# Polycyclic Aromatic Hydrocarbon Degradation by Biosurfactant-Producing *Pseudomonas* sp. IR1

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We characterized a newly isolated bacterium, designated as IR1, with respect to its ability to degrade polycyclic aromatic hydrocarbons (PAHs) and to produce biosurfactants. Isolated IR1 was identified as *Pseudomonas putida* by analysis of 16S rRNA sequences (99.6% homology). It was capable of utilizing two-, three- and four-ring PAHs but not hexadecane and octadecane as a sole carbon and energy source. PCR and DNA hybridization studies showed that enzymes involved in PAH metabolism were related to the naphthalene dioxygenase pathway. Observation of both tensio-active and emulsifying activities indicated that biosurfactants were produced by IR1 during growth on both water miscible and immiscible substrates. The biosurfactants lowered the surface tension of medium from 54.9 dN $cm^{-1}$ to 35.4 dN $cm^{-1}$ and formed a stable and compact emulsion with an emulsifying activity of 74% with diesel oil, when grown on dextrose. These findings indicate that this isolate may be useful for bioremediation of sites contaminated with aromatic hydrocarbons.

*Key words:* Biosurfactant, Polycyclic Aromatic Hydrocarbons, *Pseudomonas* sp.

## Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in soils and sediments and are of environmental concern because of their mutagenic and/or carcinogenic effects. The major sources of PAHs in the environment are the combustion of organic matter and the processing and use of fossil fuels. Biological degradation is widely accepted as the primary dissipation mechanism for most organic pollutants in the environment, but the activity of degrading microorganisms is dependent upon many factors, including contaminant uptake and bioavailability, concentration, toxicity, mobility, access to other nutrients, and activated enzymes. Mass transfer from insoluble phases is often considered to be the rate-limiting step in biodegradation of organic contaminants because the compounds must be released to the aqueous phaseprior to entering the microbial cell and subsequent intracellular transformation by the necessary catabolic enzymes. Biosurfactants are known to enhance hydrocarbon solubility and/or improve affinity of microbial cells for the substrate to facilitate their bioavailability and degradation in aqueous and soil system (Kuyukina *et al.,* 2005).

A large number of microorganisms able to use PAHs of two and three rings as the sole source of carbon and energy has been reported (Bamforth and Singleton, 2005). However, biosurfactant production by PAH-degrading microorganisms has rarely been reported. Deziel *et al.* (1996) isolated a total of 23 PAH-degrading bacterial strains from petroleum-contaminated soils, and 10 of them were able to produce biosurfactants. In one of the strains, *Pseudomonas aeruginosa* 19SJ, the production of biosurfactants from solid naphthalene was accompanied by an increase in the aqueous concentration of the compound, thus suggesting a role in promoting its solubilization. In another study, Willumsen and Karlson (1997) isolated 57 different bacteria from PAH-contaminated soils and found that many of them produced biosurfactants, although only four were able to reduce the surface tension of the culture to significant levels (35–40 mN $m^{-1}$). Burd and Ward (1996) reported the production of an extracellular surface-active factor of high molecular weight, composed of protein

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and lipopolysaccharide, by a strain of *Pseudomonas marginalis* able to grow on PAHs. Finally, according to Garcia-Junco *et al.* (2001) rhamnolipid production increased the bioavailability of phenanthrene, so promoted biodegradation activity of *Pseudomonas aeruginosa* 19SJ.

There are also several reports on improved hydrocarbon degradation by addition of biosurfactant or chemical surfactant (Rahman *et al.,* 2003; Kuyukina *et al.,* 2005). However, the effect of *in situ* biosurfactant production by hydrocarbon-degrading bacteria itself may be beneficial, promising and more practical than exogenously adding purified biosurfactant for field bioremediation application based on bioaugmentation. A hydrocarbon-degrading bacterium having the ability to produce extra-cellular biosurfactant can speed up the biodegradation by reducing mass transfer limitations and facilitating the oil-microbe contact.

In this article we describe a bacterial strain, which is having a coexisting capacity to degrade a wide range of polycyclic aromatic hydrocarbons and potentially produce biosurfactant during growth in water-soluble and insoluble carbon source including PAHs.

## Materials and Methods

### *Enrichment and isolation of bacterium*

The bacterial strain was isolated by the enrichment culture technique from soil obtained from a hydrocarbon-contaminated site at IDEA, Caracas, Venezuela. A 5 g sample of soil was inoculated into 100 ml of minimal salt medium (MSM) containing ($l^{-1}$) 6.0 g of $Na_2HPO_4$, 3.0 g of $KH_2PO_4$, 1.0 g of $NH_4Cl$, 0.5 g of NaCl, 1.0 ml of 1 M $MgSO_4$, and 2.5 ml of a trace element solution [($l^{-1}$), 23 mg of $MnCl_2{\cdot}2H_2O$, 30 mg of $MnCl_4{\cdot}H_2O$, 31 mg of $H_3BO_3$, 36 mg of $CoCl_2{\cdot}6H_2O$, 10 mg of $CuCl_2{\cdot}2H_2O$, 20 mg of $NiCl_2{\cdot}6H_2O$, 30 mg of $Na_2MoO_4{\cdot}2H_2O$, and 50 mg $ZnCl_2$] (pH 7.0). Crude oil (1%, w/v) was used as carbon source and incubated at 30 °C on a rotary shaker (200 rpm) for 4 d. After five cycles of such enrichment, 1 ml of the culture was diluted and plated on MSM agar (2%, w/v) plates containing naphthalene as sole carbon source and incubated at 30 °C. Naphthalene was provided in the vapor phase by adding crystals to Petri dish lids. The bacterial colonies obtained were further purified on Luria-Bertani agar plates. The strain was stored as frozen stock cultures at −70 °C in 25% glycerol.

