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Degradation of Aromatic and Asphaltenic Fractions by *Serratia liquefasciens* and *Bacillus sp.*

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Oil extraction and processing became a national priority for Mexican government during the decades of '50s and '60s however not taking into account the negative impact these processes have on the environment. Oil spills and oil wastes extracted during that time, remain on the soils especially around oil fields (CIMADES, 1997; Vinalay et al, 1997).

On the other hand, most bioremediation efforts have been focused on the degradation of aliphatic and lightly aromatic fractions leaving the polyaromatic and heavy fractions with no changes. This degradation implies a decrease in the content of petroleum hydrocarbons; but many of those compounds hazardous to the environment and human health remain (PEMEX, 1997). Although it has been demonstrated that crude oil is biologically degraded by widespread microorganisms (Watkinson and Morgan, 1993), some data suggest that degradation of the heavy hydrocarbons in soils, is limited when a specific microbial population involved in such degradation is not present, or when specific microorganism populations are not large enough to perform the polyaromatic and heavy hydrocarbon degradation. Also, physico-chemical conditions of the soil are important factors that affect microbial activity.

In this study, a microbial mixed culture from a highly polluted soil was obtained by enrichment culture, using the recalcitrant aromatic and asphaltenic fractions as sole carbon sources. The culture growing capability on these former mentioned fractions was evaluated along with its degradation abilities. The existence of specific microflora involved in the degradation of each fraction was studied.

MATERIALS AND METHODS

The studies were carried out with a petroleum hydrocarbon polluted soil, which was collected from a site in Cactus, Chiapas nearby the petrochemical facilities. After the collection, the soil was homogenized and its physicochemical properties were determined as described earlier (Cervantes-Gonzalez et al, 2001). The soil was used as a source of microorganisms as well as a supplement of aromatic and

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asphaltenic fractions. Colony and morphological characteristics of bacteria and fungi isolated from the mixed culture were observed by conventional techniques (Cervantes-Gonzalez et al, 2001). Only those microorganisms involved in fraction degradation were identified by biochemical assays according to Bergey's Manual (Holt et al, 1994) and an identification system Api 20E (BioMerieux, France).

Aromatic and asphaltenic fractions were extracted from a ten grams sample of polluted soil, in a Soxhlet apparatus using dichloromethane for 8 hours. Afterwards, the organic extract was concentrated until dryness. The total content of petroleum hydrocarbons was determined by weight difference (gravimetric). Dried extracts were added with 60 ml of hexane and sonicated during 10 minutes in order to precipitate the asphaltenic fraction. It was then collected by filtering through a Whatman paper No. 1. A solid sample (asphaltenes) was recovered and asphaltenic fraction was blended with KBr (infrared grade) to a ratio of 1:131 and analyzed by infrared spectroscopy in a Nicolet Nexus 470 FT-IR. Transmittance range was established at 400 to 4000 cm^{-1}.

The hexane-soluble fraction was separated (aromatic fraction) and analyzed by infrared spectroscopy (IR) and gas chromatography (GC) by a procedure described as follows: the fraction was concentrated to dryness and then suspended in 10 ml of carbon tetrachloride. Afterwards, the sample was diluted and quantified according to EPA Method 8440 (EPA, 2000). The total petroleum hydrocarbons (TPH's) quantification was performed in a Nicolet Nexus 470 AEP9900233 FT-IR E.E.S. Three hydrocarbon-characteristic peaks in the range of 400 to 4000 cm^{-1} were quantified.

One microliter of the aromatic fraction was also injected and analyzed by GC with a flame ionization detector in an Agillent gas chromatograph Series 6850 GC System, using a HP-1 capillary column of methyl siloxane (30 m x 320 μm x 0.25 μm). Method conditions were column temperature 30°C/ min, 30°-100°C at 15°/min; 100°-200°C at 7°C/min; 200°-250°C at 6°C/min; injector temperature 250°C and detector temperature 280°C. Helium was used as a carrier gas at a flow rate of 1.5 ml/min.

Inoculum culture was performed in 125-ml Erlenmeyer flasks at 28°C and 180 rpm in a mineral salt media containing (g/L), KNO_3, 1; $FeCl_3$, 0.02; $MgSO_4$, 0.2; $CaCl_2$, 1; K_2HPO_4, 1; and 600 mg of aromatic or asphaltenic fractions as the sole carbon sources and supplemented with 50 mg of yeast extract (Rojas-Avelizapa et al, 1999), pH was adjusted to 6.8. When the culture broth reached the logarithmic phase, 0.1 ml of the broth were transferred to fresh media.

