



*Dedicated to the memory of
Academician Bogdan C. Simionescu (1948–2024)*

STABILITY AND ANTIMICROBIAL ACTIVITY OF BIOSURFACTANT PRODUCED BY *PSEUDOMONAS FLUORESCENS* ICCF 392 STRAIN CULTIVATED ON RENEWABLE SUBSTRATES

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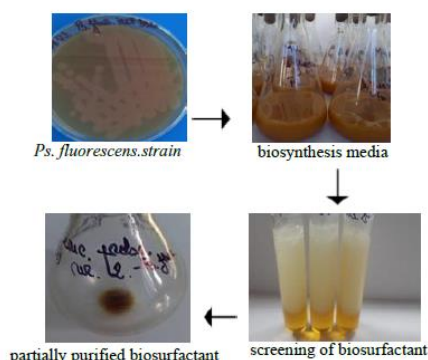
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Biosurfactants represent a vital class of surface-active agents synthesized by microorganisms, with numerous biotechnological and industrial applications. In this study, the stability and antibacterial activity of the biosurfactant produced by the *Pseudomonas fluorescens* ICCF 392 strain utilizing a low-cost mixed substrate containing 3% glycerol and 2% waste cooking oil as a carbon source were evaluated. The biosurfactant's stability was assessed at various temperatures (30°C, 37°C, 50°C, 75°C, and 115°C) and pH levels (2.0–12.0) by determining the emulsification index (E24%). The biosurfactant remained stable at temperatures ranging from 30°C to 115°C, with an E24% over 60% for emulsions formed with sunflower oil. In contrast, emulsions formed with heptane and octane showed a decrease in E24% at 115°C. In the case of pH, the biosurfactant showed optimal stability at pH 6–8, with a slight decrease in stability at pH 2–4 and pH 10–12. Moreover, antibacterial assays demonstrated significant activity against *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, and *Pseudomonas aeruginosa* ATCC 9027, suggesting its potential for diverse industrial applications.



INTRODUCTION

The global shift toward sustainable industrial practices has brought increased attention to biosurfactants, due to their biodegradability and

compatibility with green technologies. The utilization of waste materials, such as cooking oil and glycerol, aligns with the principles of the circular economy, helping to reduce resource waste and promoting more efficient industrial production.^{1–3}

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Biosurfactants are a class of secondary metabolites produced by a variety of microorganisms, such as bacteria, yeasts, and fungi.

Among bacteria, several species, including *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Burkholderia*, *Stenotrophomonas*, *Flavobacterium*, *Mycobacterium*, *Rhodococcus*, and *Arthrobacter*, have been identified as biosurfactant producers.

Due to their amphiphilic character, biosurfactants present the property to decrease the interfacial tension between hydrophobic and hydrophilic moieties, as well as between phases with varying polarity and hydrogen bonding capacities.

Based on their origin and chemical structure, biosurfactants are divided into six major groups: lipopeptides, lipoproteins, phospholipids, glycolipids, neutral lipids, and polymeric biosurfactants.⁴

According to their molecular weight, biosurfactants are classified into two categories: low molecular weight and high molecular weight, with an average molecular mass ranging from 500 to 1500 Da. Lipoproteins and lipopolysaccharides are biosurfactants with high molecular weight,⁵ while phospholipids, glycolipids, and lipopeptides are compounds with low molecular weight.^{6,7}

The most common and studied subclass of glycolipids is represented by rhamnolipids, mostly produced by the *Pseudomonas aeruginosa* strain.⁸ Nevertheless, other bacterial species, such as *P. cepacia*, *P. chlororaphis*, *P. putida*, and *P. fluorescens*, are also capable of synthesizing these kinds of bio-compounds.^{9,10}

Rhamnolipids are composed of (L)-rhamnose units linked by a glycosidic bond to one or more saturated or unsaturated β -hydroxy fatty acid chains, with carbon chain lengths ranging from 8 to 24 atoms. Mono-rhamnolipids present a single rhamnose unit, while di-rhamnolipids have two sugar units connected by an α -1,2-glycosidic bond.¹¹ Rhamnolipids demonstrate emulsion behavior due to the capacity to stabilize and form emulsions through diverse mechanisms. The emulsifying ability can be estimated using the emulsification index ($E_{24\%}$), which is one of the qualitative and semi-quantitative methods that are frequently used to confirm the presence of biosurfactant.^{12,13}

Therefore, in this study, the biosurfactant bioactivity was primarily assessed using the emulsification index ($E_{24\%}$), which is one of the most commonly used methods to evaluate the emulsifying potential of biosurfactants, particularly

for glycolipids, such as rhamnolipids. While other methods, such as drop collapse method or oil displacement assay are also relevant for a comprehensive evaluation of biosurfactant bioactivity, the emulsification index was selected based on its simplicity, reliability, and relevance to the specific properties of the biosurfactant produced by *Pseudomonas fluorescens* ICCF 392.¹⁴

To obtain biosurfactants on a large scale, several limitations must be overcome, including expensive substrates and high costs associated with isolation and purification procedures.^{15,16}

