Biodegradation **15:** 145–151, 2004.

A microbial consortium isolated from a crude oil sample that uses asphaltenes as a carbon and energy source

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Accepted 20 October 2003

Key words: asphaltenes, biodegradation, microbial consortium, mineralization, petroleum

Abstract

A microbial consortium capable of mineralizing asphaltenes was obtained from the Maya crude oil. The enrichment system was built with a glass column reactor containing mineral medium supplied with asphaltenes as energy and carbon source. The consortium growth was evaluated in Casoy agar during 40 weeks. The steady- state phase of the enriched bacterial community was observed after 10 weeks when the culture reach 10^5 to 10^6 CFU ml^{-1}. The isolates belong to bacterial genus reported for degradation of other hydrocarbons and they were identified as *Corynebacterium* sp., *Bacillus* sp., *Brevibacillus* sp. and *Staphylococcus* sp. The bacterial consortium growth was evaluated by a viable counts during 14 days exposed to different aeration, temperature, salinity, and pH conditions. The ability of the consortium to mineralize asphaltenes was evaluated using the method of ISO 9439 in glass column reactors of 20 × 3.2 cm during 13 days. Temperatures of 55 °C and salinity of 1.8% were growth limiting. The respiration of the microbial consortium using asphaltenes as a sole carbon source (800 μmoles CO_2 in 13 days) was significantly higher than those of the samples containing only the microbial consortium (200 μmoles CO_2) or only asphaltenes (300 μmoles CO_2). These results indicated the existence of asphaltenes-degradating microbes in the crude oil and confirmed that the consortium could mineralize asphaltenes in conditions of room temperature, salinity of 100 ppm, aeration of 1 l min^{-1} and pH of 7.4.

Introduction

Asphaltenes are petroleum hydrocarbons which contain nitrogen, sulfur and oxygen. Their molecular weight is between 600 and 2,000,000. The molecular structure is extremely complex. Several aromatic acyclic and heterocyclic structures are bonded by aliphatic hydrocarbons (Murgich et al. 1999; Strausz et al. 1999).

Asphaltenes cause major problems for the extraction, transport and processing of petroleum. They stick on the porous spaces on the surface of oil-wells and on the extraction and transport pipes, speeding down the oil flow (Kaminski et al. 2000; Wu et al. 2000). Also, when crude oil has a high concentration of these compounds, refining costs increase and its general economic benefit decreases (Rogel 1997; Shirokoff et al. 1997). Moreover, the complex molecular structure of asphaltenes makes them to resist biodegradation, causing their accumulation in ecosystems where petroleum and its refining byproducts are spilled in either accidental or purposeful ways (Atlas 1981; Guiliano et al. 2000). Microbial processes have long been known to be important in polluted habitats for the destruction of a large numbers of compounds (Alexander 1999). In addition it has been reported that microorganisms, exposed to asphaltenes during long periods, could have the ability to degrade these compounds. That is the case with microorganisms inhabiting in oil wells (Premuzic & Lin 1999; Margesin & Schinner 2001).

Biodegradation of asphaltenes through the use of a microbial consortium or mixed cultures isolated from soil samples and of sediments contaminated with hydrocarbons has taken place in low proportions of 0.55–35% (Venkateswaran et al. 1995; Thouand et al. 1999). However, the biodegradation of these compounds using microbial consortia isolated from petroleum has not been evaluated and these microorganisms are adapted to grow and thrive under environments with high concentrations of asphaltenes, and the effects of environmental factors on the microbial growth are not known yet.

The aim of the present work was to obtain a microbial consortium capable of using asphaltenes as a carbon and energy source from a crude oil sample and to determine the environmental factors which influenced their growth.

Materials and methods

Asphaltene extraction

The asphaltenes were obtained from Maya crude oil that contains more asphaltenes than the two other Mexican oils, Istmo and Olmeca. The Maya crude oil sample was obtained from the Blasillo-61 oil well, locating in the oil field of "El Encanto", in the state of Veracruz, Mexico. The depth of the well is 3323 m, with a temperature of 60 °C at the bottom and 20 °C at the surface. The well has 31% of water saturation and a salinity of 180,000 ppm.