Two experimental sets were prepared for the biodegradation studies either containing the aromatic or the asphaltenic fractions, both containing 25 ml of mineral salt media in 125 ml Erlenmeyer flasks. Both fractions were added to a concentration of 600 ppm. Culture broths were incubated at 28 °C and 180 rpm, during 102 hours for the aromatic fraction and 168 hours for asphaltenes. Samples

were collected from two flasks each six hours to quantify microbial growth by viable count as well as hydrocarbon degradation.

After the biodegradation phase, aromatic and asphaltenic fractions were subjected to liquid-liquid extraction using 75 ml of dichloromethane and the total content of the culture media. Organic phases were dried. Initially, degradation of aromatic and asphaltenic fraction was determined by weight difference, then by IR as described earlier. Finally, the aromatic fraction was analyzed by GC. Asphaltenic fraction degradation was determined by the changes observed (IR) in a sample spectrum at the beginning and end of the treatment. Aromatic fraction degradation was quantified as TPH's and by comparing the difference in peak areas obtained by GC at the beginning and end of the treatment (Mondello, 1989).

RESULTS AND DISCUSSION

Evaluation of the soil's physical and chemical characteristics showed it was sandy and acidic (pH 3.6). The native soil hydrocarbon content was high 232.7 g/kg of dry soil. According to reports, this hydrocarbon amount could strongly interfere with hydrocarbon bioremediation experiments (McAllister et al, 1995). By the other hand, some unpublished data (*personal communications*) suggest that a concentrations about 20 g/Kg of dry soil, hydrocarbon biodegradation can occurs. In this regard, the knowledge of microorganisms present in this soil is important, also their ability to degrade hydrocarbons under laboratory conditions with the aim to enriching the microbial population and achieving a satisfactory hydrocarbon degradation. Soil water content is also rather low (2.6%), which could represent a very low or the absence of microbial activity. Sand texture facilitates the fluid transfer allowing an appropriate aeration and so enhancing soil microbial activity (Frankenberger, 1992). Soil pH is rather low (3.6). This value is not appropriate for bacterial growth, which is reported in a range of 6-8.

Seven bacteria and two fungi composed the mixed culture isolated from the soil. It is important to point out that five out of the seven bacteria were bacilli and two cocci and four of them produce pigments. Both isolated fungi were able to produce pigments.

Results from the mixed culture growth on the aromatic fraction as the sole carbon source are illustrated in Figure 1. Only two morphologically different microorganisms were able to grow which growth curves are presented. One of them was identified as *Serratia liquefasciens* and the other corresponds to *Bacillus sp*. The curves indicated that the culture broth did not present an adaptation phase. *S. liquefasciens* showed a growth from 3.8×10^4 CFU/ml to 1×10^8 CFU/ml after 24 hr of incubation. *Bacillus sp*, presented a growth from 7.7×10^3 CFU/ml to 1×10^6 CFU/ml after 36 hr of incubation, suggesting that both microorganisms are able to use some compounds found in the aromatic fraction as sole carbon and energy sources. *Bacillus sp* growth decreased after 66 hr of incubation (Fig 1). This situation could arise due to the accumulation of metabolic

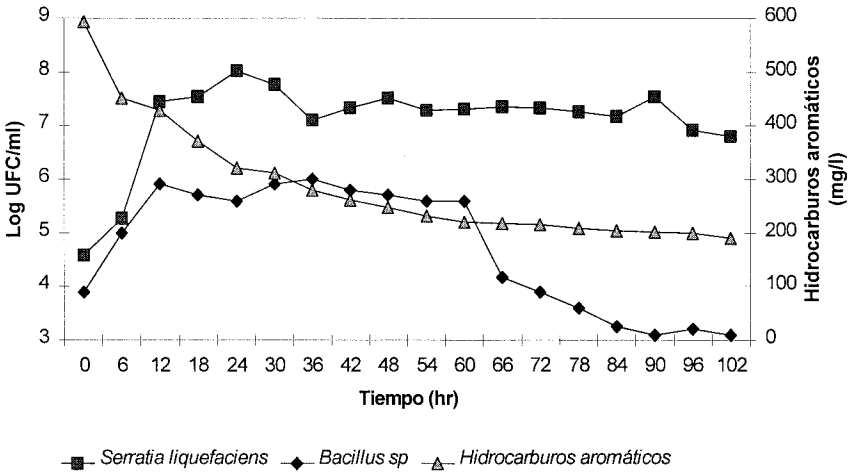

Figure 1. Growth of mixed culture on aromatic fraction as the only carbon source **CFU:** colony forming units.

waste products. For *S. liquefasciens*, its growth decreased lightly after 36 h of incubation, but it was constant during the incubation period.

