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Effectiveness of Crude Biosurfactant Mixture for Enhanced Biodegradation of Hydrocarbon Contaminated Soil in Slurry Reactor

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ABSTRACT: A crude biosurfactant mixture was produced in a 1.4 L tubular reactor by a mixed culture; after the thermal treatment of the whole culture an increase in emulsification activity and surface tension was observed, with a main effect on the first one. The emulsification index of the mixture obtained was of 60.4 %. The crude mixed biosurfactant was used to enhance hydrocarbon biodegradation of intemperized soil in a slurry reactor, for which two biodegradation assays were carried out. In the first reactor (R1), the crude mixed biosurfactant was added along with inoculum at initial stage, and the second reactor (R2) was inoculated 5 days after the initial biosurfactant addition. When the crude biosurfactant mixture was added as a pretreatment (R2), the extent and rate of hydrocarbon biodegradation was increased efficiently in the slurry phase reactor 1.3-folds in comparison to non-biosurfactant control. So, the initial hydrocarbon content (9,275 g/kg of dry soil) was reduced to 674 ± 34 mg kg-1 with about 92% of removal efficiency at the 10 days of treatment in the slurry reactor. On the other hand, the bioreactor that received the biosurfactant and the inoculum at the initial stage showed slower hydrocarbon consumption and as result hydrocarbon content was reduced by approximately 34%.

Key words: Crude, Biosurfactant, Mixed culture**,** Intemperized soil, Slurry reactor

INTRODUCTION

The success of bioremediation process depends upon the microbial ability to access the complex hydrocarbon mixtures, which are compounds with low water solubility and thus not readily available to microorganisms. A wide variety of diverse microorganisms has developed metabolic mechanisms to accomplish the breakdown of these compounds including the production of surface-active agents and emulsifiers (Moliterni, *et al.*, 212). Biosurfactants may enhance hydrocarbon biodegradation trough reduction of interfacial tension, micellar solubilization and phase transfer between soil particles and the pseudo-aqueous phase (Franzeti, *et al.,* 2010), making hydrophobic pollutants more bioavailable for microorganisms. While biosurfactants are generally equally effective in terms of solubilization and emulsification, they are also considered to be biodegradable, less toxic, and more environmentally friendly than synthetic surfactants (Mulligan. *et al.,* 2009). However, high-volume applications of biosurfactants is limited by production and purification costs, so biosurfactants compete with difficulty against the chemically synthesized compounds on the surfactant market (Muthusamy *et al.,* 2008; de Gusmão *et al.,* 2010). Since the production of high biosurfactant yields has been considered one of the obstacles in the oil industry, the use of crude biosurfactants, i.e., the use of cell free broth obtained after production has been considered as strategy for application in bioremediation.

Crude biosurfactants, produced by pure cultures of microorganisms, have been used in two ways to enhance bioavailability and biodegradation of hydrophobic compounds: as single crude biosurfactants (de Gusmão *et al.,* 2010; Benincasa, 2007; Das and Mukherjee, 2007; Abalos *et al.,* 2004; Cubitto *et al.,* 2004) or biosurfactant mixture of similar species (Hidayati *et al.*, 2011; Cameotra and Singh,2008). In our knowledge, there are not available studies about the use of crude mixed biosurfactant which was used to enhance hydrocarbon biodegradation. Thus, the aim of this work is to evaluate the effect of the addition of crude biosurfactant mixture, produced by mixed culture, to enhance hydrocarbon biodegradation of intemperized soil in slurry reactor.

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MATERIALS & METHODS

The soil sample was obtained from petroleum-contaminated site located in the central region of Mexico. This soil was exposed to hydrocarbons for several years as a result of accidental spills. Before being treated, the soil was air-dried and passed through 8-10 mesh. The sieved soil samples were kept in closed bottles at room temperature.

