Biodegradation **15:** 249–260, 2004.

Enhanced biodegradation of Casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by *Pseudomonas aeruginosa* AT10

A. Abalos[1], M. Viñas[2], J. Sabaté[2], M.A. Manresa[3] & A. M. Solanas[2,*]
[1]*Center of Biotechnology and Industrial Microbiology, University of Oriente, Santiago de Cuba, Cuba;* [2]*Department of Microbiology, University of Barcelona, Diagonal 645, E-8028 Barcelona, Spain;* [3]*Department of Microbiology and Parasitology, University of Barcelona, Diagonal 643, E-28028 Barcelona, Spain (*author for correspondence: e-mail: solanas@porthos.bio.ub.es)*

Accepted 24 March 2004

Key words: alkylated PAHs, biodegradation, biosurfactant, crude oil, isoprenoids, microbial consortium

Abstract

The biodegradation of oil products in the environment is often limited by their low water solubility and dissolution rate. Rhamnolipids produced by *Pseudomonas aeruginosa* AT10 were investigated for their potential to enhance bioavailability and hence the biodegradation of crude oil by a microbial consortium in liquid medium. The characterization of the rhamnolipids produced by strain AT10 showed the effectiveness of emulsification of complex mixtures. The addition of rhamnolipids accelerates the biodegradation of total petroleum hydrocarbons from 32% to 61% at 10 days of incubation. Nevertheless, the enhancement of biosurfactant addition was more noticeable in the case of the group of isoprenoids from the aliphatic fraction and the alkylated polycyclic aromatic hydrocarbons (PHAS) from the aromatic fraction. The biodegradation of some targeted isoprenoids increased from 16% to 70% and for some alkylated PAHs from 9% to 44%.

Introduction

Petroleum products contain thousands of individual hydrocarbons and related compounds. Their main components are usually sub-divided into saturates (*n*- and branched-chain alkanes or isoprenoids and cycloparaffins) and aromatics (mono-, di-, and polynuclear aromatic compounds containing alkyl side chains and/or fused cycloparaffin rings) (Speight 1991). Hydrocarbons constitute one of the main environmental pollutants and their abundance and persistence in several polluted environmental compartments have been reported (Casellas et al. 1995; Fernandez et al. 1992).

Microbial degradation is the main process of elimination of pollutants in the environment. Nevertheless, although most hydrocarbons are biodegradable, rates of biodegradation in the environment are limited due to their hydrophobicity and low water solubility. Thus, approaches to enhancing the biodegradation processes often attempt to increase the solubility of these hydrophobic compounds by addition of surfactants. These products increase the aqueous dispersion of poorly soluble compounds by many orders of magnitude and change the affinity between microbial cells and hydrocarbons by increasing cell surface hydrophobicity (Barkay et al. 1999; Kanga et al. 1997; Zhang & Miller 1994).

Taking into account the potential use in bioremediation technologies (Ron & Rosenberg 2002), the use of biosurfactants seems to offer more potential than chemical surfactants, due to their structural diversity, biodegradability and biocompatibility relative to synthetic surfactants (Desai & Banat 1997). The biosurfactants most widely studied are the emulsans of *Acinetobacter* spp. (Barkay et al. 1999; Foght et al. 1989) and the group of rhamnolipids produced by *Pseudomonas*

aeruginosa (Van Dyke et al. 1993; Zhang & Miller 1994).

Most studies on the influence of biosurfactants on the microbial degradation of hydrocarbons have been carried out on pure compounds such octadecane (Zhang & Miller 1992), phenanthrene (Barkay et al. 1999; Zhang et al. 1997) or fluoranthene (Willunsen et al. 1998). Studies of complex mixtures such crude oils are scarce (Foght et al. 1989) but knowledge of the influence of biosurfactants on the biodegradation of complex mixtures brings a closer approach to real situations.

Due to the complexity of oil products, biodegradation caused by mixed cultures is more effective than that caused by pure cultures (Sugiura et al. 1997). First, a broader enzymatic capability can be achieved and second, the possible formation of toxic intermediate metabolites (Casellas et al. 1998) can be counteracted by the selection of degraders of these dead end products formed mainly by cometabolism processes (Alexander 1999).

In a previous study (Viñas et al. 2002), we described three specialized consortia obtained in selected fractions from different oil products. These consortia presented different capabilities in the degradation of Casablanca crude oil and one of them, consortium AM, which was obtained by enrichment procedures on five polycyclic aromatic hydrocarbons (PAHs), achieved the highest percentage of biodegradation of the aromatic fraction. Since the aromatic fraction contains the compounds of greater interest from an environmental and toxicological viewpoint, consortium AM was chosen for further study.

Here we characterize the rhamnolipids (MA_{T10}) produced by *Pseudomonas aeruginosa* AT10 to evaluate their effectiveness in enhancing the biodegradation of Casablanca crude oil by the consortium AM.

Material and methods

Bacterial strain and culture conditions of rhamnolipid production

Strain AT10 (CECT 11769) was isolated from contaminated soil samples of the ERASOL vegetable oil refinery in Santiago de Cuba, Cuba (Abalos et al. 2000). The strain was maintained by fortnight cultures and preserved on cryovials at −20 °C. (Combourg, France). From the results obtained in a series of previous morphological and biochemical tests the isolate was identified as *Pseudomonas aeruginosa*. Strain AT10 produces rhamnolipids, named M_{AT10}, when grown in a mineral medium of the following composition (g l^{-1}): $NaNO_3$, 4.64; K_2HPO_4/KH_2PO_4 (ratio 2:1), 1;$FeSO_4{\cdot}7H_2O$, 0.0074; $CaCl_2$, 0.01; KCl, 0.10; $MgSO_4{\cdot}7H_2O$, 0.50; yeast extract, 0.10 and supplemented with 0.05 ml l^{-1} of a trace mineral solution containing (g l^{-1}): H_3BO_3, 0.26; $CuSO_4{\cdot}5H_2O$, 0.50; $MnSO_4{\cdot}H_2O$, 0.50; $MoNa_2O_4{\cdot}2H_2O$, 0.06; $ZnSO_4{\cdot}7H_2O$, 0.70. 20 g l^{-1} of waste free fatty acids (WFFA) from soybean oil production, which is mainly composed of $C_{18:1}$: 21% and $C_{18:2}$: 43.4%, was used as the carbon source. The strain was incubated at 30 °C and 200 rpm in a reciprocal shaker.