The asphaltenes were extracted from the oil sample by precipitation with n-heptane (Sigma ACS) in a proportion of 50:1 (n-heptane/sample). The sample was kept in magnetic bar agitation for 18 h. Then, it was filtrated by vacuum using Whatman No. 42 filter. The precipitated asphaltenes were transferred from the filter paper to a porcelain capsule and it was dried at 70 °C for 12 h. Finally they were kept at room temperature in an amber colored sterile container for later use.

Growth stabilization of the microbial consortium from the "Maya" crude oil

A Pyrex glass column reactor, 12 cm in diameter by 73 cm in height, filled with volcanic debris with 10 mm of diameter as support material at 3/4 of the volume, was autoclaved at 1.02 kg cm^{-2} for 30 min. Then 2.4 l of sterile mineral culture medium ISO 9439 (KH_2PO_4, 0.085 g; K_2HPO_4, 0.21 g; $Na_2HPO_4 \cdot 2H_2O$, 0.33 g; NH_4Cl, 0.005 g; $MgSO_4 \cdot 7H_2O$, 0.0225 g; $CaCl_2$, 0.0275 g; $FeCl_3 \cdot 6H_2O$ traces (ISO 1990); distilled water, 1 l; pH 7.4) supplied with asphaltenes (24 g l^{-1}) was added into the reactor. The reactor was inoculated with 24 ml of the crude oil collected under aseptic condition from the Blasillo-61 oil well. The system conditions were constant sterile aeration of 1 l min^{-1} supply by an air pump, temperature of 25 °C, pH 7.4 and salinity of 100 ppm, during 40 weeks. Viable counts of bacteria, yeast, mould and actynomyces were done in Casoy agar (Merck 5458), Sabouraud agar, Bengal red agar and Czapek agar, respectively. The number of yeasts, moulds and actinomyces were evaluated only in the first 5 weeks and bacteria counts were kept till 40 weeks. The plates were incubated at room temperature during 48 h for the bacterial community and 14 days for the other ones. The ANOVA analysis was performed to compare the variation of CFUs and it was considered to be stabilized when no significant difference was detected.

To determine the bacterial adhesion to the supporting material, 30 volcanic debris were randomly picked up from the reactor column and were washed in 100 ml of sterile tween 80 solution (1% w/v). The solution was kept in mechanical agitation for five minutes. Then the bacteria viable counts were done as above.

Isolation and identification of the microorganisms in the consortium

The bacteria that grew in the Casoy agar during the stabilization of the microbial consortium were isolated by grid inoculation in Casoy agar plates. The isolates were characterized with Gram staining and morphology observation. The first step on the identification was done by biochemical tests (McFaddin 1980): aerobic and anaerobic growth, catalase and oxidase activity, mobility in SIM medium, oxidation-fermentation of glucose and production of acid and gas in phenol red broth with glucose. The final identification was obtained by the bacterial identification kit API-50CHB and the others specific biochemical test (Sneath et al. 1986).

Incubation conditions effect on the growth of the microbial consortium

A series of 3.2 cm in diameter by 20 cm in height Pyrex column reactors were prepared in the same manner as the reactor used for the stabilization of the consortium. They contained 60 ml of ISO 9439 medium plus 0.06 g of asphaltenes. The reactors were

inoculated with 6×10^5 CFU ml^{-1} of the stabilized microbial consortium.

Aeration, temperature, salinity and pH were tested as follow: (a) Aeration of 0, 0.1 and 1 l min^{-1}; (b) temperature of 37, 55 °C and room temperature; (c) salinity of 100, 90,000 and 180,000 ppm; and (d) pH of 6.4, 7.4 and 8.4. Salinity was adjusted using NaCl and the pH was adjusted by adding 1 N NaOH or HCl. The effect of these environmental factors on the consortium growth was evaluated by viable counts on Casoy agar. Three glass column reactors were used for each factor and the viable counts were done independently for each of them. When evaluating the effect of one variable, the others were kept constant for 14 days at the following values: aeration 1 l min^{-1}, room temperature, salinity 100 ppm and pH 7.4. The results were statistically analyzed using ANOVA test.