### *Catabolic potential of strain IR1*

The ability of strain IR1 to grow on various hydrocarbons was screened by using liquid MSM containing 0.2 g of substrate/l. All PAH stock solutions were prepared in absolute ethanol. Erlenmeyer flasks (100 ml) containing 20 ml of MSM and the hydrocarbon were inoculated with 100 $\mu$l pre-culture (Luria-Bertani broth, OD 600 = 0.8, about $10^8$ cells/ml) and incubated at 30 °C and 200 rpm. Growth was followed by measuring the increase in cell density. For solid MSM the PAHs (except naphthalene) were dissolved in 5% (w/v) diethyl ether and sprayed on the surface of MSM agar. Naphthalene (1 g) was provided as crystals directly placed on the plate lid. Growth on PAHs in solid media was considered positive by the formation of a clear zone around the growing colonies or appearance of pigments.

### *Degradation of PAH compounds*

Bacterium was grown in batch culture in a 250 ml flask containing 50 ml of MSM supplemented with an individual PAH or mixed PAHs as sole carbon source. When used as single PAH in MSM, the final concentration was 200 mg/l. For a PAHs mixture 50 $\mu$g of each PAH, *i.e.* naphthalene, dibenzothiophene (DBT), pyrene and phenanthrene, were dissolved in $\alpha$-methylnaphthalene (2 ml) and 2% (v/v) of the resulting mixture was used. The experimental flasks were inoculated with 2% (v/v) inoculum ($10^5$ CFU/ml) and incubated at 30 °C in the dark on a rotatory shaker (200 rpm). At the timed interval, flasks were taken out and 1 ml culture was withdrawn for OD measurements at 600 nm and viability testing by counting colony-forming units on Luria-Bertani agar plates. The residual PAH from culture flask was extracted twice with one volume of dichloromethane and the solvent layer was separated. Evaporation of solvents was done under a gentle nitrogen stream in a fume hood. The residual PAH was dissolved in 5 ml of acetone, and 1 $\mu$l of the resultant solution was analyzed by a gas chromatograph (Boonchan *et al.*, 2000) fitted with a flame ionization detector and a 30 m DB 5.625 column (0.25 $\mu$m i.d., film thickness 0.25 $\mu$m). Un-inoculated flasks and flasks without PAH served as controls. The experimental and control cultures were conducted in triplicate and experiments were repeated at least once.

*Isolation of DNA*

Bacterial total DNA was isolated according to Chen and Kuo (1993). The culture was analyzed for the presence of plasmid DNA by the method of Kado and Liu (1981) and Hansen and Olsen (1978).

*Amplification of nahA and nahE gene*

Primers (Table I) used for *nahA* (naphthalene dioxygenase) and *nahE* (*trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase) were based on the gene sequences reported for *P. putida* G7 and *P. putida* NCIB 9816 (Habe and Omori, 2003). PCR amplification was carried out in 50 $\mu$l reaction mixtures that contained 20 mM tris-(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) (pH 8.4), 50 mM KCl, 1.25 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 $\mu$M, 2.5 U of *Taq* DNA polymerase (Invitrogen), 0.2 $\mu$M forward primer, 0.2 $\mu$M reverse primer, and 0.1 $\mu$g of template genomic DNA. PCR was performed in a PCT-100$^{TM}$ thermal cycler unit (MJ Research Inc., MA, USA) with the following temperature program: (i) 5 min at 95 °C; (ii) 30 cycles, with 1 cycle consisting of 30 s at 95 °C, 1 min at the optimum annealing temperature (*nahA* 46 °C, *nahE* 45 °C), and 2 min at 72 °C; and (iii) a final extension step of 10 min at 72 °C. PCR products were routinely visualized by running 10 $\mu$l of PCR mixture on 1% agarose gels (Bio-Rad, Richmond, CA) in 0.5 × Tris-borate-EDTA (TBE) buffer stained with ethidium bromide (0.0001%). All experiments included control reaction mixtures without added DNA. *P. putida* NCIB 9816–4 was taken as positive control and *E. coli* DH5-alpha was taken as negative control.

*Southern hybridization*

For restriction fragment length polymorphism analysis, 2 $\mu$g of total DNA was digested with mixtures of two restriction enzymes, separately, and then electrophoresed. Electrophoresis and Southern transfers onto nylon membranes were done as previously described by Sambrook *et al.* (1989). Purified PCR amplificon of *Pseudomonas putida* NCIB 9816 was used as the sequence source for the *nahA* and *nahE* gene probe. Southern hybridizations using the ECL direct nucleic acid labeling and detection system (Amersham Biosciences, NJ, USA) were made against digests of genomic DNA from IR1. *P. putida* NCIB 9816 DNA was used as a positive control. The filters were washed under high-stringency [0.1 × sodium chloride-sodium citrate buffer (SSC) (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.5% sodium dodecyl sulfate (SDS) at 68 °C], intermediate-stringency (0.1 × SSC plus 0.5% SDS at 37 °C), or low-stringency (2.0 × SSC plus 0.5% SDS at 23 °C) conditions.