Gravimetric results of the degradation of aromatic compounds by the mixed culture in liquid media are shown in Figure 1. The content of aromatic hydrocarbons decreased greatly during early treatment, coinciding with the logarithmic phase of both microorganisms. This strongly suggests that these compounds are being used. The decrease of hydrocarbons corresponded to a loss of 46% (272 mg/L) with respect to the control sample. After this period, the hydrocarbon degradation continued reaching 68% (402 mg/L) after 102 h of incubation. Abiotic loss corresponded to only 4.2 % (25 mg/L) IR results demonstrated that the removal percentage for the aromatic fraction at 24 hr corresponded to 30% (177 mg/L) rather than the 46% (272 mg/L).

In the case of the gravimetric determinations, the same situation occurs with the sample from 102 hr of incubation, in which removal corresponded to 64% (379 mg/L) rather than the 68% (402 mg/L) with respect to the control treatment. It was also interesting to note that absorption bands in the infrared spectra corresponding to C-H bonds presented differences for the treatment (data not shown).

Knowing that aromatic hydrocarbons were really degraded at a high percentage, a GC analysis was performed to determine the type of compounds that were preferentially attacked by the mixed culture and the removal percentage quantified. Results obtained by GC-FID are illustrated in Table 1, which summarizes compounds that were degraded according to their retention times. Data was obtained on the basis of the integration area of each peak. Most aromatic

Table 1. Aromatic compounds degraded during the treatment by the mixed culture obtained from the contaminated soil and analyzed by gas chromatography

Retention time (min)	Degradation (%)	Retention time (min)	Degradation (%)
21- 21-9	69	28- 28.9	44-78
22- 22.9	41-83	29-29.9	44-75
23- 23.9	43- 100	30-30.9	42-100
24-24.9	49-62	31-31.9	55-100
25-25.9	48-55	32-32.9	96
26-26.9	22-78	33-33.9	95-100
27-27.9	59-100	34-34.9	95

compounds, present in the initial sample, were degraded from 22 to 100% (130-592 mg/L) obtaining an average a removal of 67% (397 mg/L). It is important to mention that compounds whose areas were less than 100 units are not included since they were not integrated.

Biodegradation results for the aromatic fraction obtained by the three methodologies, pointed out clearly that the aromatic fraction was removed at a high percentage by the mixed culture from the polluted soil after 102 hr of treatment.

Many bacteria, filamentous fungi, yeast and some algae can metabolize hydrocarbons (Watkinson and Morgan, 1993). However, the rate and extent of biodegradation differs greatly depending on the environmental conditions, type and polluted mixture.

Data generated for this study demonstrated that the aromatic compounds in the range of C_{10}-C_{30} (data not shown) were removed in a short period of treatment (102 hr), as compared to other studies, where long periods of treatment were mentioned (Kennicutt, 1988; Potter and Duval, 2001). It is important to note that although seven bacteria and two fungi composed the mixed culture, only two were able to grow and use the aromatic fraction: *Bacillus sp* and *S. liquefaciens.*

In agreement to existing reports, *Bacillus* genera are frequently identified as active in aliphatic and aromatic hydrocarbon degradation (Cookson, 1995). In the case of *Serratia* genera, most degradation studies have been reported using *Serratia marcescens*. Fetzner and Lingens (1993) reported the metabolism of quinolein and their derivatives by this microorganism. De la Fuente et al (1991) mentioned the participation of *S. marcescens* in the oxidation of aromatic aldehydes to carboxylic acids and the oxidation of alpha pirene. There is only one report found in the literature concerning to the *Serratia* participation in biodegradation studies of petroleum hydrocarbon as reported by Salgado (1996), who made the isolation and identification of a *S. marcescens* strain in a mixed culture able to degrade Maya petroleum in liquid culture at an extent of 52%. In particular, *S.*

liquefasciens has not yet been reported, as being involved in the degradation of hydrocarbons or any toxic compounds. Further studies could demonstrate the participation of each strain in hydrocarbon degradation. However, it is important to point out that the major growth on the aromatic fraction was observed for *S. liquefasciens*.

