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Presence of opportunistic oil-degrading microorganisms operating at the initial steps of oil extraction and handling

Summary. Hydrocarbon-degrading microorganisms from natural environments have been isolated and identified using culture-dependent or molecular techniques. However, there has been little research into the occurrence of microorganisms incorporated into crude oil in the initial steps of extraction and handling, which can reduce the quality of stored petroleum. In the present study, a packed-column reactor filled with autoclaved perlite soaked with crude oil was subjected to a continuous flow of sterile medium in order to determine the presence of potential hydrocarbon degraders. Microorganisms developed on the surface of the perlite within a period of 73 days. DNA was extracted from the biofilm and then PCR-amplified using 16S rRNA bacterial and archaeal primers and 18S rRNA eukaryotic primers. No amplification was obtained using archaeal primers. However, denaturing gradient gel electrophoresis (DGGE) revealed the presence of unique bands indicating bacterial and eukaryotic amplification. Excision of these bands, sequencing, and subsequent BLAST search showed that they corresponded to *Bacillus* sp. and *Aspergillus versicolor*. The fungus was later isolated from intact perlite in agar plates. A bacterial clone library was used to confirm the presence in the biofilm of a unique hydrocarbon-degrading bacterium closely related to *Bacillus* sp. Analysis of the petroleum components by gas chromatography showed that there *n*-alkanes, aromatic hydrocarbons, and carbazoles were degraded. [**Int Microbiol** 2006; 9(2):119-124]

Key words: biofilm · oil biodegradation · crude oil · DGGE denaturing gradient gel electrophoresis

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Introduction

Characterization of oil-utilizing microorganisms has become crucial to our understanding of the biological processes responsible for oil biodegradation in natural environments and in reservoir rocks. Many ecological studies have used molecular- or culture-dependent techniques to demonstrate the occurrence of crude-oil-degrading bacteria in oil spills in aquifers [11,33], soils [7,28], and marine environments [15, 16,19,24]. Aerobic, facultatively anaerobic, and anaerobic microorganisms belonging to a great variaty of groups and detected in a wide range of environments, such as those characterized by low or elevated temperatures, acidic or alkaline pH, high salt concentrations, or high pressures, are able to degrade different oil fractions. Furthermore, several species of heterotrophic bacteria, certain yeasts, fungi, cyanobacteria, and microalgae, and some unidentified consortia, among others, play an important role in the biological treatment of polluted habitats [1,6,9,20,22]. Anaerobic microorganisms

belonging to the sulfate reducing group of bacteria, methanogenic bacteria, fermentative bacteria, or iron-reducing bacteria, and genera such as *Campylobacter*, *Oceanospirillum*, and *Thiomicrospira* have been isolated or detected using molecular techniques [20].

While all these studies have mainly focused on the isolation and identification of aerobic and anaerobic microorganisms inherent to polluted ecosystems, little research has been done on microorganisms incorporated into crude oil during the initial steps of extraction and handling, which can result in the degradation of stored oil, thus potentially reducing its usefulness, but also contribute to oil degradation in the case of a spill. In this work we used a biofilm bioreactor to determine the occurrence of potential oil-degrading microorganisms in a sample of crude oil obtained from an offshore platform. These organisms, which have the capacity to degrade hydrocarbons, can alter the quality of the stored product.

Materials and methods

Start up and growth conditions. The experiment was carried out in a fixed-biomass reactor modified from the system described by Ferrera et al. [12]. The reactor consisted of a packed column and a stirred vessel through which sterile mineral medium was pumped at a constant flow rate (2.7 ml/min) using a peristaltic pump (Watson Marlow 501U). The medium contained (per liter): NH_4Cl (300 mg), K_2HPO_4 (300 mg), $CaCl_2 2H_2O$ (200 mg), $MgCl_2 6H_2O$ (200 mg), KCl (200 mg), NaCl (30 g), Na_2CO_3 (20 mg), vitamin B_{12} (20 μg), Na_2-EDTA (5 mg), $FeSO_4 7H_2O$ (2 mg), $ZnSO_4 7H_2O$ (0.1 mg), $MnCl_2 4H_2O$ (30 μg), H_3BO_3 (0.3 mg), $CoCl_2 6H_2O$ (0.2 mg), $CuCl_2 2H_2O$ (10 μg), $NiCl_2 6H_2O$ (20 μg) and $Na_2MoO_4 2H_2O$ (30 μg). The column, which had an internal diameter (i.d.) of 15.6 mm, a length of 275 mm, and a total volume of 52.5 ml, was packed with oil-soaked perlite (Casablanca crude oil, Tarragona basin, Spain) mixed with glass beads and rings to increase porosity and improve fluid circulation. Aeration was not provided but the supplied medium had a concentration of 0.44 mg dissolved oxygen/l. All components of the system, except the crude oil, were autoclaved. Casablanca crude oil contains mainly aliphatic hydrocarbons (59.5%) and, to a lesser extent, aromatic hydrocarbons (27%). Asphaltenes and polar compounds are present only in minor amounts. Its API gravity is 33.4º.

