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Surface Active Properties of Bacterial Strains Isolated from Petroleum Hydrocarbon-Bioremediated Soil

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Abstract

Two bacterial strains identified as *Ralstonia picketti* (BP 20) and *Alcaligenes piechaudii* (CZOR L-1B) were isolated from petroleum hydrocarbon-contaminated soil following bioremediation treatment. The surface active properties, *e.g.* surface tension, emulsification and foamability of their culture filtrates were evaluated. Bacterial cell-surface hydrophobicity (BAH) as measured by analyzing cell affinity towards aliphatic and aromatic compounds was also determinated. The bacteria grew in liquid cultures containing 1% (v/v) of crude oil as carbon and energy source at 30°C under aerobic conditions. The surface tensions were reduced to 61 mN/m and 55 mN/m by *Ralstonia picketti* and *Alcaligenes piechaudii*, respectively. The emulsification index (EI24) was almost 100% for all tested compounds except diesel oil. The stability of the emulsions was determinated at 4°C, 45°C and 65°C. The emulsions were stable at 4ºC. *Ralstonia picketti* was better foam inducer (FV = 50 ml) compared to *Alcaligenes piechaudii* (FV = 10 ml). The BAH measurements revealed higher adhesion of *Alcaligenes piechaudii* cells towards different hydrocarbons compared to *Ralstonia picketti* cells. The strains were found to have a surface hydrophobicity in the following order: aliphatic hydrocarbons, BTEX, and PAHs. The ability to adhere to bulk hydrocarbon is mostly a characteristic of hydrocarbon-degrading bacteria. The strains were found to be better emulsifiers than surface tension reducers. They produce water-soluble extracellular bioemulsifiers. Both bacterial isolates have good properties to use them, mainly in the petroleum industry, *e.g.* in enhanced oil recovery and in bioremediation processes-primarily due to their emulsification property, *i.e.* emulsion forming and stabilizing capacity.

Key words: biosurfactants/bioemulsifiers, bacteria, hydrocarbons, bioremediation

Introduction

A large variety of microorganisms produce surfactants (SURFace ACTive AgeNTS) named biosurfactants (BS) which have gained attention because of their low toxicity, biodegradability, ecological acceptability, ability to be produced from renewable resources and functionality under extreme conditions. These compounds have many potential applications in agriculture, public health, food industry, health care, waste utilization, and environmental protection (Desai and Banat, 1997; Banat *et al*., 2000). At present, their use is mainly in the petroleum industry, *e.g.* in cleaning up of oil spills, removal of oil sludge from oil storage tanks, as well as enhancement of oil recovery processes from reservoirs at the level of the production well and from tar-saturated sands, and in bioremediation of water-insoluble pollutants (Banat, 1995a, b; Banat *et al*., 2000; Mulligan *et al*., 2001; Christofi and Ivshina, 2002; Singh and Cameotra, 2004; Mulligan, 2005). The bioremediation of such pollutants is limited by their poor availability to microorganisms. Nevertheless, surfactant can be used to help enhance emulsification and dispersed of immiscible compounds. A number of surfactants have been isolated from microbial cultures following growth of bacteria and fungi on a variety of aliphatic and aromatic hydrocarbons (Rosenberg and Ron, 1999). These biosurfactants, which are generally extracellular, may be relatively simple glycolipides or complex high molecular weight

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substances. Their production allows the uptake and utilization of hydrocarbons and this, in turn, leads to the growth of microbial cells which has important implications for the petroleum industry. Surface active agents produced biologically have the potential to replace traditional compounds since they are more ecologically acceptable and could be cheap to produce (Bobnolo, 1999; Rosenberg and Ron, 1999; Kosaric, 2000; Sukan and Kosaric, 2000).

