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THE POTENTIAL OF *Candida lipolytica* ICCF 214 (ATCC 16618) TO PRODUCE BIOSURFACTANS

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Abstract

Biotechnological processes, particularly those involved in biosurfactant production, can pose significant financial challenges. The viability of biosurfactant production hinges on the creation of processes that make use of cost-effective raw materials. This study focuses on exploring the potential of Candida lipolytica ICCF 214 (ATCC 16618) reference strain for biosurfactant production using a mixture of vegetable oil and other chemical compounds used for fermentation medium (1% glutamic acid, urea, or glucose) as carbon sources. Based on the obtained results, we can conclude that all three fermentation media stimulated Candida lipolytica ICCF 214 strain to produce biosurfactants, but further analysis of surface tension and antimicrobial activity of the obtained biosurfactants is required. Additionally, we observed that media with a volume of 100 ml produced higher quantities of biosurfactants, due to the good aeration of the fermentation medium. However, even the ones with 150 ml showed decent performance during the bioprocess.

Key words: *bioemulsifiers, biosurfactants, microbial fermentation, Candida lipolytica, yeasts.*

INTRODUCTION

Surfactants are molecules with both hydrophilic and hydrophobic properties, enabling them to accumulate at interfaces, reduce interfacial tensions, and form structures like micelles. These attributes make surfactants and emulsifiers essential in various industries including industrial, agricultural, food, cosmetic, and pharmaceutical sectors. Originally sourced from renewable origins like fats and oils, a majority of surfactants nowadays are derived from petrochemicals (Ambaye et al., 2021). Nonetheless, a subset known as biosurfactants is biologically synthesized by yeasts or bacteria, categorized as glycolipids, lipopeptides, fatty acids, polymeric, or particulate compounds. Biosurfactants present advantages over synthetic counterparts due to their biodegradability, lower toxicity, effectiveness in extreme conditions, and environmentally friendliness (Ambaye et al., 2021). Despite these benefits, biosurfactants are not yet economically competitive with synthetics, necessitating cost-effective production strategies to facilitate their widespread adoption. They can be produced from various substrates, predominantly renewable resources like vegetable oils and industrial wastes (Amalesh et al, 2012). Selecting low-cost substrates is essential for the economic feasibility of biosurfactant production. Key factors for successful biosurfactant production encompass the development of cost-effective processes, utilization of low-cost materials, attainment of high yields, and customization of biosurfactants for specific applications.

In the past few years, there has been a surge in interest in isolating and identifying new microbial polysaccharides and surfactants that could potentially be used in enhanced oil recovery processes. The prospect of discovering a new microbial gum, such as xanthan or gellan, or a distinctive bioemulsifier like emulsan which possesses unique properties enabling its use as a gelling agent, emulsifier, stabilizer, flocculant, lubricant, or dispersing agent, has fueled this interest.

This study endeavors to explore the potential of the microorganism *Candida lipolytica* to produce biosurfactants from a mixture of vegetable oil and other chemical compounds employed in fermentation media (1% glutamic acid, urea, or glucose) as carbon sources. The kinetics, characterization, properties, and toxicity of the resulting biosurfactant are assessed for potential environmental applications.

MATERIALS AND METHODS

Microorganism

The microorganism utilized within this study belongs to the Collection of Microorganisms of Industrial Importance (CMII-ICCF-WFCC 232), namely *Candida lipolytica* ICCF 214 (ATCC 16618), from the Institute of Chemical-Pharmaceutical Research and Development in Bucharest (ICCF BUCHAREST).

Cultural conditions and biosurfactant production

Initially, the strain of *C. lipolytica* was inoculated into test tubes containing a suitable medium for yeast, specifically YMPG (Yeast Malt Peptone Glucose), freshly prepared. The transfer of the microorganism of interest was carried out under sterile conditions, with each test tube containing about 10 ml of YMPG medium. *C. lipolytica* was transferred onto the fresh medium, and after 48 hours, under controlled temperature conditions (28°C), robust development of the microorganism was observed. In total, five test tubes were utilized for this procedure. Two cultures steps were needed to initiate the experiments, a preinoculum and inoculum phase.

- **PREINOCULUM**: was represented by the strain of *C. lipolytica* on solid (or liquid) medium.
- **INOCULUM** Phase: was prepared in liquid YMPG medium.