The selection of a suitable substrate as a carbon and nitrogen source represents an important factor because it contributes to approximately 50% of the total production cost of biosurfactants. Therefore, the use of cheap substrates in processes of submerged fermentation, such as industrial by-products (glycerol) or food industry waste (vegetable waste cooking oil), to minimize the production costs represent a viable option.^{16,17-24} Twigg *et al.*²⁵ reported the use of waste products in the biosynthesis processes to reduce the cost of biosurfactant production and enhance their sustainability by contributing to a circular economy. Also, several studies reported the use of waste cooking oil or glycerol as sole carbon sources for biosurfactant production by different *Pseudomonas* species.^{26,27} Moreover, co-utilization of these substrates could contribute to the enhancement of biosurfactant production.²⁸ So, previous studies have demonstrated the potential of glycerol and waste oils as carbon sources for biosurfactant production, but few have addressed the stability and bioactivity of biosurfactants produced from these substrates, especially when co-utilized.

This research aims to fill this gap by focusing on *Pseudomonas fluorescens* ICCF 392 and its capacity to produce stable, active biosurfactants from low-cost mixed substrates.

The selection of *Pseudomonas fluorescens* ICCF 392 strain for this study was based on its well-documented ability to produce biosurfactants with high surface activity and stability. Unlike *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* is non-pathogenic, making it a safer alternative for industrial and environmental applications.²⁸

Moreover, the biosurfactants produced by *P. fluorescens* exhibit remarkable stability across a wide range of pH and temperature conditions, underscoring its suitability for demanding industrial environments. These characteristics establish *P.*

fluorescens ICCF 392 as a promising candidate for cost-effective and sustainable biosurfactant production. Biosurfactants present a variety of industrial and environmental potential applications due to their important properties, such as low toxicity, biodegradability, biocompatibility, increased specificity, and improved environmental compatibility.^{16,29–33} By emulsifying hydrocarbons, biosurfactants can improve the solubility of oil in water, lower surface tension, and better remove oil from soil particles.³⁴

Moreover, the use of biosurfactants in pharmaceuticals, cosmetics, and the food industry is based on the property of having inhibitory activity on the growth of some pathogens.³⁵ Biosurfactants can also display antimicrobial activity against Gram-positive and Gram-negative bacteria, likely by interacting with the phosphatidylethanolamine component of biological membrane systems.³⁶ According to the literature, a lot of types of glycolipids, such as rhamnolipids produced by *Pseudomonas* species, have been recognized to date for their antimicrobial properties.^{11,37}

However, before using biosurfactants in a variety of industrial and environmental processes, an evaluation of their efficacy must be conducted. Therefore, the aim of the present study was to investigate the stability at various temperatures and pH values of the biosurfactant produced by *Pseudomonas fluorescens* ICCF 392 on a low-cost mixed substrate containing 3% glycerol and 2% waste cooking oil as a carbon source, as well as to evaluate its potential antimicrobial activity for further use in various industrial fields.

MATERIALS AND METHODS

Biosurfactant production

The use of glycerol, a biodiesel by-product, and waste cooking oil not only lowers production costs, but also reduces environmental burdens associated with waste disposal, thereby aligning with circular economy objectives.

The *Pseudomonas fluorescens* ICCF 392 strain utilized in this study is included in the Collection of Microorganisms of Industrial Importance-CMII-ICCF.

The pre-inoculum bacterial strain was cultured on M44 agar medium, consisting of 5.0% glycerol ($\geq 99.5\%$), 1.0% yeast extract, 1.0% bacto-peptone, and 2.0% agar (g/v). The strain was incubated for 48 to 72 hours at 30°C.¹⁴

The inoculum and biosynthesis media contain (g/v): glycerol 3.0 ($\geq 99.5\%$) and waste cooking oil 2.0 as carbon sources; bacto-peptone 1.0 and yeast extract 1.0 as nitrogen sources, and KH_2PO_4 0.2 as mineral salts.

Distilled water was used to prepare the culture media, and the pH was adjusted to 7.0 and autoclaved at 115°C for 30 minutes.

The inoculum was distributed into 500 mL flasks, each containing 100 mL of medium, and incubated at 30°C and 220 rpm on a rotary shaker for 24 hours.

For biosurfactant production, a 10% cell suspension of the inoculum was added to 500 mL flasks containing 100 mL of biosynthesis medium. The cultivation conditions were 30°C, at 220 rpm with an incubation time of 72 hours.

Bacterial cell growth was determined by measuring the pH and optical density of the culture media at 550 nm. The chemicals utilized in this study were from Difco (USA) and Sigma-Aldrich (Germany).³⁸ The experiments were performed in triplicate.

Biosurfactant production by *Pseudomonas fluorescens* ICCF 392 utilizing a low-cost mixed substrate

In order to reduce the costs for biosurfactant production, glycerol, a by-product from the biodiesel industry, and waste cooking oil, a waste from the food industry, were assessed as substrates in the fermentation processes.

