Chapter 2

Emulsification of Hydrocarbons Using Biosurfactant Producing Strains Isolated from Contaminated Soil in Puebla, Mexico

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/56143

1. Introduction

Among Mexico's main riches are its oil and the great expanses of land used to grow food. A large number of pipelines pass through Mexico's agricultural region carrying diesel, gasoline or crude oil, however, lack of maintenance of the pipeline installations, fuel theft, vehicle transport and even the topographical, terrain and hydrological conditions of the site cause a high incidence of contamination.

Petrolic activities have generated extensive pollution of soils worldwide, mainly in those regions where petroleum is explored, extracted, and refined. The composition of hydrocarbons on polluted soil varies according to environmental conditions and natural degradation processes. In México there are soil impacted by weathered hydrocarbons, which are predominantly saturated and aromatic, become more recalcitrant if polluted soils are not remediated, affecting the underground water, food chains, and diverse human activities.

Hydrocarbon spills on agricultural soil have direct repercussions on soil quality and its function. Some authors [1] indicate that hydrocarbon contamination reduces food crop growth by preventing water and nutrient absorption through the roots, and reducing the transport of metabolites and respiration rate.

The recovery of hydrocarbon-contaminated agricultural soil in Mexico is a complex theme because the producers harvest the crops for sustenance or sale. A remedy is therefore needed that uses sustainable biological technologies which do not pose a risk for the products of the harvests. The production of biosurfactants to recover agricultural soil used for food production is a viable alternative because of their biodegradability. Furthermore, biosurfactants have been used in the oil industry to recover oils from hydrocarbons, in the emulsification of heavy hydrocarbon fractions and in the degradation of polychlorinated biphenyls [2] and polycyclic aromatic hydrocarbons (PAH's) [3].

2. Approach to the problem

In the agricultural fields of Puebla, Mexico two hydrocarbon spills have been reported due to lack of pipeline maintenance. In 2002, a crude oil spill in the town of Acatzingo, Puebla affected a large expanse of agricultural land (approximately 50 hectares) [4]. And in San Martin Texmelucan, Puebla on December 19, 2010, the explosion caused by a crude oil spill took 30 human lives and greatly affected the agricultural land of the population [5]. The inhabitants of the affected regions still perceive damage to the soil and do not consider the land to be fully recovered [4].

In Mexico, the environmental impact of oil industry activities is rigorously controlled by the authorities (Federal Environmental Protection Agency, *Procuraduría Federal de Protección al Medio Ambiente*, PROFEPA) and therefore recuperation should take only a short time. Bioremediation processes have not given the expected results: expanses of contaminated land are heterogeneous as far as climate, water availability and oxygen availability, and the biostimulation of microbial populations is insufficient due to competing autochthonous microorganisms and inadequate nutritional balance [6, 7].

Mexico relies mainly on micro-encapsulation technology for the restoration of hydrocarbon-contaminated land, according to the National Ecology Institute (*Instituto Nacional de Ecologia*, INE) [6] using chemical substances which encapsulate hydrocarbons and prevent biodegradation. Surfactants have also been used to restore marine sediment with a recovery of 45,000 t [8]. Chemical surfactants, however, are not always environmentally biodegradable [9] and so there is a need to use biosurfactants to recover oil hydrocarbons in impacted soils.

3. Area of application

Biosurfactants are molecules with a polar region and a non-polar region, and are hence considered amphipathic, produced by extracellular or intracellular microorganisms, also can reduce surface tension at the air-water interface between two immiscible liquids or between the solid-water interface [10].

Biosurfactants have other advantages over chemical detergents since they are non-toxic and ecologically acceptable [10]. They are also highly effective at breaking down surface tension

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[11]. Several authors have reported bacterial strains isolated from hydrocarbon-contaminated soil and water which present emulsifying activity and which are capable of growing in oil using it as sole carbon source. The reported microorganisms are: *Pseudomonas aeruginosa*, *P. mendocina*, *P. aureofasciens*, *Listonella damsela*, *Bacillus sphaericus*, *B. brevis*, *Enterobacter cloacae*, *Acinetobacter calcoaceticus* var. *anitratus*, *Hafnia alvei*, *Citrobacter freundii*, *C. amalonaticus*, *Sphingobacterium multivorum*, *Staphylococcus* sp, *Neisseria* sp, *Micrococcus* sp, *Serratia rubidae*, *Alcaligenes*, *Flavobacterium*, *Nocardia*, *Achromobacter*, *Arthrobacter* [12-16]. There has been a recent rise in the study of biosurfactant for their antimicrobial characteristics as fungicide [17, 18] and as, zoospore inhibitors [19].

The use of biosurfactants for the bioremediation of hydrocarbon contaminated soil has been studied intensely since the last decade [2-3, 20]. Biosurfactants have been used by the oil industry to enhanced oil recovery [21, 22], in the emulsification of heavy hydrocarbon fractions [23], and in the treatment of wastewater with insoluble substances. They have also been used in the degradation of polychlorinated biphenyls [2]. Chemical surfactants have the advantage of being non-toxic, environmentally friendly, and biodegradable and can be produced from agricultural substrates [10].

Biosurfactants can be used as additives to stimulate bioremediation; however, the concentration of these can also be increased by the addition of bioemulsifier-producing bacteria. Bioemulsifier-producing bacteria can participate in the biodegradation of hydrocarbons and, alternatively, function as a family of bacteria that supply emulsifiers to another group of bacteria that degrade the contaminants [24].