*Biosurfactant production*

To study biosurfactant production and activity, bacteria were either grown in YPG medium [containing (g $l^{-1}$) yeast extract (5); peptone (5); glucose (15)] or in MSM separately, containing water-soluble and water-insoluble carbon source (2%, w/v). The cultures were incubated at 30 °C and 250 rpm. After 24 h the culture broth was centrifuged at 8,000 × *g* for 10 min and the supernatant was used for the measurement of surface-active properties. The surface tension of the biosurfactant was measured by the Ring method using a CSC-DuNouy tensiometer at room temperature. The culture supernatant was diluted with distilled water, and the surface tension was measured at various concentrations. Drop test and oil spread test were carried out according to Youssef *et al.* (2004). The emulsification activity ($E_{24}$) was determined by the addition of the respective hydrocarbon (kerosene, gasoline, diesel fuel, gas oil, hexadecane, and $\alpha$-methyl naphthalene) to the same volume of cell-free culture broth, mixed with a vortex for 2 min and left to stand for 24 h. The emulsification activity was determined as the per-

Table I. Specific PCR primers for the detection of *nahA* and *nahE* genes.

| Gene | Primer | Position | Product size [bp] | Sequence |
|---|---|---|---|---|
| *nahA* | *nahAf* | 1–23 | 3463 | 5′ ATGGAACTTCTCATACAA/GCC 3′ |
| | *nahAr* | 3444–3463 | | 5′ AGAAAGACCATCAGATTGT 3′ |
| *nahE* | *nahEf* | 1–23 | 990 | 5′ ATGTT/CGAATAAAATTAT/GT/GAAAA 3′ |
| | *nahEr* | 972–990 | | 5′ ACTGTATTTAGCGTGCAGC 3′ |

centage of height of the emulsified layer (mm) divided by the total height of the liquid column (mm). To study the stability of emulsion the emulsified solutions were allowed to stand at 60 °C and the emulsification index was analyzed at different time intervals. Surface-active compounds were extracted by liquid–liquid extraction from cell-free culture broth acidified with 1 N HCl to pH 2.0 (Rahman *et al.*, 2003). Supernatant fluid was mixed in a mixture of equal volume of chloroform/methanol (2:1, v/v). The solvent was evaporated and the material was used as crude biosurfactant and weighed to evaluate the yield. Determination of the carbohydrate content of the isolated biosurfactant was done by the anthrone reagent method at 620 nm (Spiro, 1966). Protein was assayed by the Bradford (1976) method using BSA as a standard. Lipid was analyzed as described by Ilori and Amund (2001). The bacterial adhesion to hydrocarbons (BATH) assay was used in order to test the hydrophobicity of the isolate according to Rosenberg and Rosenberg (1981). Specified biosurfactant production or activity was determined by analyzing the emulsification index ($E_{24}$) with diesel oil.

### *16S rRNA partial gene sequencing and bacterium identification*

Gene sequence coding for 16S rRNA was partially amplified by PCR using universal primers U1 (5′-CCA GCA GCC GCG GTA ATA CG-3′) corresponding to nucleotide positions 518 to 537 (forward primer) and U2 [5′-ATC GG(C/T) TAC CTT GTT ACG ACT TC-3′] corresponding to nucleotide positions 1513 to 1491 (reverse primer) according to the *Escherichia coli* numbering system (Weisburg *et al.,* 1991). The PCR operating conditions were as described by Lu *et al.* (2000) with minor modifications. Briefly, a reaction mixture containing approx. 50 ng of template DNA, PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 $\mu$M of each PCR primer, 0.2 mM of each deoxynucleoside triphosphate, and 5 U of Taq DNA polymerase in a total volume of 50 $\mu$l was prepared. A PCT-100™ thermal cycler unit (MJ Research Inc.) was set for a first denaturation cycle at 95 °C for 5 min and then for 45 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 20 s. A final step of 72 °C for 10 min was followed by 4 °C until the cycler shut down. The primer set generated a PCR product of approx. 996 bp. Purified *E. coli* DNA or no DNA were also run as positive and negative controls, respectively. The PCR product was separated by agarose gel electrophoresis, visualized by SYBR® Green 1 staining (Sigma, St. Louis, USA) and finally purified by using a Wizard PCR Preps Purification System (Promega Corp., Madison, USA) according to the manufacturer's instructions.

DNA sequencing reaction was performed with an ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the sequencing products were separated by capillary electrophoresis using a 310 Sequencer (Perkin-Elmer Corp., Applied Biosystems, Foster City, USA) according to standard procedures. Sequence data were analyzed with DNAMAN, version 5.2.9 (Lynnon BioSoft, Quebec, Canada) to obtain a consensus sequence. To identify the isolated bacterium, the 16S rRNA consensus sequence was then compared with 16S rRNA gene sequences from the public GenBank, EMBL, and DDBJ databases using the advanced gapped n-BLAST program, version 2.1. The program was run via internet through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast/). Sequences with more than 98% identity with a GenBank sequence were considered to be of the same species as the highest score-matching sequence on the public sequence databases.

## Results and Discussion

### *Isolation and identification of PAH-degrading bacterial strain*

A pure bacterial culture was isolated from the enriched contaminated soil samples, essentially on the basis of its ability to grow on crude oil and utilize the naphthalene vapor as sole carbon source. This Gram-negative bacterial strain grows very fast (colonies visible within 18 hours) on the MSM agar plate having naphthalene as sole carbon source and bacterial colonies accumulate the appreciable brown oxidation products within 24 hours. This is indicative of the expression of naphthalene dioxygenase and *cis*-naphthalene dihydrodiol dehydrogenasse by the strain (Ensley and Gibson, 1983). The bacteria achieve saturated growth on the MSM liquid medium within 12 hours if naphthalene is used as carbon source. Taxonomical identification of bacterial strain IR1 was performed by amplification and sequencing the

16S rRNA genes and comparing them to the database of known 16S rRNA sequences. Alignment of the 16S rRNA gene sequences of the IR1 isolate with sequences obtained by doing a Blast searching revealed more than 99.6% similarity to *Pseudomonas putida*. The 16S rRNA gene partial consensus sequence obtained from the isolate IR1 differs only by 3 mismatches in 878 bases when compared with most closely related sequences of *Pseudomonas putida* according to the Blast search results.