The results of the emulsification test were used to screen the mixed substrate as a suitable carbon source for biosurfactant biosynthesis. Emulsification activity was evaluated as emulsification index ($E_{24\%}$), according to the protocol proposed by Pathak and Keharia.³⁹

6 mL of heptane/octane/sunflower oil and 4 mL of supernatant were strongly homogenized in the test tubes, and the height of the emulsions formed was measured after 24 h. The emulsification index was determined using the following formula:

$$E_{24} = (\text{Emulsion layer height} / \text{Total liquid column height}) \times 100.$$

Stability of biosurfactant

One of the most important characteristics of biosurfactants' use in various industrial fields is

their stability in extreme environmental conditions at different temperatures and pH values.

In this case, the procedure used by Foukia *et al.*⁴⁰ to assess the stability of the biosurfactant was followed. Biosurfactant production by the *Pseudomonas fluorescens* ICCF 392 strain was evaluated through submerged fermentation in 500 mL flasks, each containing 100 mL of medium. The cultures were incubated on a rotary shaker at 220rpm and 30°C for 72 hours. The effect of pH was assessed by adjusting the pH of the supernatant obtained after centrifugation of the biosynthesis medium (9000 rpm, 20 minutes, 4°C) in the range of 2.0-12.0 using 2N HCl and 1N NaOH, and determining the emulsification index (E24%). Similarly, to investigate the stability of biosurfactants at different temperature values, the supernatant was kept at a constant temperature (30°C, 37°C, 50°C, 75°C, and 115°C) for 30 minutes, and then the emulsification index was determined.¹⁰

Isolation, and partial purification of biosurfactant

At the end of the biosynthesis process, the bacterial cells were removed by centrifugation at 9000 rpm, 4°C, for 20 minutes, and the supernatant was used for the extraction of biosurfactant.

First, the cell-free supernatant was acidified to pH 2.0 using 2N HCl and left overnight at 4°C. After, a 4-fold volume of ethyl acetate was used to extract the precipitate that was obtained by centrifugation under similar conditions. The mixture was then shaken vigorously, and it was left to settle until phase separation happened. The organic phase was collected, and the water was removed by adding anhydrous sodium sulfate. By using rotary evaporation at 40°C and low pressure, the solvent was removed, and the resulting product, considered the partially purified biosurfactant, was stored for further analysis.⁴¹ (Fig. 1).

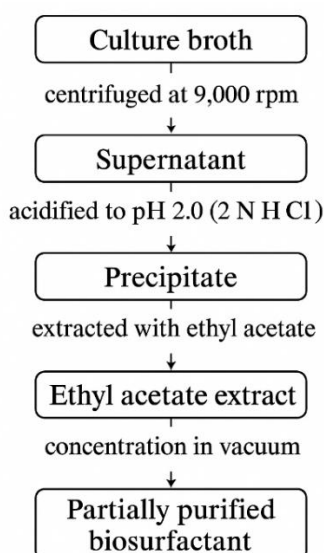


Fig.1 – Purification scheme for biosurfactant produced by *P. fluorescens* ICCF 392.

Antimicrobial activity of partially purified biosurfactant

Test microorganisms

Staphylococcus aureus ATCC 6538, *Escherichia coli* ATCC 8739, and *Pseudomonas aeruginosa* ATCC 9027 strains used for the antimicrobial study were obtained from the American Type Culture Collection (ATCC).

Antibacterial activity of partially purified biosurfactant

The partially purified biosurfactant produced on mixed substrate by the *Pseudomonas fluorescens*

ICCF 392 strain was evaluated for its antimicrobial activity against Gram-positive *Staphylococcus aureus* ATCC 6538, the Gram-negative *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027 strains using the agar diffusion (cylinder-plate) method, adapted from the agar well diffusion assay described by Niamah *et al.*⁴²

M44 agar medium was used for the evaluation of the antimicrobial property of the biosurfactant against the *Pseudomonas aeruginosa* ATCC 9027 test pathogen. In the case of *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 8739, nutritive agar medium was used, containing (g/v): meat extract 3.0, bacto-peptone 1.0, NaCl 5.0, agar 2.0. 100 mL of each

culture medium was inoculated with 1 mL of cell suspension from the stock culture (stock suspension of 10^8 CFU/mL), and then a volume of 15 mL from the inoculated media was distributed into a sterile Petri plate and allowed to solidify on a flat surface. At a distance of 28 mm from the center of the plate and at an equal distance from each other, two stainless and sterile cylinders were positioned on the medium's surface in the Petri dishes. 200 μ L of the partially purified biosurfactant sample were applied inside the cylinders, and the Petri dishes were placed in an incubator at 30–35°C for 18–24 hours. The antibacterial activity was evaluated by measuring the diameter of the inhibition zone (mm). The results were reproducible, and the average of three independent measurements was recorded.

RESULTS

Biosurfactant production by *Pseudomonas fluorescens* ICCF 392 using a low-cost mixed substrate

The high stability of the biosurfactant across pH and temperature ranges makes it a robust candidate for industrial applications in challenging environments, such as bioremediation of oil-contaminated sites, further strengthening its role in promoting sustainable environmental practices.

