Bioremediation of water contaminated with motor oil by biological surfactants produced by Streptococcus thermophilus, using cheese whey as a carbon source

Ariana Chumi-Pasato[1], *Mary Rueda-Vinces*[2], Giovanni Larriva[3], and Verónica Pinos-Vélez[4]

***Abstract* — The hydrocarbons that contaminate water are difficult to remove, among other things, due to their hydrophobic nature. A surfactant is one way to facilitate contact between the treatment agents. This research prepared a biological surfactant from whey fermentation through Streptococcus thermophilus bacteria. To optimize its production, a complete factorial design was carried out, varying the factors temperature (38, 40, and 42 °C) and time (24, 48, and 72 hours), and the response variable is the amount of surfactant produced. It was found that the highest performance was obtained at 40ºC and 48 hours. The biosurfactant was characterized to determine hemolytic activity, Parafilm, oil dispersion, emulsification index (63.64%), and surface tension (52.7 mN/m). The ecotoxicity test with Daphnia magna confirmed that the biosurfactant is environmentally friendly. Finally, a bioremediation process was applied during the 45 days when more than 50% engine oil removal was achieved.**

***Keywords:* biosurfactants; Streptococcus thermophilus; bioremediation; cheese way valorization.**

***Resumen* — Los hidrocarburos que contaminan el agua son difíciles de remover entre otras cosas por su naturaleza hidrofóbica. Una forma de facilitar el contacto entre el agente del tratamiento es usando un surfactante. En esta investigación se preparó un surfactante biológico a partir de la fermentación de lactosuero a través de las bacterias Streptococcus thermophilus. Para optimizar su producción se realizó un diseño factorial completo variando los factores la temperatura (38, 40 y 42 °C) y el tiempo (24, 48 y 72 horas) y siendo la variable respuesta la cantidad de surfactante producido. Se encontró que el mayor rendimiento se obtuvo a 40 ºC y 48 horas. Se caracterizó el biosurfactante para determinar actividad hemolítica, Parafilm, dispersión del aceite, índice de emulsificación (63.64%) y tensión superficial (52.7 mN/m). La prueba de ecotoxicidad con Daphnia magna corroboró que el biosurfactantes es amigable con el ambiente. Finalmente, aplicando un proceso de biorremediación durante los 45 días donde se alcanzó más del 50% de remoción de aceite de motor.**

***Palabras Clave:* biosurfactante; Streptococcus thermophilus; biorremediación; valorización del suero de queso.**

I. INTRODUCTION

HYDROCARBONS are a severe pollution problem because they are poorly degradable and contain toxic components. Within the hydrocarbons derived from petroleum are motor oils, whose global production uses approximately 2% of the total refined crude oil, corresponding to a consumption of nearly 38 million tons per year. In other words, 0.42 gallons of engine lubricants are produced [1] for every barrel of crude oil. Used massively, once discarded, they are a potential water contamination source [2], [3].

Used engine lubricating oil contains several chemical compounds such as heavy metals, as well as polynuclear aromatic hydrocarbons, benzene, and sometimes there may be the presence of chlorinated solvents, PCBs, etc., producing a direct effect on human health since they can become carcinogenic [4]. As a result of car washing in washing machines and lubricators, up to one million gallons of fresh water are polluted with oils, which has important effects on the exposed ecosystems [5].

In Ecuador, around 54 million liters of used oil are discarded annually, of which only 70% are for domestic use. The rest, equivalent to approximately 4 million gallons, correspond to the automotive and industrial sectors. Currently, in Cuenca, around 34 thousand gallons of used oils are collected per month from washing machines, mechanics, vulcanizers, and industries, representing 55% [6].

Due to the potential contaminants of motorcycle oil, several legislations have regulated the discharge of oils into the water since poor management of these wastes generates considerable environmental problems [7]. For example, in its official standard NOM-001-SEMARNAT-2021, Mexico established maxi-

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mum permissible limit values for fats and oils of 18 mg/l as a daily average and 15 mg/l as a monthly average [8], [9]. In Directive 75/440/EEC, Spain limits the content of these compounds in the water used for purification, where quality limit values are between 0.05 to 1 mg/l for dissolved or emulsified hydrocarbons and from 0.0002 to 0.001 mg/l. l for polycyclic aromatic hydrocarbons [10]. Within Ecuadorian legislation, Book VI of the TULSMA establishes that the maximum permissible limit for the discharge of compounds such as total petroleum hydrocarbons, oils, and greases are 20 mg/l and 70 mg/l, respectively [11].

