Technical paper

Fungi and Bacteria Isolated from Two Highly Polluted Soils for Hydrocarbon Degradation

M. E. Mancera-López,[a] M. T. Rodríguez-Casasola,[a] E. Ríos-Leal,[a] F. Esparza-García,[a] B. Chávez-Gómez,[b] R. Rodríguez-Vázquez,[a] J. Barrera-Cortés[a,*]

[a] *CINVESTAV IPN, Depto. Biotecnología y Bioingeniería, Av. IPN 2508, Col. San Pedro Zacatenco, C.P. 07360, México D.F. Tel.: +52 55 50613800/4380, Fax: +52 55 50613313, E-mail: jbarrera@cinvestav.mx.*

[b] *Instituto Mexicano del Petróleo. Depto Biotecnología, Eje Central Lázaro Cárdenas 152, Col. San Bartolo Atepehuacan, C.P. 07730, México D.F.*

Received: 03-08-2006

Abstract

Cultivable fungi and bacteria were isolated from two highly contaminated soils with total petroleum hydrocarbon (TPH) concentrations of 60,600 and 500,000 mg kg^{-1}. The aim of the study was to determine the capacity of these bacteria and fungi to degrade TPH, specifically the aliphatic hydrocarbon (AH) and polycyclic aromatic hydrocarbon (PAH) fractions, when cultivated in a mineral liquid culture (modified Rennie medium) together with a complex mixture of TPH as the unique source of carbon. Thirty-seven hydrocarbon-degrading strains were isolated, but only six strains showed a high ability to degrade PAHs, AHs and TPH. These strains were identified as *Pseudomonas pseudoalcaligenes*, *Bacillus firmus*, *Bacillus alvei*, *Penicillium funiculosum*, *Aspergillus sydowii* and *Rhizopus sp*., and they removed 79%, 80%, 68%, 86%, 81% and 67% of TPH, respectively. *P. pseudoalcaligenes* and *P. funiculosum* removed 75% of PAHs, while *B. firmus* and *P. funiculosum* removed 90% and 92% of AHs, respectively. The highest TPH removal was observed by *P. funiculosum*, which was isolated from the soil with a high TPH concentration. *A. sydowii* was also isolated from this soil; no reports were found regarding its capacity to remove PAHs, but it was able to degrade five- and six-ring aromatic compounds.

Keywords: Bacteria, bioremediation, fungi, polycyclic aromatic hydrocarbons, aliphatic hydrocarbons.

1. Introduction

Petroleum spillage is a source of severe water and soil pollution that, in addition to the known environmental problems, reduces microbial diversity through the phenomenon of selectivity.[1] The microorganisms capable of surviving in such a polluted environment are those that develop specific enzymatic and physiological responses that allow them to use the hydrocarbon compounds as substrates.[1] This response of microorganisms to organic contaminants has been studied for many years. It has been found that individual microorganisms can mineralize only a limited range of hydrocarbon substrates, so assemblages of mixed populations with an overall broad of enzymatic capacities are required to increase the rate and extent of petroleum biodegradation.[2]

At present, various microbial genera have been detected in petroleum-contaminated soil or water, which strongly suggests that each has a role in the hydrocarbon transformation process.[2] The most frequently found microorganisms are bacteria and fungi, with bacteria assuming the dominant role in marine ecosystem and fungi in terrestrial environments.[3] It has been reported that adapted communities previously exposed to hydrocarbons exhibit higher biodegradation rates than communities with no history of hydrocarbon contamination.[4] Organic compounds of low molecular weight and simple molecular structure are preferred by many microorganisms.[3] Compounds with more complex structures, such as polycyclic aromatic hydrocarbons (PAHs), with more than five benzene rings, are more resistant to microbial breakdown.[5,6]

According to Atlas, the most prevalent bacterial hydrocarbon degraders, in decreasing order, belong to the genera *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Nocardia*, *Arthrobacter* and other coryneforms, *Vibrio*, *Bacillus*, *Micrococus* and *Acinetobacter*. Other genera of bacteria able to degrade hydrocarbons include *Actinomyces*, *Aeromonas* and *Alcaligenes*.[3] Fewer fungi are known

to degrade hydrocarbons, because of reduced fungal growth in the soil due to factors such as competition with bacteria and the toxicity of the pollutants;[7] other mechanisms that limit fungal growth are antibiotic production, nutrient competition, and mycoparasitism. Fungi are of interest because of their ability to synthesize relatively unspecific enzymes involved in cellulose and lignin decay that can degrade high molecular weight, complex and more recalcitrant toxic compounds, including aromatic structures.[8]

In some Environmental Protection Agency reports, PAHs with more than five benzene rings have been considered as harbingers of cancer.[9] The impact of these compounds on human health has stimulated great interest in the identification of microbial strains with specific PAH-degrading activity.[10] The objective of this research was to study the cultivable hydrocarbon strains (bacteria and fungi) isolated from two highly polluted soils (60,600 and 500,000 mg kg^{-1} of TPH) to determine whether AH, PAH, and TPH degradation efficiency was associated with the concentration of hydrocarbons in the soils from which the strains were isolated.