The emulsification index (E24%) is a significant parameter that indicates the ability of the biosurfactant to form and stabilize emulsions, which is essential for utilization in various industries.⁴³

In this study, previously, the emulsification activity of biosurfactant produced by *P. fluorescens* ICCF 392 on a substrate containing 3% glycerol and 2% waste cooking oil as a carbon source was evaluated.

The inoculum was cultivated at 30°C for 24 hours, followed by bioprocesses performed at the same temperature for 72 hours (Fig. 2a). A gradual decrease in cell density was observed thereafter, corresponding to the transition between the end of the exponential growth phase and the beginning of the stationary phase.

The fermentation broth obtained was then centrifuged to assess the emulsifying capacity of the supernatants with heptane, octane, and sunflower oil. The supernatants of *Pseudomonas fluorescens* ICCF 392 formed stable emulsions with the tested hydrocarbons and sunflower oil (Fig. 2b). The obtained results indicate that the biosurfactant exhibited an E24% above 50% for octane, heptane, and sunflower oil, demonstrating its high emulsifying capacity.

This findings are in agreement with those of Matátková *et al.*,⁴⁴ who reported an emulsification index of 70% for sunflower oil, as well as with Joice and Parthasarathi,⁴⁵ who achieved an emulsification index of approximately 70.0% for heptane.

Similarly, the study conducted by Techaoei *et al.*⁴⁶ reported an E24% value of approximately 60% for biosurfactant produced by *Pseudomonas aeruginosa* SCMU106 using a carbon source based on glucose and corn oil. Additionally, study by Deshmukh *et al.*⁴⁷ on *Pseudomonas aeruginosa* LTR1 revealed emulsification activity with various hydrocarbons, emphasizing its potential for industrial applications. Therefore, the biosurfactant produced in this study demonstrated emulsification index values comparable to those presented in the literature for the pathogenic strain *Pseudomonas aeruginosa*, typically achieved with the use of higher-cost substrates.

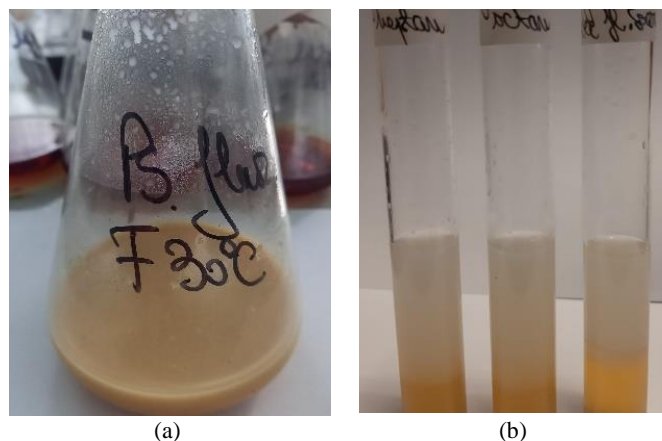


Fig. 2. – a) biosynthesis media; b) emulsions obtained with the supernatants of *Pseudomonas fluorescens* ICCF 392 with heptane, octane, and sunflower oil.

Stability of biosurfactant at different temperatures and pH values

The effect of temperature was studied by incubating the supernatant containing the biosurfactant at various temperatures between 30–115°C for 30 min. and determining the emulsification index for heptane, octane, and sunflower oil.

As presented in Figs. 3 and 4, the results obtained of $E_{24\%}$ for heptane and octane demonstrated that biosurfactant is stable between 30°C and 37°C with values of 64.28%.

An increase of temperature to 50°C, respectively 75°C, does not significantly reduce their stability, the $E_{24\%}$ values being 62.5% for heptane and 64.28% for octane in both cases.

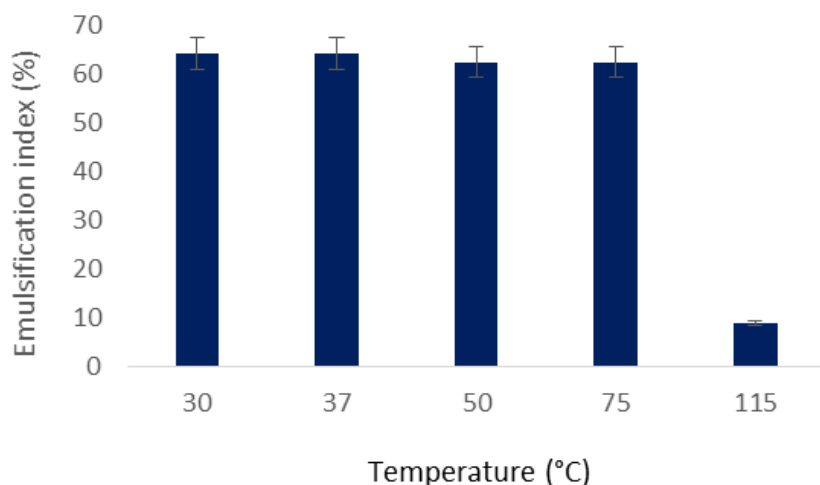


Fig. 3 – Effect of temperatures on emulsification index obtained with heptane.

