

Multifunctional, stable and low-cost lipopeptide biosurfactant produced by *Enterobacter cloacae* UCP 1597

Biosurfactante lipopeptídico multifuncional, estável e de baixo custo produzido por *Enterobacter cloacae* UCP 1597

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Abstract

Caatinga of Pernambuco is an area with a potential richness of microorganisms that produce biosurfactants, which are considered good candidates to replace synthetic surfactants in industrial applications due to their functional stability and low toxicity. In this context, current study aimed to investigate the biosurfactant production by the bacterium *Enterobacter cloacae* UCP 1597, isolated from the Caatinga soil. First fermentation was carried out in Erlenmeyer flasks containing 100 ml of medium, according to a 2³ full-factorial design (FFD). The results showed higher reduction in surface tension (28.3 mN/m) in condition 2 of the FFD, where dispersion of 38.46 cm² of burnt motor oil was also verified. Then, a second fermentation was performed in Fernbach flasks, containing 2 L of selected medium, confirming reduction in surface and interfacial tension to 30.5 and 2.3 mN/m, respectively, as well as excellent emulsifying properties. The critical micellar dilution (CMD) of the crude biosurfactant was determined (70%) and its use in phytotoxicity assay verified the absence of toxicity for cabbage seeds. The biomolecule showed a high yield (13.69 g/L) after extraction with ethyl acetate and anionic and lipopeptide nature. The stability in acid pH, high temperature and salinity, showed an acid-resistant, thermostable, and halotolerant biocompound. Thus, this lipopeptide was shown to be a multifunctional biosurfactant, since it not only has excellent surface-active properties, but it is also a good emulsifier, dispersant, and potent agent to germination of cabbage seeds. Hence, is suggested its promising application in industrial activities or environmental processes under adverse conditions.

Keywords: Anionic surfactant; Germination inducer; Lipopeptide; Non-toxic; Phytotoxicity.

Resumo

A Caatinga de Pernambuco é uma área com potencial riqueza de microrganismos produtores de biosurfactantes, que são considerados bons candidatos para substituir os surfactantes sintéticos em aplicações industriais devido à sua estabilidade funcional e baixa toxicidade. Nesse contexto, o presente estudo teve como objetivo investigar a produção de biosurfactante pela bactéria *Enterobacter cloacae* UCP 1597, isolada do solo da Caatinga. A primeira fermentação

foi realizada em frascos Erlenmeyer contendo 100 ml de meio, de acordo com um planejamento fatorial completo de 2³. Os resultados mostraram maior redução da tensão superficial (28,3 mN/m) na condição 2 do planejamento, onde também foi verificada dispersão de 38,46 cm² de óleo de motor queimado. Em seguida, foi realizada uma segunda fermentação em frascos de Fernbach, contendo 2 L de meio selecionado, confirmando a redução da tensão superficial e interfacial para 30,5 e 2,3 mN/m, respectivamente, além de excelentes propriedades emulsificantes. Foi determinada a diluição micelar crítica do biosurfactante bruto (70%) e seu uso em ensaio de fitotoxicidade verificou a ausência de toxicidade para sementes de repolho. A biomolécula apresentou elevado rendimento (13,69 g/L) após extração com acetato de etila e natureza aniônica e lipopeptídica. A estabilidade em pH ácido, elevada temperatura e salinidade, mostrou um biocomposto ácido-resistente, termoestável e halotolerante. Assim, este lipopeptídeo mostrou-se um biosurfactante multifuncional, pois além de possuir excelentes propriedades tensoativas, é também um bom emulsificante, dispersante, e potente agente para a germinação de sementes de repolho. Assim, sugere-se sua promissora aplicação em atividades industriais ou processos ambientais sob condições adversas.

Palavras-chave: Tensoativo aniônico; Indutor da germinação; Lipopeptídeo; Não tóxico; Fitotoxicidade.

Resumen

La Caatinga de Pernambuco es un área con una riqueza potencial de microorganismos productores de biosurfactantes, que se consideran buenos candidatos para reemplazar los surfactantes sintéticos en aplicaciones industriales debido a su estabilidad funcional y baja toxicidad. En este contexto, el presente estudio tuvo como objetivo investigar la producción de biosurfactantes por la bacteria *Enterobacter cloacae* UCP 1597, aislada del suelo de Caatinga. La primera fermentación se llevó a cabo en matraces Erlenmeyer que contenían 100 ml de medio, según un diseño factorial completo de 2³. Los resultados mostraron una mayor reducción de la tensión superficial (28,3 mN/m) en la condición 2 del diseño, donde también se verificó una dispersión de 38,46 cm² de aceite de motor quemado. Luego, se realizó una segunda fermentación en matraces Fernbach, conteniendo 2 L de medio seleccionado, comprobándose reducción de la tensión superficial e interfacial a 30,5 y 2,3 mN/m, respectivamente, así como excelentes propiedades emulsionantes. Se determinó la dilución micelar crítica del biosurfactante crudo (70%) y su uso en ensayos de fitotoxicidad verificó la ausencia de toxicidad para las semillas de col. La biomolécula mostró un alto rendimiento (13,69 g/L) después de la extracción con acetato de etilo y naturaleza aniónica y lipopeptídica. La estabilidad en pH ácido, elevada temperatura y salinidad, mostró un biocompuesto ácido-resistente, termoestable y halotolerante. Así, este lipopéptido demostró ser un biosurfactante multifuncional, ya que no solo posee excelentes propiedades tensoactivas, sino que también es un buen emulsionante, dispersante, y potente agente para la germinación de semillas de col. De ahí que se sugiera su prometedora aplicación en actividades industriales o procesos ambientales bajo condiciones adversas.