Biosurfactants have been tested in enhanced oil recovery and the transportation of crude oil (Banat, 1995b). They were effective in the reduction of the interfacial tension of oil and water *in situ*, and the viscosity of the oil. Emulsan® has been commercialized for this purpose (Rosenberg and Ron, 1997, 2002). Also, *Bacillus licheniforms* Jf-2 is an example, which would be well suited for *in situ* studies for enhanced oil recovery and soil decontamination (McInerney *et al*., 1990; Lin *et al*., 1994). It has been also demonstrated that the rhamnolipides produced by *Pseudomons aeruginosa* UG2 are capable of removing both aliphatic and aromatic pollutants from unsaturated soil without soil clogging (Scheibenbogen *et al*., 1994; Al-Tahhan *et al*., 2000).

This paper reports the ability of two bacterial strains, *Ralstonia picketti* (BP 20) and *Alcaligenes piechaudii* (CZOR L-1B) isolated from petroleum-contaminated and bioremediated soil to produce extracellular water soluble biosurfactants as assessed by lowering of surface tension, emulsification property and foamability.

Experimental

Materials and Methods

Site characterization. The Czechowice-Dziedzice Oil Refinery (CZOR) in Poland produced an estimated 120,000 tons of acidic, highly weathered, petroleum sludge deposited into three open waste lagoons, 3 meters deep and covering 3.8 hectares. One of the waste lagoons (0.3 hectare) was chosen for aerobic biopile bioremediation monitoring and physicochemical characterization. The waste from the lagoon was removed, and heavily petroleum contaminated soil was subjected to the bioremediation process. The biopile with active and passive aerated sections, referred further as biopile 1 or engineered, was constructed in 1997 in the smallest lagoon at the Czechowice-Dziedzice Oil Refinery (Altman *et al*., 1997; Płaza *et al*., 2003). The purpose was to evaluate novel technologies and applications for environmental restoration of soils heavily contaminated with petroleum waste by comparing bioremediation processes under active *vs*. passive aeration and the removal rates of both, easily biodegradable and recalcitrant petroleum hydrocarbons. The project focused on the application of cost-effective amendments for biostimulation, including additions of mineral NPK fertilizers and the surfactant, Rokafenol N8, to enhance hydrocarbon biodegradation.

In 2001, a second biopile was constructed to cleanup soil mixed with petroleum hydrocarbon waste (Worsztynowicz *at al*., 2001). This biopile, referred as biopile 2 or non-engineered, is situated within the second (middle) lagoon at the refinery. In this biopile contaminated soil was mixed with mineral fertilizers and wood chips, and covered with a dolomite layer. Subsequently, the dolomite layer was covered with unpolluted soil and grass seeds sown over the biopile area. A simple drainage system was built to allow natural soil aeration.

Isolation and identification of hydrocarbon-utilized bacteria. 10 g of mixed soils from biopiles 1 and 2 were inoculated in 100 ml of the mineral medium (MM). The composition of the medium used was the following (g/l): NH_4NO_3–1; $MgSO_4 \times 7H_2O$–0,2; $CaCL_2 \times 2H_2O$–0,03; K_2HPO_4–1; KH_2PO_4–1. The medium was supplemented with 1 ml of the trace elements solution (Gerhardt, 1981). 1% (v/v) of crude oil as carbon and energy source was added. The incubation was performed at 30°C for 24–48 h. Development of bacterial colonies was obtained by a serial dilution-agar plating technique on standard methods agar (SMA, Biomerieux). The isolates were initially isolated using naphthalene as a sole carbon and energy source. Then, isolates were tested for their ability to grow on the solid mineral medium (BH) with different hydrocarbons. The composition of the medium used was the following (g/l): NH_4NO_3–1; $MgSO_4 \times 7H_2O$–0,2; $CaCL_2 \times 2H_2O$–0,02; K_2HPO_4–1; KH_2PO_4–1 and 1 ml/l of the trace elements solution. Cyclohexane, hexadecane, xylene, benzene, toluene, heptane, decane, isooctane, hexane, mineral oil, pristane, and squalene were used as carbon and energy sources. All chemicals used were of analytical grade and purchased from Sigma-Aldrich Co. and Polish Chemical Reagents S.A., Gliwice. 200 µl of hydrocarbons were put on the filter paper, then the filter paper was overlaid by the BH medium with 20 g of agar (DIFCO). The incubation of Petri dishes was carried out at 30°C two weeks.

