Journal Homepage: -www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI:10.21474/IJAR01/18919
DOI URL: http://dx.doi.org/10.21474/IJAR01/18919

RESEARCH ARTICLE

REMEDIATION OF SOILS CONTAMINATED WITH HEAVY FRACTION HYDROCARBONS USING BIOSURFACTANTPRODUCED BYPSEUDOMONAS SP.

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Manuscript Info

Manuscript History
Received: 18 April 2024
Final Accepted: 23 May 2024
Published: June 2024

Abstract

This study evaluated the effectiveness of the biosurfactant generated by a strain of *Pseudomonas sp.* in the removal of heavy fraction hydrocarbons from a soil contaminated with 28,000 mg/kg, by a washing process. The efficacy of the biosurfactant was compared to that of a chemical surfactant, used at a concentration of 0.1%. After five wash cycles, the biosurfactant reached a maximum removal efficiency of 42.71%, in contrast to that of the chemical surfactant, 32.31%. In addition, bioaugmentation with the culture medium used for the production of the biosurfactant was evaluated, with a removal of 38.29% of these hydrocarbons in 90 days.

Introduction:-

Crude oil and its derivatives maintain significant global interest due to their high energy supply, and their demand continues paired to industrial and population growth (Miller and Sorrell, 2014, IEA, 2023).Exposure to these compounds carries health risks, including lung irritation, shortness of breath, and neurologic problems, and cancer risk (Kuppusamy *et al.*, 2020).Spillsrelated to hydrocarbonsmishandling cause soil contamination that affects its composition,its mechanical, physical, and biochemical properties, and the capacity for self-restoration by microbial degradation (Bazaz and Bazaz, 2021,Mitter *et al.*, 2021). In addition, the hydrophobic properties of hydrocarbons and soil capillarity intensify their adsorption and promote their persistence in this medium (Nasehi *et al.*, 2016).

Both physicochemical and biological methods are commonly used in the remediation of soils contaminated with hydrocarbons. Recently, soil washing has gained importance, especially applying surfactants and biosurfactants. Although chemical surfactants are efficient, their low biodegradability and toxicity raise environmental concerns (Badmus *et al.*, 2021). In contrast, biosurfactants offer an attractive alternative due to their biodegradability (Lima *etal.*, 2010).

This work focused on the production of a biosurfactant and the evaluation of its efficiency in the removal of heavy fraction hydrocarbons (HFH) from a contaminated industrial soil, based on the maximum concentration in industrial soils, 6,000 mg HFH per soil kg, set by the Mexican standard (SEMARNAT, 2019), as compared to a chemical surfactant. In addition, the effects of surfactants on soil microbiota were examined and direct bioaugmentation with

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the biosurfactant-producing strains,*Pseudomonas sp.*, was investigated as a possiblealternate bioremediation method.

Methodology:-

Characterization of HFH-contaminated soil

The soil characterization was conducted by examining the following parameters: pH, texture, organic matter, density, cation exchange capacity, microbiota, and initial HFH concentration, Table 1.

Table 1:- Soil characterization.

Parameter	Reference
pH	SEMARNAT, 2002
Texture	Reyes, 1996
Organicmater	Reyes, 1996
Realdensity	SEMARNAT, 2002
Apparentdensity	SEMARNAT, 2002
Cationexchangecapacity (CEC)	Reyes, 1996
Heavy fractionhydrocarbons (HFH)	SEMARNAT, 2013
Initialmicrobiota	Fernández, 2006

Study of soil washing with chemical surfactant

Initially, the removal of HFHs in the soil by washing with Tween 80, a chemical surfactant known for its affordability, and frequently used in remediation work, was studied (Cheng *et al.*, 2017). In a capped glass reactor, 50 g of soil were placed, and 116 mL of a 0.1% surfactant solution were added, aiming tocompletely saturatethe pores of the soil. This process was repeated three times on a rotary shaker at 80 rpm.

Five consecutive wash cycles were conducted, each lasting 60 minutes, with 30-minute intervals between each wash. After each cycle, a 10 g sample of the sedimented solid phase was left to dry for 24 hours to measure the amount of HFH remaining. A corresponding amount of the solution was added to the remaining soil to keep it completely flooded. For the remaining 40, 30, 20, and 10 g of soil after each sampling, 92.8, 69.6, 46.4, and 23.2 mL of solution were added, respectively.