Palabras clave: Surfactante aniónico; Inductor de germinación; Lipopéptido; No tóxico; Fitotoxicidad.

1. Introduction

Surfactants are amphiphilic molecules that have properties such as the ability to reduce surface and interfacial tension, as well as form stable emulsions between immiscible liquids with different degrees of polarity (Sarubbo, et al., 2022). Surfactants of chemical origin are derived or synthesized from petroleum sources and used as sanitizing or cleaning agents. However, the frequent use of these synthetic compounds has aroused the interest of research for the production of natural surfactants, because these surfactants have high toxicity, low biodegradability and biocompatibility, and serious problems related to the environment (Johnson, et al., 2021).

Surfactants of natural origin, known as biosurfactants, can be produced by bacteria, yeasts or filamentous fungi and have the same characteristics and efficiency as synthetic ones, but they possess advantages such as less toxicity, high biodegradability and biocompatibility, and do not present risks to the environment. In addition, they are considered the biomolecules of the 21st century, being promising for the replacement of synthetic surfactants in the global market (Sajna, et al., 2013; Gaur, et al., 2022).

Despite the various advantages, the production of biosurfactant is still limited due to the costly large-scale production costs, mainly due to the use of conventional substrates (Brumano, et al., 2016; Santos, et al., 2018; Hema, et al., 2019). An economically viable alternative is the use of agro-industrial wastes for the formulation of alternative media as carbon and nitrogen sources to obtain these biomolecules. In this context, the aim of this study was to investigate the biotechnological potential of *Enterobacter cloacae* UCP 1597 for biosurfactant production using waste soybean oil (WSO). Furthermore,

production in 2 L was carried out, and preliminary characterization of the biomolecule was performed, with special interest in the evaluation of stability under extreme conditions and phytotoxicity.

2. Methodology

2.1 Microorganism and inoculum preparation

The microorganism used in this study was the bacterium *Enterobacter cloacae* UCP 1597, previously isolated from the soil of the Caatinga in the state of Pernambuco, Brazil, identified by morphological, biochemical and molecular methods. This strain was kindly provided by the Culture Collection of the Nucleus for Research in Environmental Sciences and Biotechnology (NPCIAMB), at the Catholic University of Pernambuco (UNICAP) and has been kept in Nutrient agar medium and in 30% glycerol, at 5°C. For the inoculum preparation, *E. cloacae* was cultivated in Nutrient broth for 24 h at 28°C and 150 rpm for obtaining an optical density of 0.8 at 600 nm, corresponding to an inoculum of 10^7 CFU/ml.

2.2 Agro-industrial substrate

The alternative agro-industrial substrate used in this study was waste soybean oil (WSO), provided by a local food trade in the city of Recife, Pernambuco, Brazil (Fonseca, et al., 2022).

2.3 Biosurfactant production in 100 ml

Biosurfactant production was performed in 250 ml-Erlenmeyer flasks containing 100 ml of mineral salts medium (MSM) described by Sari, et al. (2014) (0.03% KH_2PO_4 , 0.03% MgSO_4 and 0.3% NaNO_3), supplemented with different concentrations of yeast extract, glucose and WSO, according to a 2^3 full-factorial design (FFD) (topic 2.4). The pH of the roduction media was adjusted to 6.0 and the flasks were autoclaved, at 121°C for 15 min. Then, production media were inoculated with 10% of cell suspension and incubated under orbital shaking (150 rpm), at 28°C for 72 h. After this period, the fermented media were centrifuged at 6000 g for 15 min, with subsequent filtration, and the cell-free metabolic liquids obtained were used to determine surface tension.

2.4 Full-factorial design (FFD)

To analyze the effects of the concentration of each variable (yeast extract, WSO and glucose), as well as the interaction between them, a 2^3 FFD was carried out, using surface tension as the response variable. A set of seven assays with three repetitions at the central point was performed (Table 1). Statistical analysis of the data obtained in the experiments was performed using the STATISTICA software package, version 12.0 (StatSoft Inc., Tulsa, OK, USA), and the significance of the results was tested at $p \leq 0.05$.

Table 1 - Levels of the variables studied in the 2^3 full-factorial design applied for biosurfactant production by *Enterobacter cloacae* UCP 1597.

Variables	Levels		
	-1	0	+1
Yeast extract (% , w/v)	0	0.1	0.2
Waste soybean oil (% , v/v)	2.0	4.0	6.0
Glucose (% , w/v)	0	4.0	8.0

Source: Authors.

2.5 Determination of surface tension

The cell-free metabolic liquids obtained after centrifugation and filtration were used to determine the surface tension using an automatic tensiometer model Sigma 701 (Biolin Scientific, São Paulo, Brazil), at room temperature ($\pm 25^{\circ}\text{C}$) (Kuyukina, et al., 2001). The surface tension value was obtained as an average of ten measurements, and distilled water was used as a control (surface tension = 70 mN/m).

