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Full Length Research Paper

Microbial removal of weathered hydrocarbons by well adapted-bacteria

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The effectiveness of bioremediation processes may be limited by the physical and chemical properties of the pollutant, such as availability, recalcitrance, concentration and weathering, among others. The aim of this work was to evaluate the removal of recalcitrant oil fractions (aliphatic-aromatic and asphaltenic fractions) from a weathered soil, by two bacteria adapted to a high concentration of oil hydrocarbons, isolated from a soil with a concentration of 227,000 mg of total petroleum hydrocarbons per kg soil. Kinetics of hydrocarbons removal by *Bacillus coagulans* and/or *Serratia liquefasciens* was performed in liquid culture for 168 h; hydrocarbons from soil as sole carbon and energy source (600 mg/l) were added and each of the microorganisms was inoculated for evaluation independently or as a mixed culture. The aromatic fraction was removed by *B. coagulans* at 330 mg/l; by *S. liquefasciens* at 130 mg/l; and by both microorganisms at 360 mg/l. The asphaltenic fraction was removed by *B. coagulans* at 23 mg/l; by *S. liquefasciens* at 15 mg/l; and by both microorganisms at 34 mg/l. Chromatographic analysis of the aliphatic-aromatic fraction showed the presence of branched aliphatic C6 to C26, polyaromatic substituted compounds of two and three rings, and heteroaromatic compounds of dibenzothiphene type. The compounds that were removed from the aliphatic-aromatic fraction were of all types in the range of C6 to C13.

Key words: Asphaltenes, aliphatic-aromatic fraction, weathered, biodegradation.

INTRODUCTION

The processes of exploration and production, oil refining and storage have disrupted countless populations, causing serious environmental damage, and Mexico is no exception (Culbertson et al., 2008). The National Center for Disaster Prevention states that until 2004, the volume of oil spill accident and its derivatives were estimated as 1.5 million tons per year, affecting soil, water and atmosphere. Primarily, the Southeast of Mexico contains some contaminated soil hydrocarbons with concentrations up to 450,000 mg/kg (Gallegos-Martínez et al., 2000), exceeding the permissible norm of 1000 mg/kg in Mexico. In addition, weather conditions, concentration, type of pollutants and age of pollution hold back the effective implementation of bioremediation technologies. It is well known that light oil fractions are the first to evaporate so as to decompose, and aromatics, resins and asphaltenes are the most difficult to remove. In this context, the degree of weathering a ground potential can successfully predict the biological treatment where the compounds are more easily degradable often at very low or undetectable in highly weathered sites (Wilson and Jones, 1993; Boopathy, 2000). Furthermore, the low availability and strong adsorption of aromatics soil

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particles limit the bioavailability and its use as a carbon source for microorganisms, which plays a very important role in the treatment of these sites (Ke et al., 2003).

Until recently, most of the hydrocarbons degradation studies were conducted using their model compounds or mixtures such as naphthalene, phenanthrene, anthracene, acenaphthene and fluorene. Also they used pure cultures of bacteria, consortia and fungal (Strinfellow and Aitken, 1995; Sack et al., 1997; Prenafeta-Boldu et al., 2002; Anders et al., 2007). However, lately, the degradation assessment of the aliphatic-aromatic fraction from weathered soils has been highlighted (Aurélie et al., 2009; Teng et al., 2010) as well as the removal of polycyclic aromatic hydrocarbons of the heavy oil (Vila and Grifoll, 2009), translated to the removal research in real places, where there are a large number of compounds considered as intermediate biodegradability.

Asphaltenic fraction along with resins is a group of compounds that remain unchanged throughout the period of weathering. Some authors have considered them recalcitrant to biological degradation (Bertrand et al., 1983; Rontani et al., 1985, Westlake et al., 1974; Walker et al., 1976), while others reported as high as 74% removal from 6 g/l concentration; 45.5% from 0.4 g/l and even up to 96% starting from an initial concentration of 2.5 g/l (Bertrand et al., 1983; Rontani et al., 1985; Hager et al., 2012). As can be seen, the available studies show significant differences in the results. However, it is known that asphaltene is one of the most recalcitrant compounds in nature, hence it is important to study its biodegradation.