A mixture of biosurfactants including cellular lipids produced during the degradation of heavy hydrocarbons, and additives increases solubility and facilitates hydrocarbon degradation. Cellular lipids have excellent surfactant properties and can form micelles at low concentrations, but these surfactants do not release the solubilized organic compounds to degrade them [25]. An increase in the apparent solubility of naphthalene has been observed when the concentration of glycolipids excreted by *Pseudomonas areuginosa* 19SJ exceeds the critical micellar concentration (CMC) [26].

Biosurfactants have different chemical compositions depending on the microorganism that produces them and may be lipopeptides, lipoproteins, fatty acids or phospholipids [27]. The production of biosurfactants depends on physicochemical factors (aeration, pH, substrate availability) and their evaluation will depend on kinetic factors (substrate consumption, product formation, and biomass production). Knowing the kinetics of biosurfactant production will allow the proposal of sustainable oil hydrocarbon recovery technologies for aqueous or solid systems.

Mexico has large areas of soil contaminated by oil activities; especially agricultural soils have few alternatives of sustainable technologies, therefore in this work different microorganisms were isolated from hydrocarbons-contaminated soil and the kinetics of biosurfactant production was studied to generate a proposal for the recovery of oil hydrocarbons as Maya crude oil.

4. Materials and methods

4.1. Isolation of biosurfactant-producing strains

Soil sampling was done in an agricultural area of Acatzingo, Puebla, Mexico with the following geographical coordinates 18° 57' 03.0" N 97° 46' 20.5" W. Biosurfactant-producing strains were isolated using 1 g of soil in 10 mL of pre-sterilized distilled water. The culture medium was composed of (g / L): $(NH_4)_2SO_4$ 7.7, KH_2PO_4 5.7, K_2HPO_4 2, $MgSO_47H_2O$ 2, $CaCl_22H_2O$ 0.005, $FeCl_36H_2O$ 0.0025, agar 15; distilled water 1,000 mL and preadapted to a petroleum environment using the Maya petroleum provided by the Mexican State company (PEMEX). Maya petroleum was added on sterilized filter paper (3 cm^2; with 2 g petroleum) to every lid in order to develop an atmosphere of volatile hydrocarbons inside the petri dish.

The bacteria were then isolated and grown in a liquid mineral medium (g / L): $(NH_4)_2SO_4$ 7, KH_2PO_4 5.7, K_2HPO_4 2, $MgSO_47H_2O$ 2, $CaCl_22H_2O$ 0.005, $FeCl_36H_2O$ 0.0025, Yeast extract 0.1, glucose 20. Strains presenting biosurfactant production were identified as UPAEP 6, UPAEP 8, UPAEP 9, UPAEP 10, UPAEP 12 and UPAEP 15. The following bacteria were also bought *Arthrobacter* sp ATCC 31012, *Bacillus subtilis* ATCC 21332, *Candida petrophilum* ATCC 20226.

4.2. Strain selection

The selected strains were grown in 50 mL of Lebac medium (g / L): $(NH_4)_2SO_4$ 7, KH_2PO_4 5.7, K_2HPO_4 2, $MgSO_47H_2O$ 2, $CaCl_22H_2O$ 0.005, $FeCl_36H_2O$ 0.0025, Yeast extract 0.1, glucose 20, pH 7.0; in 200 mL Erlenmeyer flasks with a 200 μL aliquot of microorganisms. Twenty-four flasks of each strain were placed in an incubator (FELISA) at 37 °C under constant agitation at 200 rpm. Three flasks were removed at each interval over a 44 and 48 h kinetic.

The parameters evaluated over time were: biomass production, pH, emulsification activity on engine oil and glucose consumption.

Biomass production was determined by taking 2 mL of culture medium and passing it through a pre-dried and pre-weighed cellulose nitrate membrane filter (0.22 μm in diameter). The filter with the biomass was then dried at 100°C for 24 h until constant weight was attained; the biomass was reported in g obtained by weight difference.

4.3. Emulsification index

Emulsification activity was determined by placing 6 mL of engine oil and 4 mL of culture medium with the biosurfactant-producing strains in a vortex [28]. They were agitated for 2 minutes and left to rest for 24 h. The percentage of emulsification was estimated according the following expression:

% Emulsifier = ((Total height of the mixture - Height of emulsified oil) / Total height of the mixture) * 100

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4.4. Glucose consumption, pH and Critical Micelle Concentration (CMC)

The glucose was determined by the AOAC 969.39 method taking a 2 mL aliquot of culture medium. If necessary it was diluted with distilled water.

The pH was determined with a potentiometer (Conductronic pH 10). In this investigation, pH was maintained close to neutrality by adding 0.1N NaOH.

The Critical Micelle Concentration (CMC) was determined according to [29].

4.5. Statistical analysis

The results were adjusted to a linear model to obtain the rate of substrate consumption (g glucose h^{-1}), the rate of biomass production (g biomass h^{-1}) and emulsification activity (% emulsifier h^{-1}). The slopes (rates) and correlation coefficients were obtained from regression linear model.

In addition, the average initial and final samples of emulsification activity were analyzed by variant analysis to find significant differences and Duncan-Waller multiple comparison tests. The statistical package used was Minitab version 13 (licensed to UPAEP, Mexico).