2.6 Dispersion test

The oil dispersion test is a method used to measure the diameter of the clear zone, which occurs after placing a surfactant-containing solution at an oil-water interface. In the present work, this test was carried out using the cell-free metabolic liquid from the condition of FFD, where the greatest reduction in surface tension was verified. Briefly, 40 ml of distilled water were placed in a 9 cm diameter Petri dish. Then, 1 ml of burnt motor oil was added to the surface of the water, followed by the addition of 0.5 ml of the metabolic liquid which was gently placed in the center of the oil layer. The oil displacement area (ODA) was obtained, according to equation 1 (Youssef, et al., 2004):

$$\text{ODA} = 3.14 \times r^2 \quad (\text{Eq. 1})$$

2.7 Production of biosurfactant in 2 L

Biosurfactant production was carried out in triplicate in 2.8 L-Fernbach flasks containing 2.0 L of the medium selected in the previous fermentation, according to the results of the FFD. The pH of the medium was adjusted to 6.0 and the flasks were autoclaved, at 121°C for 15 min. The production medium was inoculated with 10% of cell suspension and incubated at 28°C and 150 rpm, for 72 h. Then, the cultured medium was centrifuged at 6000 g for 15 min and filtered, and the cell-free metabolic liquid was used for the analyzes described below.

2.8 Assessment of surfactant properties

2.8.1 Determination of surface and interfacial tension

The surface tension was determined as described in item 2.5. Interfacial tension was measured by the Du Noüy ring method using cell-free metabolic liquid and n-hexadecane (Santiago, et al., 2021).

2.8.2 Determination of emulsification index

The emulsification index of the cell-free metabolic liquid was determined using the method described by Cooper and Goldenberg (1987). For this, 2.0 ml of cell-free metabolic liquid was added to 2.0 ml of soybean oil, WSO, motor oil or burnt motor oil in graduated tubes and the mixture was vortexed for 1 min. The mixture was allowed to stand for 24 and then, the emulsification index was calculated according to equation 2:

$$\text{EI}_{24} (\%) = \text{He}/\text{Ht} \times 100 \quad (\text{Eq. 2})$$

where: He = height of the formed emulsion; Ht = total height of the mixture.

2.9 Isolation of biosurfactant

The biosurfactant produced in the medium was extracted with ethyl acetate from the cell-free metabolic liquid (metabolic liquid: ethyl acetate 1:3; v/v) (López-Prieto, et al., 2020). The mixture was stirred vigorously for 15 min and allowed to stand at room temperature for 24 h. After this time, the organic phase was carefully separated in a volumetric flask

and evaporated at 60°C on a rotoevaporator. The precipitate obtained was subjected to lyophilization (Advantage Plus EL-85 lyophilizer, SP Scientific, USA) and then kept in a desiccator until constant weight. The weight of the biosurfactant after extraction was determined by gravimetry, and the yield expressed in g/L.

2.10 Preliminary characterization of biosurfactant

The biochemical composition of the isolated biosurfactant was determined using specific commercial kits for quantification of total proteins and enzymatic glucose (In Vitro Diagnóstica Ltda; Itabira-MG, Brazil). The total lipid content was obtained after extraction with chloroform and methanol, according to the methodology of Manocha et al. (1980). In addition, the functional groups present in the biosurfactant molecule were identified by Fourier transform infrared spectroscopy (FTIR) in the Shimadzu equipment, IR-TRACER 100, using attenuated total reflectance (ATR) accessory consisting of a “diamond/ ZnSe”. The ionic charge of the biosurfactant was also determined using the Zeta-Meter system 3.0 + ZM3-DG potentiometer (Direct Imaging, Zeta Meter, Inc., USA).

2.11 Critical Micellar Dilution (CMD)

The critical micellar concentration (CMC) of the biosurfactant was determined indirectly by determining the critical micellar dilution (CMD) (Campos, et al., 2019). Samples of the cell-free metabolic liquid were diluted in distilled water in different proportions and the surface tension values was determined for each corresponding dilution in an automatic tensiometer model Sigma 701 (Biolin Scientific, São Paulo, Brazil), using the Du Noüy ring method (Kuyukina, et al., 2001). The CMD value was given by the inflection point of the curve of the surface tension *versus* concentration of the supernatant containing the biosurfactant (Rocha e Silva, et al., 2014).

2.12 Stability of biosurfactant

The stability of the biosurfactant produced by *E. cloacae* UCP 1597 was investigated, using the cell-free metabolic liquid, in the CMD determined above. The solution was separated into small volumes, which were separately subjected to different temperatures (5, 10, 20, 40, 80 and 100°C), adjusted to different pH values (2, 4, 6, 8, 10 and 12) or concentrations of NaCl (5, 10, 15, 20 and 25%). After 1 h in the different treatments, the solutions were allowed to stand at room temperature and the surface tension was determined, as previously described (item 2.4) (Liang, et al., 2014).

2.13 Biosurfactant phytotoxicity

The phytotoxicity of the crude biosurfactant was evaluated under static conditions, through seed germination and root growth of cabbage (*Brassica oleracea* var. *capitata*), according to the methodology of Tiquia et al. (1996). First, the seeds were disinfected in a 1% sodium hypochlorite solution; and then, they were washed with sterile distilled water to remove excess hypochlorite and left at room temperature until dry. Undiluted cell-free metabolic liquid and a solution of it in distilled water at the concentration of CMD were used as test solutions. The assay was carried out in triplicate in sterile Petri dishes (15 cm) containing discs of Whatman No. 1 filter paper, to which 10 treated seeds were symmetrically added, under aseptic conditions. The plates were inoculated with 3 ml of the test solution or distilled water as a control, and incubated at 28°C for 120 h. After this period, seed germination was determined:

Relative germination of seeds (%) = (number of seeds germinated in the test solution / number of seeds germinated in the control) x 100

Relative root length (%) = (average root length in test solution/average root length in control) x 100

Germination index = [(% seed germination) x (% relative root length)]/100%.