The YMPG (DSMZ 186 medium) universal medium for yeast contained Glucose: 10g, Yeast extract: 3 g, Malt extract: 3 g, Peptone: 5 g, Agar: 17 g, Distilled water: 1000 ml pH: 6.5-7, Sterilization in autoclave at 115°C for 30 minutes. A total of 200 ml of YMPG medium was prepared for inoculation, without agar: Glucose: 2 g; Yeast extract: 0.6 g; Malt extract: 0.6 g; Peptone: 1 g. The volume was adjusted to 200 ml with distilled water. The pH of the prepared medium was determined to be 6.36. It was then adjusted to pH 7 using a 30% NaOH solution.

In the Inoculation Phase, Erlenmeyer flasks with a volume of 500 ml were utilized, each containing 100 ml of the liquid YMPG medium. Fermentation was initiated from the inoculum. In the literature, the inoculum volume varies between 2-10% (Albuquerque et al., 2006). In a first experiment we used a 5% inoculum.

The inoculation media were agitated in an orbital shaker at 220 rpm at 28°C for 48 hours. At 24-hour intervals from the start of inoculation, we monitored the evolution of the following parameters: pH and optical density (OD), read using a UV-VIS spectrophotometer at 540 nm, dilution 1:50; in the case of large inoculum volumes, we determined the biomass uplift (%).

The bioprocess

Three different fermentation media were used for this experiment.

M1 was the first fermentation medium tested for obtaining biosurfactants from the *C. lipolytica* strain. A batch of 500 ml medium was prepared, distributed as follows: two Erlenmeyer flasks with 100 ml medium each and two flasks with 150 ml medium each. The M1 medium contains: 0.1% NH_4NO_3; 0.02% KH_2PO_4; 0.02% MgSO4x7H_2O; 6% corn oil; 1% glutamic acid. After homogenizing all components, the pH was adjusted to 5.73.

M2 was the second fermentation medium tested for obtaining biosurfactants from the *C. lipolytica* strain. A batch of 500 ml medium was prepared, distributed as follows: 2 Erlenmeyer flasks with 100 ml medium each and 2 flasks with 150 ml medium each. The M2 medium contains: 0.25% $(NH_2)_2CO$ (urea), 1.36% KH_2PO_4, 0.6% $(NH_4)_2SO_4$, 7.5% corn oil. The final pH was adjusted to 5.32.

M3 was the third fermentation medium used for obtaining biosurfactants from the *C. lipolytica* strain. The M3 medium contains: 0.01% NH_4NO_3; 0.02% KH_2PO_4; 0.02% $MgSO_4$ x7H2O; 0.2% yeast extract; 10% glucose, 10% rapeseed oil (canola oil). The final pH was 5.67. The fermentation process was performed for 96 hours duration. During the fermentation process, the pH and optical density (OD) were monitored. The OD measured with a UV-VIS spectrophotometer, at 540 nm, in 1:50; 1:100 dilutions. The dry biomass parameter was determined at the end of fermentation. The quantity of the biosurfactant obtained, and the Emulsification index were also determined.

The Shoorl method, also known as the phenol-sulfuric method, is a technique used to determine the concentration of reducing sugars, such as glucose and fructose, in a solution. This method is based on the reaction between the

reducing sugars and phenol and sulfuric acid, which forms an intense color that can be measured spectrofotometrically.
The procedure involves first preparing a solution of reference sugars to create a calibration curve. Then, test samples are mixed with phenol and sulfuric acid, triggering the color formation reaction. The intensity of the formed color is then measured with a spectrophotometer at a specific wavelength, and the concentration of sugars is determined based on the light absorption.
The Shoorl method is widely used in biochemistry and microbiology laboratories for the rapid and accurate determination of sugar concentrations in a variety of biological samples and solutions.