Isolates, which grew well in the presence of hydrocarbons were identified as follows. Isolates were grown on SMA plates, and single colony of each isolate was resuspended and washed three times in 100 µl of the sterile water. The cell suspension was added to the PCR reaction mixture. PCR products were produced with whole-cell cultures described by Furlong *et al*. (2002). The PCR reaction was performed with puReTaq™ Ready-To-Go™ Polymerase Chain Reaction (PCR) beads (Amersham Biosciences) as previously described (Stefan and Atlas, 1991). Beads are premixed and predispensed complete reactions for performing PCR amplifications. With the exception of primers and template, the ambient temperature-stable beads provide all the necessary reagents to perform 25 µl polymerase chain reactions, *e.g.* stabilizers, BSA, dATP, dCTP, dGTP, dTTP, ~2.5 units of puReTaq DNA polymerase and reaction buffer. When a bead was reconstituted to a 25 µl final volume, the concentration of each dNTP was 200 mM in 10 mM Tris-HCl (pH 9.0), 50 mM KCl and 1.5 mM $MgCl_2$. A typical PCR contains <1 µg of template DNA and primers at a concentration of 0.2–1 µM. The samples were subjected to 30 cycles of 94°C for 1 min, 72°C for 2 min and 61°C for 1 min for denaturation, annealing and elongation steps, respectively. An initial denaturation step (95°C, 1 min) was used to ensure complete denaturation of the DNA. PCR amplification was performed in a Mastercycler® gradient machine (Eppendorf). The primers for the

reactions were as follows: forward primer: 27f (5'-TTCCGGTTGATCCYGCCGGA-3') and reverse primer: 1492 universal (5'-ACGGGCGGTGTGTRC-3') (Furlong *et al*., 2002). Partial sequences of rRNA genes were obtained using an ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA) with an ABI PRISM BigDye terminator sequencing Lab kit at the Genome Analysis Facility in the Botany Department at the University of Georgia, Athens. Isolates were tentatively identified by similarity to sequences in the GenBank data base using the FastA algorithm (Pearson and Lipman, 1988) of the GCG software package (Genetics Computer Group, Wisconsin).

Growth of bacteria in the liquid medium with crude oil as carbon and energy source. The medium used for isolation and cultivation of biosurfactant/bioemulsifiers-producing bacteria was as described by Abu-Ruwaida *et al*. (1991). 1% (v/v) of crude oil was used as the sole carbon source. Cultures were grown aerobically in 100 ml medium at 30°C for a period of 7 days without shaking. The emulsification of hydrocarbons was determinated by examining the presence of emulsion droplets in the cultures. After incubation the cultures were filtered by the filter paper (390, FILTRAK GmbH). Then, the culture filtrates were used to evaluate surface active properties.