16SrRNA characterization of strain AT10

Total DNA of strain AT10 was isolated from late-exponential phase cells by lysis with sodium dodecyl sulphate (SDS)-proteinase k, and treatment with CTAB (cetyltrimethylammonium bromide) as described earlier (Wilson 1987).

The nucleotide sequences of the PCR product were determined in both directions using universal primers by a DNA sequencing kit (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit version 2.0; Perkin-Elmer Biosystems, Foster City, CA) following the manufacturer's instructions. The products of the sequencing reactions were analyzed with a model 3700 DNA sequencer (Perkin-Elmer). The 16S rRNA gene sequences were aligned with published sequences from the GenBank database using BLASTn 2.2.1 (Altschul et al. 1997), FASTA 3 (Pearson & Lipman 1988) and RDP (Maidak et al. 2000) alignment tool comparison software. Nucleotide sequence data were submitted to the EMBL nucleotide sequence database and are listed under the accession number AJ549293.

Biosurfactant recovery

Rhamnolipids were recovered from the culture supernatant: cells were removed from the culture by centrifugation at 12,000 × *g* in a Kontron cen-

trifuge (Kontron, Milano, Italy) for 30 min at 4 °C. Purification of rhamnolipids was achieved, following a modification of the method of Reiling (Reiling et al. 1986), by adsorption chromatography on a polystyrene resin, Amberlite XAD2 (Sigma, St. Louis, USA). The resin (60 g) was placed in a glass column (60 × 3 cm), yielding a bed volume of 200 ml. The column was equilibrated with 0.1 M phosphate buffer, pH 6.1. The culture supernatant was acidified to pH 6.1 and applied through a sieve placed on top of the resin to prevent whirling up. The adsorption of the active compounds on the resin was monitored by measuring the surface tension (γ_{ST}) of the column outlet. The saturation of the resin was terminated when γ_{ST} of the effluent dropped below 40 mN/m. Thereafter, the column was rinsed with three volumes of distilled water until γ_{ST} of the effluent approached 72 mN/n, at which no free fatty acids remained in the column. Then biosurfactants were eluted with methanol and, finally, the solvent was evaporated to dryness under vacuum (Büchi, Flawil, Switzerland). To check the purity of the extract, that is, the absence of residual fatty acids, a 5 mg portion was resuspended in methanol and analysed by HPLC-MS (Abalos 2001). Rhamnolipids were quantified as rhamnose content by a colorimetric method, using a standard of rhamnose (Chandrasekaran & Bemiller 1980). The rhamnolipid content was calculated by multiplying the rhamnose concentration by 3.0, which represents the rhamnolipid/rhamnose correlation (Abalos et al. 2001). Rhamnolipid homologues were quantified from the molecular proportion of each of the pseudomolecular ions calculated by LC-MS (Haba et al. 2003).

Composition of rhamnolipids M_{AT10}

LC-MS. Rhamnolipid mixtures were separated and identified by liquid chromatography coupled to mass spectrometry using a Waters 2690 separation module (Waters, Milford, MA). The samples were injected (100 μl) into a Hypersil C8 WP-300 (5 μm) 150 × 4.6 mm column (Teknokroma, Sant Cugat, Spain). The LC flow-rate was 1 ml min^{-1}. An acetonitrile-water gradient was used starting with 30% of acetonitrile for 4 min, followed by a ramp of 30–100% acetonitrile for 40 min, standby for 5 min and then a return to initial conditions. Post-column addition of acetone at 200 μl min^{-1} was performed using a Phoenix 20 (Carlo Erba, Italy) because an enhancement of the sensitivity for rhamnolipids was observed. The LC effluent and acetone were mixed in a tee (Valco) and split (1/50) before entering the mass spectrometer. MS was performed with a single quadrupole mass spectrometer, VG Platform II (Micromass, Manchester, UK), equipped with a pneumatically assisted electrospray (ES) source. The negative ion mode was used. Full scan data were obtained by scanning from m/z 100 to 750 in the centroid mode using a scan duration of 2.0 s and an inter-scan time of 0.2 s. The working conditions for ES were as follows: dry nitrogen was heated to 80 °C and introduced into the capillary region at a flow-rate of 400 l h^{-1}. The capillary was held at a potential of −3.5 kV. The extraction voltage was held at −75 V. Samples were prepared by diluting in methanol 10 mg/ml of each rhamnolipid mixture, M_{AT10}.

Physicochemical characteristics of M_{AT10}

Equilibrium surface tension (γ_{ST}), interfacial tension (γ_{IT}) and cmc were measured at 25 °C by the Wilhelmy plaque technique with a Krüss K12 tensiometer. The instrument had been calibrated against Milli-Q-4 ultrapure distilled water (Millipore). Solutions with various concentrations of surfactants were obtained by successive dilutions of a concentrated sample (2×10^3 mg l^{-1}) prepared by weight in Millipore ultrapure water. To reach the equilibrium, all sample solutions had been aged in appropriate cells at room temperature (25 °C). The platinum plate and all the glassware used were cleaned in a chromic mixture. The cmc was calculated from the plots values of surface tension *versus* log surfactant concentration after equilibrium, at 25 °C. The pC_{20} is a parameter that indicates the efficiency of the performance of a surfactant. It is the amount of surfactant (mg l^{-1}) needed to decrease the surface tension of distilled water by 20 mN/m units.

Determination of the hydrophilic–hydrophobic balance (HLB)

The HLB scale (ranging from 0 to 20) is a measure of the relative contribution of the hydrophilic and lipophilic regions of the molecule, calculated from the structure of the molecule. It is quantified

according to an empirical formula that calculates the group contribution (group numbers) to the HLB number, such that the HLB was obtained from the following equation: HLB = 7 + Σ (HLB hydrophilic groups)-Σ (HLB hydrophobic groups). The group numbers assigned by Adamson for the glycolipid structure of MA_{T10} are: HLB for carboxylic groups, 2.1; glycosidic rings, 0.5; acyl groups, −0.47 (Adamson & Gast 1997).