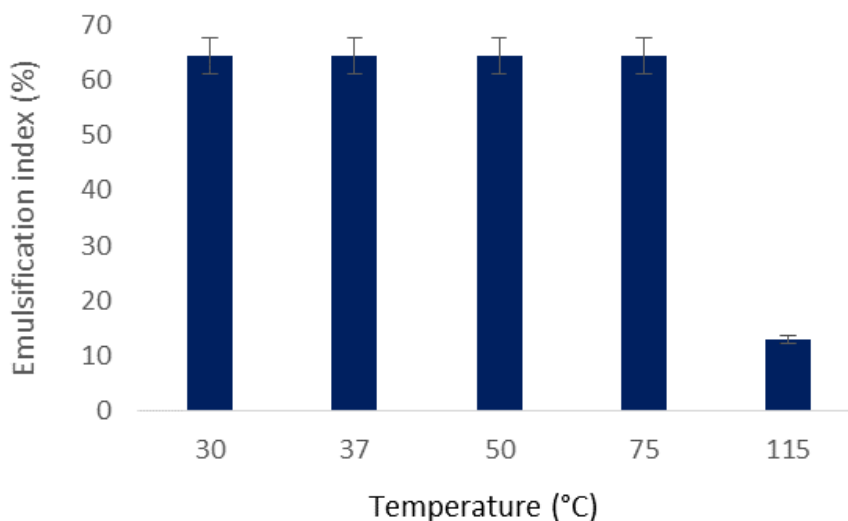


Fig. 4 – Effect of temperatures on emulsification index obtained with octane.

Instead, the exposure of the biosurfactant at the temperature of 115°C has led to a decrease of the emulsification index value of 8.92% in the case of heptane and 12.96% for octane.

It should be noted that the emulsion formed with sunflower oil was more stable, starting from the $E_{24\%}$ value of 68.51% at temperatures of 30°C, 66.66% at temperatures of 37°C and 50°C, 64.81% at the temperature of 75°C, and reaching the

$E_{24\%}$ value of 61.11% at the temperature of 115°C (Fig. 5).

Therefore, emulsions formed with sunflower oil showed better stability, with $E_{24\%}$ values ranging from 68.51% at 30°C to 61.11% at 115°C. The results obtained in this case showed that the temperature did not affect significantly the biosurfactant stability over a wide range from 30 to 115°C.

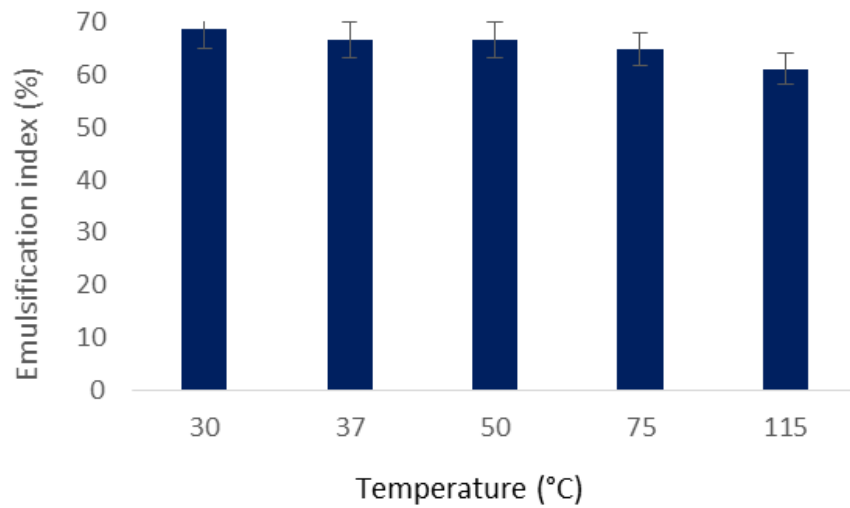


Fig. 5 – Effect of temperatures on emulsification index obtained with sunflower oil.

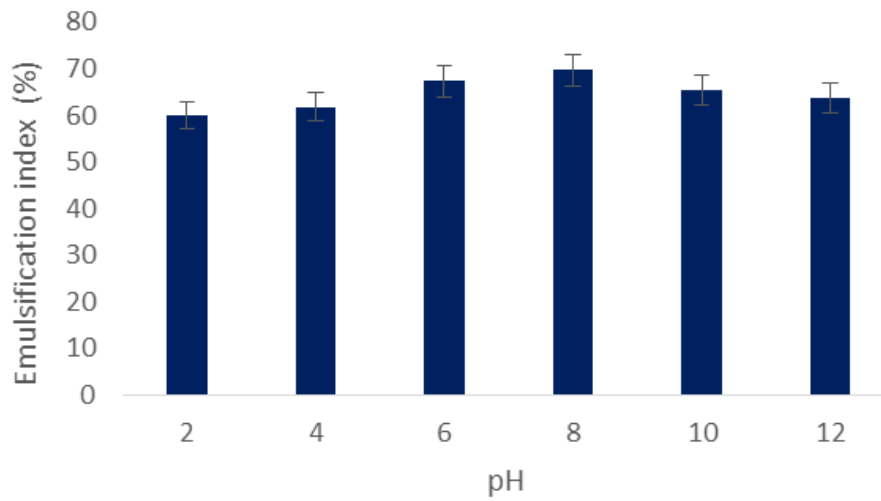


Fig. 6 – Effect of pH on emulsification index obtained with heptane.