Surface active properties. Surface tension was measured with a ring-tensiometer (Krüss Digital-Tensiometer 10, Hamburg, Germany) with a 6 cm de Nuoy platinum ring at 20°C ± 2°C. The emulsifying activity was determined using a modification of the method described by Bosch *et al*. (1988) and Willumsen and Karlson (1997). 7 ml of the culture filtrates were overlaid by 3 ml of hydrocarbons substances, and then, mixed on a vortex mixer at maximum speed for 1 min. After 24 hours the height of the emulsion layer was measured and emulsification activity was expressed as emulsification index (EI24). The EI24 was reproducible within ±2–5%. It was calculated by dividing of measured height of the emulsified layer by the height of the hydrocarbon phase, and multiplying by 100. The height of the emulsion was noted with a precision of ±1 mm. An emulsion was defined as stable if the EI24 was higher than 40% (Bosch *et al*., 1988). The emulsion stability was determined at different temperatures: 4°C, 45°C and 65°C. Foamability was measured in terms of foam volume (FV) (Das *et al*., 1998). Air at a constant flow rate (50 ml/min) was passed by measuring glass cylinder containing 20 ml of the culture filtrate. The FV was estimated as the difference between the volume occupied by the liquid-plus-foam and the volume of the liquid at rest, and was reproducible within ±5–10%. BATH (bacterial adhesion to hydrocarbons) was carried out, using the modification of the method described by Rosenberg *et al*. (1983). Bacteria were harvested from growth cultures by centrifugation (8000 × g, 10 min), then washed and suspended in 3 ml of sterilized ddH_2O. 0.5 ml of different hydrocarbons were added to cell suspension, and then vortexed in the test tube for 2 minutes. The phases were allowed to separate during 20 min, and the optical density of the lower aqueous phase was measured at 600 nm. The percentage of cells bound to hydrocarbons (% HYD) was calculated according to equation: $\%HYD = (1 - A/A_o) \times 100\%$, where A_o is the absorbance of bacterial suspension without hydrocarbons added and A is the absorbance after mixing with hydrocarbons. All the experiments were done in triplicate.

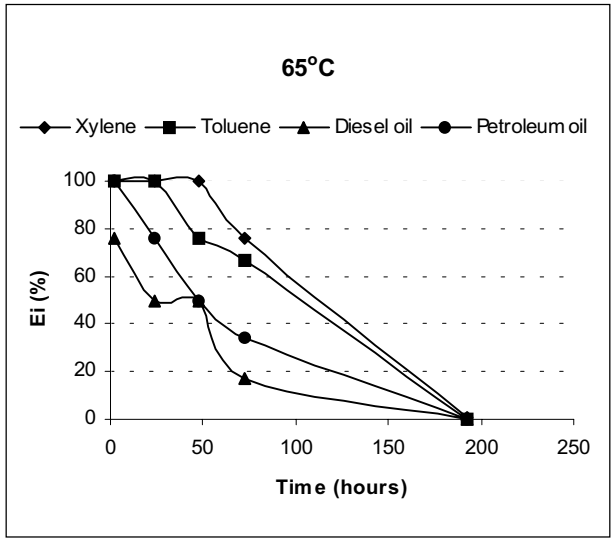
Results and Discussion

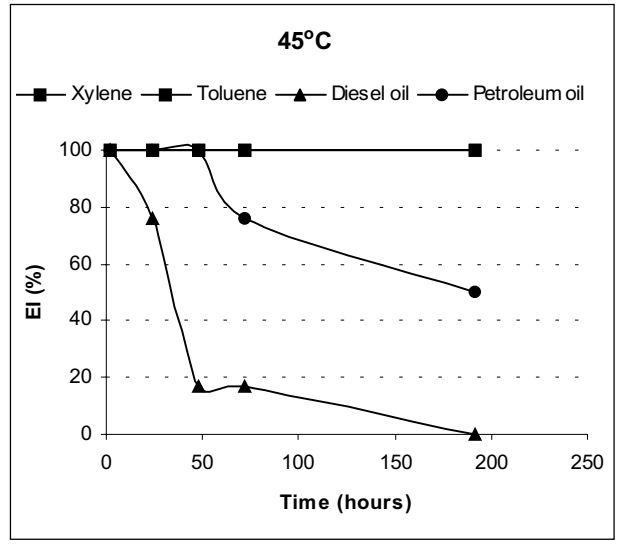
Table I presents surface active properties of *Ralstonia picketti* (BP 20) and *Alcaligenes piechaudii* (CZOR L-1B) culture filtrates, *i.e.* surface tension, emulsification and foamability. The bacteria were grown in the liquid medium containing 1% (v/v) of crude oil under aerobic conditions at 30°C. The surface tension was decreased to 61 mN/m and 55 mN/m by *Ralstonia picketti* and *Alcaligenes piechaudii,* respectively. The screening of the isolates for the ability to emulsify hydrocarbons was performed using an overlay of xylene, toluene, petroleum and diesel oils. The EI24 values for toluene, xylene and petroleum were almost 100% for both bacterial isolates (Table I). The EI 24 values for diesel oil was slightly smaller, 77% and 84% for *Ralstonia picketti* and *Alcaligenes piechaudii,* respectively. The results indicated some substrate specificity for emulsification by the bacteria. It would be advantageous for microorganisms isolated from a hydrocarbon-contaminated site to possess the ability to emulsify contaminants. The high emulsification activities were evidently due to the production of extracellular water soluble biosurfactants by isolates during the