Emulsions

Emulsions were prepared by weight of the components up to 1 g as follows: the required concentrations of MAT10 were weighed in glass tubes, mixed with the required amount of the hydrophobic component and vortexed for 1 min until complete dissolution of the paste. Then deionized water was added slowly (up to 1 g), and the tube was stoppered and mixed vigorously for 1 min. The shaking time and vortex speed were the same in all cases. Emulsions were allowed to settle at constant temperature for 24 or 48 h before the emulsion was measured and visually inspected. The degree of emulsification (E%) is the percentage of the height (cm) of the emulsion versus the total height (cm).

The selected hydrophobic components assayed were: benzene, 0.31 g; toluene, 0.31 g; all supplied by Panreac (Barcelona, Spain), *n*-alkanes, 0.31 g supplied by Petresa, Madrid, Spain); Kerosene Jetta-1 0.31 and 0.75 g; lubricating oil 0.31 g supplied by Campsa (Barcelona, Spain); Casablanca crude oil 0.5 and 0.15 g was kindly supplied by Dr. J. Grimalt (CID, CSIC, Barcelona).

The ternary phase diagram was drawn from the emulsion behaviour of a series of emulsions (see above) prepared with different proportions of the components assayed. The ternary phase diagram is represented as an equilateral triangle. Each side is divided into 100 parts to correspond to the percentage composition. The composition of the system at any point can be obtained by measuring the perpendicular distances to the three sides (Atwood & Florence 1983). In this study the combinations of the three components ranged between (w/w): M_{AT10} 2–20%; Casablanca crude oil 5–80%; deionized water 10–95%. The maximum concentrations of solubilisate forming a clear emulsion were determined by visual inspection.

Consortium AM

Subsequent enrichment cultures were established in mineral medium and a mixture of 5 PAHs with three and four aromatic rings: fluorene, phenanthrene, anthracene, pyrene, and fluoranthene. The consortium are maintaining by twice-weekly transfers in fresh medium for 5 years. Consortium AM causes the complete depletion of phenanthrene, fluorene, pyrene and anthracene in 6 days, while 90% of fluoranthene is removed in 9 days at a final concentration of 0.02% each component (Figure 1) (Viñas et al. 2002).

Casablanca crude-oil and culture media

Casablanca is a crude oil from the Tarragona Basin (Spanish Mediterranean off-shore). The composition analysis performed according to the methods described in Viñas et al. (2002) can be observed in Table 1.

The mineral medium used throughout this study contained (per liter): $K_2HPO_4{\cdot}3H_2O$, 4.25 g; $NaH_2PO_4{\cdot}H_2O$, 1.00 g; NH_4Cl, 2.00 g; $MgSO_4{\cdot}7H_2O$, 0.20 g; $FeSO_4{\cdot}7H_2O$, 0.012 g; $MnSO_4{\cdot}H_2O$, 0.003 g; $ZnSO_4{\cdot}7H_2O$ 0.003 g; $CoSO_4{\cdot}7H_2O$, 0.001 g; nitrilotriacetic acid, 0.10 g. The medium was sterilized before the addition of the crude oil. The oil was placed in an air-tight closed vial, sterilized at 121 °C for 20 min and added to the mineral medium at a final concentration of 0.5% (v/v).

Biodegradation of crude oil by consortium AM with and without rhamnolipids

The influence of addition of rhamnolipids produced by strain AT10 was evaluated on replicated batch cultures in 250 ml Erlenmeyer flasks containing 50 ml of mineral medium described above with 0.5% of Casablanca crude oil. One milliliter of cellular suspension of the consortium AM grown in mineral medium with 5PAHs, corresponding to 1.9×10^8 MPN (most probable number) of microorganisms ml^{-1}, was used as inoculum. Two sets of cultures were studied: cultures of consortium AM without rhamnolipids (hereafter AM) and cultures of AM with rhamnolipids M_{AT10} (hereafter AMRLs). The final concentration of the rhamnolipids M_{AT10} in the medium was 500 mg l^{-1}. Replicated samples and

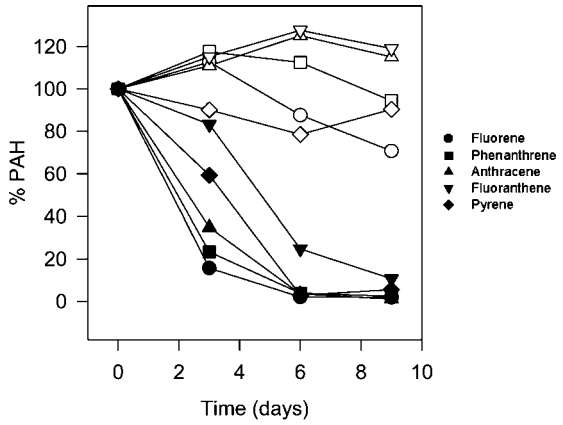

Figure 1. Degradation of a mixture of PAHs by consortium AM. The concentration of fluorene (● culture, ○ control), phenanthrene (■ culture, □ control), anthracene (▲ culture, △ control), fluoranthene (▼ culture, ▽ control) and pyrene (◆ culture, ◇ control) was determined by HPLC analyses of organic extracts.

Table 1. Characteristics of Casablanca crude oil

Saturates (F1)	57%[a]
Monocyclic aromatics (F2)	11%
Polycyclic aromatics (F3)	15%
Polars (crude oil–F1–F2–F3)	17%
n-C_{17}/pristane	0.76

[a] Data obtained from gravimetric analyses.

their corresponding controls were incubated up to 20 days in a reciprocal shaker (200 rpm, 25 °C).

Crude oil analysis

The residual crude oil from cultures and controls was extracted with dichloromethane (5 × 10 ml) by liquid–liquid extraction in duplicate samples. Before extraction, α-androstane was added to each flask as internal standard. The extracts were dried over Na_2SO_4 and concentrated in a vacuum rotary evaporator and dried under gentle nitrogen stream and analyzed as follows:

(i) Column chromatography. All the extracts were fractioned according to Aceves et al. (1988). Each extract was resuspended in 1 ml of dichloromethane and loaded in a glass column (30 × 1 cm i.d.) filled with 10 g each of 5% water-deactivated alumina (70–230 mesh, Merck). Total petroleum hydrocarbon fraction (TPH) was obtained by eluting with 100 ml of dichloromethane.