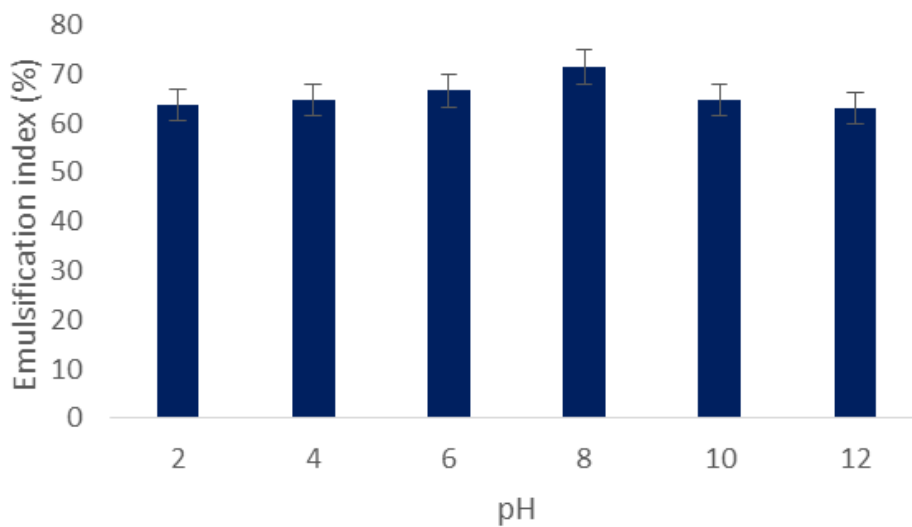


Fig. 7 – Effect of pH on emulsification index obtained with octane.

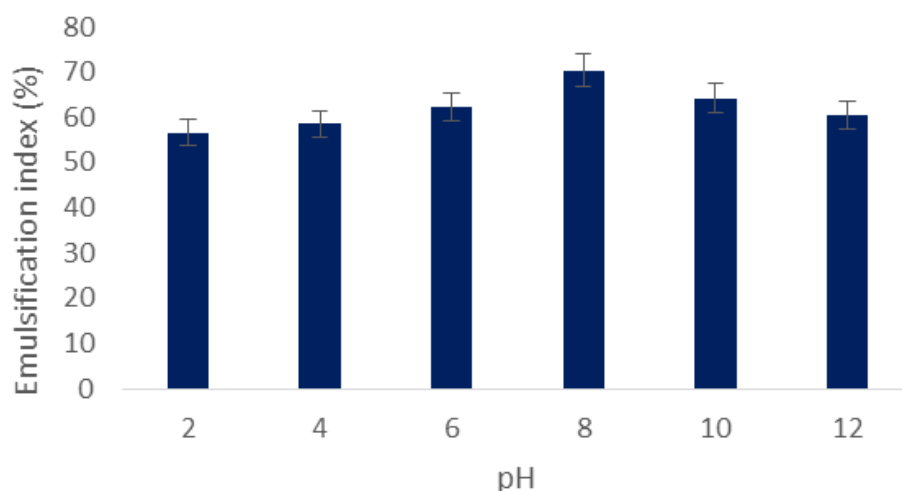


Fig. 8 – Effect of pH on emulsification index obtained with sunflower oil.

Regarding the pH, the best values of $E_{24\%}$ were obtained at pH 6 to 8. As depicted in Figs. 6, 7 and 8, in highly acidic (pH 2–4) conditions, a slight decrease in E_{24} values was obtained in the case of heptane, octane, and sunflower oil. Lowering the pH below 6 resulted in a slight decrease in stability, but still with good $E_{24\%}$ results of over 50% for heptane, octane and sunflower oil.

At pH 10–12, the $E_{24\%}$ were almost constant, the values obtained being slightly reduced compared to those obtained at the range of pH 6–8, but higher than those obtained at an extremely acidic conditions, suggesting that biosurfactant is stable between pH 2.0–12.0.

The stability of biosurfactants in the range of pH 2–12 indicates that biosurfactants have activity in both acidic and basic conditions.

Previous studies indicate that biosurfactants produced by *Pseudomonas* strains exhibit stability over a wide pH ranges, with optimal activity under slightly alkaline conditions.^{48,49}

The stability of the biosurfactant obtained is in agreement with the findings of Adebajo *et al.*⁴⁸, where the biosurfactant produced by the *Pseudomonas taenensis* strain was found to be stable at pH values from 2 to 12.