Based on this, it is important to provide knowledge related to the potential biodegradability of hydrocarbons fractions from weathered sites, considered as recalcitrant by some authors. Therefore, in this study, we evaluated the removal of aliphatic-aromatic and asphaltenic fractions from weathered soil through *Bacillus coagulans* and *S. liquefasciens* bacteria which were previously isolated from the same soil.

MATERIALS AND METHODS

Substrate and microorganisms

The total petroleum hydrocarbons (TPH) used as carbon source on degradation systems were extracted from a soil with a weathered contamination (220,000 mg TPH/kg soil) from Cactus Oil Field, located in Chiapas, Mexico. The microorganisms used were *B. coagulans*. and *S. liquefasciens*, which were previously isolated from the soil from which hydrocarbons were obtained (Rojas-Avelizapa et al., 2002).

Preparation of inoculums

The inoculum was prepared in 125 ml Erlenmeyer flasks using 25 ml of mineral medium (g/l): 1 KNO3 0.02 FeCl3, 0.2 MgSO4, 0.1 CaCl2 and 1 K2HPO4, pH 6.8. The TPH was added to the culture medium at a concentration of 600 mg/l (420 mg aliphatic-aromatic/l and 180 mg asphaltenes/l). Finally, the culture medium was inoculated with a loop of microorganism for evaluation. Cultures were incubated at 28°C and 180 rpm, making a pass every 24 h for three days; 0.1 ml of the culture was taken and grown in fresh medium in order to have younger cells.

Kinetics of growth and TPH removal

In 125 ml Erlenmeyer flasks containing 25 ml of mineral medium liquid, hydrocarbons were added to a concentration of 600 mg/l as the sole carbon and energy source and inoculated with 0.1 ml of fresh culture. The flasks were incubated at 28°C and 180 rpm during different times for 168 h. Every 6 h, two flasks were removed for analysis of residual hydrocarbons and evaluation of growth by viable count. To evaluate abiotic loss, uninoculated controls were included. The kinetics was performed in duplicate.

Extraction of hydrocarbons in liquid culture

Total petroleum hydrocarbons were quantified, and then the residual hydrocarbon content in flasks was subjected to a liquid-liquid extraction using dichloromethane as a solvent. The total content of the flask was transferred to a separation funnel (250 ml), and for this purpose, the hydrocarbons were extracted by five successive additions (30 ml) of dichloromethane and then concentrated until they got dried in a rotary evaporator. The residue obtained was resuspended in 30 ml of hexane (Burdick and Jackson) and sonicated for 10 min to precipitate asphaltenes, which were recovered by filtration on Whatman No. 1 paper and weighed. The hexane soluble fraction was analyzed by gas chromatography-flame ionization detector (GC-FID), where 1 µl was injected into an Agilent 6850 Series GC System chromatograph with a capillary column HP-1 methyl siloxane (30 m x 0.25 µm x 320 µm). The equipment was operated under the following conditions: temperature of 30°C for 1 min; 30 to 100°C/15°/min, 100 to 200°C/7°/min and 200 to 250°C/6°/min, and injector temperature of 250°C; helium was used as carrier gas at a flow of 1.5 ml/min. Aromatic hydrocarbon removal was determined by differences in areas under the curve, using the area obtained for the control (no treatment) as a reference.

Asphaltene removal percentage was calculated by difference in weight of the samples by comparing the kinetics with an untreated control. Modifications to the asphaltenes were evaluated by infrared spectroscopy (IR), wherein the sample is mixed with anhydrous KBr (IR grade) in a 1:131 ratio for analysis on a Nicolet Nexus 470 equipment. Reading was performed in the range of 4000 to 400 cm^{-1}. The observed changes due to treatment are reported as changes in the signal.

RESULTS

The results in Figure 1 show the biodegradation of the aliphatic-aromatic fraction (determined by gravimetry and GC) due to the growth of *B. coagulans* and *S. liquefasciens,* using TPH as sole carbon source. Also shown is the growth of pure strains and mixed culture. Growth curve of *B. coagulans* (Figure 1A) showed an

