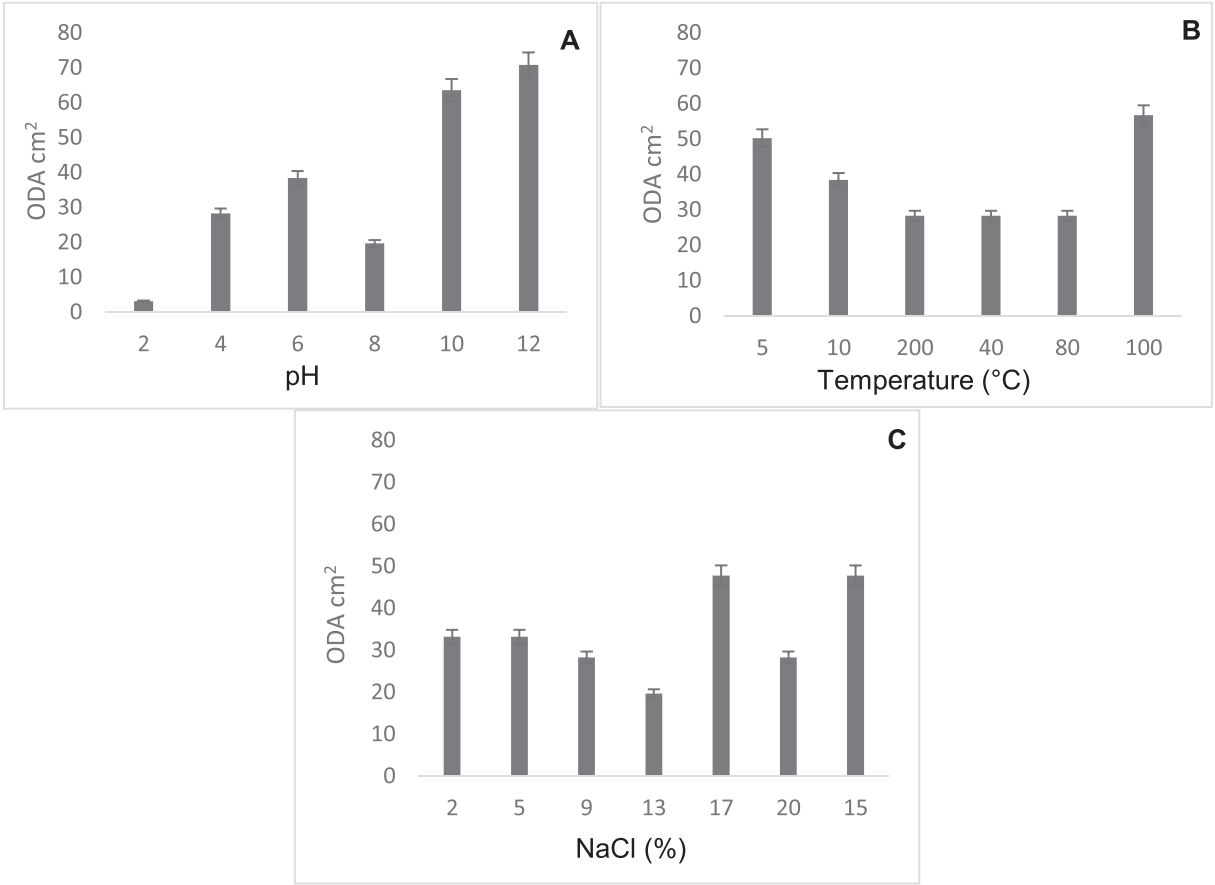

Fig. 4. Stability of the biosurfactant produced by *Mucor hiemalis* UCP 003 in different conditions by oil displacement area (ODA): A) pH; B) Temperature (C^0) and C) NaCl concentration (%).