However, a study by Dhundale *et al.*⁵⁰ on *Pseudomonas aeruginosa* strains SJS5 and SJS6 found that the biosurfactant produced by these strains was highly stable over a broad temperature range from 40°C to 80°C and a pH range from 6.0 to 12.0.

A study by Kanna *et al.*³² investigated the stability of the biosurfactant produced by *Pseudomonas putida* under varying pH (1.0–11.0) and temperature (40°C–100°C) conditions.

The results demonstrated that the biosurfactant exhibited stability within the pH range of 5.0 to 11.0, and maintained consistent surface tension across the entire temperature range, indicating its high thermostability. These results demonstrate that the biosurfactant exhibited excellent performance across a wide range of pH and temperature values, suggesting its potential for promising biotechnological applications.

Isolation, and partial purification of biosurfactant

The biosurfactant produced by *Pseudomonas fluorescens* ICCF 392 was partially purified from the culture broth, as illustrated in Fig. 1. A brown precipitate containing the biosurfactant was obtained by acidifying the culture broth with 2N HCl. This precipitate was extracted using ethyl acetate, followed by concentration in a rotary evaporator. Further, the obtained biocompound was used to test its antibacterial activity.

Antibacterial activity of biosurfactant

In order to combat pathogenic bacteria that cause infections, biosurfactants can be used as antimicrobial substances, according to Naughton *et al.*⁵² Using the agar diffusion (cylinder-plate) method⁴² the antimicrobial activity of the biosurfactant obtained after post-biosynthesis processing was analyzed against three pathogen strains, such as *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, and *Escherichia coli* ATCC 8739.

The zones of inhibition produced by the biosurfactant obtained against the microbial strains used as a test organism were recorded. Antibacterial

activity was observed against all the bacterial strains analyzed (Table 1), with different zone of inhibition measurements.

Table 1

Inhibition zones of biosurfactant produced against three pathogenic bacteria

Tested samples	Inhibition zones (mm) against <i>S. aureus</i>	Inhibition zones (mm) against <i>E. coli</i>	Inhibition zones (mm) against <i>P. aeruginosa</i>
Purified biosurfactant	24	17	30
Control (sterile distilled water)	–	–	–

* The data represent the averages of three replicates (n=3)

As we can see in Fig. 9, biosurfactant produced by *Pseudomonas fluorescens* ICCF 392 on mixed substrate showed strong antimicrobial activity, indicated by a larger zone of inhibition (24 mm) against *Staphylococcus aureus* ATCC 6538 and

against *Escherichia coli* ATCC 8739 strain (17 mm), respectively. Furthermore, in the case of the *Pseudomonas aeruginosa* ATCC 9027 strain, superior antibacterial efficacy was demonstrated, with the largest zone of inhibition observed (30 mm).

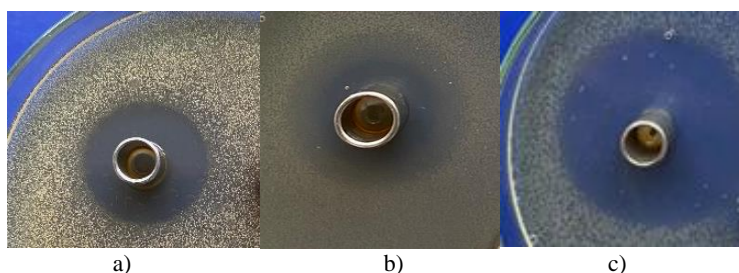


Fig. 9 – Antibacterial activity of the biosurfactant produced against three pathogenic bacterial strains: a) *Staphylococcus aureus* ATCC 6538; b) *Escherichia coli* ATCC 8739; c) *Pseudomonas aeruginosa* ATCC 9027

Considering the results obtained, it can be concluded that the biosurfactant produced by the *Pseudomonas fluorescens* ICCF 392 strain displayed significant antimicrobial activity against the test bacterial strains, showing the potential for its utilization in the medical, pharmaceutical, or food industries as antimicrobial agents, among others.

DISCUSSION

Recently, biosurfactants have received a lot of attention due to their unique and characteristic properties. The antimicrobial activity and stability over a wide range of pH levels and temperatures are important properties for most of the biosurfactants. Therefore, the current study focused on the assessment of the stability of the biosurfactant produced by the *Pseudomonas fluorescens* ICCF 392 strain on a cheap mixed substrate at different temperatures and pH values, as well as on its potential antimicrobial activity

against three pathogenic strains. According to the literature, the effect of different concentrations of carbon sources (glycerol, waste frying oil, sunflower oil, or olive oil) on biosurfactant production by *Pseudomonas* species was previously evaluated to provide the best results for biosurfactant production.⁵³⁻⁵⁶ The results obtained in the present study confirmed the ability of the *Pseudomonas fluorescens* ICCF 392 strain to produce biosurfactant on mixed substrate containing 3% glycerol and 2% waste cooking oil as carbon sources.