*Catabolic potential of strain IR1*

The bacterial strain was tested for its ability to grow on a variety of carbon sources including various low and high molecular weight PAHs as well as other simple aromatic hydrocarbons and *n*-alkanes. These chemicals represent the most common organic pollutants and are the main components of crude oils. The strain exhibited a broad substrate profile, being able to utilize pyrene, phenanthrene, dibenzothiophene etc. but does not grow on hexadecane and octadecane (Table II). The possibility that these compounds are toxic for bacteria was excluded because the strain grew on glucose containing media in their presence. This strain formed blue indigo pigmentation in the presence of indole, indicating naphthalene dioxygenase activity (Ensley and Gibson, 1983). In catechol it produced a yellow coloring suggesting a *meta*-cleavage product of catechol. When colonies were grown on PAH-coated agar plates, a zone of clearing appeared, indicating PAH degradation. With DBT on the plate as well as in liquid media the bacterium produced orange or reddish brown water-soluble product(s). The bacterium can effectively utilize the heavy crude oil as sole source of carbon and energy (data not shown).

Table II. Substrate profile of the strain IR1. Cells were grown in liquid medium with the corresponding compounds as sole carbon source as described in Materials and Methods. Growth was considered: (+++), if after 72 h, the cell density in cultures was threefold to that obtained for controls without the carbon source; (++), if after 96 h the cell density in cultures was threefold to that obtained for controls without the carbon source; (+), if after 124 h the cell density in cultures was threefold that obtained for controls without the carbon source; (–), if no growth was observed after a week. Substrate degradation ability of isolated bacteria was also confirmed by growth in agar plates containing the respective hydrocarbon as sole carbon source.

| Substrate | Growth |
|---|---|
| Naphthalene | +++ |
| Phenanthrene | ++ |
| Pyrene | +++ |
| Dibenzothiophene | +++ |
| Toluene | + |
| Phenol | – |
| Ethanol | + |
| Catechol | ++ |
| Salicylate | ++ |
| Hexadecane | – |
| Decalin(decahydronaphthalene) | + |
| 2-Hydoxyquinoline | – |
| Protocatechuic acid | + |
| $\alpha$-Methyl naphthalene | ++ |

*Degradation of PAHs*

Growth at the expense of PAHs was verified by demonstrating an increase in bacterial growth concomitant with a decrease in its concentration. Uninoculated flasks and flasks without PAHs served as controls. Since PAHs consisting of four rings or more are generally considered recalcitrant to biodegradation, we studied in details the ability of IR1 to degrade pyrene. Pyrene concentration and growth were measured. Bacterial growth and pyrene concentrations at sampling intervals are expressed as the average of those obtained for triplicate flasks. Abiotic degradation of pyrene was not detected in sterile control flasks. However, after 5 days around (68 ± 2)% pyrene was degraded by the bacterial culture (Fig. 1). The pyrene degradation is comparable to that of bacteria reported in literature (Walter *et al*., 1991; Churchill *et al.,* 1999; Sarma *et al*., 2004). However, the experimental conditions used varied in all cases. For example, Churchill *et al.* (1999) used 300 ppm of pyerene for their degradation experiment while 500 ppm (72% degradation in 2 weeks) and 200 ppm (61.5% degradation in 20 days), respectively, were used by Walter *et al.* (1991) and Sarma *et al.* (2004). The UV-visible spectra of the culture supernatant of culture grown in the presence of pyrene showed absorption maxima at 208, 216, 274 and at 220, 257 nm indicating the presence of ring fission metabolites and initial ring oxidation metabolites (Heitkamp *et al.,* 1988), respectively (data not shown). These peaks were not observed on the control flask at any time interval. The bacterial degradation of pyrene, a pericondensed PAH, has been reported by a number of groups. Most of the bacteria are actinomycetes and belong to the ge-

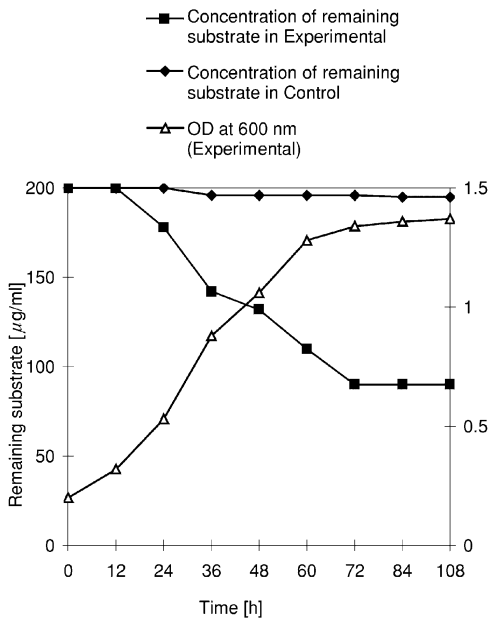


Fig. 1. Pyrene degradation and growth of strain IR1. Each value represents the mean of three samples with standard error $< 5\%$.