(ii) GC–FID: Biodegradation of saturated compound of TPH fractions were verified by Gas Chromatography with Flame Ionisation Detector (GC–FID). A Gas chromatograph Termoquest Trace 2000 was used. Compounds were separated on a capillary column DB5 (25 m by 0.32 mm [i.d.] 0.25-μm film thickness). The column temperature was held at 35 °C for 2 min and then programmed to 310 °C at a rate of 4 °C min^{-1}. This final temperature was held for 10 min. The detector and inlet temperature were set at 320 °C and 290 °C respectively. The Helium flow was 1.1 ml min^{-1} and injection volume was 1 μl.

(iii) GC–MS–SIM: Biodegradation of aromatic compounds of TPH fractions were verified by Gas Chromatography coupled to Mass Spectrometer operating in selected ion monitoring mode (GC–MS–SIM). Gas chromatography/mass spectrometry analyses were performed with a Hewlett Packard 5890 series II gas chromatograph with a 5989A mass selective detector. Compounds were separated on a HP-5 capillary column (25 m by 0.32 mm [i.d.]) with 0.25-μm film thickness and helium used as a carrier gas (10 psi). The column temperature was held at 35 °C for 2 min and then programmed to 310 °C at a rate of 4 °C min^{-1}. This final temperature was held for 10 min. Injector and transfer line temperatures were set at 280 °C, and analyzer was set at 300 °C. The

injection was in splitless mode keeping the split valve closed for 30 s.

The targets for analysis were naphthalene, phenanthrene and their alkyl derivatives C1–C4; fluorene, dibenzothiophene and their alkyl derivatives C1–C3; and fluoranthene, pyrene and chrysene. To determine the biodegradation of each analyte, the corresponding reconstructed ion chromatograms were obtained and the areas of the compounds in cultures and in controls were compared.

Results and discussion

Taxonomical characterization of strain AT10

BLAST and FASTA systems showed similarity of 99.18% to *Pseudomonas aeruginosa* AL_{98} (AJ249451) a strain degrader of natural rubber (Linos et al. 2000) and 99.18% to *Pseudomonas aeruginosa* 42A2 NCBIMB 40045 (AJ309500). High RDP match was found (0.90) in both cases.

Composition of M_{AT10}

Frequently, biosurfactants are accumulated in the culture medium as a family of different components. In the case of rhamnolipids, homologues differ in the number of rhamnose molecules as well as in the length and composition of the alkyl chain (Dèziel et al. 1999). Rhamnolipids M_{AT10} *were obtained by cultivating the strain P. aeruginosa* AT10 in an aerated submerged culture, purified and analysed by HPLC-MS as described elsewhere (Abalos 2001). Figure 2 shows the kinetics of the accumulation of the different components during the incubation. The behaviour of a particular biosurfactant varies with the composition of the mixture. The biosurfactant M_{AT10} chosen corresponds to the mixture obtained after 96 h of cultivation. It was characterized by HPLC-MS (Abalos et al. 2001) as being composed of eight different rhamnolipid molecules in the following proportion (% w/w): Rha-$C_{8:2}$ (11.83); Rha-C_{10}-$C_{12:1}$(6.43); Rha-Rha-C_{10}-$C_{12:1}$ (11.35◊); Rha-C_{10}-C_{10} (28.90); Rha-Rha-C_{10}-C_{10} (21.37); Rha-$C_{12:2}$ (4.67); Rha-C_{10}-C_{12} (2.61▽); Rha-Rha-C_{10}-C_{12} (12.81).

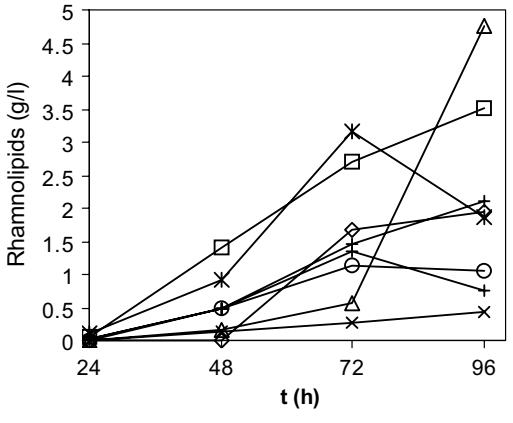

Figure 2. Time course of rhamnolipid homologues accumulation in a submerged culture of *P. aeruginosa* AT10 incubated in aerated mineral medium with free fatty acids from soybean at 30 °C followed by HPLC-MS (see Material and methods). Rha-$C_{8:2}$ (●); Rha-C_{10}-$C_{12:1}$(◆); Rha-Rha-C_{10}-$C_{12:1}$ (◇); Rha-C_{10}-C_{10} (■); Rha-Rha-C_{10}-C_{10} (□); Rha-$C_{12:2}$ (×); Rha-C_{10}-C_{12} (▽); Rha-Rha-C_{10}-C_{12} (▲).

Characterization and emulsification properties of M_{AT10}

The product M_{AT10} was a yellowish-semisolid, very soluble in alkaline solutions, methanol, trichloromethane, ethylacetate and ether, slowly soluble in water and insoluble in hexane. The rhamnolipids M_{AT10} reduce the surface tension (γ_{ST}), up to 26.8 mN/m and the interfacial tension (γ_{IT}) against hexadecane to 1 mN/m. The critical micellar concentration of M_{AT10} (CMC) was 150 mg l^{-1}. The pC_{20} found was 2.63 mg/ml this parameter indicates the efficiency of a surfactant; it is the concentration required to reduce 20 mN/cm the surface tension of the bulk phase. As an indication of the emulsification behaviour, the HLB value calculated for the rhamnolipids produced by strain AT10 was 10.07, since at the higher end of the scale (10–20) the surfactants are hydrophilic and act as solubilizing agents o/w, whereas oil-soluble surfactants with low HLB values act as w/o emulsifiers (Florence & Atwood 1998), M_{AT10} will form o/w emulsions. However, multiple emulsions (o/w/o) with crude oil were also observed, which may be due to the complex composition of the M_{AT10}. According to Clayton et al. (1992) a surfactant with an overall HLB in the range 9–11 will generally yield the best dispersion of oil droplets in the water phase. Table 2 shows the degree of emulsification caused by M_{AT10} with different hydrophobic compounds.