Also, the biosurfactant obtained is stable between 30°C and 37°C, with $E_{24\%}$ values of 64.28%. An increase in temperature to 55°C and 75°C did not significantly reduce stability, with $E_{24\%}$ values of 62.5% for heptane and 64.28% for octane. At 115°C, the emulsification index dropped drastically to 8.92% for heptane and 12.96% for octane, but not also in the case of emulsions with sunflower oil, which showed better stability, with $E_{24\%}$ values ranging from 68.51% at 30°C to 61.11% at 115°C.

Previous studies have shown that the stability of biosurfactants can vary significantly depending on the temperature to which they are exposed. In this context, stability at high temperatures is essential for the applicability of biosurfactants in industrial processes involving varied thermal conditions. Several studies in the field have also highlighted the importance of the thermal stability of biosurfactants. For example, biosurfactants produced by *Pseudomonas aeruginosa* KT 1115 demonstrated high stability at temperatures between 20°C and 80°C, with a decrease in activity at higher temperatures.⁵⁷ Additionally, *Pseudomonas* strain was reported to produce biosurfactants that remained stable at temperatures up to 70°C, with emulsification capacities diminishing at temperatures above 70°C, indicating the importance of moderate thermal stability for industrial applications.

Also, the biosurfactant obtained was stable over a broad pH (2–12), exhibiting pH stability, with the highest E24% between 30–75°C and at pH 8, and slightly decreasing values in both acidic (pH 2 and 4) and alkaline (pH 10 and 12) conditions.

Several studies have reported similar findings, indicating that biosurfactants remain stable across a broad range of pH values.^{16,48,49} The results are in agreement with those conducted by Techaoei *et al.*,⁴⁶ where rhamnolipids remained stable at neutral and basic pH. Therefore, the stability of the produced biosurfactant strain indicates its potential applicability in extreme industrial conditions, in the bioremediation of waste oils and hydrocarbons. The results of the present study are similar to those that demonstrated that biosurfactants can maintain the emulsification activity at high temperatures and pH values, highlighting the importance of this property of the biosurfactant in order to optimize their use in different industrial sectors.⁵⁸

Moreover, the partially purified biosurfactant produced by the *Pseudomonas fluorescens* ICCF 392 strain was investigated for its antimicrobial activities, and the results are listed in Table 1. The biosurfactant exhibited strong antagonistic activity against *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, and *Pseudomonas aeruginosa* ATCC 9027 strains, being able to be used in the pharmaceutical industry, among other industrial applications. The results are similar with those published by Govindammal *et al.*,⁵⁹ who confirmed that biosurfactant produced by *Pseudomonas fluorescens* demonstrated activity against *Staphylococcus aureus* and *Escherichia*

coli, and also with the findings of Alyousif *et al.*,⁶⁰ who showed the antimicrobial effect of rhamnolipid produced by *Pseudomonas aeruginosa* against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The low costs associated with the production of biosurfactant make it a very cost-effective candidate for various applications in many industries and merit further studies to scale up the production of this important bioproduct. The findings of this study highlight that biosurfactants are a sustainable alternative to synthetic surfactants, providing considerable ecological and economic advantages.^{61,62} The antimicrobial activity and stability of biosurfactants at high temperatures and different pH levels can extend the applicability of these biocompounds, offering sustainable and efficient solutions for modern industrial problems.^{17,63} This study also demonstrates how integrating waste valorization into biosurfactant production can significantly reduce environmental impact. By repurposing industrial and food waste, this approach aligns with the EU's Green Deal goals and the circular economy model, offering scalable solutions for sustainable industrial processes.

CONCLUSIONS

Biosurfactants are of significant interest for current and future applications in the era of green technology, fostering a biotechnological approach to producing a variety of bio-surfactants with wide industrial applications that are both practically important and financially viable. In this study, the efficient production of biosurfactants by *Pseudomonas fluorescens* ICCF 392 using a low-cost mixed substrate consisting of glycerol and waste cooking oil as carbon sources has been demonstrated. Additionally, the biosurfactant's stability and antibacterial activity were shown, suggesting its potential applications in bioremediation, pharmaceuticals, cosmetics, and the food industry. This research highlights the potential of biosurfactants as sustainable alternatives to synthetic surfactants with broad industrial applicability. The integration of waste materials into the production process also aligns with circular economy goals, reducing environmental impact while providing cost-effective and eco-friendly solutions. Future work should focus on optimizing production processes to further increase biosurfactant yields and production, including exploring alternative low-cost substrates and

scaling up production. Additionally, the characterization of the biosurfactant through HPLC/HPLC-MS will be included to provide a detailed understanding of its composition and properties. It would also be advantageous to explore the synergy between biosurfactants and other sustainable bioproducts in multi-component formulations for specific industrial applications. Moreover, conducting an economic analysis, including a detailed feasibility study, would provide valuable insights into the commercialization potential of biosurfactant production, taking into account the cost-effectiveness of waste-derived carbon sources.

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