Evaluation of asphaltenes mineralization

Asphaltenes mineralization was measured by CO_2 evolution using the same reactors as mentioned above. Three treatments were used: (1) stabilized consortium (6×10^5 CFU ml^{-1}); (2) asphaltenes (0.06 g); and (3) stabilized consortium plus asphaltenes (6×10^5 CFU ml^{-1} and 0.06 g respectively). The reactors were incubated at the conditions that allowed the best growth of the microbial consortium.

The CO_2 production in each system was measured every day during a period of 13 days, by the ISO 9439 device (ISO 1990) with HCl titration according to Winkler method as follow: the CO_2 produced by mineralization was quantified with titers of 0.1 M HCl of 10 ml aliquots of the solution of 0.1 M KOH plus 40 ml of distilled water. Then 2 ml of $BaCl_2$ plus drops of phenolphthalein indicator were added. The produced μmoles CO_2 was calculated as follow:

$$\mu\text{moles } CO_2 = [(B\text{-}P) \times M_{HCl} \times 1000]/2$$

where B = usage in the blank system (ml); P = usage in the problem (ml); and M_{HCl} = HCl molarity.

Results and discussion

In order to get a stable consortium from the Maya crude oil we were able to follow several enrichments and sucesions of the microbial community by the measured of CO_2 evolution. Figure 1 shows tremendous viable counts at the beginning of the selective process. Highly abundant growth of chemoheterotrophic bacteria was observed in the reactors during the first two weeks of the microbial consortium stabilization. The growth could be supported for less complex hidrocarbons presents in the sample of Maya crude oil, such as alkanes and alkenes (Morgan & Watkinson 1994). When the less persistent hidrocarbons were metabolized, the growth decreased and then stayed constant between 10^5 and 10^6 CFU ml^{-1} after the fifth week. No significant differences in the bacterial counts were detected since that time.

The growth bacteria decrease observed from the second till the sixth week (Figure 1) must be the adjustment period for the enriched bacteria which are able to degraded asphaltenes. The growth of these bacteria keep constant after asphaltenes addition at different time. The presence of yeasts, moulds and actynomyces was done because it has been reported that some of them are capable of using crude oil as sole source of carbon and energy (Fukumaki et al. 1994; Launen et al. 2000; Ravelet et al. 2000). However, these possibility was discarded after sampling for 5 weeks and giving then 14 days of incubation period. The bacteria that were able to growth in those media were the same that growth at Casoy agar. For this reason only Casoy agar was used to evaluate growth of the consortium in the following weeks.

It is possible that the environmental conditions in the Blasillo-61 oil well inhibit the growth of fungi and actinomycetes and enhance the growth of bacterial populations since it has been demonstrated that these microorganisms are dominant in the environments of other oil wells (Kampfer et al. 1993).

The volcanic debris used as support material are volcanic material composed of SiO_2 and Al_2O_4 (Schumann 1993). The bacterial adhesion at the surface of the volcanic debris was determined as 4.2×10^3 to 15×10^3 CFU ml^{-1}. The number of bacteria attached is minor compared with the bacteria in the mineral medium (1×10^5 to 1×10^6 CFU ml^{-1}). Its possible that the bacterial adhesion was limited for the aeration supply (1 l min^{-1}).