3. Results and Discussion

3.1 Biosurfactant production by *Enterobacter cloacae* UCP 1597 using WSO

According to the literature, the carbon source is the main variable to be considered in the production of biosurfactants, as it directly influences both the growth of the microorganism, as well as the structure and yield of the biosurfactant molecule (Andrade, et al., 2018; Ferreira, et al., 2020; Sarubbo, et al., 2022). In this sense, a wide variety of carbon sources have been used to obtain biosurfactants, including vegetable oils, post-frying waste oils, dairy products, corn steep liquor, cassava wastewater, among others (Araújo, et al., 2019; Mendonça, et al., 2021; Fonseca, et al., 2022; Maia, et al., 2022).

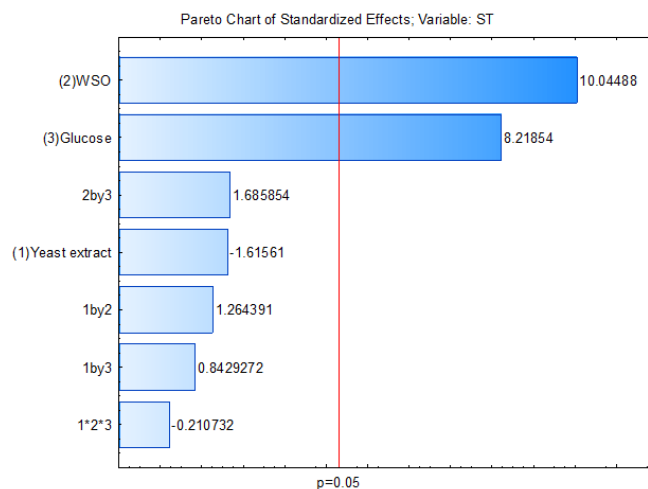
In this context, the present work investigated the biosurfactant production by *E. cloacae* UCP 1597 using a 2³ FFD in order to analyze the influence of substrate concentrations on surface tension. The data shown in Table 2 showed the greatest reduction in surface tension (from 70 to 28.3 mN/m) in condition 2 of the design, in the medium containing the lowest concentrations of yeast extract and WSO, without addition of glucose. However, the Pareto diagram represented in Figure 1 showed that only WSO and glucose had a significant and positive influence, from a statistical point of view, on surface tension. This means that at lower concentrations of these substrates, there was a reduction in the surface tension of the medium, indicating the production of the surfactant.

Table 2 - Full-factorial design applied to investigate the biosurfactant production by *E. cloacae* UCP 1597.

Conditions	Yeast extract	Waste soybean oil	Glucose	Surface tension (mN/m)
1	-1	-1	-1	31.1
2	+1	-1	-1	28.3
3	-1	+1	-1	36.0
4	+1	+1	-1	35.3
5	-1	-1	+1	35.0
6	+1	-1	+1	33.7
7	-1	+1	+1	42.6
8	+1	+1	+1	42.8
9	0	0	0	32.6
10	0	0	0	33.8
11	0	0	0	31.2

Source: Authors.

Figure 1 - Pareto chart obtained from the results of the 2^3 FFD applied to determine the influence of the independent variables on the surface tension.



Source: Authors.

The surface tension obtained in this work (28.3 mN/m) was similar to those obtained previously by *Enterobacter* sp. N18, using yeast extract and sucrose as substrates (You, et al., 2015) and *Enterobacter* sp. UJS-RC using corn steep liquor and sugarcane molasses as alternative sources (Chandankere, et al., 2020) (Table 3). Recently, Ekprasert et al. (2021) reported higher biosurfactant production by *E. cloacae* B14 using waste frying oil, compared to other agro-industrial wastes (spent coffee grounds and molasses), confirming the suitability of this alternative substrate.

Table 3 - Comparison of the surfactant properties of the biosurfactant produced by *Enterobacter cloacae* UCP 1597 in this work and the biosurfactants previously reported in the literature for representatives of the *Enterobacter* genus.