Spill treatment methods or discharge to water or soil sources are expensive and/or generate other waste due to the chemicals used, including biological, chemical, physicochemical, thermal, electrical electromagnetic, acoustic, and ultrasonic treatment methods [12]. One of the treatments that turns out to be economical and widely used because it is friendly to the environment is bioremediation, which allows the restoration of contaminated soils or waters, a very interesting biotechnological alternative [13].

Bioremediation is a process that consists of using plants, microorganisms, and enzymes that can be naturally or genetically modified to neutralize chemical and biological contaminants, reducing or eliminating their toxicity for living beings [14]. In addition to being economical, it is environmentally friendly and less invasive than other techniques [15]. For example, effective results have been seen when treating organic and inorganic contaminants with microalgae and plants in wetlands and mangroves that are solubilized in water [16], [17], [18], [19], [20]. However, in the case of hydrophobic contaminants, the efficiency of the treatments is limited by the difficulty involved in contact between the agent that performs the bioremediation and the contaminant [21], [22], [23]. For this reason, in addition to microorganisms, compounds that help increase the efficiency of contaminant removal can be implemented during the bioremediation process, such as biosurfactants, to reduce surface tension, thus allowing the mobilization and reduction of contaminants [24]. Indeed, most of the compounds in hydrocarbons are hydrophobic, making them difficult to degrade by microorganisms; therefore, when using biosurfactants, they allow these compounds to solubilize and increase biodegradation by having greater contact with the contaminant [22], [25], [26].

Some studies demonstrate the effectiveness of using biosurfactants in the bioremediation process. For example, biological surfactants produced by bacterial strains were used together with a consortium of bacteria in water samples contaminated with light crude oil, which was carried out for 90 days and increased the efficiency of the bioremediation process, obtaining 81% remediation [27]. In another study, rhamnolipid and surfactin-type biosurfactants were used together with microorganisms in water contaminated with diesel for 90 days, where the bioremediation efficiency of water and soil contaminated with diesel was improved and had a 94% remediation [28]. Hence, a duo between a biosurfactant and a bioremediation organism is interesting for treating hydrophobic contaminants such as hydrocarbons [21], [26], [28]. An interesting organism for bioremediation is Fungi because they efficiently remove contaminants. The removal mechanism with which they act by adsorption of the contaminant in their biomass or by its use in their metabolism [29], [30].

This research aims to obtain a biosurfactant through the action of *Streptococcus thermophilus* using cheese whey as a carbon source to treat water contaminated with motor oil.

II. METHODOLOGY

2.1 biosurfactant

Activation of microbial strains: The *Streptococcus thermophilus* strain of the CHR HANSEN brand, variety STl–12, was used. The activation temperature was between 37 °C to 45 °C. The strain was activated with a water-serum solution in an 8:2 ratio at an incubation temperature of 37 ºC for 24 hours.

A dilution of up to 10-5 was made for the CFU count, according to the INEN 1529-7:2013 standard. Briefly, culture media were prepared with agar in Petri dishes. Then 0.1 ml of the dilutions of the activated inoculum were placed in each dish, subsequently spreading the inoculum throughout the culture medium. The boxes were sealed with Parafilm and incubated in the oven at 37ºC for 48 h. The number of colonies was counted, then multiplied by the dilution factor and divided by the volume used using the following formula (Eq. 1):

$$\text{UFC/ml} = \frac{\text{colony count} * \text{inverse of dilution}}{\text{Volumen}} \quad (1)$$

- Additionally, the Turbidimeter equipment was used with each dilution to corroborate the results. The higher the dilution factor, the lower the turbidity value. Density, protein, lactose, and fat were determined. The Milkotester equipment (Master Pro model) and a potentiometer were used to measure the pH.
- Cheese Whey: The liquid was sterilized at 121 °C for 45 minutes. After the time was up, a filtering process separated the proteins and fats. A 100 ml aliquot of whey was taken, and the pH was adjusted to 6.8 with 1N NaOH.

Biosurfactant production: A 10% aliquot of activated inoculum was added to the sterilized whey. The fermentation process was carried out for 72 hours at 37 °C. After fermentation, the culture broth was centrifuged at 4000 rpm for 30 minutes. The precipitate was removed. The biosurfactant extraction was done using the acid precipitation method with 96% ethanol. 100 ml culture was taken as a sample; it was acidified with 5 N H_2SO_4 to obtain a pH of 2. An equal volume of ethanol was added to the sample and shaken vigorously until the components were completely mixed. This procedure was modified based on the methodology described in Santos (2017) [31]. After 8 h of rest, the appearance of a white precipitate indicated the presence of the biosurfactant. After that, the sample was separated by centrifugation at 4000 rpm for 10 minutes, leaving the white precipitate, which was washed twice with distilled water and then evaporated for 12 hours in the oven at 45 °C.