nus *Mycobacterium* (Krivobok *et al.*, 2003), *Rhodococcus* (Walter *et al.*, 1991), or *Gordonia* (Kastner *et al.*, 1994). A few pyrene-degrading strains have been identified as Gram-negative species, including *Stenotrophomonas maltophilia*, *Pseudomonas fluorescens*, *Sphingomonas paucimobilis*, and *Burkholderia cepacia* (Boonchan *et al.*, 1998). *Mycobacterium* sp. strain CH1 which can degrade pyrene was also capable of using a wide range of branched alkanes and *n*-alkanes as sole carbon and energy source. Hybridization studies showed that the enzyme system involved in PAH degradation by this strain is unrelated to the naphthalene dioxygenase pathway (Churchill *et al.,* 1999). Strain IR1 do not require any additional cofactors for cell growth on pyrene like few microbes which utilize four-ring PAHs for growth only in the presence of cofactors or surfactants (Heitcamp *et al.,* 1988; Churchill *et al*., 1999).

The DBT transformation capacity of the strain, initially indicated by the release of colored compounds in the growth medium, was confirmed by the removal of DBT from the growth medium when the compound was used as sole carbon and energy source. DBT removal was associated with corresponding microbial growth (data not shown). The DBT content of the culture decreased by $(76 \pm 4)\%$ after 72 h of incubation. The bacteria produced pinkish-red pigmentation indicating the dimer formation of the end product of DBT degradation, hydroxy-2-formyl-benzothiophene (Kodama *et al.*, 1973). When the supernatant of DBT grown culture was scanned between the wavelengths 200 and 600 nm in an UV-visible spectrophotometer, absorption maxima at 315, 475 and 390 nm were observed which correspond to the maxima of DBT metabolites reported in the literature for, respectively, the initial metabolites dihydroxydibenzothiophene, the ring cleavage product *trans*-4-[2-(3-hydroxy)-thionaphthenyl]-2-oxo-3-butenoic acid and the final metabolite 3-hydroxy-2-formyl-benzothiophene (HFBT) (Monticello *et al.,* 1985). Contrary to the results of Monticello *et al.* (1985), the accumulation of the products of DBT degradation did not inhibit the growth of the isolate IR1 and mediated DBT oxidation. We could find a time-dependent increase of the HFBT and cell growth (unpublished data). Isolate IR1 was also examined for its ability to degrade naphthalene, catechol, and phenanthrene when used separately as sole source of carbon under the conditions similar to those described above. GC analysis revealed $(75 \pm 3)\%$, $(53 \pm 2)\%$ and $(66 \pm 3)\%$ degradation of naphthalene, catechol, and phenanthrene, respectively, after one week. After one week strain IR1 could degrade $(69 \pm 3)\%$, $(72 \pm 2)\%$, $(82 \pm 4)\%$, and $(60 \pm 4)\%$ of pyrene, naphthalene, DBT, and phenanthrene, respectively, from a PAHs mixture in non-aqueous phase liquid (NAPL), *i.e.* $\alpha$-methylnaphthalene, as revealed by GC analysis. Results indicated that the isolate IR1 can effectively grow in mixtures of PAHs and the degradation is not specific to a single compound. Isolate IR1 was not capable to utilize hexadecane and octadecane as carbon source. Foght and Westlake (1988) have also reported microbes that could mineralize aliphatic or aromatic compounds but not both, suggesting that alkane and PAH biodegradation may be mutually exclusive properties in bacteria. However, there are several reports showing that the ability to degrade aliphatic and aromatic hydrocarbons is not necessarily mutually exclusive. Whyte *et al.* (1997) isolated a hydrocarbon-degrading *Pseudomonas* sp. which degraded both alkanes and naphthalene. *Mycobacterium* sp. strain CH1 which can degrade pyrene was also capable of using a wide range of branched alkanes and *n*-alkanes as sole carbon and energy sources (Churchill *et al.*, 1999).

*Isolation of plasmid*

The bacterial strain IR1 was screened for the presence of large catabolic plasmids, similar to those in NAH plasmids. However, in the initial phase, no plasmid was detected by using routine alkaline extraction methods. A large plasmid of the strain was only detected by the method of Hansen and Olsen (1978), however, the efficiency of the extraction was not good enough to thoroughly eliminate chromosomal DNA.

*PCR amplification of nahA and nahE gene and hybridization*

When the primers *nahAf* and *nahAr* were used, a single PCR fragment of the expected size (3.4 kb) was amplified in IR1 and NCIB 9816-4. No amplification was observed in the negative control and control without DNA. Similarly, when the set of primers *nahEf* and *nahEr* was used, a product of the expected size (990 bp) was produced in IR1 and NCIB 9816-4 (Fig. 2). Since the *nah* genes of the isolated strains could be amplified using primers designed from the control strains and the amplicons had the expected size, this suggests the same structural organization of the *nah* gene in both organisms. When the total DNA of strains IR1 and NCIB 9816-4 was digested separately with restriction enzymes *BamHI* and *PstI*, both of the strains produced a distinguishable pattern (data not shown). Southern blots of total DNA digested with restriction enzymes

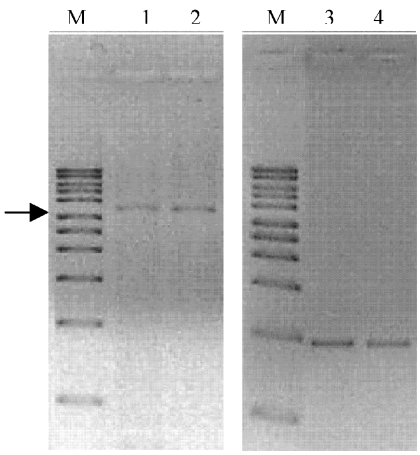


Fig. 2. Agarose gel electrophoresis of PCR products. Lane M, 1 kb DNA ladder; lane 1, *nahA* IR1; lane 2, *nahA* 9816-4; lane 3, *nahE* IR1; lane 4, *nahE* 9816-4.