4.6. Biodegradation tests of maya crude oil

A preculture of selected strains was grown in Banat broth at 30 °C under constant agitation (200 rpm) for 24 h. An aliquot of the selected strains was taken at an absorbance of 70 UK, inoculated in flasks with 50 mL of medium at a pH of 6.5 with 20,000 ppm of crude oil and incubated at 30 °C for 15 days. Following the incubation process, the samples were put in contact with HPLC grade hexane and agitated for 2 minutes. The mixture was then sonicated (Branson 1210 Ultrasonic Cleaner) for 10 minutes before being transferred to a 250 mL separatory funnel leaving the aqueous phase to decant for later use (Figure 1A). The organic phase, in which the hydrocarbons are found, was recovered by means of an asbestos filter and Na_2SO_4 anhydrous as a desiccant in a 50 mL balloon flask. The organic phase was then distilled using a Büchi Rotavapor R11 with operating temperature of 45 °C (Figure 1B).

4.7. Viability of microorganisms

In addition to the hydrocarbon degradation capacity, the viability of the strains was determined at 8, 16 and 24 days of incubation. The organic phase was therefore eliminated by centrifugation (3000 rpm for 5 minutes) and successive serial dilutions made of 10^{-6} and cultivated on plates of Lebac medium. Isolates strains were grown overnight in Lebac broth at 37 °C under constant agitation at 200 rpm. The biochemical characterization was carried out by the API 20 E, API 20 NE and API 50 CH systems (references No. 20160, 20050 and 50300; bioMérieux) following the manufacturer's recommendations. The identification was assessed by APIweb™ identification software (bioMerieux).

4.8. Biosurfactant recovery

The purification of biosurfactant was performed according to a modified technique described in [30]. With the strains with highest percentage of emulsifier. The strains were previously grown in 500 mL of Lebac medium. The biosurfactant was then extracted from the bacteria with isopropanol-ethanol (3:1) analytical grade (Merck, México) in a separatory flask. It was centrifuged at 1200 rpm for 30 minutes (Solbat), and the supernatant was eliminated. The sample was then filtered using cellulose paper grade 101 (Millipore 2.5µ M). The precipitate obtained was dried for 24 h at 60 °C in an oven (FELISA) and stored in an Eppendorf vial to determine the yield.

Figure 1. a) Emulsification of hydrocarbons. (b) Oil recovery.

5. Results

5.1. Presumptive identification of isolated microorganisms

Six microorganisms were isolated and identified according to their morphology. Table 1 shows the results of the presumptive tests for the identification of bacteria and yeasts by API galleries. The strains UPAEP 8 and UPAEP 15 were related to *Klebsiella pneumoniae* (99 and 97.6 % likelihood respectively). UPAEP 6 strain was closely related to *Klebsiella ornithinolytica* (99 %) and UPAEP 9 strain to *Klebsiella* sp (97 %). Whereas UPAEP 10 strain showed high likelihood (99 %) to *Serratia marcescens* and UPAEP 12 strain to *Candida inconspicua* (75 %).

5.2. Glucose consumption and biomass production

The kinetic characteristics of the bacteria showed similar behavior regarding rapid growth, good adaptation to hydrocarbons and rapid glucose consumption.

All strains consumed glucose in a range of 92 to 100 %. However, the glucose consumption percentage of the commercial strains was lower than the isolates studied; with the exception

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of *Bacillus subtilis* ATCC 21332 which consumed 93.6 % of glucose (Table 2). Nevertheless, the glucose consumption was inversely proportional to the biomass production during the cell growth (data not shown). The emulsification index was directly proportional at production of biomass, except *Candida petrophilum* ATCC 20226 which showed no relation. Biosurfactant synthesis and biomass production by UPAEP 6, 9, 10, and 15 (Figures 2, 4,5 and 10) strains began during the first few hours (4 to 8) as a response to substrate consumption; UPAEP 8, 12 (Figure 3 and 6) and *Arthrobacter* sp ATCC 31012 (figure 8) strains began at 20, 28 and 50 h. In contrast, *Bacillus subtilis* ATCC 21332 strain biosurfactant production occurred at the end of microbial growth (after of 76 h).

UPAEP 6 strain showed the highest increase in biomass and biosurfactant production at 24 h. Maximum biomass production occurs at 44 h and with a maximum value of 5.3 g L^{-1}. The maximum value of the biosurfactant production (80 %) at 40 h was high considering that crude oil is heavy with a density of 0.92-1.01 g mL^{-1} and an API gravity of 10.1-22.3 and viscosity can reach 10,000 cP [31] (Figure 2).

On the other hand, UPAEP 9, UPAEP 10 and UPAEP 15 strains (Figures 4, 5 and 7) showed maximum biomass production at 28, 24, and 77 h with values of 3.6, 5.3 and 9.5 g L^{-1} respectively. Biosurfactant production started from the first couple of hours and up to 49 h by UPAEP 9 and UPAEP 15 strains reached emulsification of 58 and 69 %; and at 20 h UPAEP 10 strain showed 70 % of biosurfactant production.