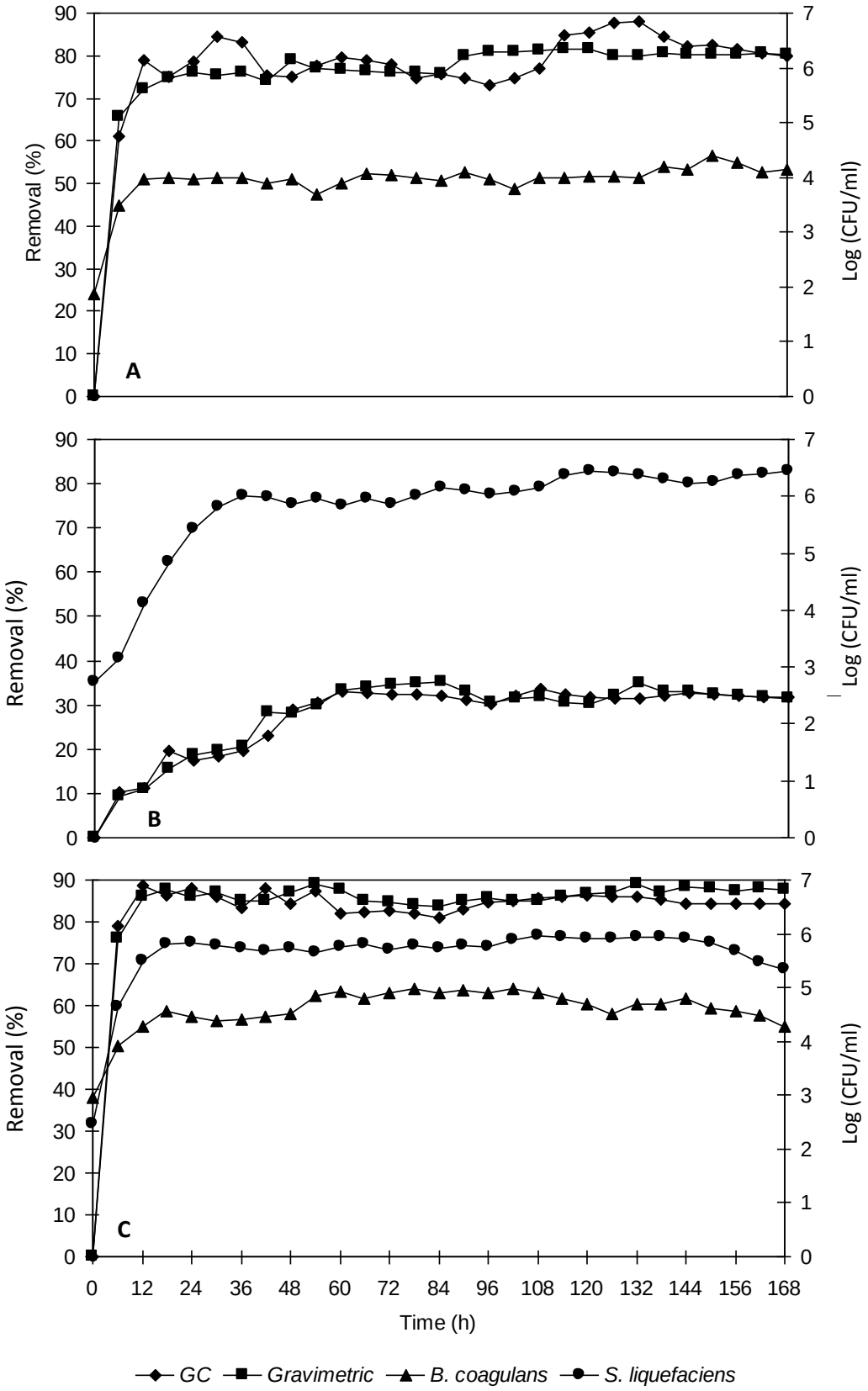

Figure 1. Curves of growth and removal of the aromatic fraction contained in the TPH by: **A)** *B. coagulans*. **B)** *S. liquefaciens*. **C)** *B. coagulans* and S. *liquefaciens* at 28°C and 180 rpm during 168 h of incubation.

exponential phase during the first 12 h, which increased its population from $0.77x10^2$ colony forming units (CFU)/ml to $9.22x10^5$ CFU/ml, and subsequently maintained a stationary phase until 168 h of incubation. During the period in which incubation was maintained, the death phase of this strain was not observed, although the fraction removal was conducted during the exponential growth phase of *B. coagulans*, leading to the removal of 330 mg/l of the aliphatic-aromatic fraction, which corresponded to 55% of initial content.