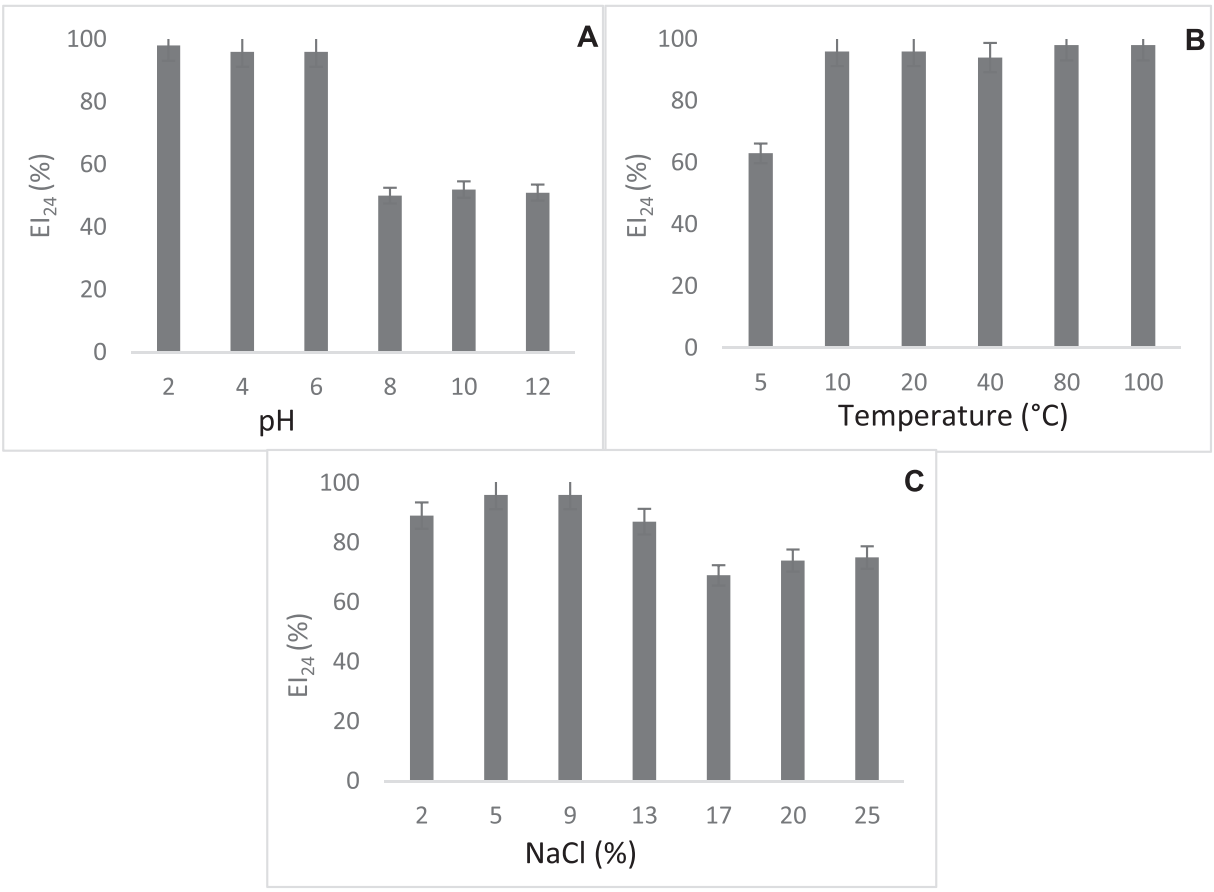

Fig. 5. Stability of the bioemulsifier produced by *Mucor hiemalis* UCP 0039 in different conditions by emulsification index -EI (%): A) pH; B) Temperature (C^0) and C) NaCl concentration (%).

vibration with C—O—C and C—O—P stretch vibrations of various oligo and polysaccharides. The presence of 1300–1500 cm^{-1} peaks indicate the presence of characteristic lipid and protein stretch $=CH_2$ and —CH [40,41]. The results presented by FT-IR spectrum are in agreement with the chemical composition, indicating the predominance of lipids and carbohydrates as similar glycolipids to BS and BE molecules.