Optimization: To optimize production conditions, a complete factorial design with three levels and two factors was carried out, with the response variable being the amount of surfactant obtained. Three replicas of each experiment were carried out. The main effects of the factor's temperature (38, 40, 42 ºC) and

time (24, 48, and 72 h) and their combination were evaluated by ANOVA statistical analysis using the R software version 4.4.0 with the R Studio interface.

2.2 Biosurfactant characterization

Hemolytic Activity was determined to check the presence of the biosurfactant through the breakdown of red blood cells. A colony of Streptococcus thermophilus strains inoculated on Blood Agar plates and incubated at 37 °C for 48-72 hours was obtained [31]. Those strains that presented a clear area around the colonies were considered positive. The presence of a diffuse green area represents a behavior α- hemolytic; if it is a clear area, it is considered β- hemolytic, and finally, if it does not present a change around the colony, it is a behavior γ- hemolytic.

After the fermentation period, the resulting liquid was subjected to a double centrifugation process for 30 minutes to detect the biosurfactant. The precipitate was discarded, and the supernatant was placed in the refrigerator for use in the different biosurfactant detection tests.

For the oil dispersion test (OSM), a modified method of Alkan et al. (2019) [32]. 25 ml of distilled water was placed in a 9 cm diameter Petri dish, and 10 uL of used motor oil was added to the center of the plate; subsequently, 20 uL of the product obtained after fermentation was added to half of the oil. The diameter of the transparent area was measured in centimeters. A theoretical comparison was performed with the diameters of the Tween 80 zone as a positive control sample. The diameter of the transparent zone (cm) was evaluated as "+" for 0.5–0.9 cm, "++" for 1–1.5 cm, "+++" for 1.5–2.1 cm and "++++" for 2.1 cm [31].

For the Parafilm test, 2 ml of the supernatant was taken, and a drop of methylene blue was added. Then, 10 µl of the mixture was taken and placed on a piece of Parafilm, making sure to leave a drop without stirring it. The appearance of the drop was examined 1 minute after being placed on the Parafilm. If the drop maintained its shape, it was estimated as negative, while if it took a flat or "collapsed" shape, it was valued as positive [31].

The emulsifying activity was measured by adding 5 ml of hexane to 5 ml of the aqueous sample and stirring at high speed in a vortex for 2 minutes. Measurements were carried out 24 hours later. The emulsion index (E24) was determined with the Eq. 2 [33].

$$\%E = \frac{\text{Aemulsion layer height}}{\text{Total height}} * 100 \quad (2)$$

A sample of the fermented liquid containing the biosurfactant was used to determine the surface tension. The test was carried out using the ring method following the NTE INEN 834 standard. The maximum thrust exerted by the fluid on the platinum-iridium ring was measured with a dynamometer. The surface tension was calculated from the diameter of the ring and the measured force [34].

An acute test was applied with Daphnia magna (a cladoceran crustacean) to determine ecotoxicity. The average effective concentration (EC50) against the biosurfactant was calculated to evaluate the strains' sensitivity. Twenty neonates under 24 hours old were exposed to different amounts of biosurfactant: 30, 25, twenty, fifteen, 10, 5, and 0 mg/L. The immobilized neonates were counted at 24 and 48 hours. The EC50 value or concentration was calculated, where 50% of the crustaceans were immobilized [35], [36].

2.3 Bioremediation of water contaminated with motor oil

The analyzed samples were prepared from distilled water and used motor oil. Each treatment was prepared by adding the biosurfactant obtained from the strains (10% v/v), the inoculum (10% v/v), and the water contaminated with motor oil for a total volume of 100 ml [27], See table I. The treatments were incubated for 15, 30, and 45 days at 40 °C, with gentle shaking at 40 rpm. In addition, total hydrocarbon analyzes were performed before and after treatment. The results were compared with a standard sample consisting of a mixture of water and motor oil.

TABLE I
DIFFERENT TREATMENTS USED FOR BIOREMEDIATION

Treatment	Water (ml)	Oils (ml)	Biosurfactant (ml)	Inoculum (mil)
Water + Oil (AA)	99	1	-	-
Water + Oil + Inoculum (AAI)	89	1	10	-
Water + Oil + biosurfactant + inoculum (AAIB)	79	1	10	10

Two methods were used to determine the percentage of motor oil removed from water: the gravimetric method based on standard 5520 of the Standard Methods for the Examination of Water and Wastewater and the UV-visible spectroscopy method.