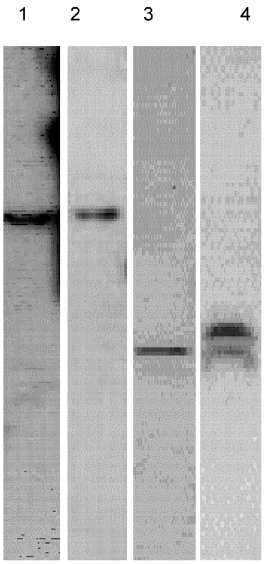


Fig. 3. Southern blot of DNA samples of *P. putida* NCIB 9816-4 and isolate IR1 digested with *Pst*1. Lane 1, 9816-4 with probe *nahA*; lane 2, IR1 with probe *nahA*; lane 3, 9816-4 with probe n*ahE*; lane 4, IR1 with probe *nahE*.

*BamHI* and *PstI* were hybridized with aromatic degradation gene probes (*P. putida* 9816-4, *nahA* and *nahE*) to assess the homology between prototype naphthalene degradative enzymes of 9816 and IR1. The strain IR1 showed hybridization signals with both of the probes in high stringency conditions (Fig. 3). No hybridization was observed in the negative control.

The bacteria used in the present study produced pinkish-red pigmentation indicating the dimer formation of the end product of DBT degradation, hydroxy-2-formyl-benzothiophene (Kodama *et al.*, 1973). Denome *et al.* (1993) reported that a single genetic pathway controls the metabolism of dibenzothiophene, naphthalene, and phenanthrene in *Pseudomona*s strain C18 and that the dibenzothiophene gene (DOX) sequence encodes a complete upper naphthalene catabolic pathway similar to NAH. This strain also formed blue indigo pigmentation in the presence of indole, indicating naphthalene dioxygenase activity (Ensley and Gibson, 1983). This strain apparently utilizes intermediate compounds of naphthalene dioxygenase pathway indicating that this bacterium utilizes naphthalene and phenanthrene via the salicylate pathway. Molecular studies further revealed that the naphthalene dioxygenase encoding gene of IR1 and 9816 have some degree of homology as evident by PCR and Southern blotting. However, Churchill *et al.* (1999) reported that in the pyrene-degrading bac-

terium *Mycobacterium* CH1 the enzyme system involved in PAH degradation by this strain is unrelated to the naphthalene dioxygenase pathway. More molecular and enzymatic studies are required to confirm the metabolic pathway of IR1.

*Biosurfactant production*

IR1 was first screened for its ability to produce surfactants by cultivation in YPG medium. The strain IR1 gave positive results in drop collapse and oil spreading tests. These qualitative tests are indicative of the surface and wetting activities (Youssef *et al.,* 2004). The strain was capable to reduce the surface tension in YPG medium from 54.9 to 35.4 dN/cm. Interestingly, most of the surface activity was confined to the culture supernatant, almost no significant surface-active potential was observed in the cells. Moreover, there was no significant effect observed on the activity of the extracellular biosurfactant when it was autoclaved.

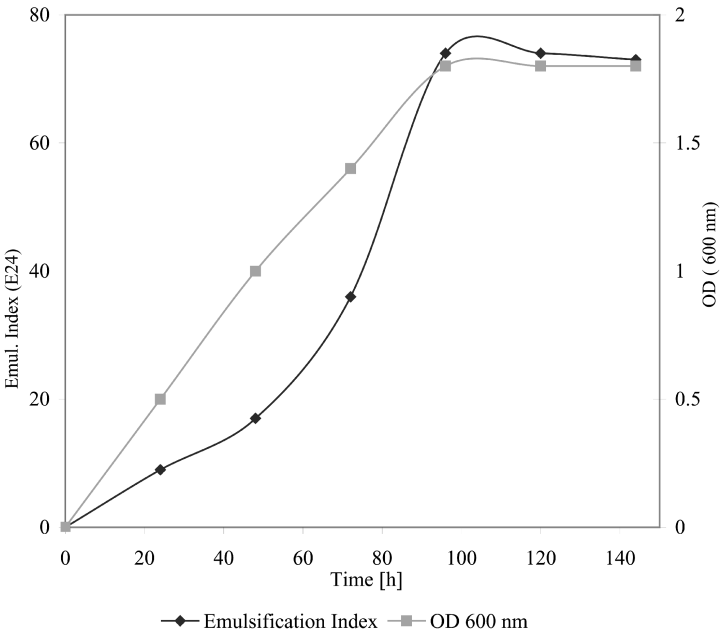


Fig. 4. Biosurfactant production during growth in YPG medium containing diesel oil. Values are the average of three cultures.