2. Materials and Methods

2.1. Soils Contaminated with Hydrocarbons

Native hydrocarbonoclast microorganisms were isolated from aged soils obtained from two different sites polluted with total petroleum hydrocarbon (TPH). The first soil was obtained from a 30-year-old waste lagoon in Poza Rica (PR), Veracruz, Mexico. The second was taken from the Santa Alejandrina (SA) swamp site near an oil refinery in Tabasco, Mexico, and had been contaminated with hydrocarbons for more than 40 years. Both soils were characterized according to their TPH concentration and hydrocarbon fractions: aliphatics (AHs), PAH, saturates (SHs) and asphaltenes (ASPHs). The following characteristics were also measured and recorded: pH, density (gravimetric method), water holding capacity, total organic carbon, assimilated phosphorus, nitrogen content and cultivable microorganism count.[11,12]

2.2. Hydrocarbon Analysis of Soil

A 1 g sample of polluted soil was mixed with 0.5 g of $NaSO_4$ anhydrous and extracted with dichloromethane for 8 h in a Soxhlet apparatus (EPA method 3540C). The extract was evaporated to dryness and measured for TPH quantification by difference in weight. [13] Next, the hydrocarbon extract was fractionated into asphaltene, aliphatic, polycyclic aromatic and saturate hydrocarbon fractions. Asphaltenes were first precipitated with *n*-pentane from 1 g TPH extracts. The asphaltene fraction was recuperated by filtration and was dried and weighed (until constant weight) for its quantification. Next, *n*-pentane was evaporated from the filtrate for fractionation of the residual hydrocarbons. The fractionation was carried out in samples of 0.1 g in a silica gel (60–200 mesh) column of 20 1.5 cm, where the separation was accomplished by successive elution with hexane (aliphatics), benzene/hexane 1:3 (aromatics) and acetone/methanol 1:1 (saturates). The hydrocarbon fractions eluted were quantified by difference in weight. Analyses of hydrocarbons were carried out in triplicate. A stock of TPH extracted from the Poza Rica Veracruz soil was prepared as described above for its use in the study of identification of hydrocarbonoclast strains.

2.3. Isolation of Strains from Contaminated Soils

Cultivable native microorganisms were isolated from the severely, long-term contaminated soils described above. Strains were isolated from polluted soil previously biostimulated with a mineral solution specific to bacterial or fungal cultivation (see below). Biostimulation was performed in 125 mL Erlenmeyer flasks, where 10 g of polluted soil was humidified with 10 mL of mineral medium. The Erlenmeyer flasks were incubated at environmental temperature for 7 days. The microorganisms were separated from the soil particles by gentle shaking of 1 g soil (dry weight) with 10 mL of sterile water for 30 min. After sedimentation, the supernatant suspension was used to prepare appropriate dilutions (from 1×10^{-1} to 1×10^{-7}) with sterile water. Aliquots of 0.2 mL were spread-plated on the appropriate medium (solid nutrient agar medium, Sabouraud maltose agar, or Czapek agar). The plates were incubated at 28 °C for 7 days. The microorganisms were allowed to spread until purification and were then conserved in a refrigerator ready for use in the production of inocula for the hydrocarbon degradation experiments.

2.4. Production of Bacterial Inocula

A loop of bacterial inoculum was placed in 25 mL of sterile nutritive broth in a 125 mL Erlenmeyer flask and incubated at 30 °C and 130 rpm for 3 days. One millilitre of the bacterial culture (1×10^5 cell mL^{-1}) was used as the inoculum in a medium containing mineral salts and a complex mixture of hydrocarbons (20,000 mg L^{-1}) extracted from the polluted soil of Poza Rica.[14] The morphology and purity of the bacterial strains was determined by Gram staining, and they were then streaked in a Petri dish.[15] The isolated bacterial cultures were characterised morphologically and biochemically using the crystal-BBL® system and complementary tests.[16]

2.5. Production of Fungal Inocula

Fungal spores cultivated in Petri dishes with Sabouraud maltose agar were suspended in 10 mL of sterile di-

stilled water. The spore suspension was then washed and filtered through sterile glass wool and kept in amber vials at 4 °C.[17] The morphology of the fungi was identified by microculture. Microcultures were prepared by setting up a small Petri dish chamber containing a V-shaped piece of glass tubing resting on several layers of moistened filter paper. A sterile block of agar medium about 1 cm square was placed on a flame-sterilized microscope slide and the slide was then placed in the moist chamber on the tubing (all material was sterilized). The fungus was inoculated near the four edges of the agar block and a sterile coverslip was put over it. After a few days, the slide was observed under the microscope, and the undisturbed fungal structures were viewed as they grew.[18]

2.6. Hydrocarbon Degradation in Liquid Culture

A 500 mg sample of TPH extracted from the Poza Rica soil was dissolved in 2 mL of hexane and added to a vial of 120 mL. After solvent evaporation at 25 °C, 25 mL of a sterile mineral medium, specific for bacteria or fungi, was added in order to obtain a TPH concentration of 20,000 mg L^{-1}. Next, 1 mL of the respective strain was inoculated onto each vial to be incubated at 30 °C and 150 rpm for 15 days.

Mineral medium (1 L) for bacteria consisted of two solutions: solution A (0.9 L) was composed of: 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.1 g KCL, 0.025 g $Na_2MoO_4 \cdot 2H_2O$, 0.014 g $Na_2FeEDTA$ and 1.0 g NH_4NO_3, and solution B (0.1 L) consisted of 0.2 g $MgSO_4 \cdot 7H_2O$ and 0.06 g $CaCl_2 \cdot H_2O$.[19] Both mineral solutions, previously prepared and fixed at pH 7, were sterilized separately at 121 °C for 18 min, before being mixed under sterile conditions.

Mineral medium (1 L) for fungi was prepared with: 0.5 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.2 g KCL, 0.2 g $MgSO_4 \cdot 7H_2O$ and 0.1 g $CaCl_2 \cdot H_2O$.[19] The mineral solution was adjusted to a pH of 6 and sterilized at 121 °C for 18 min.

2.7. Analysis of Residual Hydrocarbons in Liquid Culture

Vials were sealed for AH and PAH analysis by headspace solid-phase microextraction (HS–SPME), combined with gas chromatography (GC).[20,21] Both analyses were carried out on a Varian 3800 gas chromatograph (GC) implemented with a flame ionization detector (FID) and a non-polar 007 fused silica capillary (Quadrex Corporation) column: 25 m, i.d. 0.53 mm and film thickness 1 μm. Carrier gas was nitrogen at 0.7 kg cm^{-2}. Detector and injector temperatures were fixed to 250 and 220 °C, respectively.