Figure 1B shows the growth curve of *S. liquefasciens* also using TPH as sole carbon source, and removal curves of the aliphatic-aromatic fraction. The growth curve shows a lag phase of 6 h and then the exponential phase for the next 30 h, with a population increase from $8.8x10^3$ to $2.1x10^6$ CFU/ml. Removal of the fraction was measured by gravimetric having a loss of 30% (122 mg/l) during the first 48 h; matching with the exponential growth phase of *S. liquefasciens*. These results show a Pearson linear correlation of 0.943 between bacterial growth and substrate utilization. The results obtained by gas chromatography show similar results, obtaining a removal of 31% (126 mg/l) of the aliphatic-aromatic fraction after 48 h of incubation.

Figure 1C shows the growth curves belonging to *B. coagulans* and *S. liquefasciens* used as mixed culture. These results show that *S. liquefasciens* presented its exponential phase during the first 18 h of incubation with a growth from $1.58x10^2$ to $7.94x10^5$ CFU/ml, and then remained at stationary phase up to 144 h. Furthermore, *B. coagulans* population increased from $1.99x10^2$ to $6.3x10^4$ CFU/ml during the first 18 h of incubation. The removal of the aliphatic-aromatic fraction due to treatment by the mixed culture was 80% (326 mg/l) during the first 10 h, but at the end of the kinetics increased to about 90% (360 mg/l), mainly shifting compounds from C6 to C15.

In Figure 2, the chromatogram profiles for the removal of the aliphatic-aromatic fraction are presented to demonstrate the type of compounds removed. Thus, the chromatographic profile of Figure 2A corresponds to the initial time and reveals the total compounds present in the aliphatic-aromatic fraction before treatment; when compared with the treatment profile by *B. coagulans* (Figure 2B), it shows a significant loss of TPH, which according to the quantifications corresponded to a removal of 81% (330 mg/l). The compounds removed were from both types of aliphatic- aromatic and heteroaromatic, being observed from the chromatographic profile that was modified in concentration with the exception of a compound that has 13 carbon atoms and a retention time of 15.7 min.

Meanwhile, treatment with *S. liquefasciens* (Figure 2C) made the light compounds with retention times less than 15.0 min (C6 to C13) to be removed mostly, thereby chromatographic profile was modified in both concentration and composition. Using both bacteria as mixed culture had a synergistic effect, because the chromatographic profile showed the effect of both bacteria, that is, the consumption of almost all compounds except the one with a retention time of 15.7 min (Figure 2C). Figure 3 also shows the growth curves of both bacteria, both in pure culture and in mixed culture using the TPH as sole carbon source, in order to perform the analysis of the asphaltenic fraction removal. Figure 3A shows the growth curve of *B. coagulans* and the removal caused in the asphaltenic fraction during the treatment, which was carried out substantially during the first 36 h, where a removal of 12.5% was observed (23 mg/l). Figure 3B relates the growth of *S. liquefasciens* and the removal of the asphaltenic fraction, showing 7% (13 mg/l) removal after 60 h of incubation period covering the exponential phase and part of the stationary phase of *S. liquefasciens;* and at the end of 168 h of incubation, a removal percentage of 8% (15 mg / l) was reached.

Figure 3C shows growth curves of *B. coagulans* and *S. liquefasciens* in mixed culture in which it was observed that most of the removal of the asphaltenic fraction (18% equivalent to 34 mg/l) was carried out during the first 36 h of incubation, correlating the removal with the exponential growth of both bacteria (P=0.954 and P=925, correlation factor between growth and removal of *B.coagulans* and *S. liquefasciens*, respectively), so that hydrocarbon consumption was associated to the growth of bacteria.

DISCUSSION

In previous studies, aromatic and asphaltenic fractions degradation was reported using a mixed inoculum consisting of seven bacteria and two fungi contained in the weathered soil from which the hydrocarbon fractions used as carbon source were extracted. However, it was found that only two bacteria identified as *S. liquefasciens* and *B. coagulans* were able to grow in both fractions (Rojas-Avelizapa et al., 2002). In the present results, the involvement of each of these bacteria in the degradation process was analyzed.