Monitoring. During the entire course of the experiment, 500-ml samples were collected from the effluent; filtered through polycarbonate filters (0.2 μm), and stored at –20ºC for DNA extraction. The number of microorganisms were counted using a microscope. Samples of the effluent were taken every 2 or 3 days and fixed in formaldehyde (2% final concentration). At the end of the experiment, samples were collected from the biofilm, which covered the column-packing material (perlite and glass), for characterization. The column contents were emptied into a glass beaker containing saline solution (0.9%). The contents of the beaker were sonicated in an ultrasonic bath (Bransonic 5, Branson) for 3 min after which the volume was raised to a total of 500 ml. Aliquots of 1000 μl were removed and centrifuged for 5 min at 12,000×*g* (Hettich Mikro 20) for DNA extraction. The pellets were then stored at –80ºC until further analysis. Additional aliquots were fixed in formaldehyde (2% final concentration) for microscopy. A sample of intact filling was also stored for analysis by gas chromatography.

General analyses. DAPI (4′,6′-diamino-2-phenylindole)-stained cells [26] were counted using an epifluorescence Olympus BH microscope, following previously described statistical recommendations [17]. At least 300 cells were counted within a minimum of ten different microscope fields.

Gas chromatography. Oil samples were analyzed at the beginning (day 0) and end (day 73) of the experiment as described in García de Oteyza et al. [14]. A washing control, in which only water and sodium chloride were pumped into the system instead of medium, was carried out under the same conditions as the biodegradation experiment in order to determine which hydrocarbons were removed by wash-out with the effluent.

DNA extraction. Nucleic acid was extracted from frozen pellets and polycarbonate filters as described by Massana et al. [23].

PCR-DGGE fingerprinting. Fragments of the 16S rRNA gene suitable for denaturing gradient gel electrophoresis (DGGE) analysis were obtained using the bacterial-specific primer 358F with a 40-bp GC-clamp and the universal primer 907RM as described in Sánchez et al. [31]. For eukaryotic 18S ribosomal DNA amplification, Euk1A and Euk516r-GC primers, which amplify a fragment of approximately 560 bp, were used [10]. Archaeal 16S rRNA was amplified with primers 344F-GC and 915R [29]. DGGE was run in a DCode system (Bio-Rad) as previously described by Muyzer et al. [25]. A 6% polyacrylamide gel with a gradient of DNA-denaturant agent was cast by mixing solutions of 0% and 80% denaturant agent (100% = 7 M urea and 40% deionized formamide). PCR product (400 ng) was loaded for each sample and the gels were run at 100 V for 18 h (*Bacteria*) and at 80 V for 20 h (*Eukarya*) at 60°C in 1× TAE buffer (40 mM Tris, pH 7.4, 20 mM sodium acetate, 1 mM EDTA). Linear (40–80%, *Bacteria*; 30 to 65%, *Eukarya*) gradients of denaturant agent were used. The gels were stained with SybrGold (Molecular Probes) for 45 min, rinsed with 1× TAE buffer, transferrred from the glass plate to a UV-transparent gel scoop, and visualized under UV with a Fluor-S MultiImager (Bio-Rad). Prominent bands were excised from the gels, resuspended in Milli-q water overnight, reamplified, and purified for sequencing using a High Pure PCR Product Purification Kit (Roche).