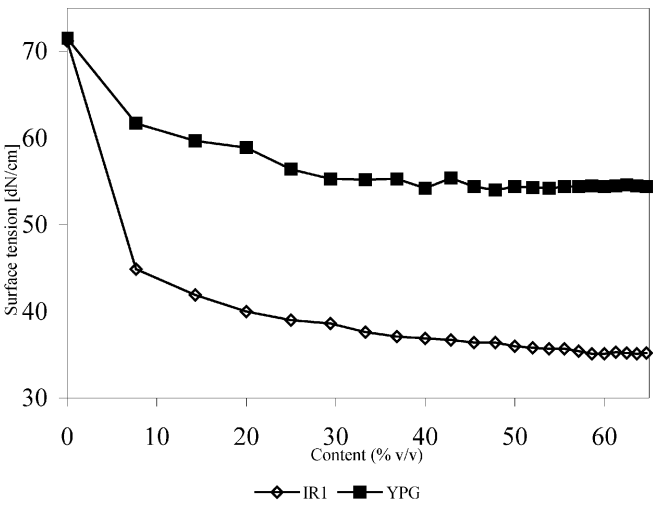


Fig. 5. Surface tension versus content of the culture supernatant. YPG is the medium used for biosurfactant production. Each value represents the mean of three readings with standard error $< 5\%$.

The reduction of the surface tension (around 20 dN/cm) is comparable to findings by other authors (Desai and Banat, 1997). This result indicates that biosurfactant production was growth associated (Fig. 4). The culture supernatant was diluted with distilled water, and the surface tension was measured at various contents (Fig. 5). These results indicate that IR1 produces biosurfactants of efficient and effective surface activity. No protein was detected in isolated biosurfactant, it however contained lipids and carbohydrates, therefore putatively it is classified as a glycolipid. Biosurfactants of glycolipid nature are common in the genus *Pseudomonas* however glycolipid-type biosurfactants have also been isolated from *P. putida* (Tuleva *et al.,* 2002). Emulsification activity is an indicator used extensively to quantify biosurfactant produced by bacteria (Rahman et *al.,* 2003). Emulsification activities of the culture supernatant were measured with water immiscible substrates. Results show that culture supernatant was having high emulsification activities against diesel [(74 ± 4)%], kerosene [(51 ± 3)%], gas oil [(56 ± 4)%], gasoline [(42 ± 2)%], *n*-hexane [(21 ± 2)%], hexadecane [(32 ± 2)%] and 2-methylnaphthalene [(67 ± 3)%]. Emulsions were found stable at room temperature for 72 h without any significant change in emulsification index.

*Biosurfactant production during growth on PAHs*

The strain IR1 was capable to produce the biosurfactant when it grew on various PAHs. We studied in detail the biosurfactant production during growth in MSM containing pyrene as sole source of carbon. Biosurfactant production was seen within 30 h of incubation [(26 ± 1)%] and peak of biosurfactant activity [$E_{24}$, (68 ± 4)%] was obtained after 70 h as indicated by diesel-water emulsification ($E_{24}$) activity. The surface tension of the culture supernatant was lowered to 33.1 dN/cm and culture supernatant of isolates grown in pyrene also gave positive results in drop collapse and oil spreading tests. Results show that culture supernatant was having high emulsification activities against kerosene [(59 ± 2)%], gas oil [(66 ± 4)%], gasoline [(35 ± 2)%], *n*-hexane [(30 ± 2)%], hexadecane [(21 ± 2)%] and 2-methylnaphthalene [(71 ± 2)%]. There was no significant effect observed on the activity of the extracellular biosurfactant when it was autoclaved. We could also detect biosurfactant during the growth, separately on DBT [(54 ± 2)%], naphthalene [(45 ± 3)%], and phenanthrene [(56 ± 2)%], PAHs mixture in NAPL [(67 ± 2)%] in MSM. The biosurfactants produced during growth on PAHs also showed glycolipidic nature. The hydrophobicity of the cell surface was tested using the BATH assay. No significant change in the hydrophobicity was observed in the pyrene and glucose grown cells. The hydrophobicity of the cells grown in pyrene and glucose was (46.5 ± 3.1)% and (40.1 ± 1.2)%, respectively. This indicates that the biosurfactant plays a minor role in changing the cell surface hydropbobicity to improve the affinity of microbial cells for the substrate to facilitate their bioavailability. Presumably, degradation of the different types of hydrocarbons by IR1 was due to the solubilization and increased bioavailability of hydrocarbons by extracellular biosurfactant.

*In situ* biosurfactant production by hydrocarbon-degrading bacteria may be beneficial and more practical than exogenously adding purified biosurfactant for field bioremediation application based on bioaugmentation. This strain may prove to be a promising microorganism for bioremediation by removing PAH–containing pollutants from contaminated sites. However, this is not appropriate to note at this stage of research but as evident from the results this strain does not attack hexadecane and octadecane, it opens the possibility to explore its usefulness for de-aromatization of petroleum products and crude oil.

Bamforth S. M. and Singleton I. (2005), Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. J. Chem. Technol. Biotechnol. **80**, 723–736.

Boonchan S., Britz M. L., and Stanley G. A. (1998), Surfactant-enhanced biodegradation of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia*. Biotechnol. Bioeng. **59**, 482–494.

Boonchan S., Britz M. L., and Stanley G. A. (2000), Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. Appl. Environ. Microbiol. **66**, 1007–1019.

Bradford M. M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. **72**, 248–254.

Burd G. and Ward O. P. (1996), Involvement of a surface-active high molecular weight factor in degradation of polycyclic aromatic hydrocarbons by *Pseudomonas marginalis*. Can. J. Microbiol. **42**, 791–797.

Chen W. P. and Kuo T. T. (1993), A simple and rapid method for the preparation of Gram-negative bacterial genomic DNA. Nucleic Acids Res. **21**, 2260.

Churchill S. A., Harper J. P., and Churchill P. F. (1999), Isolation and characterization of a *Mycobacterium* species capable of degrading three- and four-ring aromatic and aliphatic hydrocarbons. Appl. Environ. Microbiol. **65**, 549–552.