The four groups from the stabilized microbial consortium are shown in Table 1. All of them belonged to the genera that are able to degrade petroleum hydrocarbons. The strains of *Bacillus* and *Brevibacillus* are capable of degrading benzene and other polyaromatic hydrocarbons (Smith 1994). Some strains of *Staphylococcus* can use linear alkanes as a source of carbon and energy (Morgan & Watkinson 1994). *Corynebacterium* is a genus with a great metabolic diversity, and it can degrade branched alkanes, branched hydro-

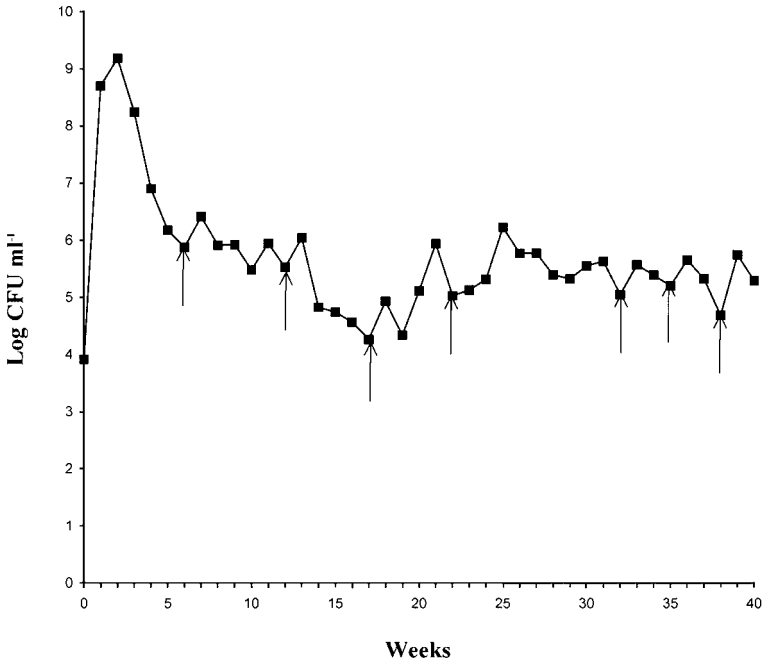

Figure 1. Growth stabilization of microbial consortium isolated from Maya crude oil. The weekly viable counts represent the mean of three replicate. (↑) time for adding asphaltenes (2.4 g) and mineral medium culture.

Table 1. Identification of the chemoheterotrophic bacteria strains that integrate the isolated and stabilized microbial consortium

Strains isolate	Group	Biochemical test	Microscopic morphology	Genus
50-GP, 60-GP, 61-GP and 84-GP	I	Acid production from glucose, arabinose, xylose, rhamnose, fructose, galactose, mannose, lactose, maltose, sucrose, trehalose, raffinose, salicin and starch. Hydrolysis of esculin, urea and tyrosine. Nitrate reduction	Nonsporing Gram-positive rods	*Corynebacterium* sp.
58-GP and 67-GP	II	Bacterial identification kit API-50CHB (bioMérieux)	Endospore-forming Gram-positive rods	*Bacillus* sp.
76-GP and 78-GP	III	Bacterial identification kit API-50CHB (bioMérieux)	Endospore-forming Gram-positive rods	*Brevibacillus* sp.
63-GP and 64-GP	IV	Growth on 10% NaCl agar, lysine descarboxilasa, ornitine descarboxylasa. Hydrolisis of arginine, urea and gelatine. β-galactosidasa. Indol and Voges-Proskauer test, utilization of citrate. H_2S production	Gram-positive cocci	*Staphylococcus* sp.

carbons with methyl substitutions, dibenzothyophene, benzothyazol and anthracene (De Wever & Verachert 1997; Omori et al. 1992). The identified strains are found interacting together inside the reactor to degrade and use asphaltenes as their only carbon and energy source.