The procedure followed for the gravimetric method consisted of evaporating the water sample with oil from the different proposed treatments in a rotary evaporator and then extracting the oil that remains as a residue with an amount of 10 ml of n-hexane (EMSURE brand), subsequently dry the solvent and weigh. The HTS is calculated with Eq. 3. P1 corresponds to the weight of the empty container in grams, P2 to the weight of the container with the sample residue in grams and V to the sample volume initially measured in ml.

$$\text{HTP(mg/L)} = \frac{(P2-P1) * 1000000}{V} \quad (3)$$

The concentration of hydrocarbons was carried out by UV-visible spectrophotometer (THERMO SCIENTIFIC model GENESYS 180). First, a calibration curve was obtained by measuring the absorbance of a series of solutions of known concentrations, with n-hexane as a standard substance, and worked in a wavelength range between 200 to 400 nm in the case of hydrocarbons [37]. Subsequently, the samples were evaporated in the rotary evaporator and extracted with n-hexane to obtain the concentration of the used motor oil sample.

III. RESULTS AND DISCUSSION

3.1 Obtaining the biosurfactant

After cultivation, ovoid-shaped *Streptococcus thermophilus* bacteria were obtained, and the majority were grouped, forming elongated chains, which developed into pairs known as diplococci [38]; these can be seen in Figure 1a. In the colony count with the dilution of 10^{-4} after 48 hours, $UFC/ml = 1.34\ x\ 10^{7}$

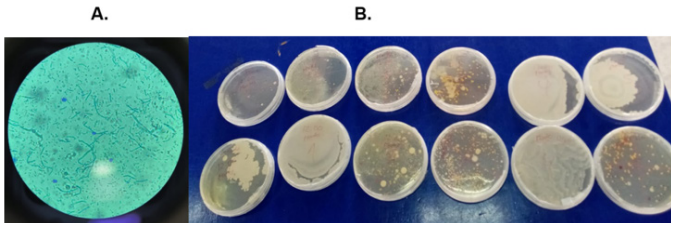

Fig. 1. a. Structure of the studied strain observed under a microscope at 100x. b. Colony count of Streptococcus thermophilus in Petri dish.

Figure 1b shows the growth of Streptococcus thermophilus colonies in the Petri dishes, in which whitish circles of different sizes correspond to the colonies of the bacteria studied. The culture medium used was enriched Agar, so the count values are lower compared to other studies in which M-17 Agar or MRS medium were used [38], [39]. Its characteristic white color was visualized with diameters less than 3 mm.

From measuring the parameters of the fresh cheese whey with the Milkotester equipment, values of 0% fat, 3% protein, 4.5% lactose, and a 1.019 g/cm3 density were obtained. The serum, before being sterilized, presented microorganisms such as Bacillus. Most of the values obtained are close to those reported for other serums, which are between 0.85-1.25% for proteins, 0.94-5.2% for lactose, 0.25-0.7% for fat, 6.45-6.66 for pH, 1.025-1.027 g/cm^3 for density, 3.71% lactose content and 6-7.3% for total solids of sweet whey obtained from homemade cheese production [40]. Only the percentages of proteins and fats differ considerably, presumably due to a poor process in obtaining the cheese.

According to the Ecuadorian standard INEN 2594, which corresponds to the requirements of liquid whey, values of the physicochemical parameters for sweet whey are established of 5% lactose content, 0.8% milk protein, 0.3% milk fat, 6.4-6.8 pH [41]. The measured pH of the serum studied was 6.4, classifying the serum as sweet and being within the established ranges. The fat percentage of the studied whey reflects a lower value than that referred to in national regulations (0.3%), which can be explained by the long resting time of the whey, where the fats form a cream on the surface that is then removed, causing that whey contains little fat. The amount of milk protein is above the norm value (0.8%); this phenomenon is due to the early cutting of the curd, which, being soft, causes premature clot breaks, causing the release of protein and other components in the whey. The amount of lactose present in the study whey was 4.5%, a value close to other studies that reached around 5%, which turned out to be an excellent culture medium for the growth of *Streptococcus thermophilus*, the main source of energy for the bacteria.