According to the results, both pure bacteria were able to remove the aliphatic-aromatic fraction. However, *B. coagulans* was more efficient than *S. liquefasciens* in reducing the content of the aromatic fraction in the culture medium. Furthermore, *B. coagulans* fully degraded C6 to C26 compounds while *S. liquefasciens* degraded only C6 to C8 compounds (Figure 2A, B). The entirely degraded compounds correspond to monoaromatic and polyaromatic compounds, some substituted of two and three rings and heteroaromatic compounds of the dibenzothiophene type, which clearly correspond to the type of hydracarbons found in weathered contaminations. Alkylated polyaromatic compounds have been less studied than unsubstituted; however they are much more abundant than unsubstituted polyaromatic (Vila and Grifoll, 2009). So, their degradation by these soil native

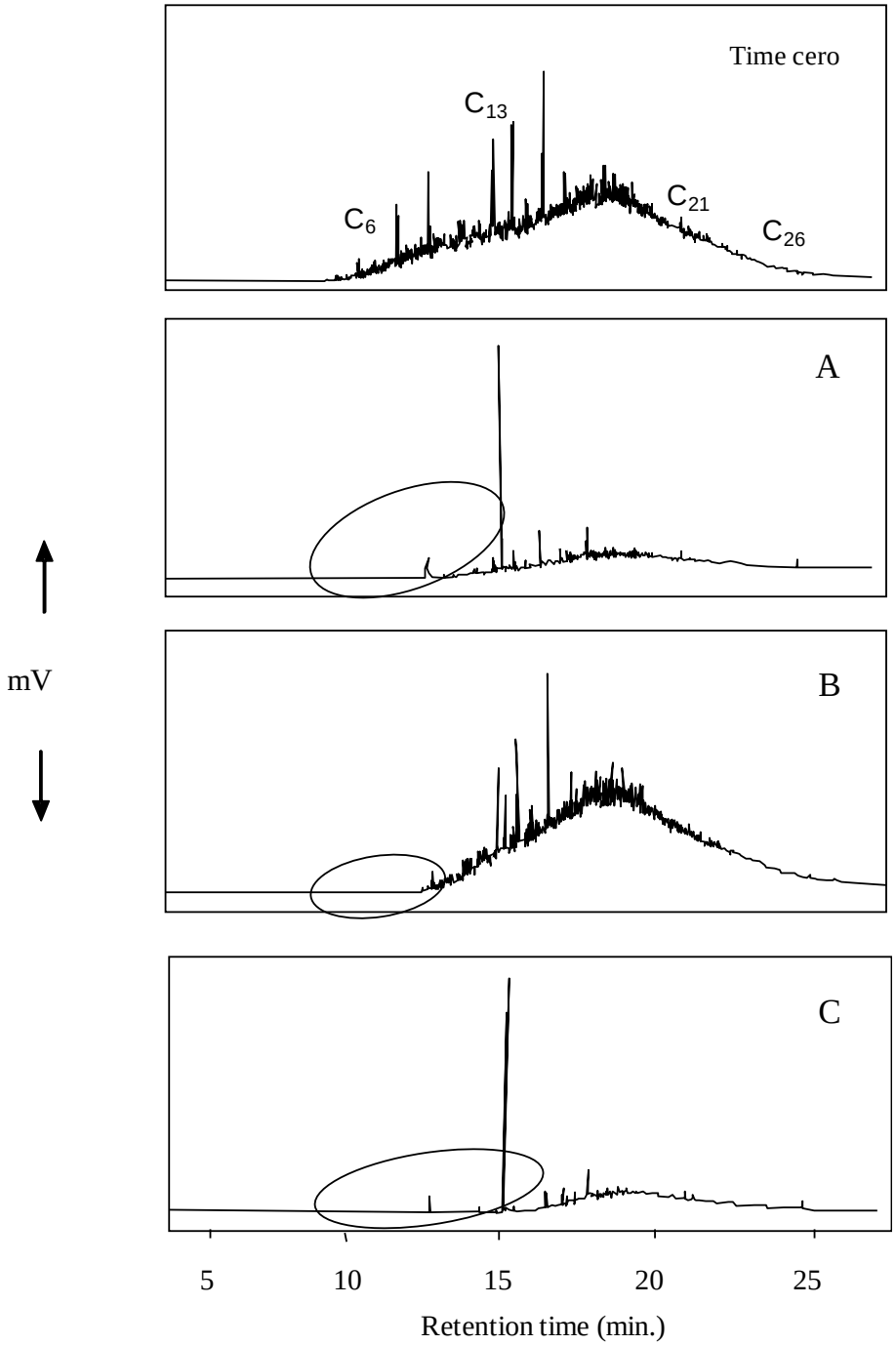

Figure 2. Chromatographic profile of removal of the aromatic fraction contained in the TPH used as source of carbon. **A)** *B. coagulans*. **B)** *S. liquefaciens*. **C)** *B. coagulans* and *S. liquefaciens* at 28°C and 180 rpm during 144 h of incubation.

microorganisms involves their real biodegradability; though the problem of bioavailability in the soil matrix remains a barrier to be overcome by various physico-chemical processes.