Table I
Surface tension, EI24, FV of *Ralstonia picketti* and *Alcaligenes piechaudii* culture filtrates

Strains	Treatment (C-source)	Surface tension (mN/m)	Emulsification EI24 (%)		Foamability FV (ml)
Ralstonia picketti (BP-20)	crude oil	61.1 ± 0,3*	toluene xylene petrolum oil diesel oil	100 ± 4 100 ± 3 100 ± 2 77 ± 1	50 ± 3
Alcaligenes piechaudii (CZORL-1B)	crude oil	55.2 ± 0,2	toluene xylene petrolum oil diesel oil	100 ± 2 100 ± 3 100 ± 3 84 ± 1	10 ± 2

Control: water – 72,1 ± 0,4; * – mean ± SD





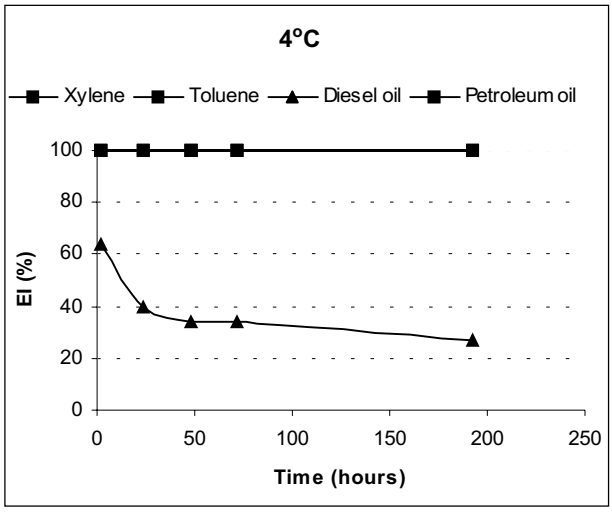


Fig. 1. The stability of the emulsion produced by *Alcaligenes piechaudii* evaluated by EI values (mean values) at different temperatures.

incubation utilizing the crude oil as C-source. The strains isolated were better emulsifiers than surface tension reducers. The above properties make the organisms a potential candidates for the enhanced oil recovery and bioremediation processes.

Results on % FV showed appreciable emulsion forming capacity of the culture filtrates having values 50 and 10 for *Ralstonia picketti* and *Alcaligenes piechaudii,* respectively (Table I).

Figures 1 and 2 illustrate the emulsion stability evaluated by EI values at 4°C, 45°C and 65°C for *Ralstonia picketti* and *Alcaligenes piechaudii*. The stability of the emulsion was the best at 4°C for both isolates. The higher temperature, the worse emulsion stability. The results has confirmed that the ability of biosurfactants to form a stable emulsion is not always associated with surface tension lowering (Willumsen and Karlson, 1997).