UPAEP 8 (Figure 3), UPAEP 12 (Figure 6) and *Arthrobacter* sp ATCC 31012 (Figure 8) strains showed slow biosurfactant production in contrast with the isolated strains. Biosurfactant production started only at 20, 28 and 50 h, and reached a maximum value of 65, 37 and 30 % respectively (at 40, 49 and 72 h). *Arthrobacter* sp ATCC 31012 showed slow growth, the highest biomass production was of 8.5 g L^{-1} at 55 h. Anyhow, UPAEP 12 strain showed a highest increase in biomass production between 28 and 46 h with a final value of 7 g L^{-1} (77 h) and UPAEP 8 strain showed maximum biomass production of 6.6 g L^{-1} at 24 h.

However, *Bacillus subtilis* ATCC 21332 strain (Figure 9) showed maximum biomass production in the first 10 h with 4.6 g L^{-1}. Maximum biosurfactant production (27 %) is observed at the end of the kinetic (70 h).

The *Candida petrophilum* ATCC 20226 strain (Figure 10) showed an important decrease in glucose up to 70 h (data not shown). Biosurfactant production began at 20 h. No relation to substrate consumption or to biomass production was observed. The maximum emulsification percentage obtained was 80 % after 70 h.

The initial pH of the culture medium was 7.0 and lowers during the cellular growth of the studied isolates, therefore was adjusted with NaOH 0.1N to obtain a pH closer to neutrality (data not shown). Thus, the final pH of the culture medium ranged from 6.07 to 7.37 (Table 2). It is interesting to observe, that the drop in pH occurred just before the biosurfactant synthesis, possibly due to a prior synthesis of organic acids as precursors of biosurfactants by UPAEP 6, UPAEP 8, UPAEP 9, UPAEP 10 and UPAEP 15 strains. Yet, the pH was maintained between 6.5 and 6 with few changes during the entire kinetic by UPAEP 12 strain, and *Arthrobacter* sp ATCC 31012 showed only a small drop at 49 h. *Bacillus subtilis* ATCC 21332 and

Candida petrophilum ATCC 20226 strains remained the pH close to neutrality during the entire kinetic.

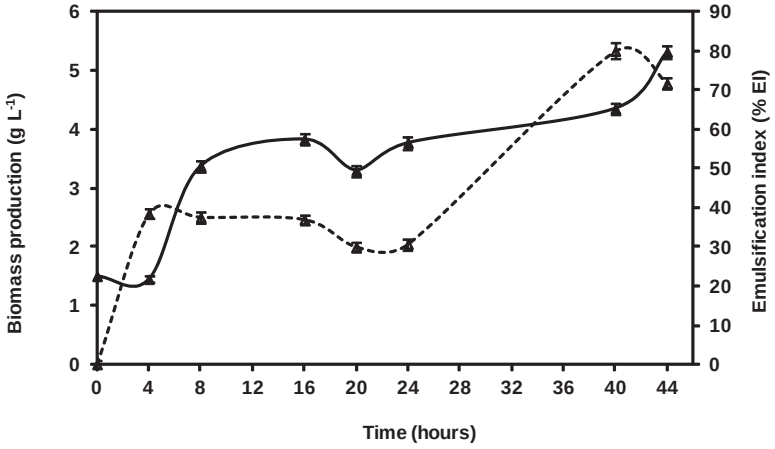

Figure 2. Bacterial growth by bacteria strain UPAEP 6 associated to biomass production (▲), and Emulsification Index EI (%) (Δ). Results are the averages of triplicate experiments ± standard deviation.

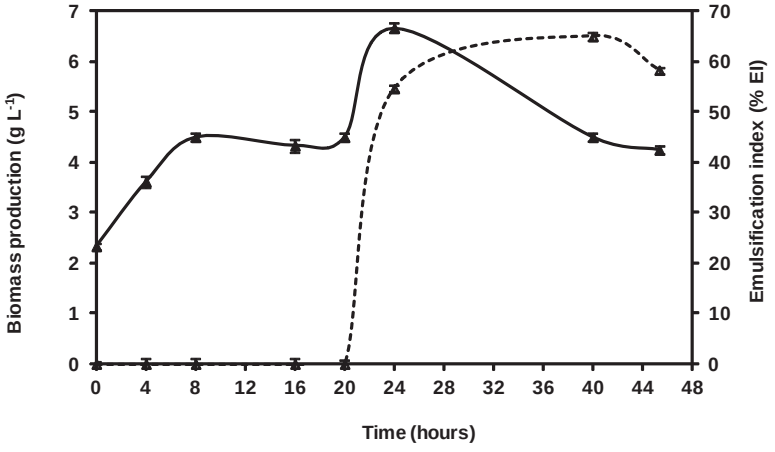

Figure 3. Bacterial growth by bacteria strain UPAEP 8 associated to biomass production (▲), and Emulsification Index EI (%) (Δ). Results are the averages of triplicate experiments ± standard deviation.

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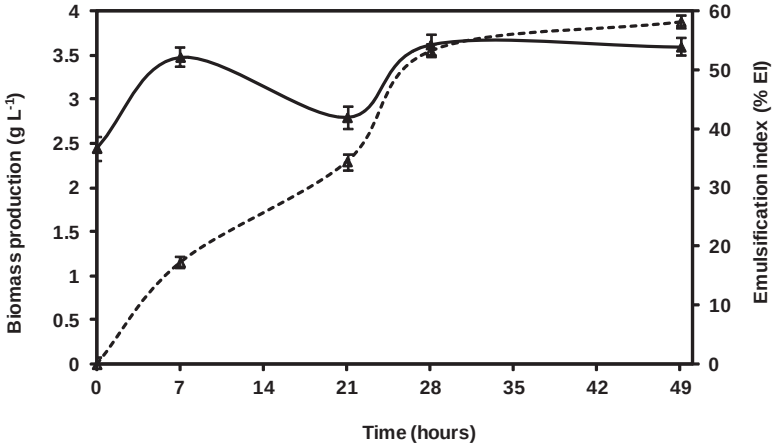

Figure 4. Bacterial growth by bacteria strain UPAEP 9 associated to biomass production (▲), and Emulsification Index EI (%) (Δ). Results are the averages of triplicate experiments ± standard deviation.