The AH fraction was microextracted with fibre coated with 85 μm polydimethylsiloxane, and previous heating of TPH samples (in a hermetically sealed vial) to 80 °C. The time required for absorption was 3 min, with 30 s for desorption, on gas chromatography

(GC). The temperature regimen for the GC column was: 50 °C, 6 °C min^{-1} up to 230 °C, maintained for 36 min. A standard mixture of AHs in the range C_{12}–C_{30} was used to identify the range of hydrocarbons present in the TPH samples; the removed AHs were quantified on the basis of chromatograms obtained before the hydrocarbon biotreatment.

PAH fraction microextraction was carried out with fibre coated with 100 μm polydimethylsiloxane. In this case, TPH samples were heated to 100 °C (vial hermetically sealed) for 15 min. The required adsorption and desorption times were 10 and 1 min, respectively. In order to obtain a good separation and quantification of the PAH compounds, two different sequential temperature regimes were applied to the capillary column: (a) 80 °C for 7 min, with increments of 4 °C min^{-1} up to 228 °C, maintained for 5 min; (b) 4 °C min^{-1} up to 270 °C, maintained for 3 min. Quantification of PAH was carried out with a mixture of known hydrocarbons to form ten standard PAH: naphthalene, acenaphthylene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benzo(k)fluoranthene, benzo(a)pyrene, inden[1,2,3-cd]pyrene; the mixture was analysed under the same conditions as the gas samples. TPH were extracted with hexane from the liquid culture and deposited in 120-mL vials. The hexane was evaporated to dryness and the vials were measured for TPH quantification by difference in weight.[13]

3. Results

3.1. Hydrocarbon Composition

Table 1 shows the characteristics of the two highly polluted soils: SA with 500,000 mg of TPH per kg^{-1} of dry soil, approximately 90% more TPH than PR soil. As shown in the table, SA soil had low AH and PAH concentrations (32 ± 4% and 5 ± 2%, respectively) and a high ASPH concentration (53 ± 5%) compared with PR soil. Considering the relatively high PAH concentration in PR (21%) compared with SA (5%), PR soil was selected for extraction of the complex hydrocarbon mixture to be used in the identification of hydrocarbonoclasts. An additional advantage of this TPH extract was the AH and PAH composition, as shown in Figures 1 and 2. For AHs, the following composition was determined: 11% $<C_{12}$, 23% C_{12}–C_{20}, and 66% C_{20}–C_{30}. For PAHs, molecular structures with between two and six-rings were detected with the following percentages: 57% two rings, 18% three rings, 3% four rings, 10% five rings and 12% six rings (Figure 2). Assuming that ASPHs are recalcitrant compounds, the maximal hydrocarbon removal expected would be 65%.[4] With this speculation, the hydrocarbon removal percentage reported here was calculated on the basis of the degradable hydrocarbon fraction.

Table 1. Initial hydrocarbon composition of the Poza Rica and Santa Alejandrina soils

Hydrocarbons	Polluted soil	
	Poza Rica (PR)	Santa Alejandrina (SA)
TPHs (mg k^{-1} of soil)	60,600 ± 5000	500,000 ± 8000
Degradable hydrocarbon concentration		
Aliphatic hydrocarbons (%)	40 ± 5	32 ± 4
Polycyclic aromatic hydrocarbons (%)	21 ± 3	5 ± 2
Saturated hydrocarbons (%)	4 ± 2	10 ± 2
total	65	47
Recalcitrant hydrocarbon fraction		
Asphaltenes (%)	35 ± 4	53 ± 5

It is important to remember that, for complex mixtures of petrochemical hydrocarbons, laboratory studies in aqueous media to obtain fundamental data on biodegradation are the first step in the controlled screening of potentially useful bacterial species.[22]

3.2. Physicochemical Characteristics of the Polluted Soils

The characteristics of the soils used for micoorganism isolation (PR and SA) are shown in Table 2. The differences between the two soils augur the finding of two different microflora. According to the literature, neutral soils enhance the growth of bacteria, while acidic soils enhance the growth of fungi.[7] According to these reports, PR soil should have a microflora dominated by bacteria. The characteristics of the SA soil describe a soil of low quality,[23] with the possibility of isolating an interesting hydrocarbonoclast microbial population for PAH removal, principally composed of fungi.

3.3. Strains Isolated from Soils

Thirty-two bacteria were isolated from the polluted soils, twenty-one from PR soil and eleven from SA soil. Microscopic analysis showed the predominance of certain morphological characteristics, such as circular form (21), convex elevation (12), entire margin (22), colour beige