Biomass determination

For biomass determination, 5 ml samples were mixed in pre-weighed tubes with chilled distilled water and centrifuged at 5000 rpm for 20 min. Following two washing cycles, the resulting cell pellet was dried in an oven at 90°C for 24 h. All the assays were carried out in triplicate and did not vary more than 5%.
Further, in order to separate biomass from the medium and preserve biosurfactants / substances of interest in the supernatant, with pH values ranging between 2 and 4, the pH was adjusted using a 30% NaOH solution to reach a pH of 7-7.5. Subsequently, the fermentation media was centrifuged at 7500 rpm for 20 minutes at 4°C. After centrifugation, biomass was separated from the supernatant, and the percentage of dry biomass was determined. The biomass was transferred from the centrifuge tubes to initially weighed Petri dishes and dried at 37°C for 24 – 48 hours until constant weight. The supernatant was refrigerated to facilitate the separation of the vegetable oil (corn and rapeseed oil used in preparing fermentation media). Prior to refrigeration, a simple filtration was performed. The volumes of supernatant obtained after centrifugation were measured.

Isolation of the Biosurfactant

The biosurfactant was extracted using different solvent system methods. Following the protocol, the cell-free broth was acidified with 6 M HCl to reach pH 2.0 and then precipitated with two volumes of methanol (96 %Purity). After 24 h at 4 °C, samples were centrifuged at 5000 g for 30 min, washed twice with cold methanol and dried at 37 °C for 24–48 h. The yield of isolated biosurfactant was expressed in g/L. In the second method, the biosurfactant was recovered from the cell-free broth by cold acetone precipitation, as described by Ilori et al. (2005). Three volumes of chilled acetone were added and allowed to stand for 10 h at 4 1C. The resulting precipitate was collected by centrifugation and then evaporated to dryness to remove any residual acetone. Subsequently, the precipitate was re-dissolved in sterile water. The third method was that described by Amézcua-Veja et al. (2007). A sample of the cell broth was placed in a separatory funnel and an equal volume of ethyl acetate was added. The broth and ethyl acetate mixture formed two phases. The upper phase was transferred to a round-bottom flask. The lower phase was extracted with an equal volume of ethyl acetate three times for complete recovery of the biosurfactant. The round-bottom flask was placed on a roto-evaporator and the ethyl acetate was evaporated under vacuum at 80 °C. The residue obtained was washed twice with hexane and dried in the oven until a constant a constant weight was achieved.
From the three extraction methods, experiments were conducted with the first two methods. Consequently, 10 ml of supernatant from samples 1, 3, and 5 were precipitated with two volumes of methanol (96% purity, reagent from Merck), and another set of 10 ml samples were precipitated with three volumes of acetone (97% purity, reagent for analysis, Merck). After 24 hours, it was found that the method with methanol yielded a greater amount of precipitate compared to the samples where acetone was used as the solvent.
For the seven samples, precipitation was performed with 100 ml of cold methanol, brought to pH = 2 with 10% HCl.
For each sample, 100 ml of supernatant with a pH of 2 and 200 ml of cold methanol were added. They were left for 24 hours at 4°C, then centrifuged at 5000 rpm for 20 minutes at 4°C. The resulting precipitate was washed twice with methanol and centrifuged again. The resulting precipitate was dried for 24-48 hours at 37°C, or by drying in an oven at 105°C for 4 hours, until a constant weight was reached.

The emulsification index

Four milliliters of supernatant were taken from each of the seven aforementioned samples, and the emulsification activity was monitored using different substrates (sunflower oil, corn oil, and motor oil). Six milliliters of each oil were added to each sample for emulsification index analysis. After 24 hours, the stability of the formed emulsion was observed by agitating the samples using a vortex mixer for two minutes.

The emulsification index was calculated by dividing the height of the emulsified layer by the total height, multiplied by 100.

RESULTS AND DISCUSSIONS

The pH evolution in two of the *C. lipolytica* inoculum cultures was evaluated (Figure 1).

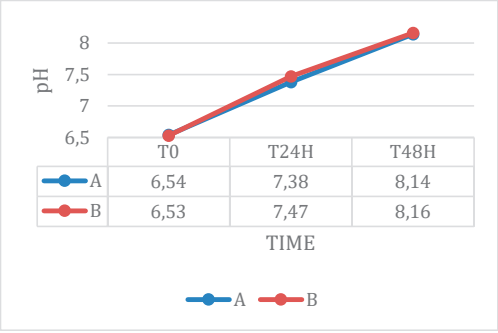

Figure 1. The pH evolution for the inoculum A and B

The pH levels can influence enzyme activity, nutrient availability, and product formation, among other factors. Maintaining optimal pH conditions is essential for maximizing the efficiency and yield of the fermentation process. Tracking pH evolution allows for adjustments to be made as needed to ensure the desired outcomes are achieved.