Isolation and propagation of the biosurfactant-producing strain.

The biosurfactant-producing population of hydrocarbonclast bacteria was isolated and subcultured from HFH-contaminated soilby plate dilution in a MERCK-brand specific medium for *Pseudomonas sp.* (110988 Millipore P base). After the total count, a colony was taken and reproduced by plate dilution method to ensure the selection of a single species.

Biosurfactant production and extraction

In afull 4 L bioreactor a Minimal Salt Medium (MSM) (Çakmak *et al*., 2017), was prepared using the following components in g/L: K_2HPO_4 (10.0), NaH_2PO_4 (5.0), $NaNO_3$ (2.0), $MgSO_4{\cdot}7H_2O$ (0.2), $CaCl_2{\cdot}2H_2O$ (0.01), $FeSO_4{\cdot}7H_2O$ (0.08), and 1% v/v of motor oil (hydrocarbon) as the only carbon source. It was inoculated with the isolated strain of *Pseudomonas sp*. and kept in incubation with aeration at 37 C.

After a maturation of six days, the biosurfactant was extracted. Sixteen samples of 9 mL each were centrifuged for 10 min at 4000 rpm. The upper phase was collected in an Erlenmeyer flask and the pH was adjusted to 2. They were kept at 4 C for 12 hours. The samples were gathered in a separation funnel and a liquid-liquid extraction was performed with a 2:1 chloroform-ethanol mixture. The organic phase was recovered, and the solvent evaporated, leaving only the biosurfactant.

Drop displacement test and E_{24} emulsion index.

The drop displacement test was performed as follows (Habib *et al*., 2020): a layer of distilled water and a drop of motor oil were poured into a Petri dish. A drop of the biosurfactant was dripped on the oil to seeits possible dispersion due to the effect on the surface tension in the water, which would confirm the presence of thebiosurfactant.

To calculate the E_{24} emulsion index, the same amount of oil and surfactant were served in a 10 mL test tube, stirred for a few minutes and left to stand for 24 hours; distilled water was usedas a control. After the process, emulsion layers heights and total height were measured to determine the percentage of E_{24} using the following equation (Hajimohammadi *et al.*, 2016):

$$\%E_{24} = \frac{\text{Emulsion height}}{\text{Total height}} \times 100$$

FTIR Analysis

The biosurfactant was characterized by FTIR, with a Perkin Elmer Frontier equipment with ATR, calibrated in an interval of 4000 to 400 cm^{-1}, eight scans were performed. Theresulting spectrum was exported and graphed in Excel for later analysis.

Study of soil washing with biosurfactant

The soil affected with HFHwas washed with the biosurfactant. The same conditions and process used with the chemical surfactant were used. A concentration of 0.1% was kept and five washing cycles were performed, each lasting 60 min,116 mL of the biosurfactant wash solution was used to flood the soil pores to maximum capacity. Soil samples of 10 g were taken after each wash cycle and the aqueous phase was separated for later treatment.

Effects on the microbial population

Samples washed with both surfactants were stored at room temperature and were left to rest for 15 days,then the count of colony-forming units was conducted in each one.

Bioaugmentation

A culture medium inoculated with *Pseudomonas sp.* was added to the contaminated soilto obtain a density of 10^{12} CFU per gram of soil, by irrigation. Sampling was conducted every 15 days to findHFH contentafter a period of 90 days.

Phytotoxicity

After bioaugmentation treatment, a phytotoxicity test was conductedwith radish seeds (*Raphanus sativus*) (Luo *et al.*, 2018, Sivkov and Nikiforov, 2021).In 30 g samples of soil previously treated with chemical surfactant, biosurfactant and clean soil, three scattered radish seeds were buried at a depth of 2 cm and irrigated every 48 hours. Seed growth was checked for 15 days.

Results and Discussion:-

The results of the initial characterization of the soil are summarized in Table 2.

Table2:- Results of initial soilcharacterization.