Table 2. Emulsification (%) of hydrophobic substrates with rhamnolipids M_{AT10}

Substrate	M_{AT10}: Substrate:H_2O			$^{*}E_{24}$ (%)	$^{*}E_{168}$ (%)
Benzene	15	31	54	–	–
Toluene	15	31	54	77	30
n-alkanes[a]	15	31	54	75	–
Lubricating oil[b]	15	31	54	100	100
Kerosen[c]	15	31	54	100	100
	5	75	20	50	50
Casablanca oil	15	31	54	100	100
	5	15	80	60	60

[a] *n*-alkanes (%): C_{12} : 0.9; C_{13}: 97.8; C_{14}: 0.9.
[b] composition (%): total paraffins: 18; total naphtenes: 60.4; total aromatics: 8.1; thiophenes: 3.5.
[c] Jetta-1, Campsa, Spain.
[d] E_{24} and E_{186} emulsion measured at 24 and 168 h respectively.

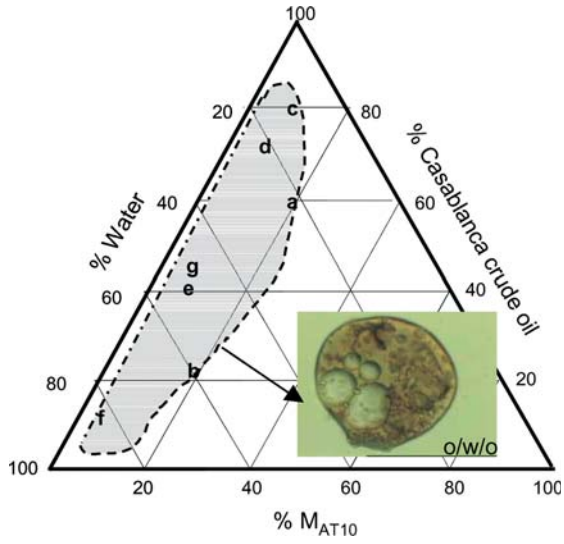

Figure 3. Phases diagram of the ternary system oil–rhamnolipids-water. The shaded zone correspond to the compositions oil–rhamnolipids-water studied. All compositions formed multiple emulsions o/w/o (microscopic view). 100% of each component corresponded to the vertex of the triangle. (a) 20:60:20; (b) 20:20:60; (c) 10:80:10 (d) 10:70:20; (e) 10:40:50; (f) 5:15:80; (g) 5:45:50.

M_{AT10} was very effective with complex mixtures. Although Casablanca oil and kerosene were partially emulsified when 5% of M_{AT10} was added, complete emulsification of Casablanca crude oil, mineral oil or kerosene, was achieved when 15% of M_{AT10} was added to the mixture (Table 2). The emulsions were stable after 168 h. In the case of *n*-alkanes or toluene 75% of emulsification was observe.

To examine the solubilization behaviour of Casablanca crude oil in the multicomponent biosurfactant solutions (M_{AT10}), the diagram of the ternary phase (Figure 3) is indicated when two of the components are immiscible. Solubility data are expressed as a solubility (emulsification) versus concentration to describe the effect of varying all three components of the system (Florence & Atwood 1998). Figure 3 shows the different equilibrium phases obtained after mixing different quantities of the components (M_{AT10}:Casablanca crude oil:water) in the required proportions (see Material and methods). The shaded area of the phase diagram corresponds to the emulsification range. The degree of emulsification was assayed within the range of 5–20% of M_{AT10}, and depended on the proportion of the components. When the proportion of the components was 20:70:10; 20:60:20 or 20:20:60, (those proportions are represented along the line of 20% of rhamnolipids, in Figure 3) the degree of emulsification was 70–73% . Hundred percent of emulsification was found when the proportion was 10:70:20 or 10:40:50, whereas the 10:80:10 or 10:50:40 mixture gave an emulsification of 80.6% and 60% respectively. Seventy percent emulsification was observed when M_{AT10} was 5%, in the proportions 5:45:50 and 5:35:65, whereas 60% of emulsification was found when the proportion was 5:15:80.

From the result found we conclude that M_{AT10}, is a good emulsifier, since up to 80% of crude oil could be emulsified. M_{AT10} added in a proportion 1:10:89 (M_{AT10}:Casablanca crude oil:water) was used to enhance the biodegradation of Casablanca crude oil in cultures of the bacterial consortium AM.

Microscopy observations (Figure 3) indicated that the emulsions were oil in water in oil (o/w/o). These multiple emulsions may be due to the proportions of the rhamnolipid molecules in M_{AT10} which influence the surface-active properties of the biosurfactant mixture and affect the micelle structure. The presence of micelles increases the apparent solubility, facilitating the uptake of the oil into the cell. This process, in combination with