Denome S. A., Stanley D. C., Olson E. S., and Young K. D. (1993), Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* strains: complete DNA sequence of an upper naphthalene catabolic pathway. J. Bacteriol. **175**, 6890–6901.

Desai J. D. and Banat I. M. (1997), Microbial production of surfactants and their commercial potential. Microbiol. Molecul. Biol. Rev. **61**, 47–64.

Deziel E., Paquette G., Villemur R., Lepine F., and Bisaillon J.-G. (1996), Biosurfactant production by a soil *Pseudomonas* strain growing on polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. **62**, 1908–1912.

Ensley B. D. and Gibson D. T. (1983), Naphthalene dioxygenase: purification and properties of a terminal oxygenase component. J. Bacteriol. **155**, 505–511.

Foght J. M. and Westlake D. W. (1988), Degradation of polycyclic aromatic hydrocarbons and aromatic heterocycles by a *Pseudomonas* species. Can. J. Microbiol. **34**, 1135–1141.

Garcia-Junco M., De Olmedo E., and Ortega-Calvo J. J. (2001), Bioavailability of solid and non-aqueous phase liquid (NAPL)-dissolved phenanthrene to the biosurfactant producing bacterium *Pseudomonas aeruginosa* 19SJ. Environ. Microbiol. **3**, 561–569.

Habe H. and Omori T. (2003), Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. Biosci. Biotechnol. Biochem. **67**, 225–243.

Hansen J. B. and Olsen R. H. (1978), Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**, 227–238.

Heitkamp M. A., Franklin W., and Cerniglia C. E. (1988), Microbial metabolism of polycyclic aromatic hydrocarbons isolation and characterization of a pyrene-degrading bacterium. Appl. Environ. Microbiol. **54**, 2549–2555.

Ilori M. O. and Amund D. I. (2001), Production of a peptidoglycolipid bioemulsifier by *Pseudomonas aeruginosa* grown on hydrocarbon. Z. Naturforsch. **56c**, 547–552.

Kado C. I. and Liu S. T. (1981), Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**, 1365–1373.

Kastner M., Breuer-Jammali M., and Mahro B. (1994), Enumeration and characterization of the soil microflora from hydrocarbon-contaminated soil sites able to mineralize polycyclic aromatic hydrocarbons (PAH). Appl. Microbiol. Biotechnol. **41**, 267–273.

Kodama K., Umehara K., Shimizu K., Nakatami S., Minnoda Y., and Yamadam K. (1973), Identification of microbial products from dibenzothiophene and its proposed oxidation pathway. Agricul. Biol. Chem. **37**, 45–50.

Krivobok S., Kuony S., Meyer C., Louwagie M., Willison J. C., and Jouanneau Y. (2003), Identification of pyrene-induced proteins in *Mycobacterium* sp. strain 6PY1: evidence for two ring-hydroxylating dioxygenases. J. Bacteriol, **185**, 3828–3841.

Kuyukina M. S., Ivshina I. B., Makarov S. O., Litvinenko L. V., Cunningham C. J., and Philp J. C. (2005), Effect of biosurfactants on crude oil desorption and mobilization in a soil system. Environ. Int. **31**, 155–161.

Lu J.-J., Perng C.-L., Lee S.-Y., and Wan C.-C. (2000), Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. J. Clin. Microbiol. **38**, 2076–2080.

Monticello D. J., Bakker D., and Finnerty W. R. (1985), Plasmid-mediated degradation of dibenzothiophene by *Pseudomonas* species. Appl. Environ. Microbiol. **49**, 756–760.

Rahman K. S. M., Rahman T. J., Kourkoutas Y., Petsas I., Marchant R., and Banat I. M. (2003), Enhanced bioremediation of *n*-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. Bioresour. Technol. **90**, 159–168.

Rosenberg M. and Rosenberg E. (1981), Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. J. Bacteriol. **148**, 51–57.

Sambrook J., Fritsch E. F., and Maniatis T. (eds.) (1989), Molecular Cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sarma P. M., Bhattacharya D., Krishnan S., and Lal B. (2004), Degradation of polycyclic aromatic hydrocarbons by a newly discovered enteric bacterium, *Leclercia adecarboxylata*. Appl. Environ. Microbiol. **70**, 3163–3166.

Spiro R. G. (1966), Analysis of sugars found in glycoproteins. Methods Enzymol. **8**, 7–9.

Tuleva B. K., Ivanov G. R., and Christova N. E. (2002), Biosurfacatnt production by a new *Pseudomonas putida* strain. Z. Naturforsch. **57c**, 356–360.

Walter U., Beyer M., Klein J., and Rehm H.-J. (1991), Degradation of pyrene by *Rhodococcus* sp. UW1. Appl. Microbiol. Biotechnol. **34**, 671–676.

Weisburg W. G., Barns S. M., Pelletier D. A., and Lane D. J. (1991), 16*S* ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173**, 697–703.

Whyte L. G., Bourbonniere L., and Greer C. W. (1997), Biodegradation of petroleum hydrocarbons by psychrotrophic *Pseudomonas* strains possessing both alkane (alk) and naphthalene (nah) catabolic pathways. Appl. Environ. Microbiol. **63**, 3719–3723.

Willumsen P. A. and Karlson U. (1997), Screening of bacteria, isolated from PAH contaminated soils, for production of biosurfactants and bioemulsifiers. Biodegradation **7**, 415–423.

Youssef N. H., Duncan K. E., Nagle D. P., Savage K. N., Knapp R. M., and McInerney M. J. (2004), Comparison of methods to detect biosurfactant production by diverse microorganisms. J. Microbiol. Methods **56**, 339–347.