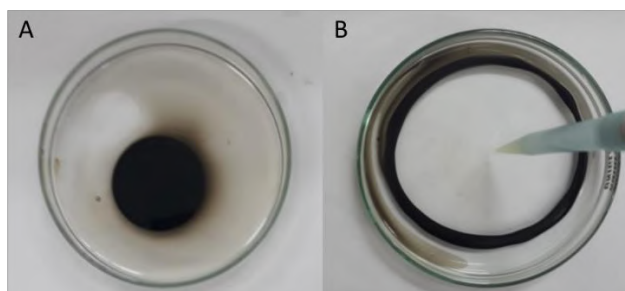
Microorganism	Substrates	Fermentation conditions	Surface tension (mN/m)	Interfacial tension (mN/m)	EL ₂₄ (%)	Yield (g/L)	References
<i>Enterobacter cloacae</i> UCP 1597	Yeast extract and WSO	100 ml of medium, 28°C, 150 rpm, 72 h.	28.3	-	-	-	Present study
		2 L of medium, 28°C, 150 rpm, 72 h	30.5	2.3	51.3 (soybean oil) 52.2 (WSO) 77.5 (motor oil) 93.4 (burnt motor oil)	13.69	
<i>E. cloacae</i> BAGM01	sucrose and yeast extract	1 L of medium, 30°C, 150 rpm, 144 h.	34.5	-	71% (kerosene)	-	Curriel-Maciel, et al. (2021)
<i>Enterobacter</i> sp. UJS-RC	sugarcane molasses and corn steep liquor	50 ml of medium, 40°C, 200 rpm, 120 h	28.3	-	75.1% (hexadecane)	4.4	Chandankere, et al. (2020)
<i>E. cloacae</i> MIS-1	molasses and ammonium sulfate	40°C, 160 rpm, 36 h	34	-	-	-	Hosseini & Tahmasebi (2020)
<i>E. hormaechei</i> MIS-2			31	-	-	-	
MIS-C (consortium)			31	3.2	~69% (hexadecane)	1.53	
<i>E. cloacae</i> B14	glucose	100 ml of medium, 30°C, 150 rpm, 96 h	-	-	42.2 (spent engine oil)	-	Ekprasert, et al. (2019)
<i>E. cloacae</i> C3	glucose, glutamic acid and yeast extract	250 ml of medium, 30°C, 150 rpm, 72 h	32.0	-	55% (diesel and vegetable oils) 50% (hexane) 45% (kerosene)	1.0	Jemil, et al. (2018)
<i>Enterobacter</i> sp. N18	sucrose and yeast extract	37°C, 150 rpm, 48 h	27.9	-	-	0.15	You, et al. (2015)
<i>E. cloacae</i>	olive oil and ammonium sulfate	100 ml of medium, 40°C, 150 rpm, 48 h,	53.6	15.2	-	1.22	Darvishi, et al. (2011)
<i>Enterobacter</i> sp. MS16	sunflower oil cake	100 ml of medium, 30°C, 120 rpm, 168 h	34	-	70.50 (diesel) 60.10 (kerosene) 58.90 (hexadecane)	1.5	Jadhav, et al. (2011)

Source: Authors.

3.1.1 Dispersion test

The dispersion test is an effective method commonly used for the detection or confirmation of biosurfactant production. The appearance of a transparent area in the center of the oil indicates the presence of a surfactant compound, and the diameter of the transparent area is directly related to the surfactant activity (Satpute, et al., 2010; Rahman, et al., 2019). In this context, the dispersion test of burnt motor oil was performed in current study, as shown in Figure 2. After the addition of the cell-free metabolic liquid (crude biosurfactant), a halo of 7.0 cm was visualized, corresponding to ODA of 38.48 cm². Similar results have been previously reported for biosurfactants produced by *E. cloacae* B14 (Ekprasert, et al., 2019) and *E. cloacae* MIS-1 (Hosseini & Tahmasebi, 2020). Microbial surfactants with good dispersant properties are promising alternatives to replace synthetic dispersants in fabric dyeing and oil spill bioremediation processes, among others (dos Santos, et al., 2021; Khubaib, et al., 2021).

Figure 2 - Dispersion test of burnt motor oil using biosurfactant produced by *Enterobacter cloacae* UCP 1597 in condition 2 of FFD. Visualization of the Petri dish containing distilled water and burnt motor oil before (A) and after application of the cell-free metabolic liquid (crude biosurfactant) (B).



Source: Authors.

3.2 Biosurfactant production

3.2.1 Surfactant properties

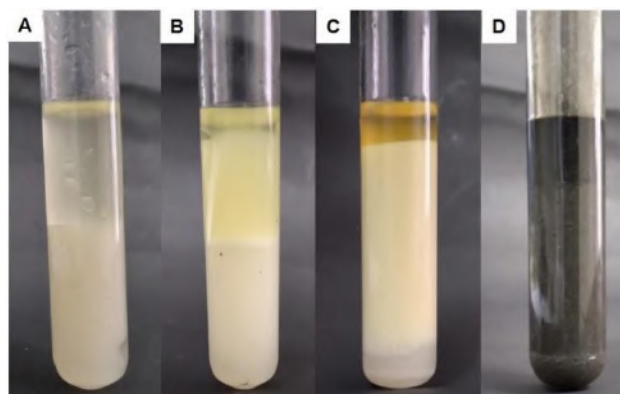
The large-scale production and application of microbial surfactants demands the performance of scale-up studies in order to obtain higher yields and make the process economically viable (Almeida et al., 2017; de Medeiros et al., 2022). In present work, production was carried out in 2 L of the medium containing 0.2% yeast extract and 2% WSO, with a slight increase in surface tension (30.5 ± 0.2 mN/m) when compared with fermentation conducted in 100 ml of medium (28.3 mN/m). Faccioli et al. (2022) recently described a similar behavior, when they increased biosurfactant production by *Pseudomonas cepacia* CCT 6659 from 100 ml to 1.2 L of medium. These authors suggested that this increase may be related to factors intrinsic to the scaling process, as the increase in volume leads to an increase in the total surface area for complete saturation with biosurfactants. However, the surface tension value obtained is similar to that previously reported for other studies with *Enterobacter* sp., in production volumes from 50 ml to 1 L (Table 3).

On the other hand, the reduction of interfacial tension is considered as another parameter that indicates the production of biosurfactant (dos Santos, et al., 2021). In this sense, excellent interfacial tension of 2.3 mN/m was verified with n-hexadecane, since values below 7 mN/m are referred as good in the literature (Luna, et al., 2013; Pinto, et al., 2022).