The texture analyses indicated that the soil contained (by weight) 47 % sand, 28 % clay and 25 % silt. According to that, this soil can be classified as sandy clay loam, by using the USDA textural triangle (NOM-021-RECNAT-2000; Ewis *et al.,* 1998). The organic matter content was 3.94% and the pH was 7.42. Initial hydrocarbon concentration was 9,275 g/kg of dry soil, free from any asphaltenes. Other important soil characteristics, that could influence hydrocarbon biodegradation, are showed on Table 1.

Table 1. Physicochemical properties of contaminated soil

Moisture (%)	4.31 ± 0.10
Electric conductivity (dS/m)	0.39 ± 0.01
Apparent density (g/mL)	2.88 ± 0.03
Real density (g/mL)	2.28 ± 0.05
Total nitrogen (%)	0.03 ± 0.001
Total carbon (%)	2.29 ± 0.08

A mixed culture composed of 7 bacterial strains (*Achromobacter glicosidans*, *Bacillus cereus*, *B. subtilis, Pseudomonas pseudoalcaligenes*, and two unidentified strains) was used as inoculum (García-Rivero *et al.* 2007). This culture was isolated from a hydrocarbon contaminated soil sample, and was stored in glycerol at –20°C, with periodic reseeding on fresh medium.

The biosurfactant production culture was carried out in a 1.4L tubular reactor with a working volume of 1L. In this case, a preculture was developed by inoculation of the mixed culture in a mineral medium (García-Rivero *et al.,* 2007) containing 5 g/L^{1} of diesel oil, incubating at 150 rpm and 30°C for 5 days. The reactor received 500 mL of the preculture broth (containing 1.23x10^{5} UFC/mL) and 500 mL of mineral medium, and it was incubated at room temperature for 3 days. The whole broth was autoclaved at 121°C (15 lbs pressure) for 15 min, to ensure elimination of any microbiological activity, subsequently the emulsification index tension and surface tension was quantified (sample BsT). In a second trail the thermally treated **broth** was centrifugated for 15 min in order to remove cellular debris, and the biosurfactant was quantified in the supernatant (sample BsTC).

Emulsification activity of samples was evaluated according to Cooper and Goldenberg (1987), 6 mL of diesel fuel was added to 4 mL of samples (BsT or BsCT), in a screw cap tube and vortexed at a high speed for 2 min. The emulsion index (EI_{24}) was calculated by dividing the height of the emulsion layer by the total height of the mixture and multiplying by 100. Surface tension was determined with a CSC-DuNOUY 70535 tensiometer.

For evaluating the effect of biosurfactant in biodegradation assays, soil was treated in a slurry reactor, because this is one of the best options for the bioremediation of soils polluted by recalcitrant pollutants under controlled environmental conditions (Robles-González, *et al.,* 2010). The soil suspension was prepared by mixing mineral medium and 20% (w/v) of soil. In our experience (García-Rivero *et al.,* 2007), this soil concentration allows to achieve an adequate mixing.

The hydrocarbon biodegradation assays were performed in 1.2 L glass reactors that were operated in batch mode, at room temperature and continuously mixed by a magnetic stirring bar, and were periodically opened in order to avoid the CO_2 accumulation. All reactors contained 720 mL of liquid phase and 180 g of contaminated soil. The first reactor experiment (R1), was inoculated with 90 mL of mixed culture, subcultured on mineral medium. For the second reactor (R2), 90 mL of the subcultured microorganisms were added after 5 days of initial biosurfactant addition. Both R1 and R2 were added with 30% (V/V) of the biosurfactant solution at the beginning of the assays. The third reactor served as a non-biosurfactant control. The cell density of the bacterial inoculums used in the three reactors contained 2.93x10^{7} UFC/mL.

The hydrocarbons were extracted from soil samples by the conventional Soxhlet extraction, USEPA 3540 (USEPA 1996) as follows: 10 g of soil were treated using 160 mL of methylene chloride. After that extract was treated with cold *n*-pentane to remove asphaltenes (García-Rivero *et al.,* 2007). The hydrocarbon concentration in the extract (free from any asphaltenes) was determined by a Varian star 3900 gas chromatography (GC), equipped with a flame ionization detector. The capillary column used was Altech Phase ATTM-1. The initial column temperature was 45°C and was increased to 340 at a rate of 10°C/min. Temperatures of injector and detector were 340 and 350°C respectively. The carrier gas was Helium at a constant flow rate of 1 mL/min.