Clone library and RFLP analysis. The bacterial 16S rRNA gene was amplified between positions 27 and 1492 (*Escherichia coli* 16S rRNA gene sequence numbering) and cloned as described in Ferrera et al. [13]. Two clones with different band patterns were chosen for partial sequencing. The coverage of the clone library was calculated according to the following equation: $C = 1 - (n/N)$, where n is the number of unique clones and N is the total number of clones examined [30].

rRNA sequencing. Sequencing reactions were carried out by "Sistemas Genómicos" services (Spain) with the primers 907RM for bacterial DGGE bands, Euk1A for eukaryotic DGGE bands, and 27F, 907RM and 1492R for cloned 16S rRNA genes. The sequencing service used the ABI PRISM BigDye Terminator cycle sequencing kit and reactions were run in an automatic ABI PRISM 3100 sequencer from Applied Biosystems. Sequences were submitted to a BLAST search [3] to determine phylogenetic affiliation, and to the CHECK-CHIMERA program from RDP [21] to identify potential chimeric artifacts.

Accession numbers. Three 16S and two 18S rRNA gene sequences obtained from the biofilm were sent to the EMBL database [http://www. ebi.ac.uk/embl] and were assigned the accession numbers AJ639851 to AJ639855.

Isolation and growth conditions. When the column was emptied, a sample of the intact perlite was deposited onto agar plates containing the previously described mineral minimum and incubated at 25ºC to isolate potential petroleum degraders. The DNA of the isolated microorganism was extracted as described above. PCR was done with primers Euk1A and EukB, which amplified almost the entire 18S rRNA gene of the isolate [10]. The PCR program involved an initial denaturation at 94ºC for 3 min, 30 cycles of denaturation at 94ºC for 45 s, annealing at 55ºC for 1 min, extension at 72ºC for 3 min, and a final extension at 72ºC for 5 min.

Results and Discussion

Microbial growth in the bioreactor. The experiment lasted 73 days during which samples of the effluent were fixed periodically for cell counting by microscopy. Total cell numbers in the effluent were determined periodically in order to monitor the stability of the oil-degrading biofilm, assuming that changes in the microbial growth rate of the biofilm would be reflected in the number of cells in the effluent. The results showed that cell number increased progressively during the first month, reaching a stable average of 4.3×10^5 cells/ml. Cell production (6.53×10^7 cells/h) from the stabilized biofilm was calculated as the product of the cell number and the pumping rate. Microscopy showed a low morphological diversity, since only rods and some filaments were detected in the late stages of the experiment.

Isolation of crude-oil-degrading microorganisms. Particles of perlite from the column filling were deposited onto agar plates and incubated under oxic conditions. After 2 days, fungal growth was observed over and around the perlite particles. DNA was extracted from a pure culture of this fungus and the 18S rRNA gene was amplified with primers Euk1A and EukB. Partial sequencing with both primers revealed that the closest match, determined by a BLAST search, corresponded to *Aspergillus versicolor* AB008411 (98.8% similarity, 1317/1333). The amplification product of the fungus was compared using DGGE to samples of the effluent and the column in order to verify that the microorganism was indeed present during the experiment (Fig. 1).

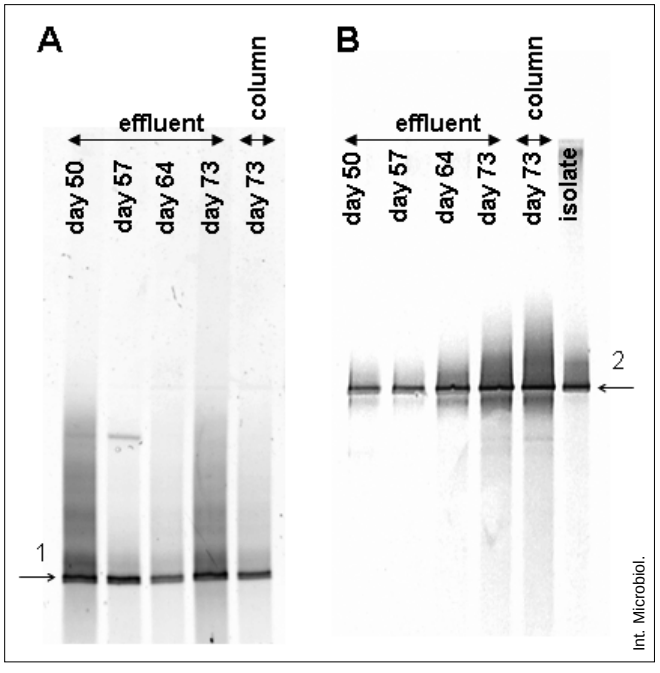

Fig. 1. Negative images of DGGE gels with PCR-amplified segments of 16S and 18S rRNA genes using bacterial (**A**) and eukaryotic primers (**B**) from samples obtained from the effluent over time and from the column biofilm. The amplification product of the fungus isolated on agar plates was also run in order to verify that it was indeed present during the experiment.