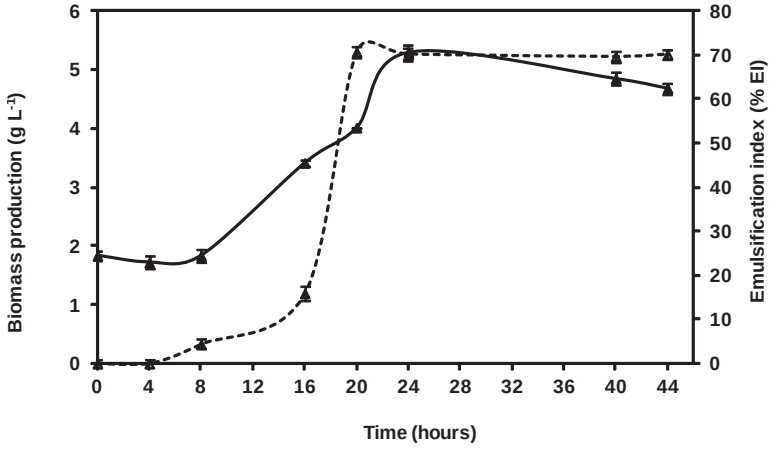

Figure 5. Bacterial growth by bacteria strain UPAEP 10 associated to biomass production (▲), and Emulsification Index EI (%) (Δ). Results are the averages of triplicate experiments ± standard deviation.

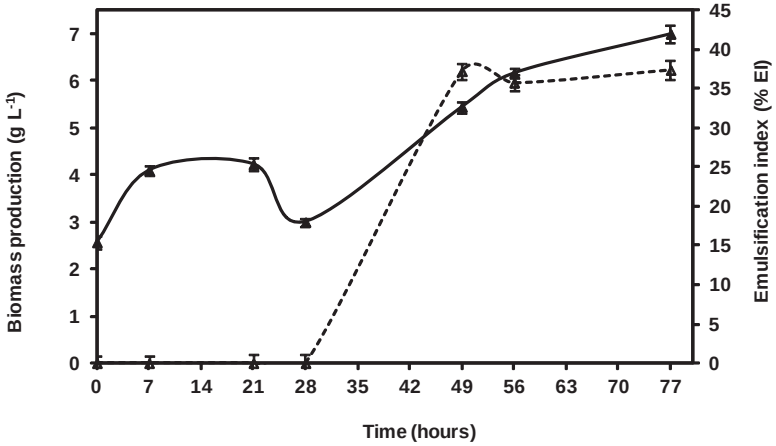

Figure 6. Bacterial growth by bacteria strain UPAEP 12 associated to biomass production (▲), and Emulsification Index EI (%) (Δ). Results are the averages of triplicate experiments ± standard deviation.

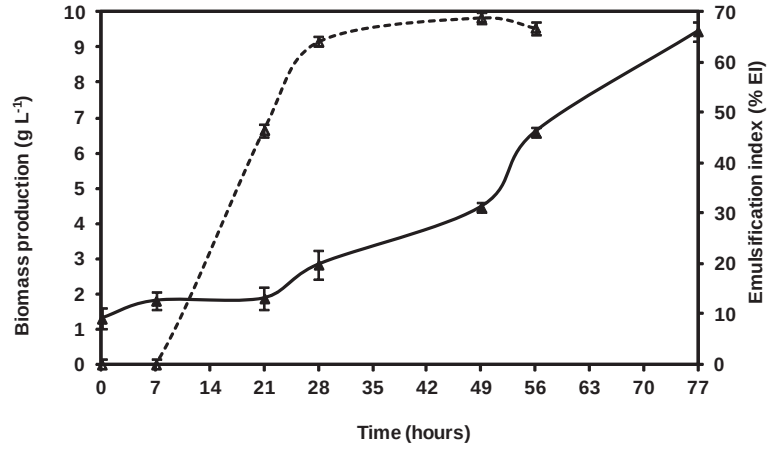

Figure 7. Bacterial growth by bacteria strain UPAEP 15 associated to biomass production (▲), and Emulsification Index EI (%) (Δ). Results are the averages of triplicate experiments ± standard deviation.