In the context of a fermentation process, optical density is an important tool in monitoring and controlling biotechnological processes.

The OD evolution in two of the *C. lipolytica* inoculum cultures is presented in Figure 2.

It was observed that in the first 24 h from inoculation both cultures were in exponential growth phase, while after 48 hours of inoculation, the culture was fully grown.

Considering the pH and optical density values, inoculum B demonstrated superior growth compared to A, therefore it was chosen to start the BIOPROCESS (Figure 3).

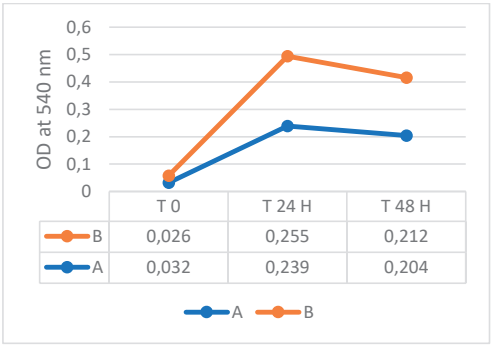

Figure 2. The Optical density (OD) read at 540 nm for the inoculum A and B

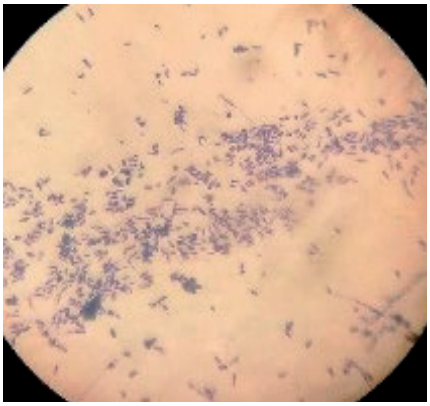

Figure 3. Microscopic aspects of *C. lipolytica* culture stained with methylene blue

The inoculation of the three fermentation media marked as **M1, M2**, and **M3,** has been carried out using inoculum B, which demonstrated superior growth compared to A, has been performed. An initial sampling was at time T 0, followed by an initial 96 hours fermentation process (the flasks were stirred at 200 rpm, 28°C). Depending on the observed values of the key process parameters, it was decided whether to potentially extend the fermentation process to either 120 hours or 144 hours.

For medium M3, the sugar content was additionally determined using the Shoorl method after the inoculation phase.

It was noted that M3 originally had a sugar content of 7.85 g%. Medium M3 was initially supplemented with 10% glucose monohydrate.

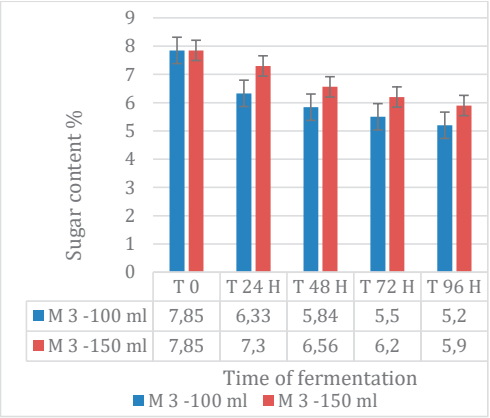

Figure 4. Sugar content (%) for medium M3

Observations indicated that the culture in medium M3 metabolized approximately 0.3 % glucose after 48 hours of fermentation, indicating the possibility of prolonging the bioprocess for an extended period of time, such as 144 hours.

The sugar content refers to the concentration or amount of sugars present in a solution or medium. In the context of fermentation processes, sugar content is often monitored as it serves as a crucial substrate for microbial growth and metabolism. Yeasts and other microorganisms typically utilize sugars as a source of energy for fermentation, converting them into various products such as alcohol, organic acids, or gases.

Measuring the sugar content helps to understand the progress of fermentation, as the consumption of sugars and the production of fermentation by-products can be monitored over time. This information is vital for optimizing fermentation conditions, controlling product quality, and maximizing yields. Various methods, such as spectrophotometry, chromatography, or enzymatic assays, can be employed to determine the sugar content accurately.

In figure 5 is presented the pH evolution from T0 until the completation of fermentation for the bioprocess (96 h). In Figure 6 is presented the evolution of optical density, read at 540 nm, using a UV-VIS spectrophotometer.