The effect of different environmental factors on the growth of the stabilized microbial consortium are presented in Figure 2. These data showed that a salinity of 90,000 and 180,000 ppm significantly limited the growth of the bacteria in the consortium. At 55 °C the growth of the consortium decreased after 12 days exposure. The aeration and pH do not have a significant effect on the microbial consortia growth within the tested limits. These results mean that the bacterial consortium obtained is capable of using asphaltenes as a carbon and energy source in different environmental conditions. Furthermore, the existence of lower numbers of bacteria under 55 °C and 180,000 ppm salinity indicates how the asphaltenes biodegradation is limited in the oil well (60 °C, 180,000 ppm of salinity). The oil well environment is characterized by elevated temperature, acidic or alkaline pH, high salt concentration and high pressure (Eren 1946). These results also demostrate that the conditions we used for the stabilization of the consortium makes the enrichment of a bacterial populations adapted to the environmental conditions of the oil well surface (environmental temperature and NaCl salinity of 100 ppm).

The bacterial consortium grow in the first four days using 0.06 g of asphaltenes as a carbon and energy source (Figure 2). The asphaltenes concentration can only sustain a maximum of 1×10^8 CFU ml^{-1}.

Our results were different to the ones reported in previous work. Stampleton et al. (1998) showed that small variation in temperature and pH are limiting factors for the growth of microorganisms with the ability to degrade petroleum hydrocarbons. The isolated bacteria in the microbial consortium can use asphaltenes as their only source of carbon and energy when the pH variation was from 6.4 to 8.4 and when the temperature variation was from room temperature to 37 °C.

The environmental conditions that were used to grow the stabilized microbial consortium and to evaluate its asphaltene mineralization abilities were as follows: 1 l min^{-1} aeration, room temperature, 100 NaCl ppm salinity and pH of 7.4. In Figure 3 it is clear that the highest evolution of CO_2 was obtained from the treatment of asphaltenes plus the microbial consortium. It has been demonstrated that asphaltenes can be oxidized by the metabolic activity of microorganisms isolated from sediment polluted with petroleum hydrocarbon, producing structures with keto and hydroxyl groups after ten days of incubation (Rontani et al. 1985). The elevated production of CO_2 in the treatment of microbial consortium plus asphaltenes indicates that these compounds are used as a source of carbon and energy. According to Kanaly & Harayama (2000) and Sukesan & Watwood (1998), the bacteria able to degrade asphaltenes should use the asphaltenes as their only carbon and energy source, in a similar way that an organic compound can be biodegraded (Leahy & Colwell 1990). The bacteria of the consortium have the capability to mineralize the asphaltenes extracted from the Mayan crude oil, under the evaluated environmental conditions. This capability makes

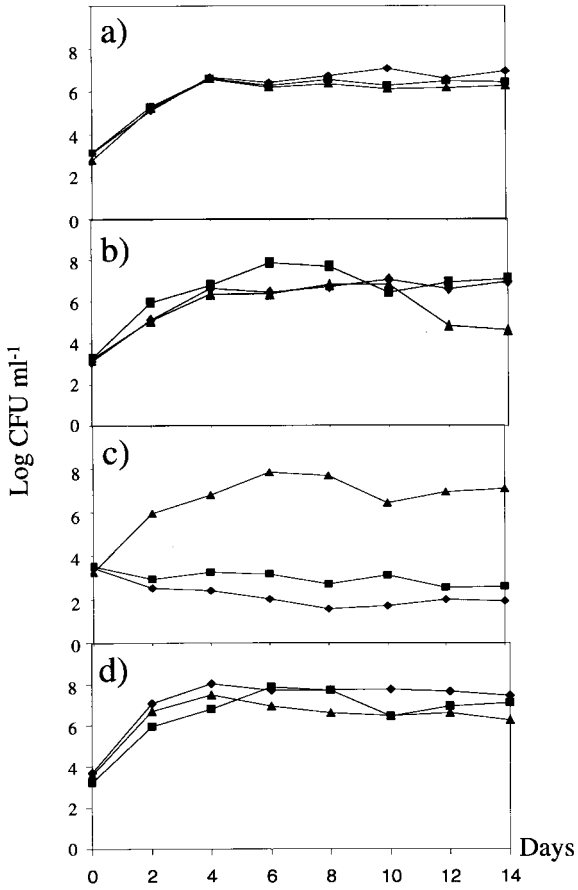

Figure 2. Effects of environmental factors on growth of the stabilized microbial consortium. (a) Aireation: 11 min^{-1} (♦), 0.11 min^{-1} (■), 0.1 min^{-1} (▲). (b) Temperature: room temperature (♦), 37 °C (■, 55 °C (▲). (c) Salinity: 180,000 ppm (♦), 90,000 ppm (■), 100 ppm (▲). (d) pH 8.4 (♦), 7.4 (■), 6.4 (▲).