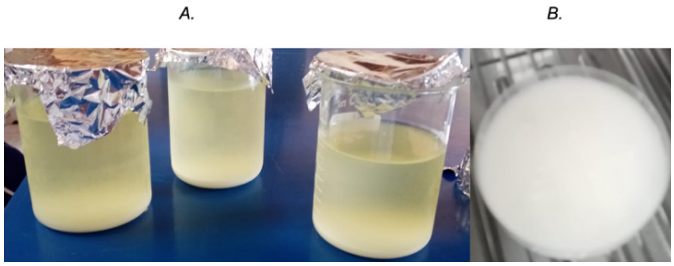

Fig. 2. a. Drying of the biosurfactant. b. Biosurfactant observed under a microscope.

The presence of biosurfactants was verified with quantitative tests such as surface tension and emulsification index. The biosurfactant concentration was 1 g/L, a value similar to that obtained in other studies, where values were between 0.8 and 1.2 g/L [42]. The resulting white powder was observed under a microscope, giving crystalline structures, as shown in Figure 2B.

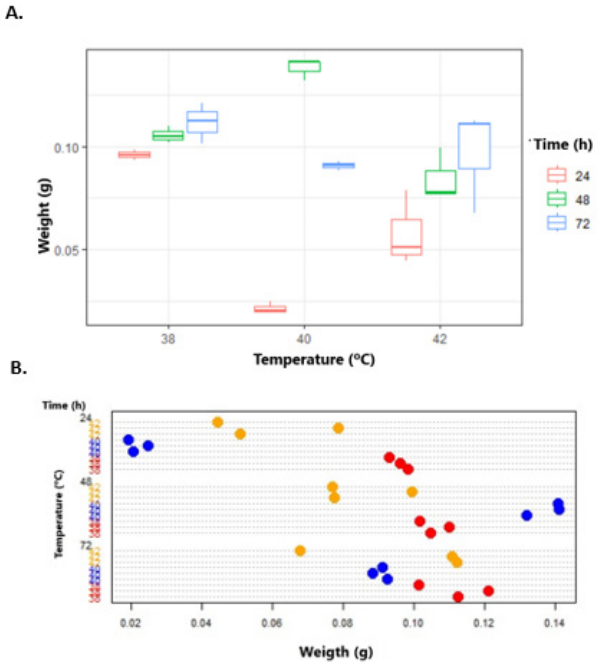

Fig. 3. a. Box plot of the interaction temperature: time vs. Weight. b. Time/temperature plot concerning weight.

For optimization, tests were carried out at different temperatures and times. The weights obtained are shown in Fig. 3, which correspond to the dry whitish residue of surfactant. In Figure 4, you can see the dot scatter plot of the interaction of temperature and time concerning weight. Each color represents a temperature tested (red for 38 °C, blue for 40 °C, and yellow for 42 °C) and the weights obtained. It is observed that the best surfactant production was found at a longer time and temperature. Studies indicate that the influence of time is fundamental for production because bacteria sometimes require more time to process the nutrients present in the carbon source to produce the desired metabolite [31]. It also depends on the microorganisms since each type has its metabolism.

It is important to determine the production time and temperature since it was shown that they influence the concentration of the biosurfactant; during the treatment, the bacteria can run out of the substrate and begin to feed on what they produce, affecting the final amount obtained from the biological surfactant [43], [44]. The optimal time and temperature choice was also based on reducing costs and production times and obtaining a high biosurfactant concentration. Furthermore, the growth temperature range of the chosen bacteria is between 37 °C and 45 °C. The highest yield was a weight of 0.14 g for the conditions of 40 °C for 48 h.

Once homoscedasticity and normality were confirmed through two-way ANOVA statistical analysis, it was confirmed that the factors time p($8.41e^{-08}$) and temperature p(0.00076) and their interaction p($4.63e^{-06}$) were statistically significant. As shown in Figure 3, the temperature and time in which a greater average weight (0.14 g) was obtained were 40 °C and 48 hours, respectively. Therefore, these values have been chosen as the optimal ones, which give better performance in producing biological surfactants. This is also evident when comparing the means of the different combinations of the factors. The total amount obtained from the optimized biosurfactant was 5.6 g/L, which, when compared with the value of the unoptimized biosurfactant (1 g/L), can be said to have increased the amount produced.

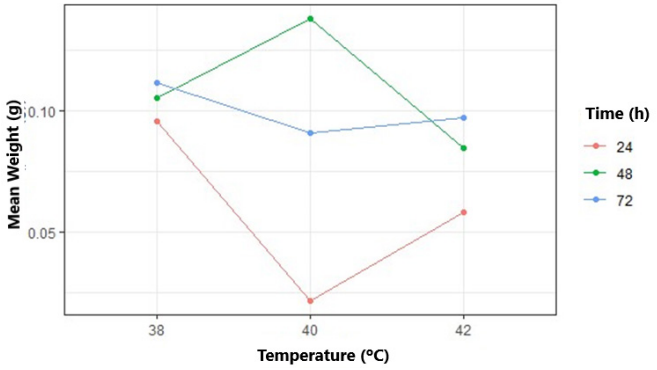

Fig. 4. Factor interaction graph.