Molecular analyses. DGGE analysis with bacterial and eukaryal primers from several samples of the effluent and the column (day 50 to day 73) showed that there were no substantial changes in the bacterial composition over time once the biofilm was stable (Fig. 1). There was no amplification using archaeal primers.

DGGE analysis with PCR products showed the presence of only one intense band of bacteria and one of eukarya. Partial sequencing of each band revealed that the closest match determined by a BLAST search corresponded to *Bacillus* sp. AY082367 (100% similarity, 559/559) and *Aspergillus versicolor* AB002064 (100% similarity, 533/533), verifying that the fungus isolated from the agar plates was indeed the main eukaryote present in the column. Since the microorganism corresponding to *Bacillus* sp. could not be isolated in pure culture, a clone library was obtained with the bacterial 16S rRNA gene in order to obtain additional information about the bacteria implied in petroleum degradation. Fingerprinting techniques such as DGGE allow easy and quick comparison from related microbial assemblages, but the sequences obtained from DGGE bands are short (less than one-third the total length of small subunit rRNA) and of variable quality. For this reason, bacterial diversity was assessed with a clone library. The 91 resulting clones showed two different RFLP patterns. A representative clone of each pattern was sequenced with primers 27F, 907R, and 1492R to obtain the entire sequence of the 16S RNA gene. The closest relative according to a BLAST search corresponded in both cases to an uncultured *Bacillus* sp. clone AY082367 (99% similarity, 1446/1455). Eighty-nine clones corresponded to unique RFLP band patterns. The coverage of the library was 100%.

It is remarkable that both microorganisms (*Bacillus* and *Aspergillus* sp.) are able to form spores. This could explain their resistance to the hostile oil environment. Many species belonging to the genus *Bacillus* have been isolated from petroleum-contaminated environments. For example, Sorkoh et al. [32] obtained 368 isolates belonging predominantly to the thermophilic *B. stearothermophilus* from oil-polluted areas in Kuwait . Other strains of *Bacillus* have been isolated from oil-polluted environments under high temperature [2] as well as from deep-sea ecosystems [4], contaminated seawater and marine sediments [34], and soils [28].

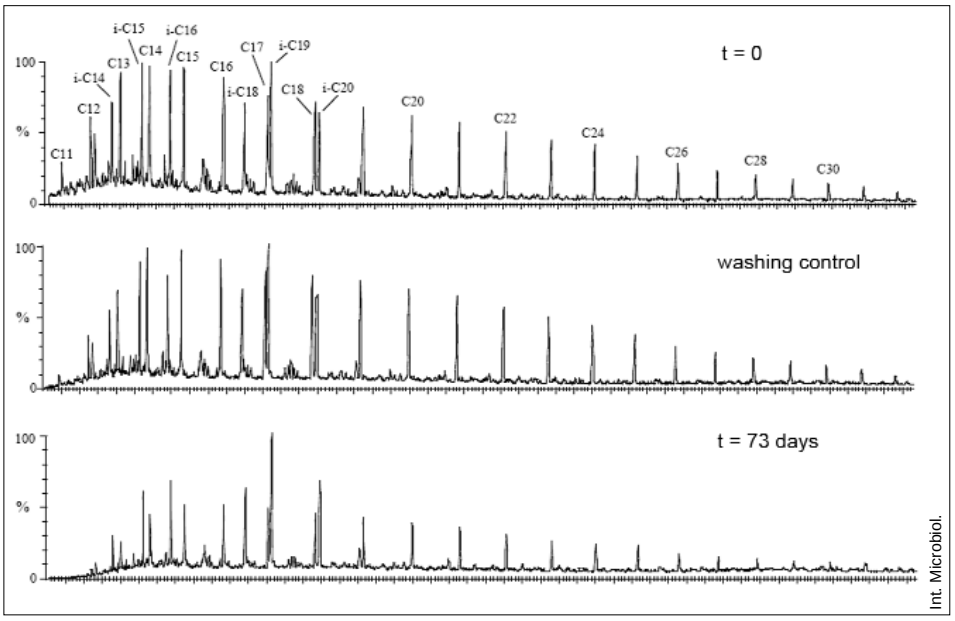

Fig. 2. Aliphatic hydrocarbons in the original crude-oil sample (t = day 0), the washing control, and at the end of the experiment (t = day 73).