The changes in cell surface hydrophobicity as measured by analyzing cell affinity towards aliphatic and aromatic hydrocarbons are summarized in Table II. The differences in the affinities of isolates for hydrocarbons were demonstrated. Significant increase in cell surface hydrophobicity was observed towards aliphatic hydrocarbons. Generally, the strains were found to have a surface hydrophobicity in the following order: aliphatic hydrocarbons, BTEX, and finally PAHs. *Alcaligenes piechaudii* showed higher affinity towards aliphatic and aromatic hydrocarbons than *Ralstonia picketti*. The percentage cell transferred to aliphatic hydrocarbons (hexane, heptane, decane, hexadecane) ranged from 87% to 67%. Lower affinity towards PAHs (naphthalene, anthracene, phenantrene, pyrene and chrysanthene) was observed. The values of BAH ranged from 15 to 46% and from 18 to 42% for *Alcaligenes piechaudii* and *Ralstonia picketti*, respectively. A previous study has suggested that the ability to adhere bulk hydrocarbon is a characteristic of hydrocarbon-degrading bacteria, and affinity for hydrocarbons may vary among hydrocarbon-degrading

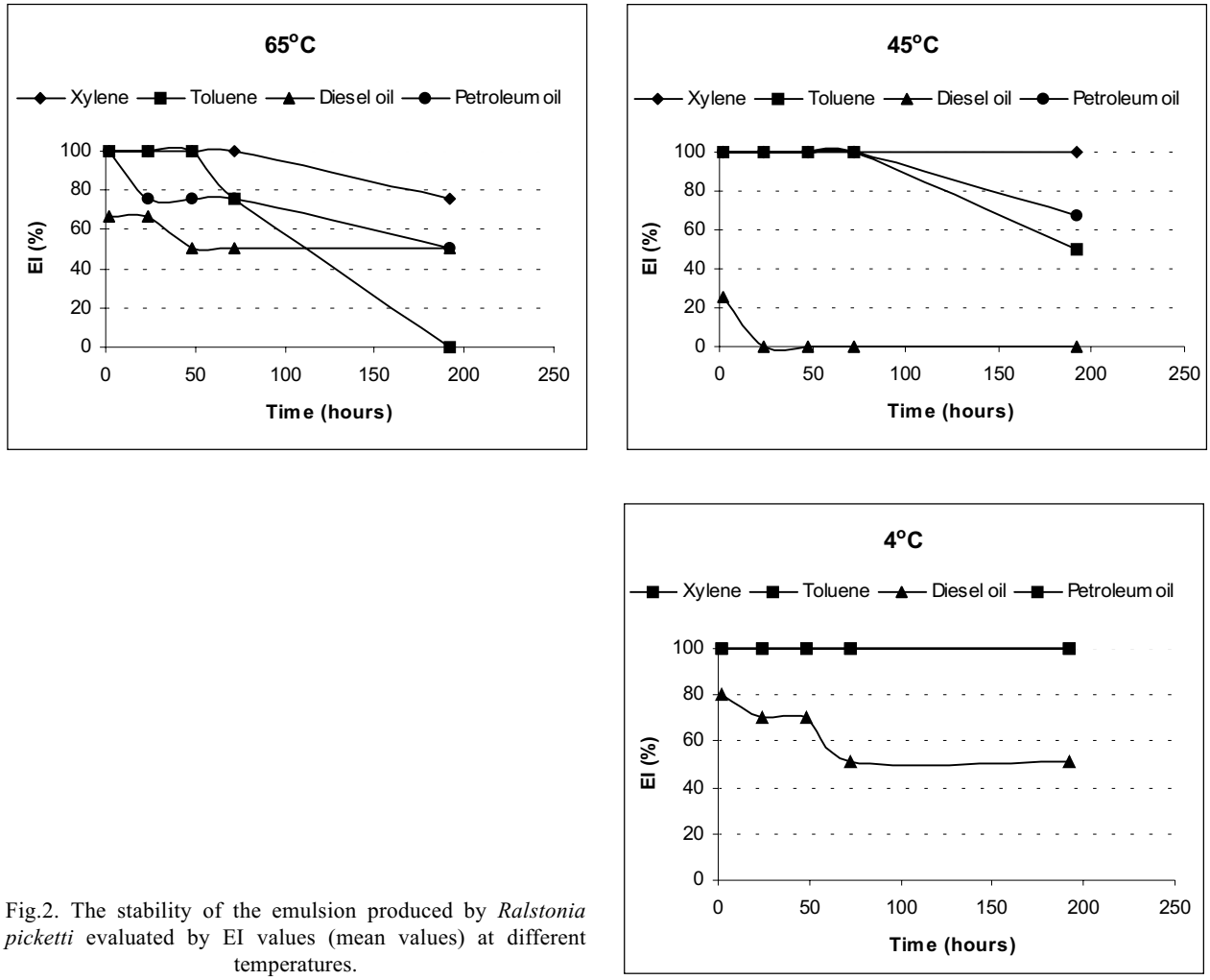


Fig.2. The stability of the emulsion produced by *Ralstonia picketti* evaluated by EI values (mean values) at different temperatures.

bacteria (Prabhu and Phale, 2003). The increase in cell hydrophobicity contributes to the increase of the cell interaction with hydrocarbons, and ultimately increase of biodegradation rate. Biosurfactants modulate the hydrophobicity of the cell surface, which appears to be an important factor for cell adhesion. Further studies on the relationship between adherence to hydrocarbons and growth of hydrocarbon-degrading bacteria are underway. These properties, biosurfactant production and modulation of the cell surface hydrophobicity play an important role in efficient hydrocarbon assimilation/uptake, which mechanism is not fully understood.