The interaction graph shows that the highest surfactant production was obtained at 40 ºC, at 48 h. Also, it can see the interactions between time and temperature.

3.2 Biosurfactant characterization

After 48 hours of incubation at 37 °C, whitish growth areas corresponding to Streptococcus thermophilus were observed. The test was considered positive due to a transparent halo around the colonies generated by the lysis of red blood cells, indicating a decrease in surface tension caused by the secretion of biosurfactants. The light area represents that the bacteria strains studied have a high potential for biosurfactant production [31], and the larger the halo diameter, the greater the concentration of the biosurfactant that is produced [45]. In the case of the strains studied, the observed halo was considered to have β hemolytic behavior since the hemolysis was total or complete due to the presence of a transparent halo.

The hemolytic activity test is performed to verify the absence of production of hemolysins without surfactant properties [46]. It is important to perform these tests to identify false positives, as in one study, the strains showed hemolytic activity, had negative results in the other tests performed (droplet collapse and oil dispersion), and a low reduction in surface tension (greater than 60 mN/m), due to the presence of compounds other than biosurfactants that caused the lysis of red blood cells.

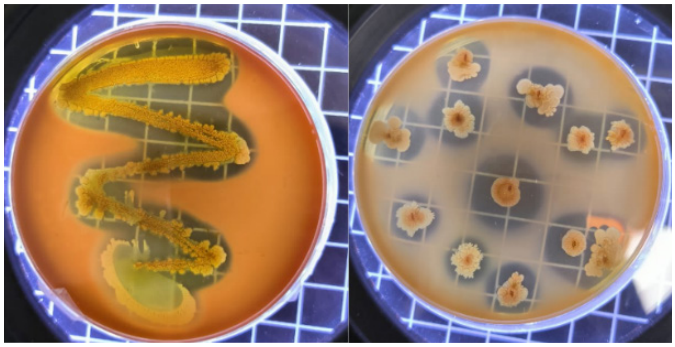

Fig. 5. Hemolytic activity test.

Figure 5 shows the results of the hemolysis test, where the transparent halos formed due to the red blood cell membrane rupture caused by the biosurfactants are observed. The seeding was done by puncture and streaking; the halo was formed similarly in both cases.

The oil dispersion test resulted in a halo diameter of 6.5 cm (++++), confirming the presence of biosurfactant in the sample. The results were compared with those obtained in another study where crude oil was used for the test with a diameter of 5.9 cm for the biosurfactant obtained from lactic acid bacteria [32]. It can be estimated that neither value differed much. In addition, Tween 80 was taken as a positive control sample, this being a chemical surfactant. This test, in addition to helping confirm the presence of the biosurfactant, also demonstrated its ability to break surface tension. There is a linear relationship between the concentration of the biosurfactant and the diameter obtained in the oil dispersion, where the larger the diameter, the higher the concentration, and in turn, it is inversely proportional concerning the surface tension, where the larger the diameter, the lower the surface tension [47].

The Parafilm test studies change in surface tension through the behavior of a drop on a hydrophobic surface [31]. After the estimated time of 1 minute, the shape of the drop placed on the Parafilm was verified. The test was considered positive when a collapse of the placed drop was observed. Additionally, a drop of distilled water was placed on the Parafilm as a blank and was observed to maintain its round shape.

As in the oil dispersion test, there is also a relationship with the concentration of the biosurfactant, where a total and immediate drop collapse will result in a higher product concentration. It may also be due to the carbon source used for production. If fats or oils are used, due to their hydrophobic nature, it is more difficult for bacteria to obtain their nutrients than when sugars are used as a carbon source. Therefore, the concentration of the biological surfactant will vary [31]. After

24 hours, the height of the emulsion was measured, obtaining a value of 2.8 cm out of a total height of 4.4 cm. The emulsification index at 24 h was 63.64%, and it was observed that after a while, the emulsions remained stable since their height did not vary significantly. These results are similar to those of another study where a%E was obtained. 50% at 24 hours and remained stable a week later [32]. A positive test is the presence of emulsion and an emulsification index greater than 40% that remains stable over time. In addition, it was reported that the emulsion is formed depending on the carbon source, as is the case with glucose and acetate of sodium as carbon sources in the production of the biosurfactant, which generated a high positive response when carrying out this test, contrary to what happens with other sources such as diesel where the emulsion was not formed [31].