Aerobic heterotrophic culturable bacteria from mixed culture in the slurry reactor during hydrocarbon biodegradation assays were estimated at different

times. 0.1 mL of serial dilute soil samples were plated in Trypticase Soy Agar. Triplicate samples were incubated at 30°C during 48 h before colonies were counted.

All treatments and analysis of the resulted samples were developed in triplicate with standard deviation (SD) represented by error bars in graphs and (±) numerical values in tables. The statistical mean differences were developed using the SAS® system with Tukey´s Test.

RESULTS & DISCUSSION

For detecting the biosurfactant produced by the mixed culture, we choose qualitative analytical techniques related to properties of this chemical specie. For doing this we compared the values obtained after analyzing culture media before inoculation (fresh broth) with those obtained with final culture broth (whole culture broth) and the subsequent treatments applied to this. The obtained values are shown in Table 2.

Table 2. Surface tension and IE_{24} values obtained for whole culture broth during the 3 days fermentation

Sample	Surface tension (dynes/cm)	IE_{24} (%)
Fresh broth	35.6 ± 1.7^{b}	5.7 ± 0.2^{d}
Whole culture broth	34.5 ± 1.7^{b}	17.3 ± 0.8^{b}
BsT	30.8 ± 1.5^{c}	60.4 ± 3.0^{a}
BsTC	37.6 ± 1.8^{a}	6.9 ± 0.2^{c}

Note: Tukey's test was conducted comparing the surface tension and emulsification index with samples of the broth with different treatments. Different letter indicates significant differences ($p \leq 0.05$).

Surface tension value of whole broth was comparable to the fresh broth, which could indicate that the biosurfactant mixture produced in the culture did not have surfactant activity, meanwhile the increase in the emulsification index observed for the whole broth suggest the presence of biosurfactant with emulsification properties. On the other side, thermal treatment of the whole culture broth increased both surfactant and emulsification activity, with a more significant effect on the last one. However, the subsequent centrifugation of the broth modified both properties, bringing the surface tension and the IE_{24} to the values obtained for the fresh broth. These results suggest that thermal treatment produced changes that weakened cellular membrane, allowing biosurfactants remained associated with cellular debris (observed on BsT sample), and because of that the surfactant and emulsification activities decreased after biomass was removed by centrifugation (BsTC sample).

In order to prove that the decrease in surface tension and the increase in IE_{24} produced by the crude extract is the result of biosurfactant production by the mixed culture, we used a yeast culture broth as negative control. This broth was treated following the methodology used to crude extract biosurfactant (thermal treatment and centrifugation). In this case, none of the samples of yeast culture showed emulsification or superficial tension activity, so we can affirm that the biosurfactant activity reported is resulting from the production of biosurfactant agent by the mixed culture.

The emulsification activity of the crude biosurfactant mixture obtained in this work was within the range of values (E_{24} = 30 -71%) reported for different types of biosurfactants produced by single microorganisms (Biria *et al.,* 2010; Nayak *et al.,* 2009; Reis *et al.,* 2004). In fact, it was comparable with the few available reports about production of biosurfactants by bacterial consortium. For example, Darvishi *et al.,* (2011) and Rahman *et al.,* (2003) reported an emulsification activity of 83.4 and 65 % for heavy crude oil and diesel fuel, respectively.

Finally, the procedure proposed in this work to ensure the inactivation of biomass, also increases biosurfactant content in the broth and it may be cheaper than a multistage procedure for biosurfactant recovery. For instance, releasing biosurfactants from cell surface by sonication (Gusmão *et al.,* 2010; Hwang *et al.,* 2008) or extraction with phosphate-buffered saline (Rodrigues *et al.,* 2006) require an additional step to remove biomass.