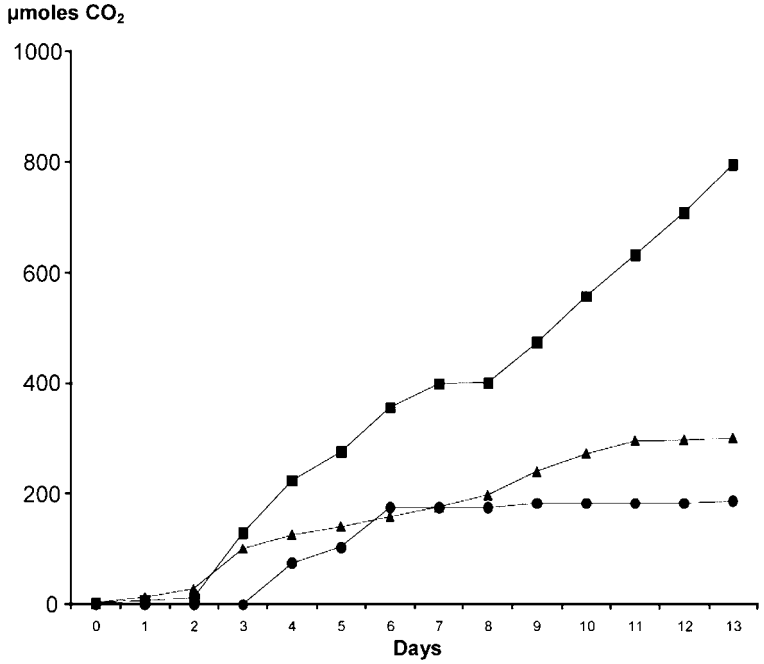

Figure 3. Acumulated producction of CO_2 for the stabilized microbial consortium with asphaltenes as only carbon and energy source. Asphaltenes plus consortium (■), asphaltenes (●), consortium (▲).

it possible to eliminate the asphaltenes present in environment using the microbial consortium.

It should be stressed, however, that an organic compound as the asphaltenes with such a complex structure shows different susceptibility to mineralization. The lateral aliphatic chains are most susceptible than the aromatic structures, and the polycondensed structures are the most difficult to degrade (Rontani et al. 1985). This mineralization process is enhanced when the hydrocarbon structures are exposed in the asphaltene molecules. Even though, in the crude oil the micelar structure of the asphaltenes are present (Acevedo et al. 1999), this micelar structure was eliminated with n-heptane during their extraction from the crude-oil sample.

Moreover, when the micelar structure is eliminated of the asphaltenes these can liberate a small quantity of CO_2 without the metabolic activity of microorganisms (Figure 3).

On the other hand, the production of CO_2 for the microbial consortium without asphaltenes could be the mineralization of organic compounds liberated from the bacterial lisis. In both cases, the CO_2 produced is significantly smaller than the system with the consortium plus asphaltenes.

Conclusions

A asphaltenes-degradating bacterial consortium was obtained from a crude oil sample using a glass column reactor under controlled incubation conditions. The isolated and stabilizated consortium have the capability to grow and mineralize the asphaltenes extracted from crude oil. The results revealed the posibility to use these microbes for the reduction of asphaltenes in ecosystems where they accumulate and cause pollution problems.

Acknowledgements

We thank Dr. EnTao Wang for helpful commentary on the manuscript. This work is part of the proyect Degradación de hidrocarburos de alto peso molecular DEPI970520, Escuela Nacional de Ciencias Biológicas, México.

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