Three models of hydrocarbon transport to microbial cells are generally considered (Kosaric, 2000): (1) interaction of cells with more water-soluble hydrocarbons, (2) direct contact of cells with large hydrocarbon drops – in this mechanism microbial cells attach to the surface of hydrocarbon drops that are much larger than cells; the availability of substrate surface area for cell attachment is a limiting factor for microbial growth; biosurfactants/bioemulsifiers produced by hydrocarbon utilizing bacteria cause the dispersion of hydrocarbon droplets in the aqueous medium and thereby increase the surface area; addition of biosurfactants to the hydrocarbon medium stimulat growth of microorganisms, and (3) microbial cells interact with particles of solubilized, microemulsified hydrocarbons.

Depending on the specified organism, hydrocarbon uptake may take place through one or combination of the presented mechanisms (Prabhu and Phale, 2003).

Hydrocarbon uptake/degradation problem is closely involved with cell surface-active properties, *eg.* ability of cell to attach to hydrocarbon or to produce surface active compounds. Both these mechanisms involve modulation of cellular physiology, which will lead either to changes in cell surface properties like hydrophobicity or secretion of surface-active compounds into the medium or a combination of both.

The results indicate that surface active properties play an important role in characterization of biosurfactant-producing bacteria which have ability to degrade different hydrocarbons.

Table II
Comparison of *Ralstonia picketti* and *Alcaligenes piechaudii* hydrophobicity (%) towards different hydrocarbons

Hydrocarbons	% cell transfered to hydrocarbon phase (mean values)	
	Ralstonia picketti (BP-20)	*Alcaligenes piechaudii* (CZORL-1B)
Hexane	41	87
Hexadecane	48	81
Heptane	37	83
Decane	31	67
Isooctane	37	5
Cyclohexane	1	82
Xylene	*	54
Toluene	*	53
Benzene	*	68
Naphtalene	32	15
Anthracene	42	35
Phenanthrene	37	34
Pyrene	40	34
Chrysanthene	18	46

* A > Ao
Standard deviation values were in the rang of ± 1–6%

The recent developments in the field of production and recovery of biosurfactants indicate that biosurfactants will be providing promising substitutes to chemically synthesized surfactants in the future. However, development of novel approaches which are industrially applicable and economically acceptable requires comprehensive studies, especially in the area of surface active properties which play an important role in bioprocess engineering.

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