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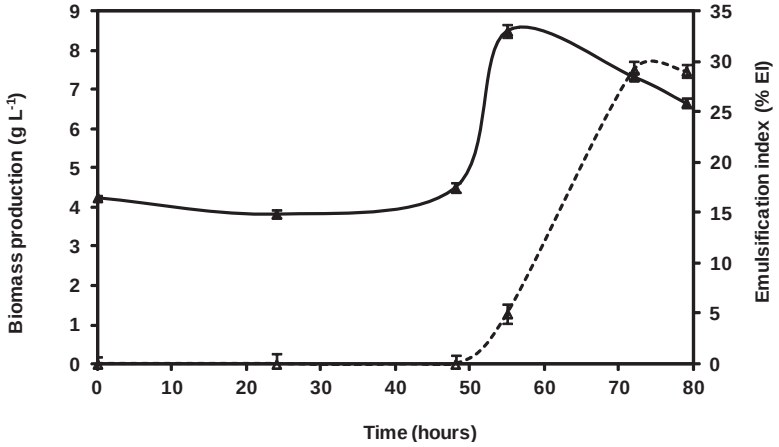

Figure 8. Bacterial growth by bacteria strain commercial *Arthrobacter* sp ATCC 31012 associated to biomass production (▲), and Emulsification Index EI (%) (Δ). Results are the averages of triplicate experiments ± standard deviation.

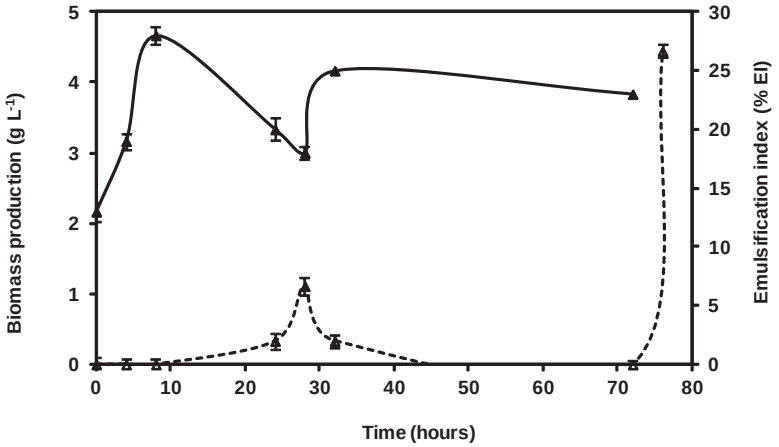

Figure 9. Bacterial growth by bacteria strain commercial *Bacillus subtilis* ATCC 21332 associated to biomass production (▲), and Emulsification Index EI (%) (Δ). Results are the averages of triplicate experiments ± standard deviation.

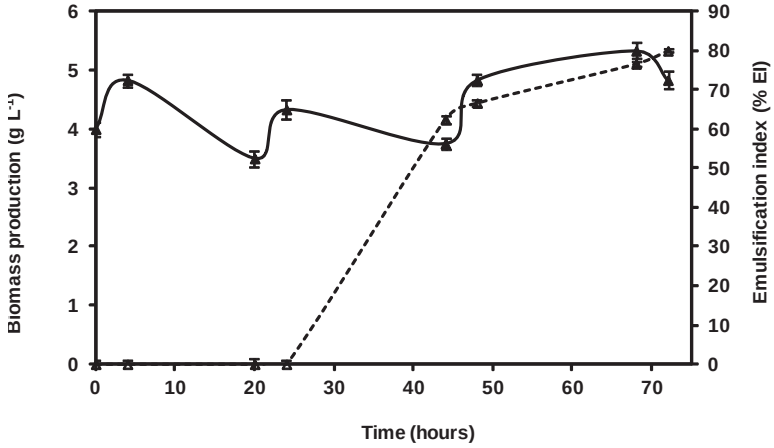

Figure 10. Bacterial growth by bacteria strain commercial *Candida petrophilum* ATCC 20226 associated to biomass production (▲), and Emulsification Index EI (%) (Δ). Results are the averages of triplicate experiments ± standard deviation.

5.3. Production rates

Table 3 shows the results of the estimated rates. The UPAEP 6 strain showed the highest biomass production rate with 0.178 g h^{-1}. The strains with best biosurfactant production rates were UPAEP 10 and UPAEP 8 with 2.5 and 2.39 % h^{-1}, respectively. Significant differences were found in the variance analysis of the emulsification final values with 70% (*Serratia marcescens*) and 80% (*Klebsiella pneumonia*). The highest rates of emulsification were for UPAEP 8 and the yeast *Candida petrophilum* ATCC 20226 (80%). CMC results of the selected strains are similar to that reported for Tergitol (0.0149 mg L^{-1}) and 10 times less than *Serratia marcescens* subsp. *marcescens*.

The capacity of these bacteria to degrade toxic compounds depends on the contact time with the compound, the environmental conditions in which they develop and their physiological versatility.

5.4. Biodegradation tests of maya crude oil

Once the strains had been evaluated, the next step was to evaluate the removal percentage of Maya crude oil (20,000 ppm) using UPAEP 8 (*Klebsiella pneumoniae*) and UPAEP 10 (*Serratia marcescens*). These two bacteria showed a greater than 80 % degradation for Maya crude oil (Figure 11,12 and 13).

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Bacterial strain UPAEP	Classification	% likelihood
6	*Klebsiella ornithinolytica*	99
8	*Klebsiella pneumoniae*	99
9	*Klebsiella* sp	75
10	*Serratia marcescens*	99
12	*Candida inconspicua*	75
15	*Klebsiella pneumoniae*	97.6

Table 1. Identification of the bacterial strains was by the API galleries.