Parameter	Result	Interpretation
pH	7.36 ± 0.02	Neutral soil[1]
Texture	Sand 31.33% ± 0.5% Clay 1.66% ± 0.5% Silt 67.01 % ± 0.5%	Loamy-silty soil[1]
Organic material	25.58% ± 0.2%	High[1]
Real density	2.4 g/cm^3 ± 0.1	Medium[2]
Apparent density	1.11 g/cm^3 ± 0.1	Medium[2]
CEC	71.33 meq/100 g soil ± 1.2	High[3]
HFH	28,000 mg/kg drysoil	4.6 times the maximum regulated concentration, 6,000 mg/kg[4]
Initialmicrobiota	$1.9x10^8$ CFU	-

Based on: [1](SEMARNAT, 2002), [2](Department of Agriculture, 2019), [3](Reyes, 1996), [4] (SEMARNAT, 2019)

Biosurfactant production

Bacterial growth within the biosurfactant culture medium is shown in Figure 1. In the interval from onset to day six, a latency or lag phase wasseen, followed by the exponential phase until day 21. Then the stationary phase took place, where the population reached a maximum of $2.5x10^9$ CFU.

40 mL of product was obtained for every 250 mL of culture medium.

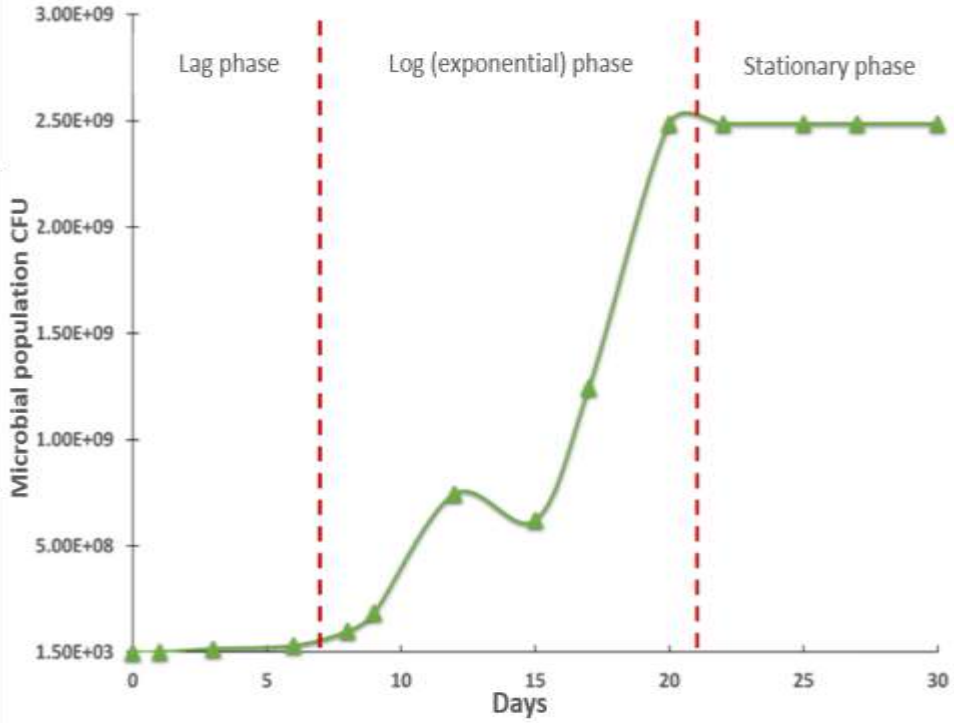

Figure 1:- Growth of the microbial population in the culture medium.

Biosurfactant characterization

Following the droplet dispersion test (Figure 2a), the product was able to disperse oil into the aqueous medium showing the presence of biosurfactant. The reduction in surface and interfacial tension between the oil layer and the water surface is evident (Habib *et al.*, 2020).

a **B**

Figure 2:- Droplet dispersion test and E_{24} index, (a)water-oil-dispersion and(b) biosurfactant-oil.

In the E_{24} index test (Figure 2b), the product formed an emulsion height of 2 cm in a total of 3 cm, resulting in an emulsion index of 66%. In contrast, using the control an emulsion height of 1.5 cm was obtained for a total height of 3 cm, giving an emulsion index of 50%.