The presence of oil-utilizing fungi in soils has also been investigated. Radwan et al. [27] found species of *Aspergillus* and *Penicillium* in oil-polluted Kuwaiti desert samples, and *Aspergillus terreus* and *Fusarium solani* were isolated from oil-contaminated areas in a natural soil [8]. Species belonging to *Aspergillus*, *Penicillium*, *Beauveria*, *Acremonium*, *Cladosporium*, *Fusarium*, and *Trichoderma* were obtained from an agricultural soil in France [6]. In addition, screening of aerobic culturable hydrocarbon-degrading microorganisms isolated from petroleum-polluted soils and cyanobacterial mats from Indonesia resulted in the collection of 21 fungi belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Amorphoteca*, *Neosartorya*, *Paelomyces*, *Talaromyces*, and *Graphium* [7].

Although *Bacillus* spp. have been frequently isolated from hydrocarbon-polluted sites, as stated above, members of this genus have never been shown unequivocally to directly degrade hydrocarbons. In fact, *Bacillus* spp. are known to act as secondary degraders, i.e., they assimilate the metabolites produced by true primary-hydrocarbon degraders [5]. This explains why this strain could not be cultivated on mineral medium plates in the absence of a carbon source other than crude oil. Consequently, the biodegradation activity observed in our experiment could be linked only to the fungus.

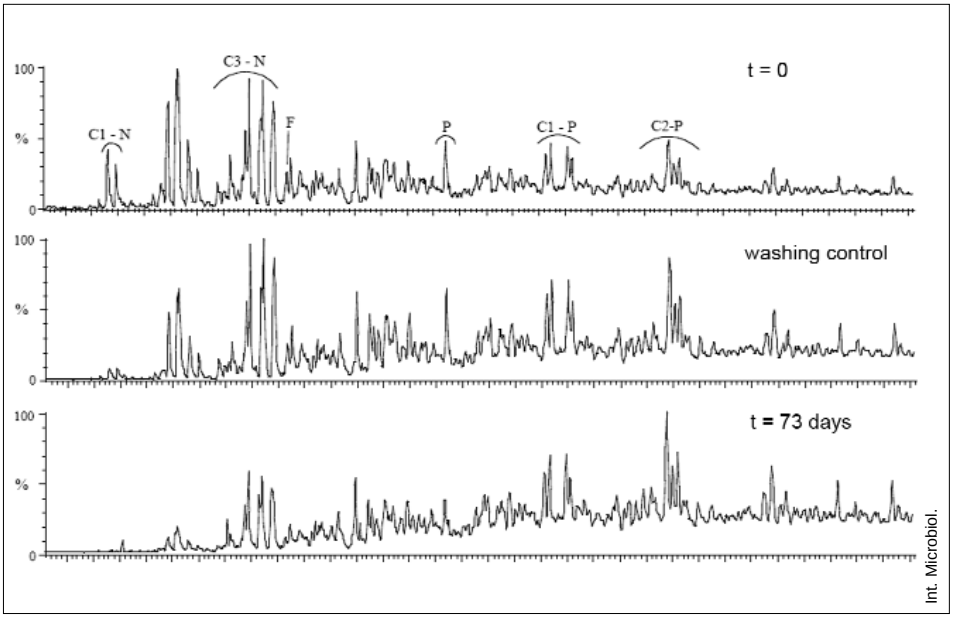

Fig. 3. Aromatic hydrocarbons in the original crude oil sample (t = day 0), the washing control, and at the end of the experiment (t = day 73).

Hydrocarbon distribution. An assessment of the compositional changes in crude oil was based on hydrocarbon (aliphatic and aromatic) and resin analysis of the perlite at the beginning and end of the experiment. A washing control without nutrients was also done to measure which hydrocarbon compounds may have been washed out, since changes in crude-oil composition after water washing has been reported [18].