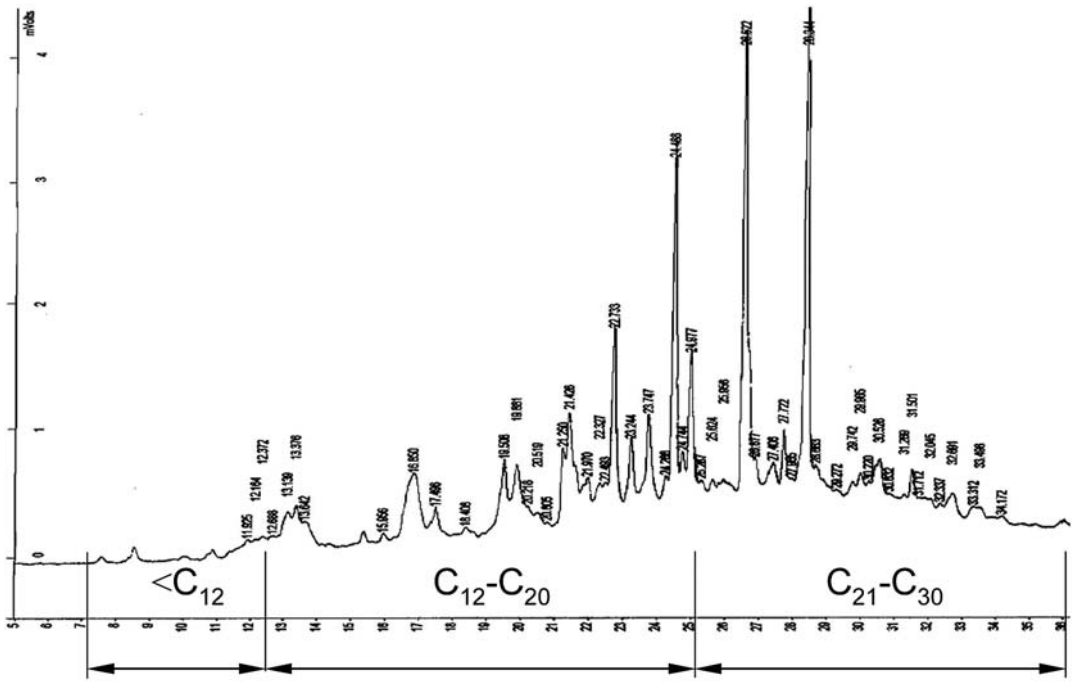

Figure 1. Chromatogram of the aliphatic hydrocarbon (AH) fraction contained in a complex hydrocarbon mixture extracted from the PR soil containing 60,600 mg kg^{-1} TPH. GC was implemented with an FID and a non-polar 007 fused silica capillary column: 25 m, i.d. 0.53 mm and film thickness 1 μm.

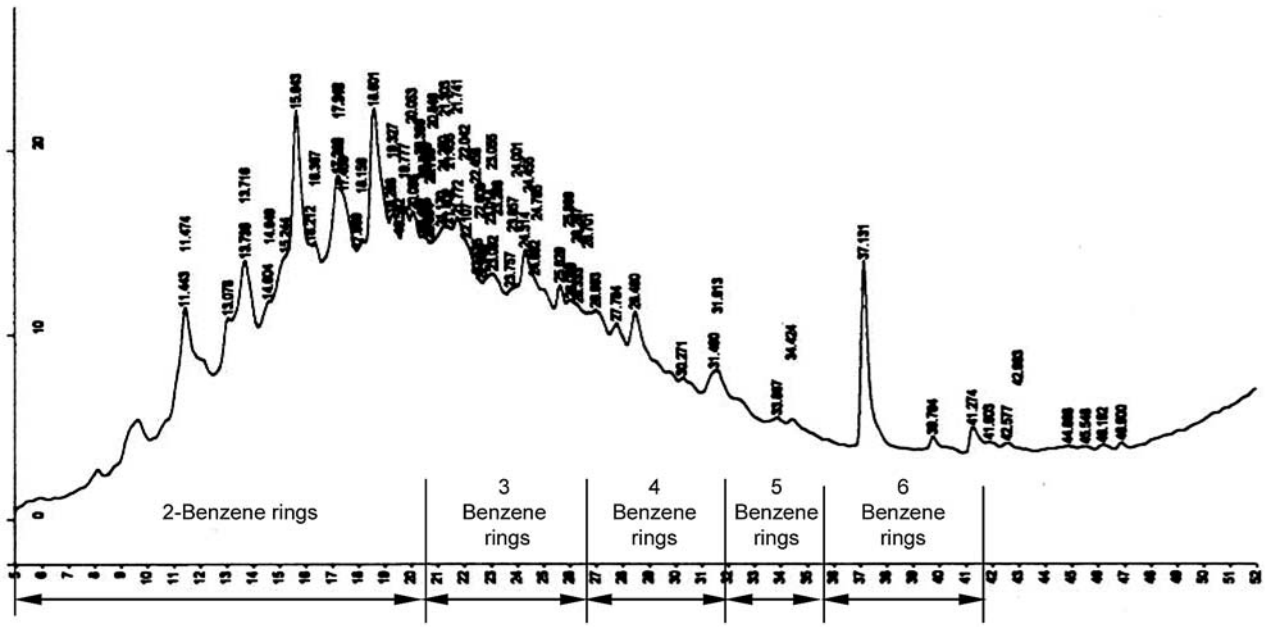

Figure 2. Chromatogram of the polycyclic aromatic hydrocarbon (PAH) fraction contained in a complex hydrocarbon mixture extracted from the PR soil containing 60,600 mg kg^{-1} TPH. GC implemented with an FID and a non-polar 007 fused silica capillary column: 25 m, i.d. 0.53 mm and film thickness 1 μm.

Table 2. Physicochemical characteristics of the Poza Rica and Santa Alejandrina soils.

Parameter measured	Poza Rica (PR)	Santa Alejandrina (SA)
pH	7.7 ± 0.03	3.8 ± 0.02
Density (kg m^{-3})	1600 ± 100	2700 ± 200
Water retention capacity (%)	70 ± 5	ND*
Total organic carbon (%)	12.2 ± 0.2	5.5 ± 0.5
Available phosphorous (%)	0.04 ± 0.01	0.005 ± 0.001
Nitrogen content (%)	0.19 ± 0.01	0.05 ± 0.01
Cultivable microorganism (UFC·$g^{-1}$of soil)	87×10^2	5×10^2

* It was not possible to determine the water holding capacity due to the high TPH concentration of this soil.

(13), oily appearance (16) and small size (0.3 mm) (15). Most of the bacilli were short (10) and large (12), and approximately 67% stained Gram-negative. According to literature, most hydrocarbonoclast strains are identified as Gram-negative.[24]

It was observed that fungi isolated from the same site had, in general terms, the same characteristics. The principal difference was the pigmentation, which evolved with fungal age.