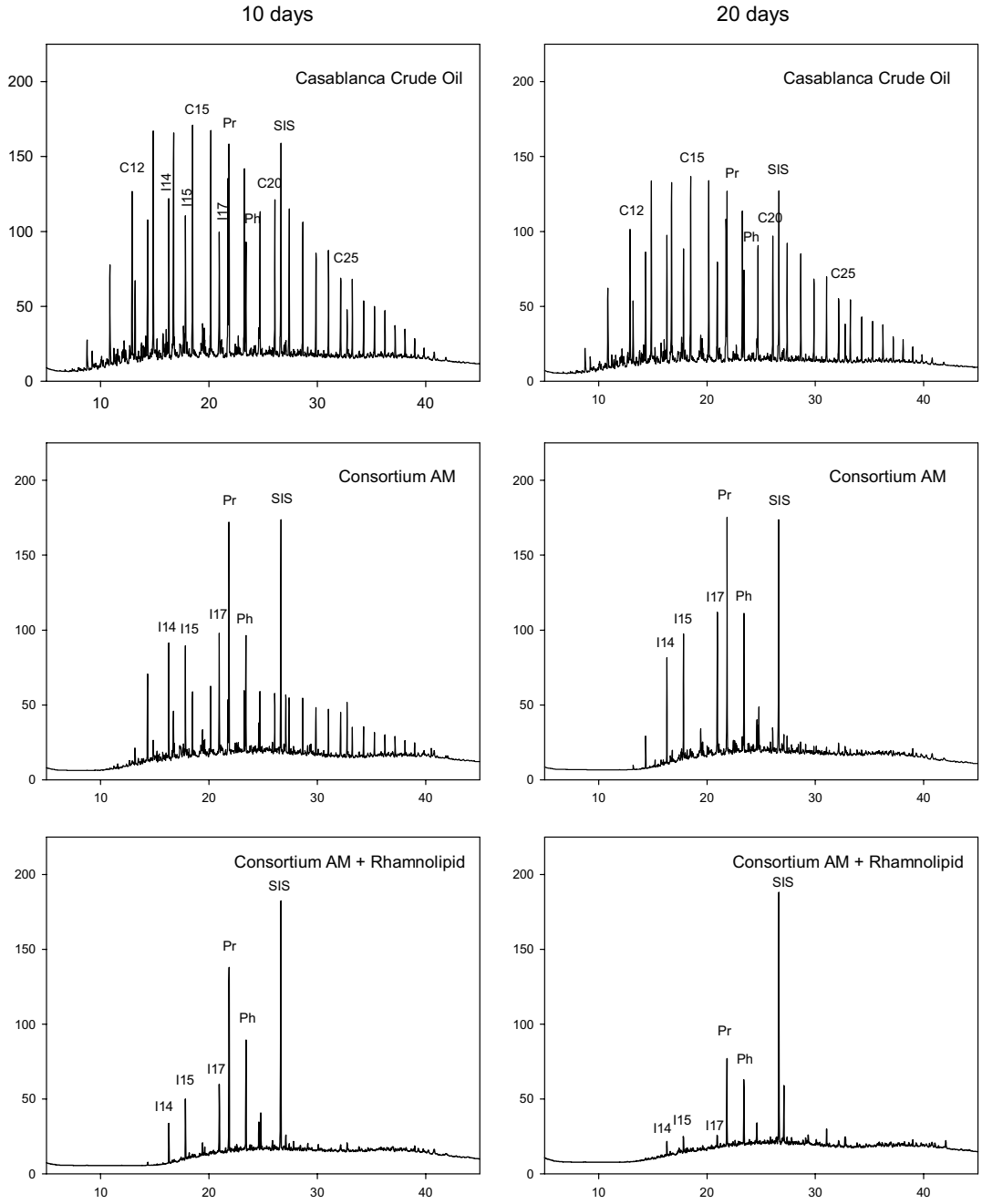

Figure 4. Chromatographic profiles (GC–FID) of TPH extracts from 10 and 20 days. C12–C25, *n*-alkanes containing 12–25 carbons; Pr, Pristane; Ph, phytane; I15–18, isoprenoid alkanes containing 15–18 carbons, SIS, surrogate internal standard.

the direct contact of the soluble hydrophobic compounds, may have significant influence on bacterial growth and degradation of organic compounds present in each phase (Selkelky & Shreve 1999).

Biodegradation of the Casablanca crude oil by consortium AM with and without M_{AT10}

The biodegradation activity of consortium AM corresponding to TPH fraction of Casablanca

Table 3. Percentage (%) of biodegradation of targeted isoprenoids by microbial consortia AM and microbial consortia with biosurfactant (AMRLs)

Molecule	AM 10 days	AMRLs 10 days	AM 20 days	AMRLs 20 days
Farnesane	19.8	82.2	49.5	85.7
IP16[a]	20.86	70.1	28.89	82.3
norpristane	13.08	54.3	14.42	88.7
Pristane	1.44	0	5.11	58.1
Phytane	0.94	9.1	0	39
Total[b]	10.16	37.14	16.25	70
Total[c]	32.9	60.63	61.86	71

[a] 2,6,10-trimethyltridecane.
[b] Total biodegradation of isoprenoids was calculated by comparing the sum of all GC-FID isoprenoid area in cultures and controls.
[c] Total biodegradation of TPH fraction was calculated by comparing the sum of all resolved peaks in GC-FID chromatograms in cultures and controls.

Table 4. Percentage (%) of biodegradation of targeted PAHs by microbial consortia AM and microbial consortia with biosurfactant (AMRLs)

Molecule	AM 10 days	AMRLs 10 days	AM 20 days	AMRLs 20 days
Naphthalene (Naph)	11.5	100	100	100
C1-Naph	13.77	100	100	100
C2-Naph	11.06	89.5	100	100
C3-Naph	0	66.8	87	95.49
C4-Naph	0	0	11	17
Fluorene (Fl)	0.18	100	100	100
C1-Fl	0	75.8	87	89
C2-Fl	0	0	18	14.63
C3-Fl	0	0	9	44.33
Phenanthrene (Phe)	36.09	100	100	100
C1-Phe	0	100	96	100
C2-Phe	0	28.8	48	56.38
C3-Phe	0.3	4.8	20	38.95
Dibenzothiophene (DBT)		ND	ND	ND
C1-DBT	0.49	86.9	75	81.04
C2-DBT	0.88	18.7	40	47.78
C3-DBT	8.46	8.8	29	34.59
Pyrene (Py)	ND	ND	ND	ND
C1-Py	0	8.3	0	35.35

C1-C4 represent carbon numbers of the alkyl groups in alkylated PAHs. Compounds Naph, C1-Naph, C2-Naph, C3-Naph, C4-Naph, Fl, C1-Fl, C2-Fl, C3-Fl, C1-DBT, C2-DBT, C3-DBT, Phe, C1-Phe, C2-Phe, C3-Phe and C4-Phe were characterized by the signals at m/z 128, 142, 156, 170, 184, 166, 180, 194, 208, 198, 212, 226, 178, 192, 206, 220 and 234, respectively. DBT was characterized by the signals at m/z 139 + 152. The percentage of biodegradation was calculated by comparing in cultures and controls the areas shown by the targeted compounds in reconstructed ion chromatograms.

crude oil was 32.9% and 61.86% at 10 and 20 days of incubation respectively (Table 3). Most of this percentage corresponds to *n*-alkanes (Figure 4). Cultures with rhamnolipid (AMRLs) achieved 60.6% and 71% biodegradation of the TPH fraction at 10 and 20 days of incubation, but in this case major biodegradation of isoprenoids or branched-chain alkanes was achieved (Figure 4). The degradation observed for some chosen targeted isoprenoids including pristane and phytane (Table 3) showed that consortium AM degraded 16.25% in 20 days, whereas the addition of M_{AT10} increased the percentage of biodegradation of this group of compounds to 70%, indicating that the solubilization of these complex hydrophobic compounds was effective.