3.5. Thermal, ionic and pH stability evaluated by dispersing ability

The dispersion ability of the BS and BE were estimated by the dispersed area of the oil (ODA) after changes in the physicochemical properties of the biomolecule-containing metabolic liquid. According to the results, BS maintained stability at alkaline pH (10 and 12) (Fig. 4A), at a concentration of 17 to 25% NaCl (Fig. 4B) and at a temperature of 5°C (Fig. 4C), when comparing the action of the BS present in the metabolic liquid without changes (control) (ODA = 50.24 cm^2).

These results indicate that the BS of *M. hiemalis*, under the conditions of stability of this study, is capable of keeping the insoluble oil particles suspended in water, preventing them from aggregating to each other. Additionally, the action of BE in the metabolic fluid without modification (control) was not significant as it displaced only 19.63 cm^2 of ODA using burned motor oil. Therefore, for the BE, the stability test for dispersing ability was not carried out.

3.6. Bioemulsifier stability evaluated by emulsification index (EI_{24})

Stability was evaluated after exposure of the metabolic liquid containing the BE to elevated temperatures, NaCl concentrations and pH. According to Fig. 5, it is possible to notice stability of emulsions formed with burned motor oil in acid pH (pH 2 to 6) (Fig. 5A). Regarding the NaCl concentration it is possible to observe stability up to 13% (w/v). In reference to the influence of temperature the stability from 10°C at all temperatures is evidenced (Fig. 5C). In these, emulsification index values remained around 90% (EI_{24} of metabolic liquid without modifications, control).

3.7. Effect on viscosity of hydrophobic compounds

Fig. 6 demonstrates the effect of BS and BE on viscosity of motor oil and soybean oil. After the addition of BS, it was possible to observe a reduction in the motor oil viscosity from 83 to 55 cP (corresponding

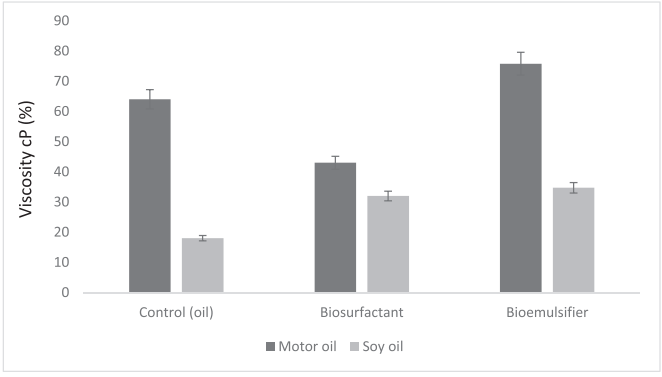

Fig. 6. Influence of biosurfactant and bioemulsifier produced by *Mucor hiemalis* on the viscosity -cP (%) of hydrophobic compounds.

to 43%). On the other hand, the soybean oil viscosity increased from 23 to 43 cP (corresponding to 34%). These effects indicate that BS can be used in different areas of industry, as it has the ability to increase the viscosity of synthetic oil as well as reduce the viscosity of vegetable oil.

In contrast, after the addition of the BE, the viscosity of motor oil increased from 83 to 96.8 cP (corresponding to 75.8%) and that of soybean oil increased from 23 to 45.2 cP (corresponding to 34.7%). The increase in oil viscosity due to the influence of BE can be justified by its action on the dispersion of droplets of metabolic liquid in the oil preventing coalescence.

4. Conclusions

Mucor hiemalis has shown to be an efficient microorganism capable of producing both biosurfactant and bioemulsifier of glycolipid composition in a single culture medium, using a carbon source of renewable origin (waste soybean oil). The biosurfactant and emulsifier production has been described in the first time in the same medium and under different physical conditions by *Mucor hiemalis*. Moreover, it can be affirmed that the two biomolecules produced meet the basic requirements of sustainability and add value, as these are the main raw material for obtaining compounds produced daily in various industrial sectors. In this sense, the production of bioemulsifier in the absence of agitation favors its industrial scale production by reducing the operational cost.

Conflict of interest

All authors declare no conflict of interest.

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