3.4. Bacteria: Degrading Strains

All of the bacteria grew in mineral medium with 20,000 mg of TPH L^{-1}. However, only three of the 32 isolated bacteria were able to degrade at least 60% and 73% of PAHs and AHs, respectively (Table 3). These three bacteria were identified as *P. pseudoalcaligenes* (B01PR), *B. firmus* (B05PR) and *B. alvei* (B21PR). They were isolated from soil with 60,600 mg kg^{-1} of TPH, and it was found that these bacteria had already been reported to degrade organic compounds. *B. firmus* cultivated in liquid culture had transformed 4-chloroaniline to 4-chloroacetanilide and 4-chloropropionanilide.[25] *P. pseudoalcaligenes* cultivated in liquid culture had degraded polychlorinated biphenyls; Gibson reported 100% degradation, while Edita reported 56%.[26,27] *B. alvei* has been reported to degrade chloride pesticides (p-p-DDT, DDE, Lindane and Endrin) with biodegradation efficiencies of 80–100%, as well as anthracene (66–85%) and phenanthrene (61–69%) hydrocarbons; the latter results were obtained in solid fermentation (contaminant concentration 0.91–1.07 mg L^{-1}) using activated carbon and zeolite 5A as solid support.[28,29]

Table 3. Hydrocarbon removal by bacterial strains.

Strain	Identified as	AH (%)	PAHs (%)	PAHs (%)/AH (%)	TPHs (%)
B01PR	*Pseudomona pseudoalcaligenes*	82 ± 5[b]	75 ± 5[a]	0,91	79 ± 5[a]
B05PR	*Bacillus firmus*	90 ± 6[a]	65 ± 5[b]	0.73	80 ± 4[a]
B21PR	*Bacillus alvei*	73 ± 6[c]	60 ± 3[b]	0.82	68 ± 2[c]

Conditions of culture: Culture media modified (Rennie, 1981) for hydrocarbonoclastic bacteria; temperature 30 °C, 150 rpm, treatment duration: 15 days. Different letters are significant different (P(F) = 0.05).

Table 3 shows the percentages of TPH removal determined for the three bacteria cited as better at removing complex mixtures of hydrocarbons. As seen in the table, *P. pseudoalcaligenes* had the highest capacity to remove PAHs (75%), whereas *B. firmus* was the best at removing AHs (90%). The genera of these two species have been classified as among the best at degrading hydrocarbon compounds; *Bacillus* is the second best among the coryneform types.[3] Das tested the capacity of *B. subtilis* and the *P. aeruginosa* M and NM strains to remove crude petroleum oil in liquid culture; removal of 38%, 54% and 46%, respectively, were reported.[30] These percentages are lower than those obtained with *B. firmus* and *P. pseudoalcaligenes* in this study, though the species are members of the same genera. It is possible that the TPH composition was the determinant of the results. Rambeloarisoa showed that compounds such as SHs, aromatics and polar compounds present in different crude oil samples were degraded at different degrees by the same organisms.[31]

The percentages of hydrocarbon removal observed in the present study could be considered low because the residual hydrocarbon concentration (7,878 mg kg^{-1} TPH with *P. pseudoalcaligenes*) was above the maximal value accepted by NOM-EM-138-ECOL-2002, which is 1,000 mg kg^{-1} for TPH, or 0.08 mg kg^{-1} for carcinogenic compounds such as benzo[a]pyrene. Farinazleen[2] reported that a limited number of xenobiotic compounds can be mineralized by single isolates, but often a consortium of bacteria is required for complete degradation. It is thought that greater hydrocarbon removal could be achieved if a consortium of selected bacteria was used.[2]

Table 4 shows the hydrocarbon fractions removed in terms of carbon number for AHs or ring number for PAHs. All of the microorganisms had the capacity to remove all of the hydrocarbon structures, though in low percentages in the case of aromatic compounds of five or six rings. It seems that these structures were better removed by *P. pseudoalcaligenes* and *B. firmus*, but further studies are needed to verify the results. The compounds preferentially degraded by all of the bacteria were $<C_{12}$ and $<C_{20}C_{30}$ AHs, and two- and three-ring PAHs. It is important to remark that, during hydrocarbon degradation, more complex structures are transformed into simpler structures, so the information presented in Table must be analysed carefully.

It should be noted that it was expected that bacteria isolated from the SA soil would show the greatest TPH removal. Rahman had reported that extreme pH conditions are expected to have a negative influence on the ability of microbial populations to degrade hydrocarbons. The contrary results found in this work suggest that the bacteria had problems adapting to the higher pH of the PR soil (Table 2).[32]

3.5. Fungi: Degrading Strains

All of the fungi grew in mineral medium with 20,000 mg of TPH L^{-1}. However, only three of the five isolates degraded at least 60% and 72% of PAHs and AHs, respectively (Table 5). These three fungi were identified by microculture as *P. funiculosum* (H02SA), *A. sydowii* (H03SA) and *Rhizopus* sp. (H01PR). The percentages of AHs and PAHs degraded by *P. funiculosum* were significantly greater ($P(F) < 0.05$) than those of the other two species. No significant difference was observed in the degradation of PAHs by *P. funiculosum* and *A. sydowii*, the strains most efficient at removing PAHs. In Table 5, the ratio %PAH/%AH >0.8 indicates the efficiency of these fungi in removing PAHs.

The three fungi identified as the most efficient at removing hydrocarbons have already been reported as being able to degrade organic compounds. *Rhizopus* sp. degraded 60% of pentachlorophenol (12.5 mg L^{-1}) in a solid

Table 4. TPHs removed by bacteria in terms of carbon or benzene ring number.

	AH			PAH				
	< C12	C12-C20	C20-C30	2-rings	3-rings	4-rings	5-rings	6-rings
P. pseudoalcaligenes	0.84	0.78	0.83	0.92	0.8	0.7	0.26	0.25
B. firmus	0.95	0.83	0.92	0.82	0.75	0.4	0.11	0.17
B. alvei	0.72	0.59	0.78	0.78	0.75	0.21	0.1	0.03

Table 5. Hydrocarbon removal by fungi.