Research with different microorganisms in different carbon sources found that both variables are relevant when performing the test since it is easier for one strain to adapt to a specific medium than another. Regarding the emulsification index, an investigation reported a higher value for glucose and oil for the *Ralstonia taiwanensis* strain. In contrast, the Pseudomonas veronii strain had a low index in the same substrates. Another investigation that used a synthetic substrate for the strain of Streptococcus thermophilus obtained a lower%E than when using lactic whey, repeating the same with other strains of lactic acid bacteria where the index increased when using whey as a carbon source [32].

The surface tension test result showed a surface tension value of 52.7 mN/m, compared with other studies, which obtained a similar result of 48.85 mN/m [32], using cheese whey as a carbon source and *Streptococcus thermophilus* as a biosurfactant-producing bacteria. Taking pure water as a reference as a control value of 72 mN/m, there is a considerable reduction in surface tension, thus confirming the presence of biosurfactants.

Other studies that used different types of bacteria, obtained surface tension values of between 42 to 71 mN/m, using oil and glucose as culture medium. Likewise, in another study, bacteria and yeast were used to produce a biological surfactant, which managed to reduce the surface tension from 72 to 36 mN/m for the bacteria and 42 mN/m for the yeast [46], [48].

A selection criterion for biosurfactant-producing strains is surface tension values less than 40 mN/m; likewise, higher values are considered emulsifiers [31], [49]. Strains that show values greater than 60 mN/m of surface tension are not considered producers of biological surfactants since there is an inversely proportional relationship between the concentration of the biosurfactant and the surface tension; as long as there is a high reduction in surface tension, the greater will be your concentration [47]. It should be considered that there is an important relationship between the reduction of surface tension with the carbon source, having significant variances between one medium and another, regardless of the strains used, and different carbon sources exert different effects on the production of the biosurfactant [31].

In the different treatments in water for the removal of the oil with the biosurfactant, a concentration of 30 mg/L was used, which is equivalent to 10% of the solution; at the beginning of the study, the test was carried out at that concentration, resulting in the death of 7 neonates of the 20 placed, at 48 hours. But then, by continuing with the standard for the test, they were carried out at different concentrations of the biosurfactant to find out EC50 value. When carrying out the test, it was observed that after 24 hours of having placed the *Daphnia magna* in the solutions with the biosurfactant at a lower concentration, there is low mortality. From 10 mg/L, the mortality increases until all of them die at 48 hours. The EC50 value (mean effective concentration) can be seen in Figure 6, which shows that at a concentration of 14.366 mg/L, p($8.653e^{-10}$), 50% of the neonates die, obtained from the statistical analysis where the value is significant, having a reliability of 97%, and it can be said that the biosurfactant has a low toxicity. Other studies obtained an EC50 of between 3 and 120 mg/l in the evaluation of the toxicity of anionic chemical surfactants, which are generally used in detergents, tested after 24 hours with *Daphnia magna*, demonstrating a low to intermediate toxicity[50]. Although these values do not represent the toxicity of a biological surfactant, they can be used as a reference and allow comparison of both types since they are marketed and widely used.

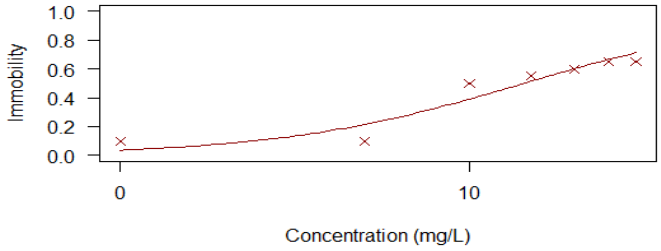

Fig. 6. Biosurfactant concentration concerning immobility rate.

3.3 Application in water bioremediation

The treatments were exposed to different conditions, such as placing them in an incubator with constant shaking at 40 °C and using a bacteria-fungus consortium, in which it was proposed to use the fungus Aspergillus niger. Bioremediation with fungus relies on improved detoxification and degradation of toxic pollutants through intracellular accumulation or enzymatic transformation to less toxic or nontoxic compounds [21], [23].

The different treatments showed a considerable reduction of oil in the water during the incubation time, except the treatment that contained only water with used motor oil, which had a 5% reduction due to losses in the container when performing other tests. Other studies reported a 7% reduction in the standard sample because the water they used was not sterile [27].