The infrared spectrum (Figure 3)shows characteristic bands of the functional groups present in the structure of rhamnolipids. As reported by Sharma *et al.*, (2019), the band of 3432.06 cm^{-1} is associated with the stretching of O-H bonds, while at the wavelength of 2931.24 cm^{-1} are found the C-H bonds, indicating the presence of long carbon chains with strong absorbance. At the wavelength of 2848.76 cm^{-1}, there are stretches corresponding to the C=C-H bonds, possibly present within the structure of the rhamnose. The 1746.84 cm^{-1} and 1643.12 cm^{-1}bands are associated with the stretches of the -COOH bonds. The interval between 1500cm^{-1} and 1000 cm^{-1} is related to deformations in the structure of carbohydrates present in rhamnolipids. Other authors, such as Ratna and Kumar,

2022, Pathania and Jana, 2020, and Eraqi *et al*., 2016, confirmed the obtention of rhamnolipids produced by microorganisms isolated and reproduced in the culture medium.

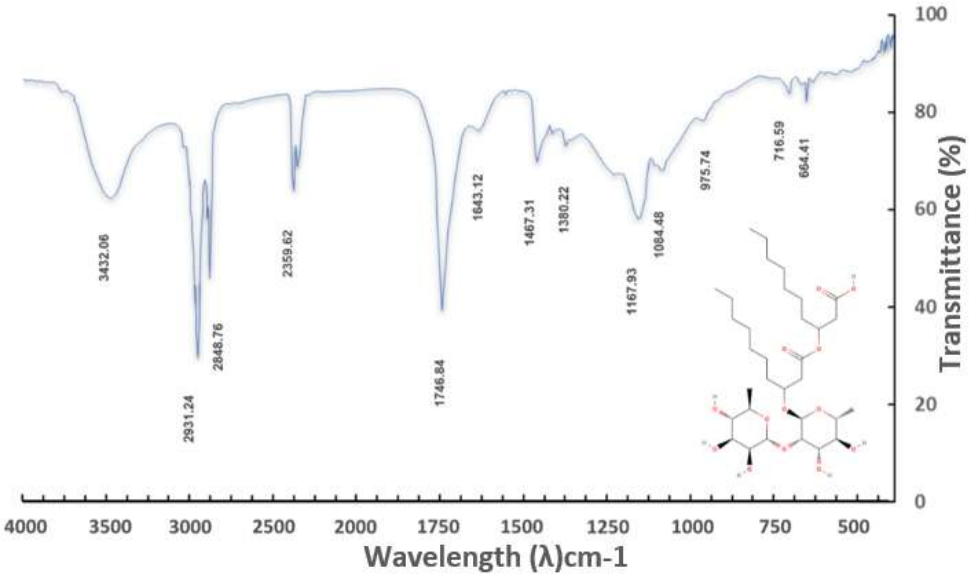

Figure 3:- FTIR spectrum of the extracted biosurfactant.

Soil washing using Tween 80 and biosurfactant.

There was an equivalence in both Tween 80 surfactantand the biosurfactantin the first three washing cycles, with identical maximums (Figure 4). The first three wash cycles presented an average removal of 13%, 19%, and 24%, respectively, for both surfactants. After cycle 3, a pronounced trend towards greater removal corresponding to the biosurfactant is observed. In cycle 4, the difference in removal is 5%, and in cycle 5, this difference increased to 10% with a tendency to increase in removal in subsequent cycles. When using Tween 80, a removal of 32.31% was obtained, compared to the biosurfactant, with which a removal of 42.71% was obtained, that is, a 10% higher removal. In both cases, five washing cycles were conducted. However, in the control test using water, only 8.79% removal was achieved. In all cases the concentration of pollutants remained above the maximum regulated concentration.