Figure 2 shows the chromatograms for the aliphatic hydrocarbons of the original crude oil, the washing control, and a sample obtained at the end of the experiment. The results show that there was biodegradation of nearly 70% of all *n*-alkanes. Concerning the aromatic hydrocarbons, all the lighter compounds were strongly degraded (Fig. 3). Over 90% of the naphthalenes, up to the C_2-naphthalenes, were degraded, but some degradation continued up to the C_4 homologues. Furthermore, fluorene, phenanthrene, and their lighter homologues were also biodegraded; these changes were not due to abiotic processes since the compounds were still present in the washing control. In addition, although Casablanca crude oil is not rich in resins, biodegradation of other compounds, such as nitrogen-containing carbazoles, was not found (not shown).

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Presencia de microorganismos oportunistas degradadores de petróleo en la extracción y manipulación iniciales de petróleo

Resumen. Algunos autores han aislado e identificado microorganismos degradadores de petróleo utilizando técnicas moleculares o dependientes de cultivo. Sin embargo, se ha investigado poco la presencia de microorganismos que entran en contacto con el petróleo en las fases iniciales de su extracción y manipulación, circunstancia que puede reducir la calidad del petróleo almacenado. Mediante un reactor con una columna cargada con perlita esterilizada en autoclave y empapada de petróleo, y sujeto a un flujo continuo de medio estéril, determinamos la presencia de posibles degradadores de hidrocarburos. En la superficie de la perlita se desarrollaron microorganismos en un período de 73 días. Se extrajo el DNA del biofilm, y se amplificó por PCR con cebadores para el 16S rRNA de bacterias y arqueas y con cebadores para el 18S rRNA de eucariotas. No se obtuvo ampliación del DNA de arqueas, pero el análisis por electroforesis en gel con gradiente desnaturalizante (DGGE) reveló la presencia de bandas únicas en la amplificación de bacterias y eucariotas. La escisión de estas bandas, la secuenciación y la subsiguiente búsqueda en BLAST demostraron que correspondían a *Bacillus* sp. y a *Aspergillus versicolor*. El hongo fue aislado posteriormente en placas de agar a partir de perlita intacta. También se llevó a cabo una biblioteca genética bacteriana, que confirmó la presencia de una única bacteria degradadora de petróleo en el biofilm, muy próxima a *Bacillus* sp. El análisis de los componentes del petróleo por cromatografía de gases mostró que había habido degradación de *n*-alcanos, hidrocarburos aromáticos y carbazoles. [**Int Microbiol** 2006; 9(2):119-124]

Palabras clave: biofilm · biodegradación · petróleo · DGGE (electroforesis en gel con gradiente desnaturalizante)

Presença de microorganismos oportunistas degradadores de petróleo na extracção e manipulação iniciais de petróleo

Resumo. Alguns autores isolaram e identificaram microorganismos degradadores de petróleo utilizando técnicas moleculares ou dependentes de cultivo. No entanto, tem-se investigado pouco a presença de microorganismos que entram em contato com o petróleo nas fases iniciais de sua extracção e manipulação, circunstância que pode reduzir a qualidade do petróleo armazenado. Mediante um reator com uma coluna carregada com perlite esterilizada em autoclave e embebida de petróleo, e sujeito a um fluxo contínuo de meio estéril, determinamos a presença de possíveis degradadores de hidrocarbonetos. Na superfície da perlite desenvolveram-se os microorganismos ao longo de 73 dias. Extraiu-se o DNA do biofilm, e amplificou-se por PCR com "primers" para o 16S rRNA de bactérias e arqueias e com "primers" para o 18S rRNA de eucariotas. No caso dos "primers" para arqueias não se obteve amplificação do DNA. No entanto, a análise por electroforesis em gel com gradiente desnaturalizante (DGGE) revelou a presença de bandas únicas na amplificação de bactérias e eucariotas. A cisão destas bandas, a seqüenciação e a subseqüente procura em BLAST demonstraram que correspondiam a *Bacillus* sp. e a *Aspergillus versicolor*. O fungo foi posteriormente isolado em placas de agar a partir de perlite intacta. Também se usou uma biblioteca genética bacteriana, que confirmou a presença de uma única bactéria degradadora de petróleo no biofilm, muito próxima a *Bacillus* sp. A análise dos componentes do petróleo por cromatografia de gases mostrou que tinha havido degradação de *n*-alcanos, hidrocarbonetos aromáticos e carbazois. [**Int Microbiol** 2006; 9(2):119-124]

Palavras chave: biofilm · biodegradação · petróleo · DGGE (electroforesis em gel com gradiente desnaturalizante)