While the *n*-alkanes are the most biodegradable components of the saturated fraction of petroleum products, the group of isoprenoids are more recalcitrant. This has been attributed to the branched chemical structure (Britton 1984) or to a repressed action of *n*-alkanes present (Morgan & Watkinson 1994). Nevertheless, an important characteristic to take into account is their extremely low water solubility, which limits their availability to biodegrading microorganisms. The results obtained with consortium AM in the presence and absence of rhamnolipids indicate that the low degradability of isoprenoids obtained by consortium AM without M_{AT10} was due mainly to their low availability.

The reconstructed ion chromatograms for targeted compounds from the aromatic fraction (Table 4) demonstrated that at 10 days of incubation the addition of rhamnolipids increased the biodegradation of all selected compounds. Consortium AM presented a preferential degradation of parental PAHs in detriment to their branched derivatives. Naphthalene and its methyl and dimethyl derivatives were biodegraded only 11–13.7% and the more branched tri- and tetramethyl

derivatives were not degraded. The presence of a methyl substituent in a PAH renders the molecule more resistant to microbial metabolism and biodegradation decreases with an increase in carbon atoms in the side chain (Solanas et al. 1984; Sugiura et al. 1997).

In contrast to the behaviour of consortium AM alone, AMRL cultures showed a complete removal of dimethyl naphthalene, a depletion of 89.5% of trimethyl naphthalene and a removal of 66.8% of trimethyl naphthalene. Taking into account that the aqueous solubilities of the substituted naphthalenes decrease with an increase in methyl substitution (Verschueren 1977) it seems that the addition of rhamnolipids enhanced solubilization of these less water-insoluble compounds. In this respect, Kanga et al. (1997) described that a glycolipid biosurfactant presented a greater solubilization potential of high branched naphthalenes than synthetic surfactants because they formed a large micellar volume which can better accommodate these higher molecular weight substituted derivatives. Taking these results into account, an increment of quantities solubilized and therefore available to the microorganisms may allow a more rapid depletion of these branched PAHs.

At 20 days of incubation, the biodegradation of the aromatic fraction for both types of culture, AM and AMRLs, showed similar results. Nevertheless, the removal of some higher branched PAHs such trimethylfluorene or trimethylphenanthrene increased when rhamnolipids were present and only AMRL cultures can degrade methylpyrene (35.35%). As described for methylnaphthalenes, the solubility of other alkylated PAHs also decreases with an increase in methyl substitution (Verschueren 1997).

These results have a great interest from an environmental point of view. While parental polycyclic aromatic hydrocarbons (PAHs) have acutely toxic effects and can present mutagenic, teratogenic or carcinogenic properties (International Association of Research Chemists 1983) their alkylderivatives can have even more serious consequences for public health. Alkylated PAHs are the main group of compounds within the mutagenic, lower molecular weight fraction of PAH in energy-related materials (Griest et al. 1979). Also, fluorene, phenanthrene and anthracene, have been found to be inactive as mutagens in the Ames assay, while methylated derivatives of these compounds are mutagenic toward *S. typhimurium* TA 100 (LaVoie et al. 1979).

The results obtained have potential applications in the bioremediation of contaminated sites by accelerating the biodegradation rates of these more hydrophobic pollutants. The research is an on-going effort to study the application of rhamnolipids of strain AT10 in bioremediation of hydrocarbons contaminated soils.

Conclusions

Rhamnolipids from strain AT10 of *Pseudomonas aeruginosa* enhanced the biodegradation of Casablanca crude oil by a microbial consortium. An increase in biodegradation rate of total petroleum hydrocarbons was achieved when rhamnolipids were present. However, rhamnolipids were particularly effective in stimulating the biodegradation of the group of isoprenoids from the aliphatic fraction and on the alkylated polycyclic aromatic hydrocarbons from the aromatic fraction.

Acknowledgements

This research was funded by the grants from the Spanish Government's National Plan for Research (REN2001-3425/TECNO; PPQ-2000-0105-P4 and 2001SGR 00143) and CIRIT 2001SGR 00143. M. Viñas is a recipient of a pre-doctoral fellowship from the Comissionat de Recerca i Innovació Tecnològica (CIRIT) of the Generalitat de Catalunya. We are grateful to Pilar Teixidó (Serveis Científico-Tècnics, University of Barcelona) for the GC-MS data. The authors declare that the experiments discussed in this paper complied with current Spanish law.

References

Abalos A, Deroncelé V, Espuny J, Bermúdez R & Manresa A (2000) Surface active rhamnolipids accumulation by *Pseudomonas aeruginosa* AT10 from a vegetal oil refinery wastes. Revista Cubana de Química. XII, 24–29

Abalos A, Pinazo A, Infante R, Casals M, García F & Manresa A (2001) Physico chemical and antimicrobial properties of

new rhamnolipids produced by *Pseudomonas aeruginosa* AT10 from soybean oil refinery wastes. Langmuir 17: 1367–1371

Aceves M, Grimalt J, Albaigès J, Broto F, Comellas L & Grassiot M (1988) Analysis of hydrocarbons in aquatic sediments. II. Evaluation of common preparative procedures for petroleum and chlorinated hydrocarbons. J. Chromatogr. 436: 503–509

Adamson A & Gast A (1997) Emulsions. Foams and aerosols. In: Physical Chemistry of Surfaces (pp 500–528). Wiley and Sons, New York

Alexander M (1999) Biodegradation and bioremediation, 2nd edn. Academic Press, San Diego, CA

Altschul SF, Madden TL, Schäffer AA, Zang J, Zang Z, Miller W & Lipman DL (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acid Res. 25: 3389–3402

Atwood D & Florence, AT (1983) Phase behaviour of surfactants. In: Surfactant Systems, Their Chemistry, Pharmacy and Biology (pp 40–70). Chapman & Hall, London

Barkay T, Navon-Venezia S, Ron EZ & Rosenberg E (1999) Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier alasan. Appl. Environ. Microbiol. 65: 2697–2702

Britton LN (1984) Microbial degradation of aliphatic hydrocarbons. In: Gibson DT (Ed) Microbial Degradation of Organic Compounds (pp 89–129). Marcel Dekker, New York

Casellas M, Fernandez P, Bayona JM & Solanas AM(1995) Bioassay-directed chemical analysis of genotoxic components in urban airborne particulate matter from Barcelona (Spain). Chemosphere 30: 725–740