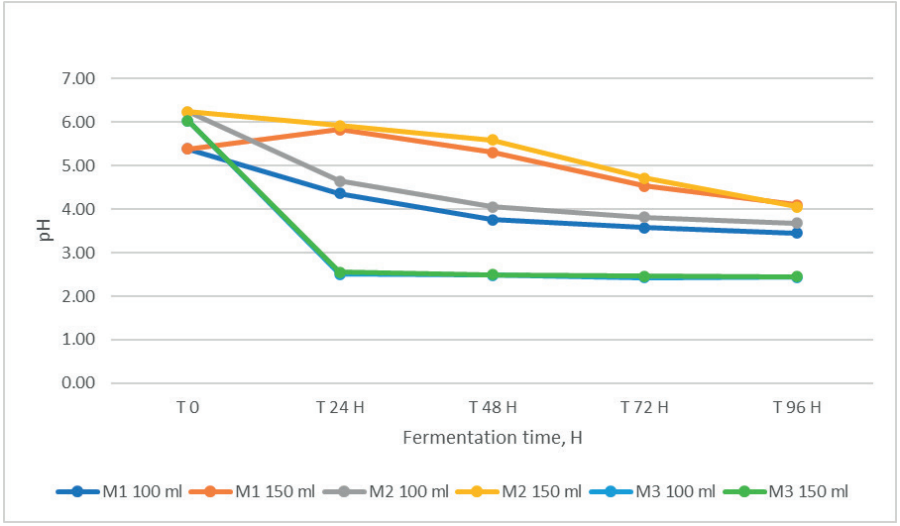

Figure 5. The pH evolution from T 0 until the completation of fermentation for the bioprocess (96 h).

Figure 6. The evolution of optical density, read at 540 nm, using a UV-VIS spectrophotometer

At the end of fermentation, pH and OD values were determined from the duplicate Erlenmeyer flasks, in which no samples were taken during the bioprocess. These measurements were compared with values determined both during the fermentation process and at its end. This procedure was carried out to identify any significant differences in process parameters.

After analyzing the parameters measured at the end of fermentation, we grouped together the flasks where there were no significant differences. Thus, from the 12 flasks with three different fermentation media, the following resulted after grouping: seven samples suitable to further investigations. From those with 100 ml of medium, they were combined with those made in duplicate for M1, M2, and M3. From those with 150 ml, only M1 and M2 were combined, and for M3, separate ones were made for both samples with 150 ml (the one in duplicate from which no samples were taken and the one from which samples were taken for analysis during the fermentation process).

This resulted in a total volume of medium after combining the flasks.

In Table 1 are presented the values for dry biomass (Figure 7), expressed in g/L, for each of the experimental substrate and fermentation volume.

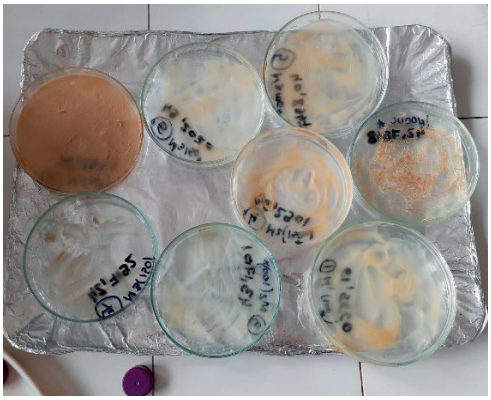

Figure 7. Dry biomass

Table 1. Calculation of dry biomass, g/L

Reunited medium	Dry biomass (g/L)
M 1 100 ml	8.07
M 1 150 ml	12.28
M 2 100 ml	2.32
M 2 150 ml	3.00
M 3 100 ml	4.65
M 3 150 ml	4.31
M 3 150 ml *	1.32
INOCULUM A	4.73

Biosurfactant yield

Candida strains have been shown to produce biosurfactants with different yields, which are dependent of the medium and culture conditions.

Crude biosurfactant of 13.99 g/L was extracted from the 96h culture of *C. lipolytica ICCF 214* showing a growth-associate production. Similarly, a biosurfactant yield of 4.5 g/L was extracted from the 144h culture of *C. sphaerica* (Rufino et al., 2013). On the other hand, the yield of another biosurfactant produced by *C. sphaerica* was 9 g/L after 144 h of experiment, which is in accordance with the values previously reported in the literature (Sarubbo et al 2007; Rufino et al., 2013). reported a yield of 8 g/L for a biosurfactant produced by *C. lipolytica* using canola oil and glucose as substrates. In Table 2 is presented the values for biosurfactant content, expressed in g/L.