Strain	Identified as	AH (%)	PAHs (%)	PAHs (%)/AH (%)	TPHs (%)
H02SA	*Penicillium funiculosum*	92 ± 5[a]	75 ± 0.5[a]	0.81	86 ± 6[a]
H03SA	*Aspergillus sydowii*	88 ± 4[b]	70 ± 2[a]	0.93	81 ± 5[b]
H01PR	*Rhizopus sp.*	72 ± 4[b]	60 ± 4[b]	0.83	67 ± 3[b]

Conditions of culture: Modified culture media, Rennie (1981), for hydrocarbonoclastic fungi; temperature 30 °C, 150 rpm, treatment duration: 15 days. PR and SA: origin of the polluted soil (PR, Poza Rica; SA, Santa Alejandrina swamp). Different letters are significant different (P(F)=0.05).

Table 6. TPHs removed by fungi in terms of carbon or benzene ring number.

	AH			PAH				
	< C12	C12-C20	C20-C30	2-rings	3-rings	4-rings	5-rings	6-rings
P. funiculosum	0.89	0.9	0.93	0.9	0.85	0.75	0.3	0.22
A. sydowii	0.63	0.73	0.78	0.85	0.81	0.65	0.25	0.2
Rhizopus sp	0.8	0.72	0.71	0.8	0.61	0.51	0.06	0.1

fermentation incubated at 30 °C for 24 h, with sugarcane bagasse as the solid matrix.[31] A *P. funiculosum* strain isolated from the soil at a former gasworks site degraded 57% of the pyrene contained in a liquid culture (50 mg L^{-1}) at 22°C over 28 days.[27] *A. sydowii* isolated from pesticide-treated wheat straw was able to hydrolyze (in 3 weeks) 300 mg kg^{-1} of organophosphate pesticides contained in a soil treated with 1,000 mg kg^{-1} of these contaminants.[28] There have been internal reports on the isolation of *A. sydowii* from marine sediments, but none were found on its capacity to remove PAHs.[32]

P. funiculosum and *A. sydowii*, which showed the greatest capacity (81–86%) to remove 20,000 mg of TPH L^{-1}, were isolated from soil containing 500,000 mg kg^{-1} of TPH. Their greater capacity to remove TPHs could possibly be due to adaptation of these fungi to the pollutant composition, as well as to the enzymatic systems of the fungi. Alexander reported that microorganisms isolated

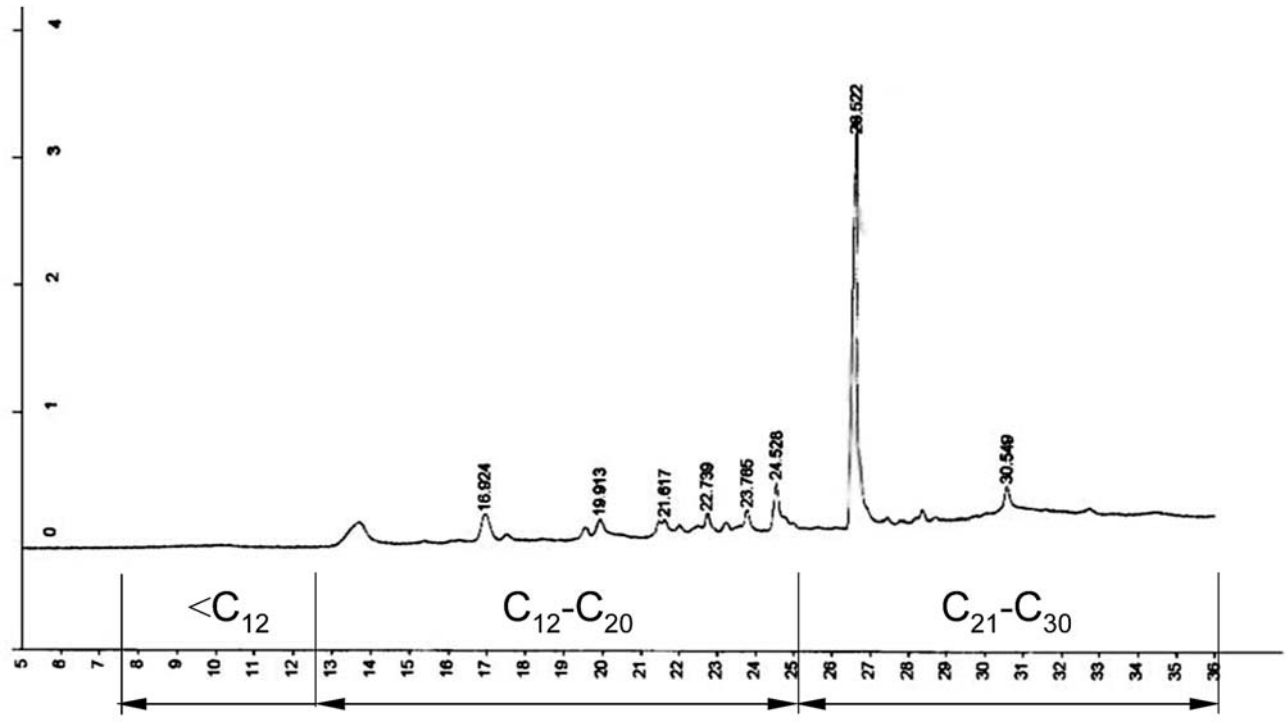

Figure 3. Chromatogram of the residual aliphatic hydrocarbon (AH) fraction analysed after biotreatment with *A. sydowii.* GC implemented with an FID and a non-polar 007 fused silica capillary column: 25 m, i.d. 0.53 mm and film thickness 1 μm.

from highly polluted soils could degrade organic compounds of high molecular weight.[33]

Atlas reported that, in pure cultures, specific AH and PAH fractions have been removed by up to 90% and 75%, respectively.[34] However, it is well known that these hydrocarbon removal percentages can diminish or increase depending on the fermentation type (solid, liquid or slurry) and microorganisms or microflora involved (pure cultures or co-cultures; bacteria-bacteria, fungi-fungi or bacteria-fungi), as well as on the characteristics and concentration of the pollutant involved.[35] In the present work, the results seem to be satisfactory regarding the high complexity and high concentration of the hydrocarbon mixture used (Figures 1 and 2). Figures 3 and 4 show the chromatograms of the residual AH and PAH concentrations determined by GC after complex hydrocarbon mixture biotreatment with *A. sydowii*. Comparison of these two chromatograms with those obtained before the biotreatment (Figures 1 and 2), shows clear diminishment of the peaks, principally of $<C_{12}$ and C_{12}–C_{20} AHs, and of two-rings and three-ring PAHs.