Fig. 1 show the residual hydrocarbon measured in the slurry reactor during the biodegradation process when biosurfactant mixture (BsT sample) was added at the initial time (reactor R1) and as pretreatment (reactor R2). Hydrocarbon content rapidly dropped to 674 and 2,578 mg/kg in the first 10-days of culture in reactor R2 and in non-biosurfactant control, respectively. Reactor R1 showed a slower reduction, and as a result, on the 20th day hydrocarbon level showed its maximum reduction, reaching the 52 % from the original value.

In fact, compared to the control, adding the biosurfactant at the same time than the inoculum, cause a negative effect on hydrocarbon degradation by microorganism. Biosurfactants could be biodegraded before their expected action takes place, either due to biosurfactants interfering with direct uptake of hydrocarbons or simply because of the fact that these molecules may be treated as an alternative carbon

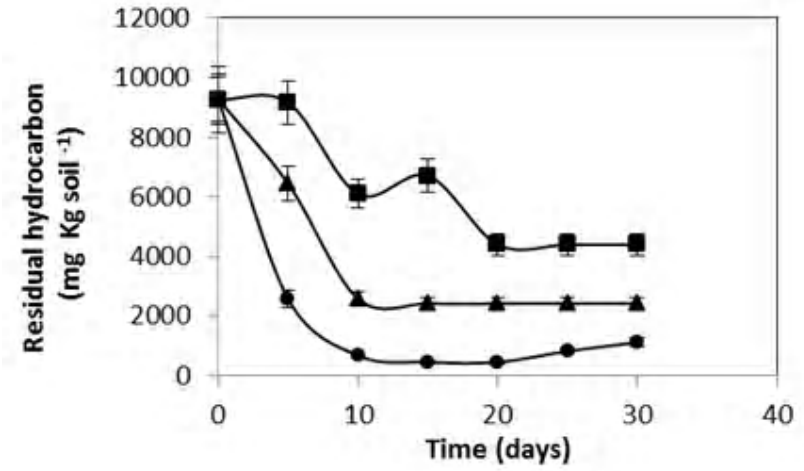

Fig. 1. Residual hydrocarbon in slurry treatment when crude biosurfactant mixture was added at initial time (Reactor R1) (■), biosurfactant mixture added as a pretreatment (Reactor R2) (•) and non-surfactant control (▲)

source (Ławniczak *et al.,* 2013). Similar results of no stimulation of hydrocarbon removal were reported in PAH's (Marcoux *et al.,* 2000) and diesel oil degradation (Chrzanowski *et al.,* 2012).

Biodegradation extent obtained in reactor R2 is in agreement with works in which the surfactant was used as a crude extract. For example, Abalos *et al.,* (2004) demonstrated that addition of a crude rhamnolipids extract accelerates the biodegradation of total petroleum hydrocarbons from 32% to 61% at 10 days of incubation. Similar results were obtained by Owsianiak *et al.,* (2009), applying rhamnolipids in diesel degradation by a microbial consortium. However, in our work biosurfactant addition increased hydrocarbon biodegradation 1.3-fold in comparison with control. This could be explained by the lack of nutrients. Recent findings have clearly confirmed that if the availability of carbon sources is high, it requires a sufficient amount of essential nutrients, such as nitrogen or phosphorus, to achieve an efficient bioremediation process (Ławniczak *et al.,* 2013). The biodegradation rate of hydrocarbons in the first days of cultures varied among the three assays with a similar behavior than the above described for biodegradation extent.The biodegradation rates are showed in Table 3. For reactor R2 this rate was 1.3-fold higher than the obtained in the non-biosurfactant control, while the rate of hydrocarbon reduction in reactor R2 was the smallest and it was 0.5-fold lower than the rate observed in non-biosurfactant control. Results obtained indicated that biosurfactant addition, before inoculation, increase the bioavailability of hydrocarbons and consequently the biodegradation extent and the initial consumption rate. Even better results could be expected when combined the addition of both nutrients and biosurfactant mixture.