For asphaltenic fraction biodegradation, the results obtained were plotted in two growth curves (Fig 2), which showed that the cultures grew fast. Growth for *S. liquefasciens* was from 7.9 x 10^4 CFU/ml to 7.9 x 10^7 CFU/ml after 60 hr of incubation. *Bacillus sp* showed a growth of 8.7 x 10^2 CFU/ml to 2.9 x 10^6 CFU/ml after 24 hr of incubation. It was also noted that *Bacillus sp* viability fell at 60 hr of incubation, which could be due to the accumulation of metabolic products secreted by this bacteria or by *S. liquefasciens* which had a constant growth for all incubation period.

Results of gravimetric determination of asphaltene biodegradation are illustrated in Figure 2. The fraction was degraded at an extent of 50% (296 mg/L) after 48 hr of treatment by the mixed culture. This removal percentage is in well agreement with the exponential phase of the mixed culture and remained with no changes for the incubation period. Comparing the results of asphaltene degradation by microbial pathway and abiotic loss, it was observed that asphaltenes were degraded at 50% (296 mg/L) since the abiotic loss was 3.6 % (21.6 mg/L).

The analysis of the asphaltenic fraction by IR permitted the identification of those absorption bands that suffer modifications due to the biological treatment. Table 2 summarizes the main absorption bands that were modified to some extent. Most bands showed a decrease in absorbance, which could indicate a modification in the asphaltene structure. Bands that suffered major modifications are those in the range of 1500 cm^{-1}. At 2400 cm^{-1} an important loss was observed. The results demonstrated that the asphaltenic fraction was removed to 50% by the mixed culture after 168 hr of treatment.

Table 2. Main absorption bands of asphaltenic fraction identified and degraded by infrared spectroscopy

Wavelength (cm^{-1})	Zero time Abs. (nm)	Final time Abs. (nm)	Bond type
1460	0.1466	0.1290	C=C aromatic ring
1508	0.1338	0.0955	C=C aromatic ring
1546	0.1230	0.0940	C=C aromatic ring
1564	0.1278	0.0942	C=C aromatic ring
1873	0.0238	0.0066	Ni
2326	0.0348	0.0094	Ni
2853	0.0091	0.1366	C-H in CH_2
2930	0.1354	0.1472	C-H in CH_2
3580	0.0620	0.0462	Ni

Ni = non identified

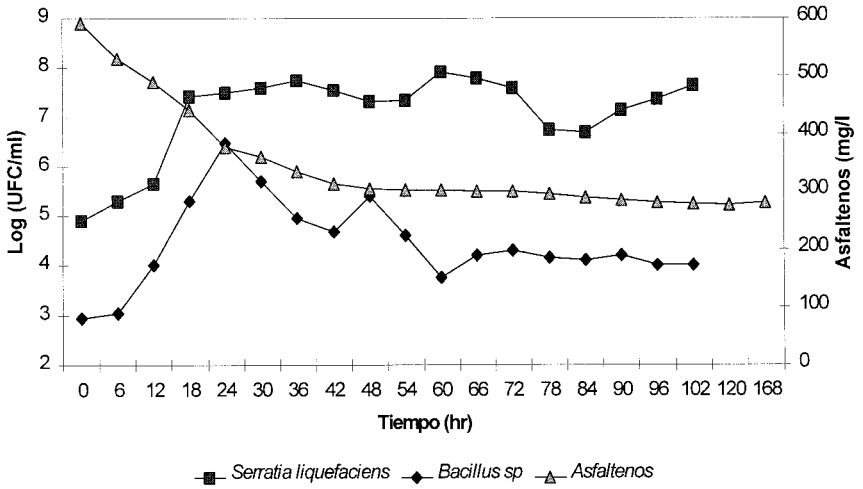

Figure 2. Growth of mixed culture on asphaltenic fraction as the only carbon source **CFU:** colony forming units.

Degradation pathways of asphaltenes have not been defined due to the complex structure of these compounds and the scarce information available on the subject (Rontani et al, 1985). Some authors consider that these compounds are recalcitrant to a microbial attack. However, other research groups have reported that asphaltenic fraction was partially degraded by a marine mixed bacterial population with saturated hydrocarbons as cosubstrates (Rontani et al, 1985). The results presented in this study are very promising as they demonstrate that asphaltenes can be degraded by a microbial pathway. The mixed culture had the ability to remove approximately 50% (292 mg/l) of asphaltenes during a short period of time (168 hr), which represents an important contribution. However, attention must be drawn to the type of asphaltenes used during the aforementioned studies, since these compounds vary according to their origin. Another important contribution of this work was the participation of *Bacillus* and *S. liquefasciens* in the degradation of asphaltenes, which has not been reported before.

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