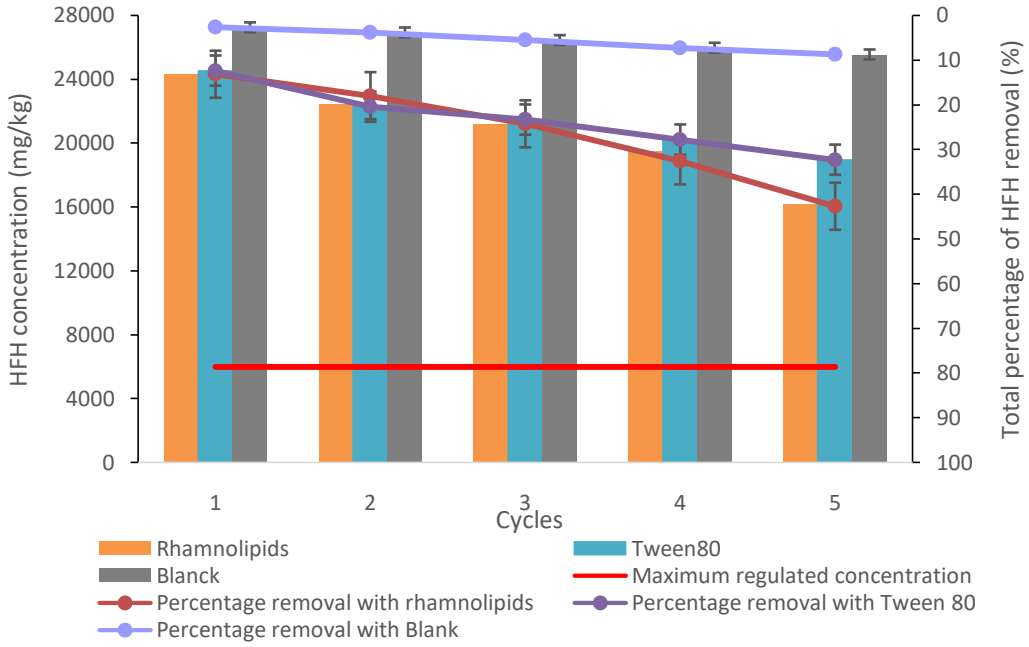

Figure 4:- Comparison of washing with chemical surfactant and biosurfactant.

When using Tween 80, as shown in Figure 5, an average of 14 washing cycles with an average of 1500 mg/kg of removal was estimated to obtain a maximum HFH that was below the maximum regulated concentration for industrial soils, of 6,000 mg/kg. Using the biosurfactant, nine wash cycles would be required if the average removal trend per wash cycle of 2,800 mg/kg is kept (Figure 6). By using fewer washing cycles, the raw material and the amount of energy used would also be reduced.