Table 2. Biosurfactant content obtained with precipitation of methanol, g/L, 96 h

Reunited medium	Sample notation	Biosurfactant content (g/L)
M 1 100 ml	1	2.12
M 1 150 ml	2	0.43
M 2 100 ml	3	3.12
M 2 150 ml	4	4.47
M 3 100 ml	5	4.00
M 3 150 ml	6	2.13
M 3 150 ml *	7	1.89

In Table 3 are presented the values for biosurfactant content, expressed in g/L.

Table 3. Biosurfactant content obtained with precipitation of acetone, g/L 96 h

Reunited medium	Sample notation	Biosurfactant content (g/L)
M 1 100 ml	1	12.45
M 1 150 ml	2	4.45
M 2 100 ml	3	9.45
M 2 150 ml	4	13.99
M 3 100 ml	5	5.74
M 3 150 ml	6	-
M 3 150 ml *	7	2.82

In Table 2 and Table 3 are presented the values for biosurfactant content (g/L), the purification has been with methanol and acetone. Can observe that the precipitation with acetone produced for M2 the largest quantity of biosurfactant content.

Emulsification index

Biosurfactant production is often assessed by measuring emulsification. Although a direct correlation has been found between surface activity and emulsification activity and the emulsion index has been used as a screening method, the ability of a molecule to form a stable emulsion is not always associated with lowered surface tension activity. Sunflower oil, corn oil and motor oil were the substrates. The water–oil emulsions were compact and remained stable for more than six months at room temperature, suggesting that the addition of the biosurfactant to a remediation process may enhance the availability of the recalcitrant hydrocarbon. Different oil-degrading microorganisms produce surface active substances and some make a stable oil-in-water emulsion. These microorganisms can be categorized into two groups - those that produce a low-molecular-weight surfactants, which typically but do not form stable emulsions, and others that produce polymers that primarily act as emulsion stabilisers but have minimal impact on surface tension. A few bacteria and yeasts have both types of properties. The present data suggest that *C. lipolytica* ICCF 214 could be included in the latter category of microorganisms, as this strain was able to produce a biosurfactant that could reduce the surface tension and make stable emulsions with motor oil, as will be shown later. In figure 8 is presented the emulosification index. Table 4 presents the hydrophobic substrates tested for emulsification by the cell-free broth containing the biosurfactant from *C. lipolytica* ICCF 214.

Figure 8. Emulsification index

Table 4. Emulsification index (EI) %

Reunited medium	Sample notation	EI -24 H %- substrat sunflower oil	EI -24 H %- substrat corn oil	EI -24 H %- substrat motor oil
M 1 100 ml	1	15	60	65
M 1 150 ml	2	30	30	65
M 2 100 ml	3	No emulsion formed	No emulsion formed	15
M 2 150 ml	4	No emulsion formed	No emulsion formed	7
M 3 100 ml	5	42	86.3	43
M 3 150 ml	6	47	77.7	50
M 3 150 ml *	7	58	55	60

In the Table 4 we can observe that the best result for emulsification index (%) was for medium M3, using the corn oil substrate. Albuquerque et al (2006) obtained the similar index emulsification.

CONCLUSIONS

Based on the obtained results, we can conclude that all three fermentation media produced biosurfactants using the Candida lipolytica ICCF214 strain, but further analysis of surface tension and antimicrobial activity of the obtained biosurfactants is required. Additionally, we observed that media with a volume of 100 ml produced higher quantities of biosurfactants, indicating good aeration of the fermentation medium. However, even the ones with 150 ml showed decent performance during the bioprocess

- The biosurfactant isolation method using two volumes of methanol will be employed, and other solvent extraction methods (such as chloroform) will be explored.
- Regarding the emulsification index (E24), medium M3, with a volume of 100 ml, exhibited the largest emulsified layer, prompting further analysis of the chemical composition of the obtained product.
- Other new fermentation media or microorganisms will be attempted to optimize the bioprocess for biosurfactant production.

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