The genus *Bacillus* has been reported as part of some communities associated with degradation of petroleum hydrocarbons. It is particularly mentioned that the Gammaproteobacteria are dominant right after a process of contamination, and are subsequently displaced by Bacilli and Alfaproteobacterias (Bordenave, 2007), reported to be involved in the processes of some advanced degradation. *S. liquefasciens* has been

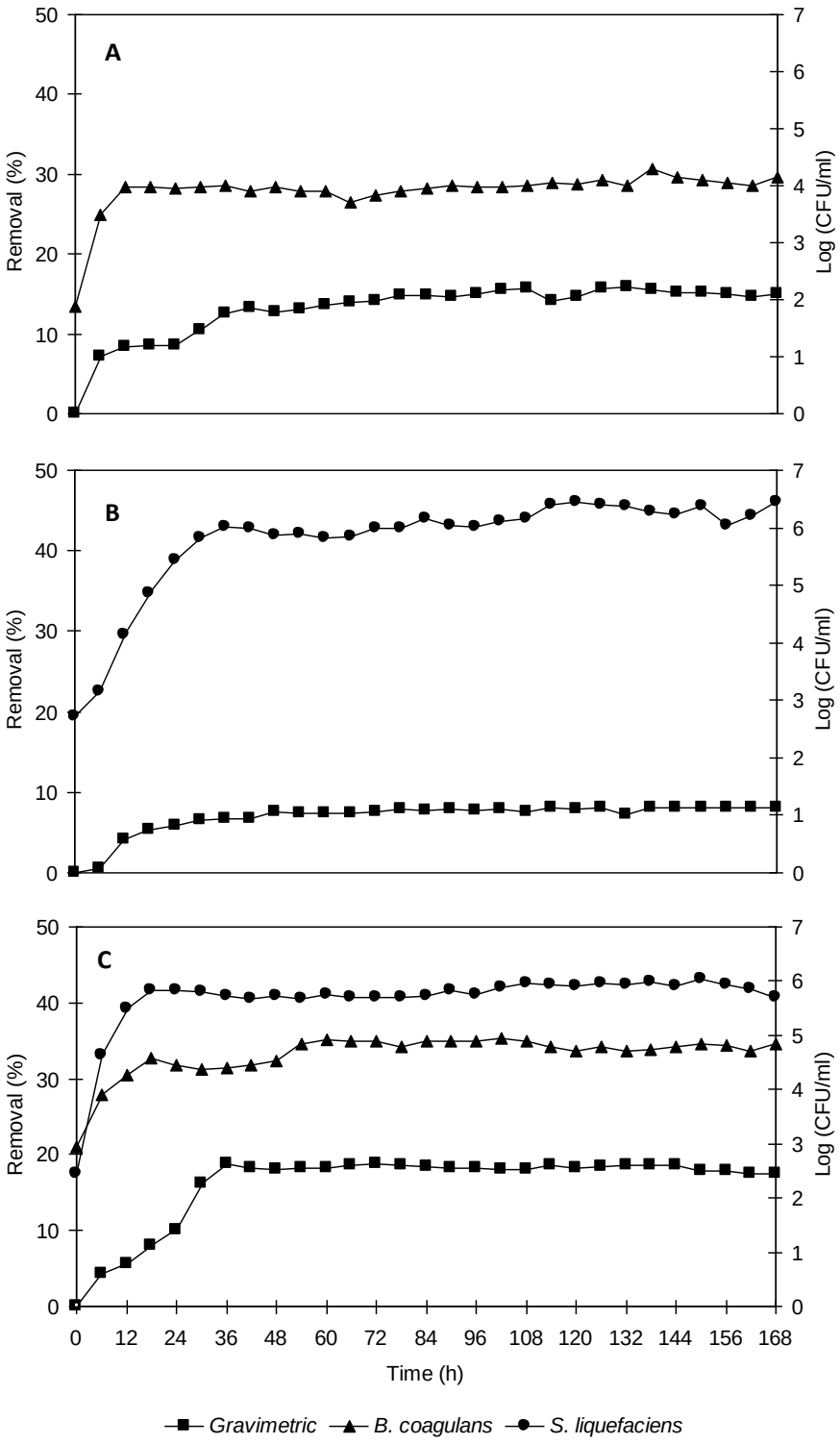

Figure 3. Curves of growth and removal of the asphaltenic fraction contained in the TPH. **A)** *B. coagulans*. **B)** *S. liquefaciens*. **C)** *B. coagulans* and *S. liquefaciens* at 28ºC and 180 rpm during 168 h of incubation.

reported along with *Bacillus* in removal processes of polycyclic aromatic hydrocarbons (PAH) in soils (Mohsen et al., 2009). Furthermore, it has been speculated that lignolitic enzymes play an important role in the degra-

dation of PAH (Haritash and Kaushik, 2009), which have also been detected both in *Bacillus* and in *S. liquefasciens*. Therefore, both bacteria have characteristics of removing aromatic compounds as demonstrated in this investigation.

The use of both bacteria as mixed culture showed a minimal increase in degradation of the aliphatic-aromatic fraction compared with that only obtained by *B. coagulans*. However, this higher degradation was statistically significantly ($P < 0.05$). Although it is suggested that the degradation of the two bacteria is not numerically additive when mixed culture is used, the result of the degradation is an effect of both bacteria. There are few reports on the degradation of the aromatic fraction that evaluates the role of each of the microorganisms involved. Generally, this degradation is usually carried out by consortia from different oils with different degrees of weathering, which leads to the variation in the composition of the fraction (Walker et al., 1976; Sudarat et al., 2000; Alonso-Gutierrez et al., 2009).

Regarding the asphaltenic fraction, it is known that such compounds are very recalcitrant and complex molecules with a high molecular weight. Only few studies have reported their biodegradation. Pineda-Flores et al. (2004) reported the use of asphaltenes as a carbon source by a consortium of bacteria of the genera *Corynebacterium*, *Bacillus*, *Brevibacillus* and *Staphylococcus*. Particularly, the genus *Bacillus* has been reported in asphaltene degradation processes (Hager et al., 2012) and as a member of the native biota of a highly polluted site of asphalts (Kim and Crowley, 2007).