3.2.2 Emulsifying properties

The determination of EI_{24} is often used to identify biosurfactants that have good emulsifying properties (Andrade, et al., 2018; Pele, et al., 2019; Santiago, et al., 2021). This method is evaluated by the ability to maintain at least 50% of the original volume of the emulsion after 24 hours of formation (Lima, et al., 2017). In this context, the present study determined the EI_{24} using the cell-free metabolic liquid obtained in the 2 L fermentation. As shown in Table 3 and evidenced in Figure 3, the best results were verified for burnt motor oil > motor oil > WSO > soybean oil, confirming that the ability to form stable emulsions depends not only on the intrinsic properties of the biosurfactant, but also on the type of hydrophobic component used in the test (Uzoigwe, et al., 2015; Rahman, et al., 2019). Therefore, the biosurfactant produced by *E. cloacae* UCP 1597 showed excellent emulsifying properties, mainly with motor oil and burnt motor oil, when compared with other biosurfactants produced by *Enterobacter* sp. (Table 3).

Figure 3 - Emulsions obtained by crude biosurfactant produced by *E. cloacae* UCP 1597 with soybean oil (A), WSO (B), motor oil (C) and burnt motor oil (D) after 24 h incubation at room temperature.



Source: Authors.

Furthermore, the stability of the formed emulsions was verified after one week of incubation at room temperature, since the EI_{24} decreased by less than 5% for each hydrophobic compound tested (data not shown). The ability to form stable emulsions is one of the most important characteristics to consider in a biosurfactant and suggests its application in the formulation of detergents, cosmetics and food products (Rulli, et al., 2019; Rodríguez, et al., 2022).

3.3 Isolation of biosurfactant

Biosurfactant recovery currently represents 60-70% of the total cost of production, and makes the process expensive compared to obtaining chemical surfactants. Thus, the use of appropriate techniques is essential to allow these biomolecules to be economically and competitively integrated in the market (Thavasi & Banat, 2019; Sarubbo, et al., 2022). Several techniques have been reported to recover biosurfactants after fermentation, the most reported of which is liquid phase solvent extraction using a variety of organic compounds (Venkataraman, et al., 2021; Eras-Muñoz, et al., 2022). In this work, the biosurfactant produced by *E. cloacae* UCP 1597 was extracted with ethyl acetate, obtaining a yield of 13.69 g/L, higher than those previously described for biosurfactants produced by this genus (Table 3). Achieving high biosurfactant yields largely depends on the selection of suitable substrates, as well as production conditions, as widely described in the literature (Pele, et al., 2019; Ferreira, et al., 2020; Gaur, et al., 2022).

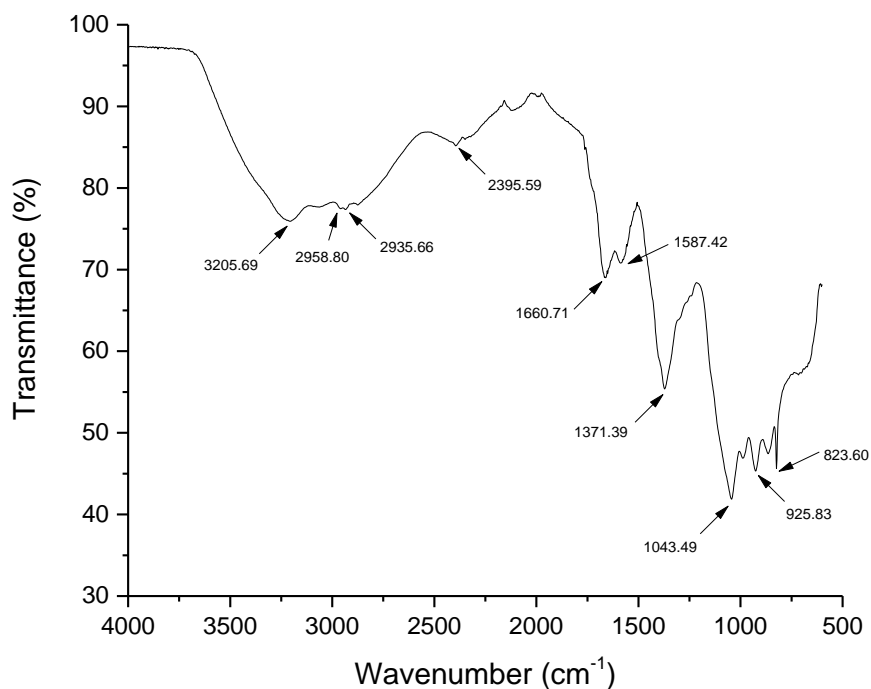
3.4 Preliminary characterization of biosurfactant

Zeta potential determines the ionic charge of particles and it is used to predict and control the stability of colloidal suspensions and emulsions. According to the analysis performed in the present study, the biosurfactant produced by *E. cloacae* UCP 1597 showed anionic character (-24.52 mV), in agreement with those produced previously by *E. cloacae* B14 (Ekprasert, et al., 2019) and *E. cloacae* BAGM01 (Curiel-Maciel, et al., 2021). Anionic surfactants have a negative charge in their polar part, as well as strong foaming, detergent and wetting power. Therefore, they are used in the formulation of cosmetic products such as creamy soaps and cleansing lotions (Chua, et al., 2019; Yorke, et al., 2021).