Strain UAPEP	Initial pH		Final pH *		% Glucose consumption **	
6	7.0	± 0.2	7.37	± 0.09	99.7	± 0.9
8	7.0	± 0.1	6.65	± 0.11	99.9	± 0.9
9	7.0	± 0.1	6.25	± 0.14	95.8	± 1.0
10	7.0	± 0.1	6.64	± 0.06	99.7	± 0.9
12	7.0	± 0.1	6.07	± 0.14	92.0	± 0.5
15	7.0	± 0.2	6.86	± 0.24	100	± 0.1
Strain ATCC						
31012	7.0	± 0.1	6.22	± 0.15	66.96	± 0.6
20226	7.0	± 0.1	6.45	± 0.12	76.48	± 0.5
21332	7.0	± 0.1	6.64	± 0.13	93.61	± 0.5

* pH values for isolates incubates in Lebac medium for 44 and 48 h at 37°C under constant agitation at 200 rpm (see Methods); each value represents the average of three replicates ± standard deviation.

* * Glucose consumption percentage is the difference between initial and final glucose concentration; each value represents the average of three replicates ± standard deviation.

Table 2. Changes of pH and Glucose consumption by Biosurfactants-producing bacterial strains during the bacterial growth.

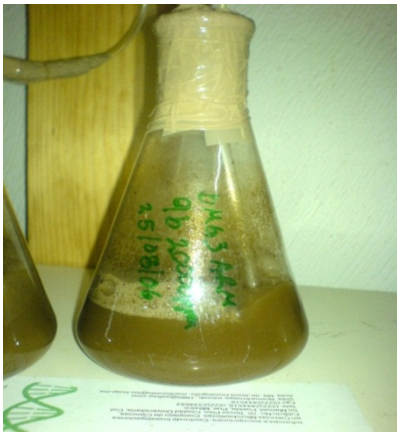

Figure 11. Maya oil Bioemulsification. Experiment with 20,000 ppm of petroleum and biosurfactan-producing micro-organisms.

Strain UAPEP	Rate Biomass production (g h^{-1})	R^2	Emulsification Activity (% h^{-1})	R^2	Rate substrate consumption (g glucose h^{-1})	R^2	Emulsification Index Final value * (%)	CMC (mg L^{-1})
6	0.178	0.76	1.72	0.51	0.86	87.1	$65^{b,c}$	0.0016
8	0.074	0.68	2.39	0.93	0.277	88.5	80^{a}	0.0047
9	0.018	0.80	1.13	0.86	0.336	70.0	49^{c}	0.0014
10	0.074	0.81	2.5	0.82	N.D.**	N.D	70^{b}	0.0014
12	0.05	0.72	0.01	0.41	0.218	87.0	58^{c}	0.0010
15	0.100	0.86	1.39	0.64	0.404	78.0	70^{b}	0.062
Strain ATCC								
31012	0.071	0.83	1.16	0.66	0.428	84.2	40^{c}	0.005
20226	0	0.21	1.32	0.88	0.390	97.6	80^{a}	0.005
21332	0.031	0.78	0.19	0.74	0.380	80.0	27^{d}	0.0015

* Final value Means with different letters are significantly different (P<0.05).

* * It was not determined.

Table 3. Biosurfactants-producing bacterial strains isolated from polluted soil with hydrocarbons.

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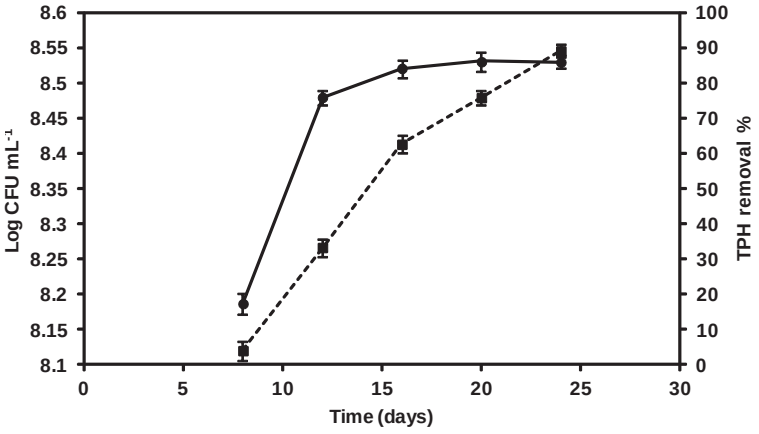

Figure 12. Removal of TPH by bacteria *Klebsiella pneumoniae* (UPAEP 8 strain) isolated from contaminated soil. Strain was grown at 30 °C, and 20000 ppm of mayan crude oil. Removal of TPH (■). Cell growth of strain with 20000 ppm of mayan crude oil (●). Results are the average of triplicate experiments ± standard deviation.

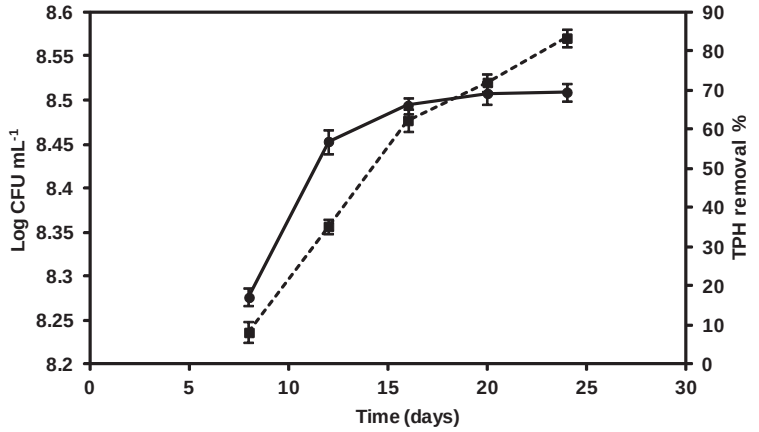

Figure 13. Removal of TPH by bacteria *Serratia marcescens* (UPAEP 10 strain) isolated from contaminated soil. Strain was grown at 30 °C, and 20000 ppm of mayan crude oil. Removal of TPH (■). Cell growth with 20000 ppm of mayan crude oil (●). Results are the average of triplicate experiments ± standard deviation