Casellas M, Grifoll M, Sabaté J & Solanas AM (1998) Isolation and characterization of a 9-fluorenoned-degrading bacterial strain and its role in synergistic degradation of fluorene by a consortium. Can. J. Microbiol. 44: 734–742

Chandrasekaran EV & Bemiller JN (1980) Constituent analysis of glycosaminoglycans. In: Wrhiste L and Wolfrom ML (Eds) Methods in Carbohydrate Chemistry (pp 89–97). Academic Press, New York

Clayton JR, Payne JR & Farlow JS (1992) Oil Spill Dispersants: Mechanisms of Action and Laboratory Tests. CRC Press Inc., Boca Raton, FA

Desai JD & Banat IM (1997) Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biol. Rev. 61: 47–64

Déziel E, Lépine F, Dennie D, Boismenu D, Mamer O & Villemur R (1999) Liquid chromatography/mass spectrometry analysis of mixture of rhamnolipids produced by *Pseudomonas aeruginosa* strain 57RP grown on mannitol or naphthalene Biophys Biochim. Acta 1440: 244–252

Fernandez P, Grifoll M, Solanas AM, Bayona JM & Albaigés J (1992) Bioassay-directed chemical analysis of genotoxic components in coastal marine sediments. Environ. Sci. Technol. 26: 817–829

Florence AT & Atwood D. (1998) Emulsions. In: Physico-Chemical Principles of Pharmacy (pp 252–307) Palgrave, New York.

Foght JM, Gutnick DL & Westlake DWS (1989) Effect of emulsan on biodegradation of crude oil by pure and mixed cultures. Appl. Environ. Microbiol. 55: 36–42.

Griest WH, Tomkins BA, Epler JL & Rao TK (1979) Characterization of multialkylated polycyclic aromatic hydrocarbons in energy-related materials. In: Jones PW and Leber P (Eds) Carcinogenesis, Vol. 4 (pp 395–409). Raven-Press, New York

Haba E, Abalos A, Jáuregui O, Espuny MJ & Manresa A (2003) Use of liquid-chromatography-mass spectroscopy for studying the composition and properties of rhamnolipids produced by different strains of *Pseudomonas aeruginosa*. J. Sur. Det. 6: 155–161

International Association of Research Chemists (1983) IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans. Polynuclear aromatic compounds. Part. 1. Int. Assoc. Res. Chem. Monogr. 32: 355–364

Kanga SA, Bonner JS, Page CA, Mills MA & Autenrieth RL (1997) Solubilization of naphthalene and methyl-substituted naphthalenes from crude oil using biosurfactants. Environ. Sci Technol 31: 556–561

LaVoie EJ, Bedenko V, Hirota N, Hecht SS & Hoffmann D (1979) A comparison of the mutagenicity, tumor initiating activity and complete carcinogenicity of polynuclear aromatic hydrocarbons. In: Jones PW and Leber P (Eds) Carcinogenesis, Vol. 4 (pp 705–721). Raven Press, New York

Linos A, Reichelt R, Keller U & Steinbuchel A (2000) A gram-negative bacterium, identified as *Pseudomonas aeruginosa* AL98, is a potent degrader of natural rubber and synthetic cis-1,4-polyisoprene. FEMS Microbiol. Lett. 182: 155–161

Maidak BL, Cole JR, Lilburn TG, Parker Jr CT, Saxman PR, Stredwick JM, Garrity GM, Olsen GJ, Pramanik S, Schmidt TM & Tiedje JM (2000) The RDP (Ribosomal Database Project) continues. Nucleic Acids Res. 28: 173–174

Morgan P & Watkinson RJ (1994) Biodegradation of compounds of petroleum. In: Ratledge C (Ed) Biochemistry of Microbial Degradation. Kluwer Academic Publishers, Dordrecht

Pearson WR & Lipman DJ (1988) Improved tools for biological sequence analysis, Proc. Natl. Acad. Sci. USA 85: 2444–2448

Reiling HE, Thanei-wyss U, Guerra-Santos LH, Hirt R, Käppeli O & Fiechter A (1986) Pilot plant production of rhamnolipid biosurfactant by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. 51: 985–989

Ron EZ & Rosenberg E (2002) Biosurfactants and oil bioremediation. Curr. Opin. Biotechnol. 13: 249–252

Sekelsky AM & Shreve GS (1999) Kinetic model of biosurfactant-enhanced hexadecane biodegradation by *Pseudomonas aeruginosa*. Biotechnol. Bioeng. 63: 402–409

Speight JG (1991) The Chemistry and Technology of Petroleum. John Wiley & Sons, New York

Solanas AM, Parés R, Bayona JM & J Albaigès J (1984) Degradation of aromatic petroleum hydrocarbons by pure microbial cultures. Chemosphere 13: 593–601

Sugiura K, Ishihara M & Shimauchi T (1997) Physicochemical properties and biodegradability of crude oil. Environ. Sci. Technol. 31: 45–51

Van Dyke MI, Couture P, Brauer M, Lee H & Trevors TJ (1993) *Pseudomonas aeruginosa* UG2 rhamnolipid biosurfactant: structural characterization and their use in removing

hydrophobic compounds from soil. Can. J. Microbiol. 39: 1071–1078

Verschueren K (1997) Handbook of Environmental Data on Organic Chemicals. Van Nostrand Reinhold Company, New York

Viñas M, Grifoll M, Sabaté J & Solanas AM (2002) Biodegradation of a crude oil by three microbial consortia of different origins and metabolic capabilities. J. Ind. Microbiol. Biotechnol. 28: 252–260

Wilson K (1987) Preparation of genomic DNA from bacteria. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. (Eds) Current Protocols in Molecular Biology (pp. 2.4.1–2.4.2). John Wiley, New York

Willumsen PA, Karlson U & Pritchard PH (1998) Response of fluoranthene-degrading bacteria to surfactants. Appl. Microbiol. Biotechnol. 50: 475–483

Zhang YM & Miller RM (1992) Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (Biosurfactant). Appl. Environ. Microbiol. 60: 2101–2106

Zhang YM & Miller RM (1994) Effect of a *Pseudomonas* rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. Appl. Environ. Microbiol. 60: 2101–2106

Zhang YM, Maier WJ & Miller RM (1997) Effect of rhamnolipids on the dissolution, bioavailability and biodegradation of phenanthrene. Environ. Sci. Technol. 31(8): 2211–2217