On the other hand, the determination of the biochemical composition of the biosurfactant indicated the lipopeptide nature (lipids 60.95%, proteins 28.5% and carbohydrates 3.55%), which was confirmed by FTIR spectroscopy of the isolated biomolecule. According to the infrared spectrum shown in Figure 4, a wide absorbance band was observed, starting at approximately 3600 cm^{-1} and reaching a maximum at 3205.69 cm^{-1} . Absorbance in this region is a result of stretching vibrations -CH and -NH, and is a characteristic of carbon-containing compounds with amino groups (de Farias, et al., 2011;

Jemil, et al., 2017). The peaks detected at 2958.80 and 2935.66 cm^{-1} indicated the presence of $-\text{C}-\text{CH}_3$ bands or long alkyl chains (Jemil, et al., 2019). Two other absorbance peaks were observed at 1660.71 and 1587.42 cm^{-1} , suggesting the presence of peptide groups and C=O bonds (caused due to C=O stretching vibrations), respectively (Das, et al., 2008). The intense band at 1371.39 cm^{-1} may be a result of deformation and bending vibrations of $-\text{C}-\text{CH}_2$ and $-\text{C}-\text{CH}_3$ groups in aliphatic chains (Dehghan-Noudeh et al., 2015). The carbonyl ester group was detected from the absorbance peak at 1043.49 cm^{-1} (Jemil, et al., 2019). Thus, the production of a lipopeptide biosurfactant was confirmed, in correspondence with others previously produced by *Enterobacter sp.* (You, et al., 2015; Jamil, et al., 2018; 2019).

Figure 4 - Infrared spectrum of the biosurfactant produced by *E. cloacae* UCP 1597.



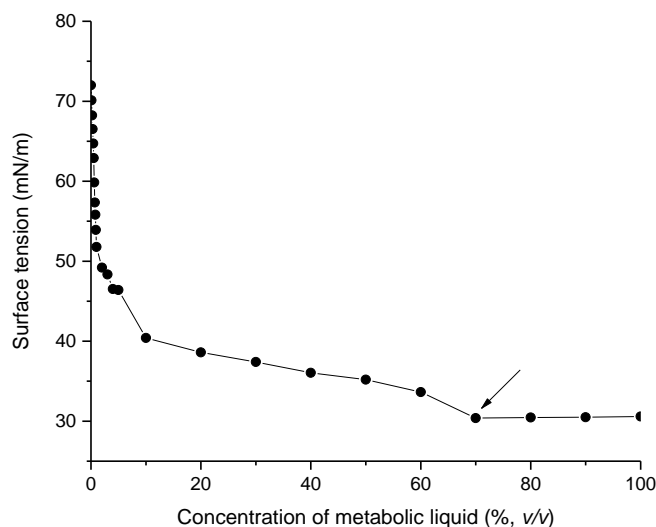
Source: Authors.

3.5 Critical Micellar Dilution (CMD)

The critical micelle concentration (CMC) indicates the minimum concentration of biosurfactant required for maximum surface tension reduction, and is a commonly used parameter to assess the efficiency of surfactants (Bergström, 2015; Sarubbo, et al., 2022). However, as the isolated biosurfactant did not show good water solubility, it was only possible to determine the CMC of the biomolecule indirectly from the crude biosurfactant, that is, the cell-free metabolic liquid. Thus, the concentration of the biosurfactant was estimated by the critical micellar dilution (CMD) technique (Rocha & Silva, et al., 2014; Campos, et al., 2019).

As shown in Figure 5, the surface tension was gradually reduced from 72 to 30.5 ± 0.2 mN/m, with increasing concentration of the metabolic liquid (0-100%). However, from 70% (dilution 30:70) there was no significant reduction in surface tension, indicating that the CMD was reached.

Figure 5 - Critical micellar dilution of the biosurfactant produced by *E. cloacae* UCP 1597.



Source: Authors.

3.6 Biosurfactant stability

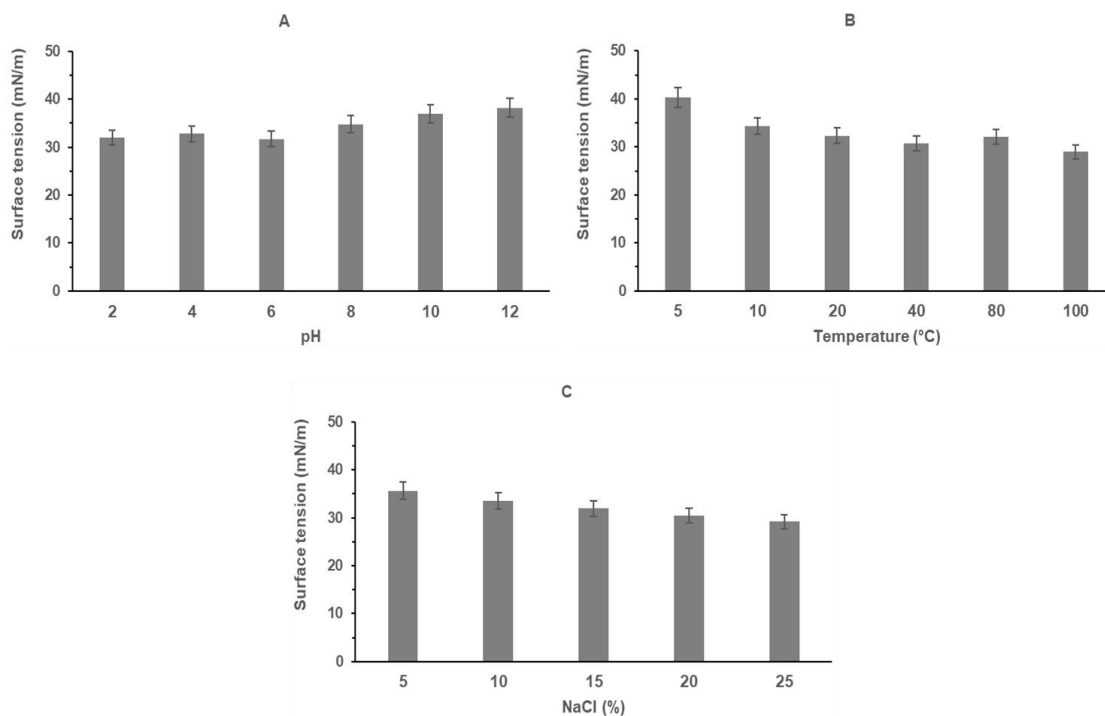
The action of biosurfactants can be influenced by the variation of physical and chemical factors, such as pH, temperature and salinity (Khopade, et al., 2012; Negin, et al., 2017). Therefore, it is essential to assess the stability of the biosurfactant produced depending on the type of application (Belhaj, et al., 2019; Purwasena, et al., 2019). In this sense, stability studies of the crude biosurfactant produced by *E. cloacae* UCP 1597 were carried out at CMD, and the results are shown in Figure 6.