Table 3. Effect of crude biosurfactant mixture addition on biodegradation of hydrocarbons in slurry reactor

Treatment	Hydrocarbon degradation(%)*	Hydrocarbon degradation rate**(mg/kg soil d)
R1	52^{c}	318.01
R2	87^{a}	860.15
Control	73^{b}	669.66

Note: Same letter indicates no significant differences
*Values obtained after 30-days of culture
**Estimated in the first 10-days of culture

The microbial counts of heterotrophic microorganisms in soil are shown in fig. 2, the heterotrophic population displayed a rapid growth phase in the three reactors, followed by a decrease; behavior that coincided with residual hydrocarbon profile. Nevertheless, heterotrophic population appears to be stable in the last 15 days only in reactor R2, probably because biosurfactant addition increased the carbon source availability, namely organic matter or hydrocarbons. In the other hand, in R1 reactor the reduced availability of hydrocarbons could provoke a slow initial growth phase, but the added biosurfactant and organic matter could be enough to allow microbial growth.

Although applications of biosurfactant for the enhanced degradation of hydrocarbons have been reported, the results showed in this work add some knowledge in this area, such as the reactor based studies and the introduction of mixed culture microbial community for crude biosurfactant production. These results can be significant for engineering applications

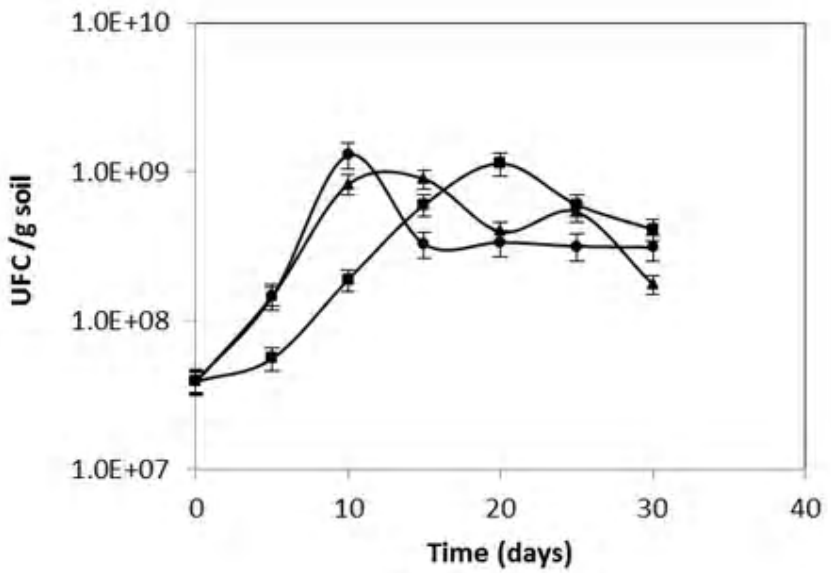

Fig. 2. Growth of heterotrophic microorganisms in soil samples of the slurry reactors when surfactant was added at initial time (Reactor R1) (■), surfactant was added as a pretreatment (Reactor R2) (●) and non-biosurfactant control (▲).

where an economical biosurfactant production could be possible and the process generates a little residue volume.

CONCLUSION

Mixed culture was efficient in producing substances with biosurfactant characteristics when they are cultivated using diesel fuel as the only carbon source. The thermal treatment of the final culture broth weakened the cell membrane which helped to increase the surfactant content, with a more significant effect on emulsification activity. It was demonstrated that this activity was caused by the biosurfactant produced and not by the cellular debris obtained as a result of the thermal treatment. Adding biosurfactant mixture as a pretreatment to contaminated soil is better than adding this with the inoculums to improve the hydrocarbon biodegradation process in a slurry reactor. The previous addition could increase availability of hydrocarbons, favoring biosurfactant cell-substrate interactions, otherwise biosurfactants could be used as an alternative substrate instead of encourage hydrocarbon removal. However for understand this phenomenon more special assays are required.

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