The different treatments had an initial concentration of total petroleum hydrocarbons of 8780 mg/L. After the 45 days proposed in the study, final concentrations were between 5348 and 3012 mg/L. The treatment with a higher percentage of removal was that of water, oil, consortium of microorganisms, and biosurfactant (AAIB), achieving 57.68% oil removal. In comparison, the treatment with a lower percentage of bioremediation was the control sample of water and oil, having a 5% reduction. These results were compared with studies that reported removal of 50% in the first 30 days and 81% at 90 days; the treatment with biosurfactant and mixed culture had the highest removal [27]. These results are also compared with

another study, where a high percentage of hydrocarbon removal was obtained (92%) in the third week of experimentation, considering the hydrocarbon concentrations were lower (38.30 mg/l). Likewise, in another investigation, a 39.4% removal of heavy crude oil was obtained for 100 days, where natural mineral nutrients were combined with rhamnolipid biosurfactants. With these values, it is possible to analyze what influences the removal percentage, such as the type of contaminant, the concentration, and the days the treatment lasts [51].

The better removal obtained with the combination of biosurfactant with bioremediation with fungi is because the biosurfactant makes the contaminant available for use by the fungus. This is demonstrated when comparing treatments with (AAIB) or without (AAI) biosurfactants. In all cases, the use of biosurfactants favors bioremediation.

Table 2 shows the results of the weights of the different treatments from day 0 to 45 days.

TABLE II
RESULTS OF THE DIFFERENT TREATMENTS USED FOR BIOREMEDIATION

Days	Micro-organisms	AAI(g)	HTP (mg/L)	% Removal	AAIB (g)	HTP (mg/L	% Removal
0	Bacteria	0.878	8780	0	0.878	8780	0
15		0.6265	6265	25.15	0.6453	6453	23.27
30		0.6009	6009	27.71	0.524	5240	35.4
45		0.5348	5348	34.32	0.4182	4182	45.98
0	Consortium: Bacteria-Fungus	0.878	8780	0	0.878	8780	0
15		0.6107	6107	26.73	0.5202	5502	32.78
30		0.583	5830	29.5	0.4251	4251	45.29
0		0.5075	5075	37.5	0.3012	3012	57.68

Note. AAI: Water, oil and inoculum. AAIB: Water, oil, inoculum, and biosurfactant

The results open the way for this treatment to be used on an industrial scale. Cheese whey is a frequent waste in the local industry. This would justify implementing a system to obtain biosurfactants, which can be used in biological systems such as water treatment plants. Additionally, it can be used in combination with bioremediation to eliminate hydrophobic contaminants such as car wash water or other aqueous waste contaminated with hydrocarbons.

Lubricating oils, in addition to containing hydrocarbons in their composition, also contain other contaminants such as heavy metals, chlorinated solvents, and residual engine dirt, making them difficult to degrade. That said, with the results obtained in this research, it can be concluded that using the biosurfactant and the bacterial consortium removes the hydrocarbon and other types of contaminants in the sample [52]. For example, there is a study where biosurfactants produced by P. aeruginosa were used to remove lead and mercury in marine intertidal sediments, resulting in 62% and 50% of Pb and Hg, respectively. The ionic character of surfactants provides for their use in removing heavy metals due to the affinity of the cations for negatively charged surfactants [53].

CONCLUSIONS

Whey is the main byproduct of the dairy industry and due to its high level of nutrients, it has a high contaminant load and, far from being used, is disposed of in water sources or sewage systems without prior treatment. That said, using whey as a carbon source made it possible to obtain a biological surfactant through whey fermentation by the action of *Streptococcus thermophilus* bacteria. Its extraction was obtained using the acid precipitation method, which was modified since, from the beginning, the solvents used were methanol and chloroform; their risk and cost are known; it was decided to replace them with 96% ethanol, obtaining similar results.

It was demonstrated that time, temperature, and their interaction play a fundamental role in the production of the biosurfactant, where it was evident that at a temperature of 40 ºC and 48 hours of fermentation, the ideal conditions are achieved where *Streptococcus thermophilus* produces a greater amount of biological surfactant improving process performance.

The biosurfactant could be identified thanks to the different qualitative and quantitative tests, thus confirming the product's presence. Furthermore, the ecotoxicity tests and results concluded that the biological surfactant is not dangerous to aquatic fauna if used in water bodies.

The efficiency of the biosurfactants was evaluated in the different treatments carried out for the bioremediation process. The product obtained and the microorganisms that degraded the oil in the contaminated water had a favorable result. Given that the oil is a complex sample, it is recommended that the bioremediation time be increased to improve the biodegradation of contaminating compounds.

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