Table 6 shows an analysis of the AHs and PAHs removed by the hydrocarbonoclast fungi. The removed hydrocarbon fractions are given in terms of the carbon number for AHs and the ring number for PAHs. It was observed that all of the carbon compounds conforming to AH were removed at a higher percentage by *P. funiculosum*. Aromatic compounds with two or three rings were removed by all of the fungi at approximately the same percentages as those attained by the bacteria. For four-, five- and six-ring PAHs, a slightly greater removal percentage was observed, with respect to that achieved by the bacteria (Table 4). These results agree with those of Canet, who argued that fungi produce enzymes that enhance the breakdown of strong molecular bonds.[36]

It can be said that the highest removal percentages of aromatic compounds with 5 or 6 rings were achieved by fungi isolated from the SA soil; that is, the soil polluted with 500,000 mg kg^{-1} of TPHs.

4. Conclusions

Three bacteria, *P. pseudoalcaligenes*, *B. firmus* and *B. alvei*, and three fungi, *P. funiculosum, A. sydowii* and *Rhizopus sp*., from 37 strains of microorganism isolated from highly polluted and aged soils, were identified as efficient in degrading a complex mixture of weathered hydrocarbon compounds at an average range of 67–86%. *P. pseudoalcaligenes* and *P. funiculosum* demonstrated the greatest removal of PAHs (75%), whereas *B. firmus* and *P. funiculosum* removed the most AHs (90% and 92%, respectively). High hydrocarbon degradation was observed by *P. funiculosum* isolated from soil with a high TPH concentration (SA soil). Another strain isolated from this soil was *A. sydowii*, for which not reports were found regarding its capacity to remove PAH. This species was found to degrade aromatic compounds with five or six-rings.

It is well known that assemblages of mixed populations with overall broad enzymatic capacities are required to increase the rate and extent of TPH biodegradation. It is suggested that the removal percentages of TPH could be increased by using consortia including the hydrocarbonoclasts presented here, considering their great capacity to remove TPH from complex mixtures of hydrocarbons.

5. Acknowledgement

Authors wish to thank to CONACYT and SEMARNAT for the financial support of this work through the project SEMARNAT-2002-C01-0154. The proactive input of the Editor and anonymous referees of the Acta Chimica Slovenica is gratefully acknowledged.

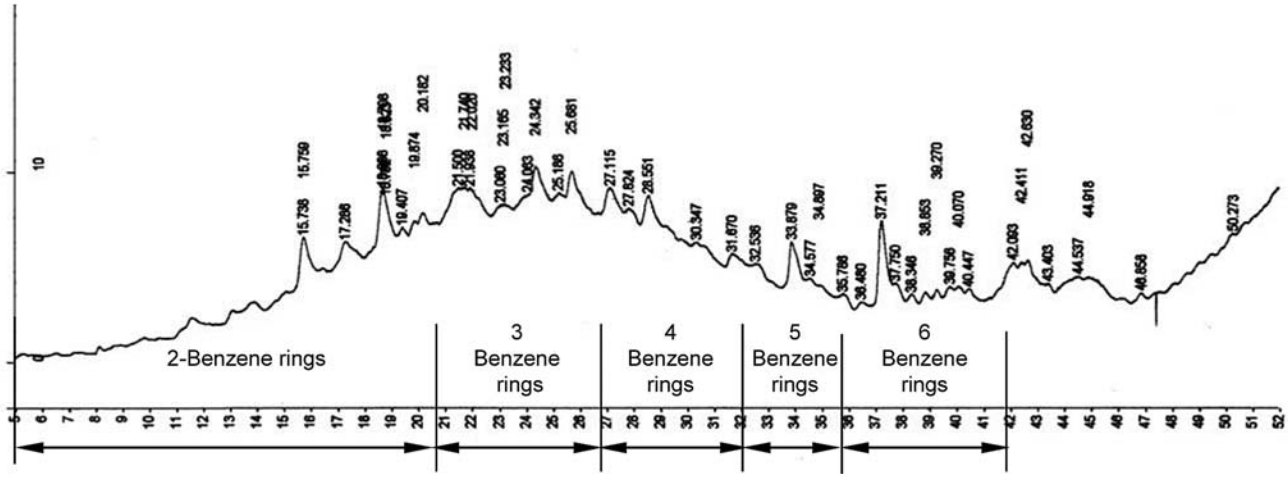

Figure 4. Chromatogram of residual polycyclic aromatic hydrocarbons (PAHs) fraction after biotreatment with *A. Sydowii.* GC implemented with an FID and a non-polar 007 fused silica capillary column: 25 m, i.d. 0.53 mm and film thickness 1 μm.