An important point is the detailed analysis of the modifications these molecules undergo after biodegradation process. In this study, analysis of the infrared spectra showed changes in the absorption bands at different links of the structure of the asphaltene at the end of the degradation period, thus suggesting that it was caused by its use as a carbon source, because controls had no modification. The results show that in the three biodegradation processes (individual pure strains and mixed culture), these modifications were due to symmetric CH_2 bending (1467 cm^{-1}), asymmetric CH_2 bending (1450 cm^{-1}), symmetric CH_3 bending (1378 cm^{-1}), weak symmetric stretch C=C-C=C (1640 cm^{-1}), C=C stretching (1642 cm^{-1}), aldehyde C=O stretching (1724 cm^{-1}), C=C stretching in aromatic ring (1460, 1508, 1546, 1564 and 1605 cm^{-1}) and =CH links outside the plane (741 cm^{-1}). Regarding this, Rontani et al. (1985) reported the analysis of asphaltenes by IR and observed variation after a treatment by means of a microbial consortium in the absorbing areas of 3640 to 3100 cm^{-1} and 1720 to 1690 cm^{-1}. These correspond to -OH and -C=O stretches, respectively, showing significant oxidation with this substrate. During the kinetics, it was found that *S. liquefasciens* and *B. coagulans* removed about the same amount of asphaltenes, not showing significant difference between the results; neither was there significant difference with obtained degradation when the two bacteria were used as mixed culture ($P < 0.05$). The low degradation of asphaltenes (18% equivalent to 34 mg/l) is attributed to the use of TPH extracted from weathered soil formed by aliphatic and aromatic fraction; with the two available fractions, the one with the easiest assimilation was largely degraded. However, when using pure asphaltenic fraction (600 mg/l), the pure culture obtained a removal of 48% (288 mg/l) (data not shown).

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