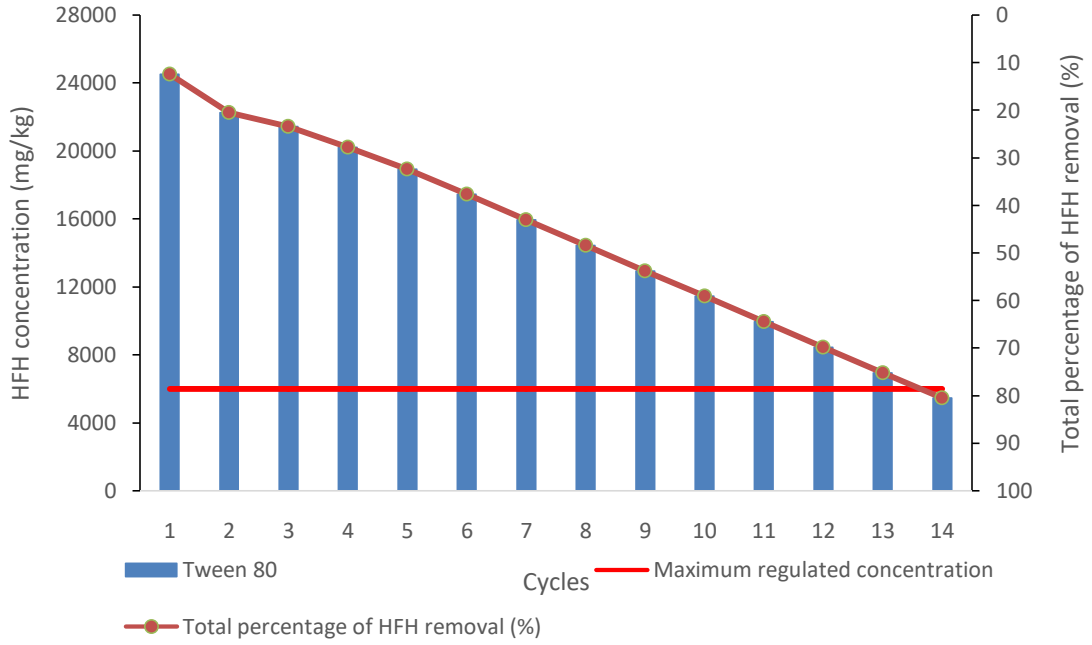

Figure5:- Estimation of washing cycles with Tween 80 to reach the HFHstandard.

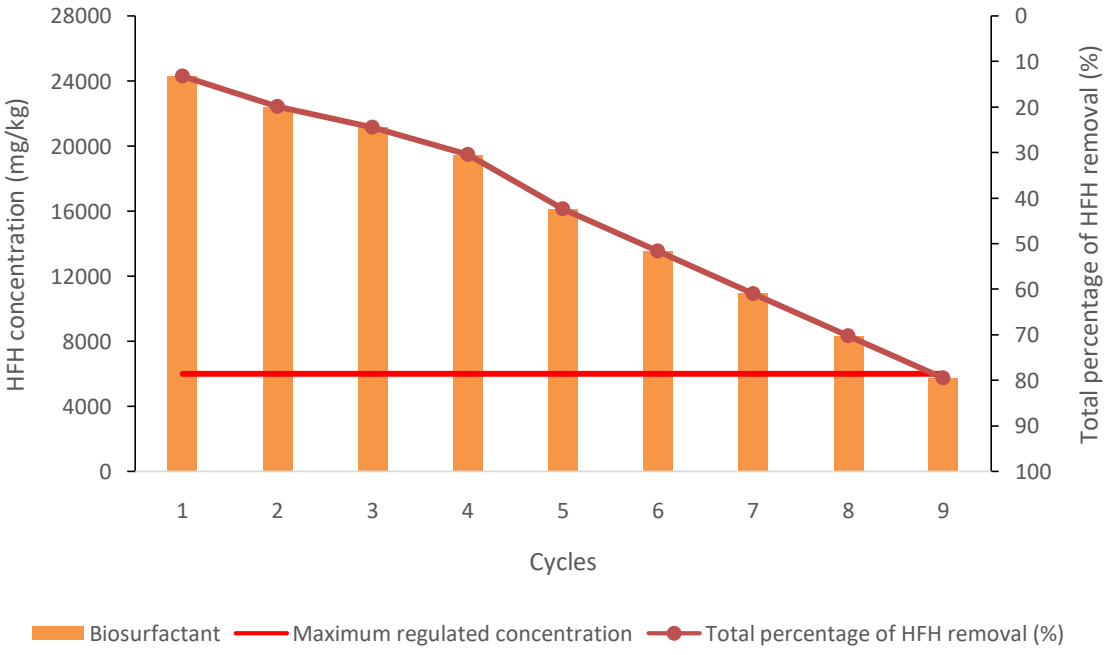

Figure 6:- Estimation of washing cycles with Tween 80 to reach the HFHstandard.

Bacterial population in soil after the washing process

The quantification of soilbacteria after washing treatment with each surfactant is shown in Figure 7. At the end of treatment, the microbiota population decreased by almost 93% with the use of the chemical surfactant. In contrast, the use of biosurfactant notoriously promoted the growth of bacteria in the soil, resulting in a 5.3-fold increase in the initial population of the affected soil.