6. References

1. M. R., Atlas, A. Horowitz, M. Krichevky, K. A. Bej, *Microbiol. Ecol.* **1991**, *22*, 249–256.
2. G. Farinazleen, Z. Raja, A. Rahman, S. Abu, B. Mahiran, *Int. Biodeterior. Biodegrad.* **2004**, *54*, 61–67.
3. R. M. Atlas, C. E. Cerniglia, *BioScience* **1995**, *45*, 332–338.
4. J. G. Leahy, R. R. Colwell, *Microbiol. Rev.* **1990**, *54*, 305–315.
5. C. Cerniglia, G. White, H. Heflich, *Appl. Environ. Microbiol.* **1985,** *50*, 649–55.
6. I. D. Bossert, R. Bartha, *Bull. Environ. Contam. Toxicol.* **1986,** *37*, 490–495.
7. P. Meysami, H. Baheri, *Adv. Environ. Res.* **2003**, *7*, 881–887.
8. J. C. Colombo, M. Cabello, A. M. Arambarri, *Environ. Pollut.* **1996**, *94*, 355–362.
9. S. Lundstedt, Analysis of PAHs and their transformation products in contaminated soil and remedial processes. Sweden: Department of Chemistry, Environmental chemistry Umeå University. **2003,** 1–55.
10. R. M. Atlas, *Microbiol*. **1981**, *45*, 180–209.
11. D. J. Muñoz, A. Mendoza, F. López, A. Soler, M. M. Hernandez, UNAM: Manual de métodos de análisis de suelos; UNAM, México, **2000**.
12. K. Alef, P. Nannipieri, P. Academic Press: San Diego, California, US, Methods in Applied Soil Microbiology and Biochemistry; US, **1995**.
13. S. Mana, J. P. Busalmen, S. R. de Sánchez, *Int. Biodeterior. Biodegrad.* **2001**, *47,* 233–238.
14. M. Trzesicka, O. Ward, *Can. J. Microbiol*. **1995**, *41*, 470–476.
15. J. Ingraham, A. Ingraham, Editorial Reverté, Introducción a la microbiología. Barcelona, España, **1998**.
16. S. L. Ávila, S. M. Estupiñán, *Caldasia* **2006,** *28*, 67–78.
17. A. Verdin, S. Lounes, R. Durand, *Int. Biodeterior. Biodegrad.* **2004**, *53*, 65–70.
18. S. Ryuji, K. Yasuhiro, I. Megumi, K. Masayoshi, T. Tomomasa, I. Tetsuyoshi, K. Susumu, F. Kazuhiro, *Microbiology* **2003,** *149*, 2501–2511**.**
19. R. Rennie, *Can. J. Microbiol*. **1981**, *27*, 8–14.
20. R. Marce, F. Borrull, *J. Chromatogr. A* **2000,** *885*, 273–290.
21. F. Bothe, K. Dettmer, W. Engewald, *Chromatogr. Suppl.* **2003**, *57*, 199–206.
22. K. Plohl, H. Leskovšec, M. Bricelj. *Acta Chim. Slov.* **2002**, *49*, 279–289
23. C. Mollea, F. Bosco, B. Ruggeri, *Chemosphere*. **2005**, *60*, 636–643.
24. S. Peressutti, H. M. Alvarez, O. H. Pucci, *Int. Biodeterior. Biodegrad*. 2003, *52*, 21–30.
25. P. Wallnoefer, L. Pflanzenschutz, B. Pflanzenbau, *Environ. Res. Progr.* **1980**, 828–833.
26. D. T. Gibson, D. L. Cruden, J. D. Haddock, G. J. Zylstra, J. M. Brand, *J Bacteriol.* **1993**, *14,* 4561–4564.
27. E. Ryslava, K. Zdenek, T. Macek, H. Novakova, K. Demnerova, M. Mackova, *Fresinius Environ. Bull.* **2003,** *12,* 296–301.
28. E. Bestawy, A. Mansy, A. Koweidy, *J, Microbiol.* **2002**, *55*, 320–335.
29. A. Bilyk, T. Traczewska, *Environ. Protec. Eng*. **1999**, *25*, 123–129.
30. K. Das, A. K. Mukherjee, *Biores. Technol*. **2007**, *98,* 1339–1345.
31. D. Cortés, J. Barrios, A. Tomasini, *Process. Biochem.* **2002,** *7*, 881–884.
32. C. Raghukumar, Diversity and adaptations of deep-sea microorganisms. *National Institute of Oceanography*. Dona Paual, Goa 403 004. *Internal Report.*
33. M. Alexander, *Biodegradation and Bioremediation*, Academic Press Inc., E. U. A., **1999**.
34. R. M. Atlas, *J. Chem. Technol. Biotechnol.* **1991**, *52*, 149–156.
35. B. Chávez-Gómez, R. Quintero, F. Esparza-García, A. M. Mesta-Howard, F. J. Savala-Díaz de la Serna, C. H. Hernandez-Rodríguez, T. Guillén, H. M, Poggi-Varaldo, J. Barrera-Cortés, R. Rodríguez-Vázquez, *Biores. Technol.* **2003,** *89*, 177–183.
36. R. Canet, J. G. Birnstingl, D. G. Malcom, J. M. López, A. J. Beck, *Biores. Technol.* **2001,** *76*, 113–117.

Povzetek

Namen raziskav je bil določitev razgradljivosti ogljikovodikov v nafti (TPH) s pomočjo bakterij in gliv, gojenih v modificiranem Rennie-jevem mineralnem mediju. Kulture so bile izolirane iz onesnaženih tal z visoko koncentracijo alifatskih (AH) in poliaromatskih (PAH) ogljikovodikov. Ločili smo 26 različnih aktivnih kultur, od katerih jih je bilo 5 sposobnih visoke stopnje razgradnje PAH, AH in TPH. Rezultati kažejo, da sta *P. pseudoalcaligenes* in *A. sydowii* odstranili 71 in 93 % TPH, *P. pseudoalcaligenes* in *P. funiculosum* sta razgradili 75 % PAH, medtem ko sta *B. firmus* in *A. sydowii* uničili 90 in 88 % AH. Najbolj učinkoviti so bili mikrobi izolirani iz tal z najvišjo koncentracijo TPH.