According to Figure 6A, the cell-free metabolic liquid (crude biosurfactant) showed small changes in the reduction of surface tension at pH 2-8, being considered stable in this range since the variation did not exceed 5 mN/m (Fai, et al., 2015). However, at pH 10 and 12 there was a greater increase in surface tension, proving to be sensitive to alkaline environments. Likewise, Jamil et al. (2018) found a slight decrease in the activity of the biosurfactant produced by *E. cloacae* C3 at pH 10, which may be explained by the fact that the basic medium influences the solubility of the biomolecule and, therefore, its ability to reduce surface tension.

On the other hand, the thermostability of the biosurfactant at 10-100°C (Figure 6B) was evidenced, in agreement with the studies performed by Jamil et al. (2018) and Curiel-Maciel et al. (2021). At 5°C, there was an increase in surface tension (~40 mN/m), suggesting that this surfactant has a Krafft temperature (also known as critical micellar temperature) (Perfumo, et al., 2018) at low values, similar to the biosurfactant lipopeptide produced by *Bacillus subtilis* N3-1P (Zhu, et al., 2020). According to Nakama (2017), the Krafft point is closely related to the structure of surfactants (*i.e.*, hydrophilic and lipophilic groups) and ionic character.

In addition, the biosurfactant produced by *E. cloacae* proved to be halotolerant, evidencing efficiency in the reduction of surface tension up to 25% of NaCl (Figure 6C). According to the literature, biosurfactants commonly tolerate saline concentrations of up to 10% (w/v), while synthetic surfactants are inactivated with $\geq 2\%$ NaCl (Santos, et al., 2016; Pele, et al., 2019). However, some studies have highlighted the stability up to 25% of NaCl of the biosurfactants produced by *Bacillus subtilis* HOB2 (Haddad, et al., 2009) and *Serratia marcescens* UCP 1549 (dos Santos, et al., 2021), where the authors suggested the usefulness of these biomolecules in industrial or environmental processes with high ionic strength.

Figure 6 - Stability of the biosurfactant produced by *E. cloacae* UCP 1597 against variations in pH (A), temperature (B) and salinity (C).



Source: Authors.

3.7 Biosurfactant phytotoxicity

The evaluation of the efficacy and toxicity of a biosurfactant is considered an important factor in predicting its potential application and effect on an ecosystem. This evaluation is fundamental, mainly because it is a bioproduct with possibilities of insertion in the market (da Silva et al., 2021). Previously, et al. (2014) reported the use of CMD to assess the toxicity of crude *Pseudomonas cepacia* CCT6659 biosurfactant. In the present work, undiluted cell-free metabolic liquid and CMD were used to investigate the toxicity of crude biosurfactant produced by *E. cloacae* UCP 1597 in cabbage (*B. oleracea* var. *capitata*) seeds.

The results shown in Table 4 showed that the solutions tested did not show inhibitory effect on seed germination or root elongation in the analyzed vegetable. Consequently, the biosurfactant produced by *E. cloacae* UCP 1597 was considered as non-toxic, since the GI value $\geq 80\%$ has been used as an indicator of the absence of phytotoxicity (Tiquia, et al., 1996). This conclusion was supported by previous studies performed by Araujo et al. (2019) and Deivakumari et al. (2020), who studied the phytotoxic effect of biosurfactants produced by *Serratia marcescens* UCP 1549 and *Pseudomonas aeruginosa* DKB1, respectively, and obtained similar results against cabbage seeds. However, to our knowledge, this is the first report in the literature assessing the toxicity of biosurfactant produced by an *E. cloacae* strain.

Table 4 - Phytotoxicity of the crude biosurfactant produced by *E. cloacae* UCP 1597 using cabbage (*Brassica oleracea* var. *capitata*) seeds.

Test solutions	Seed germination (%)	Root elongation (%)	Germination index (%)
Undiluted cell-free metabolic liquid	134	93.3	125
Cell-free metabolic liquid at CMD	120	92.9	111.48

Source: Authors.

4. Conclusion

Our results demonstrated of the biotechnological potential of *Enterobacter cloacae* UCP 1597 in the production of an anionic and non-toxic lipopeptide biosurfactant, with high potential as inoculant on agriculture, considering its excellent induction of germination of cabbage (*Brassica oleracea* var. *capitata*) seeds and broad stability. We also verified the excellent emulsifying and dispersant properties of the biomolecule, highlighting the multifunctional character of the biosurfactant produced.

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