6. Discussion

Serratia genus have been reported by other authors as biosurfactants-producing bacterial capable degrader oily compounds [32, 33]. According to [34] bacteria with high capacity to produce biosurfactant promising remain very still, because many companies wish to replace chemical biological to chemical surfactants. The biosurfactant production rate for *Serratia marcescens and Klebsiella pneumonia* 2.39 and 2.5 (% h-1) respectively show the significant potential for industrialization of the strains. Biosurfactants-production remains a topic of industrial interest [35] emulsified 20% of 1500 mg / L of octadecane, while the present work with the best strains emulsified 80 and 90% of Mayan crude oil at an initial concentration of 2000 mg/L. According to [34] states that the genus *Pseudomonas* is the most promising from the industrial point of view, among other things because of the chemical nature of the rhapnolipids, in work [35] are employed *Pseudomonas aeruginosa* ATCC 9027, however the strains studied in this work were even better at the emulsification even using oil that is more complex relative to octadecano.

All the selected strains presented emulsifying activity, the majority associated with the growth of microorganisms and a decrease in pH. Some authors [19] reported that for the *Pseudomonas* species, an association has been found in the synthesis of different metabolites (fatty acids, lipopeptides, peptides and amino acids), which can be used for cellular synthesis and biosurfactant production. Although this work is focused on the degradation of recalcitrant hydrocarbons such as Maya crude oil, there is wide interest in biosurfactant production due to its applications in various fields. Other authors [32] performed a chemical and antimicrobial characterization of pseudofactin II, a biosurfactant secreted by *Pseudomonas fluorescens* BD 5 identified as a new cyclic lipopeptide with broad-spectrum bactericidal activity.

The bacteria used the Maya crude oil as sole carbon source, associated with high biomass content and a very high capacity to emulsify hydrocarbon compounds in relatively short operating times (15, 17 and 24 days) compared to those reported by other authors [36-38]. The values of the production kinetics of are very important considering of the scaling the process, *Klebsiella pneumoniae* showed up to 90 % removal and is a promising strain for future biodegradation studies.

The results will allow the use of these cultures as possible inoculants, in real bioremediation experiences where large quantities of inoculants are required. Crude oil biodegradation has been studied extensively because of the high variability of crude oil amount, incubation times and methodologies used to quantify degradation.

7. Future work

In Mexico, particularly on agricultural land, biological techniques which leave no chemical residue and with low-toxicity are required to recover impacted soil. The impact on agricultural

http://dx.doi.org/10.5772/56143

soil and its recovery for farmers is a major problem. Sustainable biological techniques may be an alternative and raise the expectations of farmers hoping to plant their crops without risk. Biosurfactants have shown their potential in bioremediation of contaminated soil and water with oil and its derivatives. Because of its low toxicity and biodegradability these are considered as an accepted alternative and environmentally friendly.

However, the *in situ* production of these compounds by microorganisms in natural environments are link to many factors including the type of contaminant, nitrogenous compounds content, interaction with native microorganisms and some others. It is important to perform tests on real soil before the scaling tests since several studies have reported inconsistent results. Therefore the use of microorganisms producing biosurfactants in bioaugmentation processes requires a careful study; new research on the scaling processes to optimize biosurfactants production must be conducted.

The rhamnolipids produced by *Pseudomonas auriginosa* biosurfactants have been extensively studied, but there are other organisms that produce substances with emulsifier, such as those produced by the serrawettin by *Serratia marcescens* this it is a bacteria which has been described as plant growth promoting rhizobacteria (PGPR), which refers to the promotion of growth when plants are inoculated, because it has the ability to produce indole-3-acetic acid (IAA). Due to the activities of the oil industry in Mexico, agricultural soils are contaminated with hydrocarbons, leading to impairment of soil properties and the consequent decline in agricultural production. Technologies should be applied for the recovery of the ground with the least environmental impact. The plant-assisted bioremediation (phytoremediation) is an alternative for the *in situ* treatment of soil contaminated with hydrocarbons. The UPAEP 10 strain of *S. marcescens* is capable of producing biosurfactants and degrades crude oil which is needed for investigating the ability of promoting plant growth in order to develop rhizoremediation technologies.

8. Conclusions

This study showed microorganism isolated of contaminated soils with high capacity of degrading recalcitrant compounds. In México there is a great need to develop clean technologies due to oil spill accidents in agricultural soils. Biosurfactant production by native strains as *Klebsiella pneumoniae* (UPAEP 8 strain) and *Serratia marcescens* (UPAEP 10 strain) showed emulsification rates of up to 80 %, and CMC values were similar than commercial detergents; therefore may be a promising way for recovery of weathered soils with heavy hydrocarbon particles. The implementation of clean technologies will allow farmers to continue producing their products of the harvests harmless and safe for sale and consumption.

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