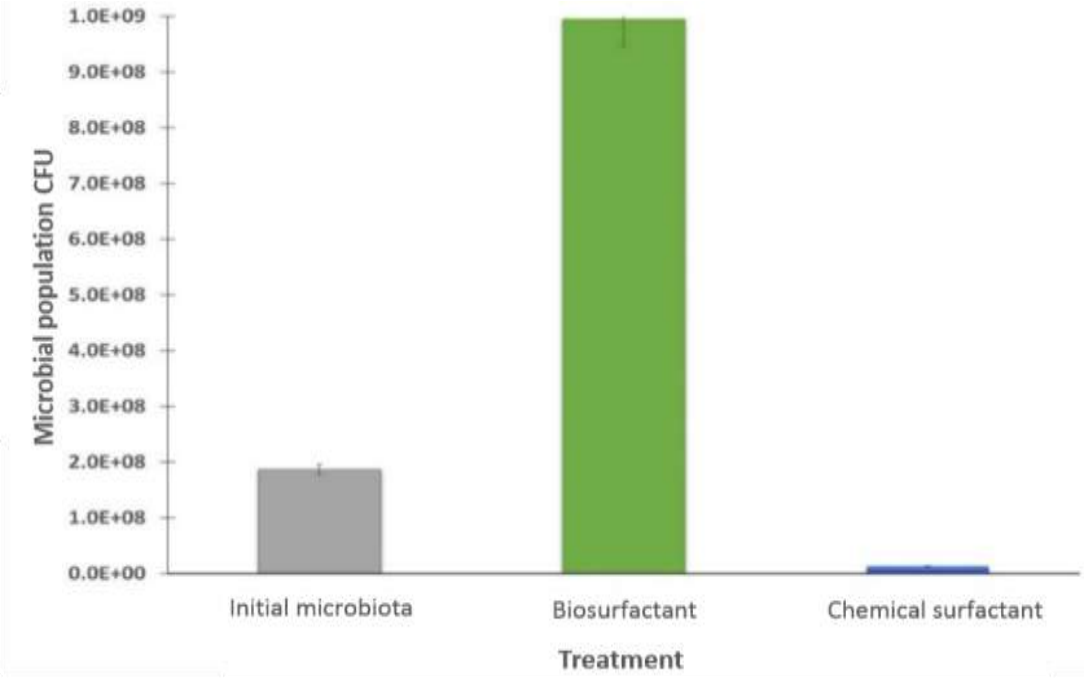

Figure 1.7:- Comparison of bacterial growth after washing processes.

The favorable results obtained with biosurfactant can be attributed to the addition of a culture medium rich in minimal salts, as well as the presence of bacteria in the surfactant (Cisneros *et al.*, 2016). On the other hand, the chemical surfactant is inhibitory of the growth of the native soil microbiota (López *et al*., 2018).

At the end of the washing treatment with both surfactants and after a period of 30 days, the soil concentrations of HFH were obtained and summarized in Table 3. The HFH content in the soil after washing,within the estimated margin of error, remained unaffected in the case of Tween 80. However, when using the biosurfactant it decreased in 1.308 mg/kg. This could be attributed to the enhanced activity of hydrocarbonclast bacteriain the soil (Liao *et al.*, 2016).

Table 3:- HFH concentration after washing with each surfactant.

Surfactant	InitialHFH (mg/kg)	HFHafter washing (mg/kg)	HFH 30 days later (mg/kg)
Tween 80	28,000	18,953	19,231
Biosurfactant	28,000	16,140	14,832

Soilbioaugmentation

The results obtained in the bioaugmentation experiment are shown in Figure 8. After 9 days of treatment, HFHs decreased by 9% and a downward trend remained constant, reaching a maximum degradation of 38.29% after 90 days of treatment. It was seen that degradation increased with increasing numbers of hydrocarbonclast bacteria. The average rate of degradation resulted innear 5.7% over the 90 days, meaning that with this trend, it would take 285 days to reach the maximum regulated concentration of 6,000 mg HFH/ soil kg. In contrast to soil washing, bioaugmentation stands for a significant reduction in economic, energy, and ease terms by using exclusively a growing medium enriched with *Pseudomonas sp*., although it requires a relative long term to achieve the remediation goals.

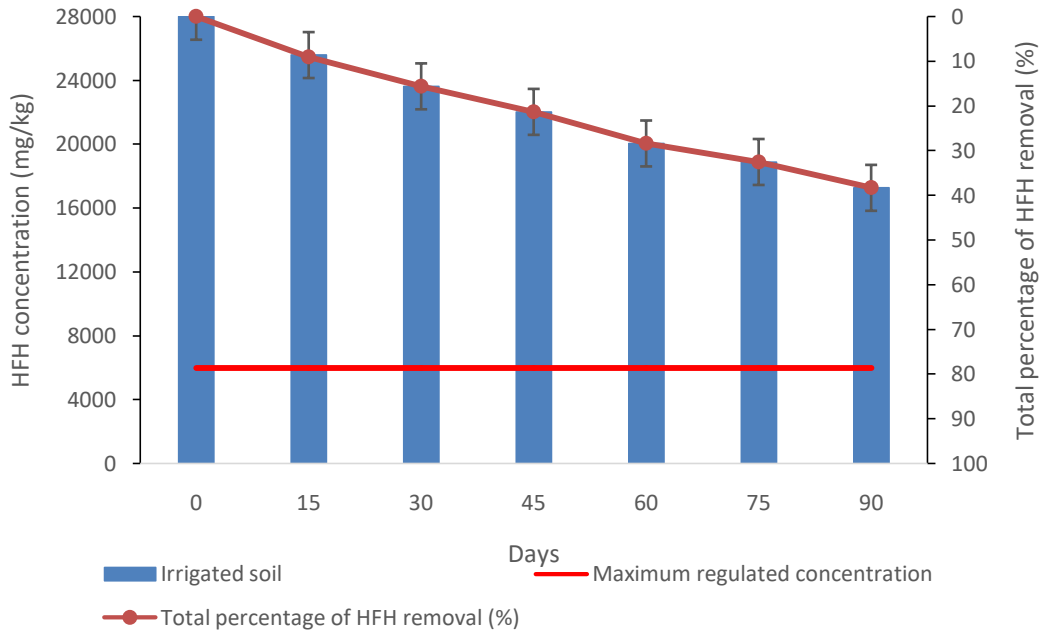

Figure 8:- HFH biodegradation after bioaugmentation with Pseudomonas sp.

Phytotoxicity

After watching the germination and growth of radish seeds, the total height of seedling growth above the soil was recorded to define the effects of the initial and residual contaminant on the soil. The results obtained are presented in Table 4. By germinating the seeds in the various soils, the clean soil resulted in the germination and growth of all the seeds. Germination of two and none seeds was seen in soils washed with biosurfactant and Tween 80, respectively. Despite germinating two seeds in the soil washed with rhamnolipids, the total height of the seedlings only reached 6 cm. This could be explained by the persistent presence of hydrocarbons in the soil and the negative effects they cause. However, the presence of a highconcentration of microorganisms in the soil may have contributed to the germination of the seeds, albeit at a slower rate.

Table 4:- HFH concentration after washing with each surfactant.

Soil	Initialseeds	Sproutedseeds	Total height(cm)
Tween 80	3	0	0
Biosurfactant	3	2	5 and 6
Blank	3	3	8, 6, and 6

Conclusions:-

In this study, the feasibility of isolating and subculturing a native strain of *Pseudomonas sp*., which produces biosurfactant, from soil contaminated with HFH, was proved. The isolated strains showed the ability to produce biosurfactant in a specific culture medium that used hydrocarbon as the sole carbon source. Droplet dispersion and emulsion index E_{24} tests showed that this biosurfactant can change surface tension and create hydrocarbon emulsions.

The FTIR analysis showed a molecule with characteristics identical to rhamnolipids. Experimentation with this biosurfactant in the soil washing method resulted in a 42% decrease in HFHs in five continuous washing cycles, compared to the use of Tween80, a chemical surfactant, with which only a maximum removal of 32% was achieved. However, after five washing cycles, the amount of HFH did not decrease sufficiently to reach the maximum regulated concentration in soils of industrial origin, which is stablished by the current Mexican standard.

The use of the applied biosurfactant promoted the growth of beneficial microorganisms for the soil, while an affectation of the microbiota was seen when using Tween 80, which could alter the properties of the contaminated soil. Also, bioaugmentation with an inoculum of the isolated *Pseudomonas sp.* was proven as an effective technique

to remediate de HFH contaminated soil, although it could take a longer period of time to achieve the desired concentration.

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