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Rhamnolipid production by a novel thermophilic hydrocarbon-degrading *Pseudomonas aeruginosa* AP02-1

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Abstract Thermophilic bacterial cultures were isolated from a hot spring environment on hydrocarbon containing mineral salts media. One strain identified as *Pseudomonas aeruginosa* AP02-1 was tested for the ability to utilize a range of hydrocarbons both *n*-alkanes and polycyclic aromatic hydrocarbons as sole carbon source. Strain AP02-1 had an optimum growth temperature of 45°C and degraded 99% of crude oil 1% (v/v) and diesel oil 2% (v/v) when added to a basal mineral medium within 7 days of incubation. Surface activity measurements indicated that biosurfactants, mainly glycolipid in nature, were produced during the microbial growth on hydrocarbons as well as on both water-soluble and insoluble substrates. Mass spectrometry analysis showed different types of rhamnolipid production depending on the carbon substrate and culture conditions. Grown on glycerol, *P. aeruginosa* AP02-1 produced a mixture of ten rhamnolipid homologues, of which Rha-Rha-C_{10}-C_{10} and Rha-C_{10}-C_{10} were predominant. Rhamnolipid-containing culture broths reduced the surface tension to ≈28 mN and gave stable emulsions with a number of hydrocarbons and remained effective after sterilization. Microscopic observations of the emulsions suggested that hydrophobic cells acted as emulsion-stabilizing agents.

Introduction

Increased interest in oil biotechnology has stimulated attempts to isolate novel microorganisms to exploit in various fields such as petroleum production (Van Hamme et al. 2003), biorefining (Le Borgne and Quintero 2003), bioremediation (Korda et al. 1997; Dua et al. 2002) and enhanced oil recovery (Banat et al. 2000). Microbial surface-active compounds (biosurfactants) have been one of these interesting areas of research predicted to have significant potential in commercial applications especially as substitutes for synthetic surfactants in oil and other industries (Desai and Banat 1997; Banat et al. 2000; Mulligan 2005). The range of potential petroleum-related industries for biosurfactant applications includes oil storage cleaning, enhancing oil recovery, mobilizing heavy crude oil, transporting petroleum in pipelines and managing oil spills both inland and at sea (Banat et al. 1991, 2000; Banat 1995a,b; Maier and Soberón-Chávez 2000; Ron and Rosenberg 2002; Mulligan 2005).

Pseudomonads are the best-known bacteria capable of utilizing hydrocarbons as carbon and energy sources and producing biosurfactants which enhance the uptake of such immiscible hydrophobic compounds (Al-Tahhan et al. 2000; Beal and Betts 2000; Noordman and Janssen 2002; Rahman et al. 2002).

Although the potential for biosurfactant production is dictated by the genetic traits of microorganisms, environmental conditions and nutrients can significantly influence the level of expression as well as the chemical characteristics. Rhamnolipid compounds are frequently the main biosurfactants produced by *Pseudomonas aeruginosa* as a mixture of mono- and di-rhamnolipids which have quite different physico-chemical properties (Benincasa et al. 2004).

Carbon source in particular can affect the composition of the mixture and hence the activity and functions; however, a few studies have focused on the biosurfactant production by microorganisms growing on substrates other than hydrocarbons (Lang and Wullbrandt 1999; Makkar and Cameotra 2002). Thus, optimization of fermentation conditions is needed to increase the yield of product and also improve the specificity to overcome a number of drawbacks which limit industrial production and utilization of microbial surfactants.

Since *P. aeruginosa* is an important opportunistic pathogen of humans, biotechnological applications of its

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biosurfactants may be limited. Nevertheless, it is well known that *P. aeruginosa* virulence depends on a large number of cell-associated and extracellular factors finely regulated by cell-to-cell signalling systems (Van Delden and Iglewski 1998). Therefore, although a careful investigation of such molecules is necessary, rhamnolipids have been used without risks and nowadays represent the most frequently employed biosurfactants in bioremediation and environmental field applications.

In the present study, we described the isolation and characterization of a novel thermophilic strain of *P. aeruginosa* able to utilize hydrocarbons ranging from *n*-alkanes to polycyclic aromatic hydrocarbons as a carbon source and can degrade both crude and refined oils. This strain produces rhamnolipid biosurfactants during the growth on hydrocarbons and both water-miscible and immiscible carbon substrates. Both physical and chemical characteristics of the rhamnolipid biosurfactants produced and possible functional properties as well as potential uses were investigated.

Materials and methods

Bacterial isolation and characterization

A standard enrichment technique was used to isolate thermophilic hydrocarbon-degrading microorganisms from muddy sediments collected in sulphataric hot springs in Viterbo, Italy.

A few grams of sample was added to 250-ml flasks containing 100 ml of basal mineral medium (BMM) and was incubated at 45°C. Filtered diesel oil at 2% (v/v) or crude oil at 1% (v/v) concentration was provided as carbon and energy source. BMM contained (in g/l) 0.1 $MgSO_4$, 0.9 $(NH_4)_2SO_4$, 0.7 KH_2PO_4, 1.5 K_2HPO_4, 0.5 $MgCl_2$ and 10 ml of trace element solution prepared as described by Balch et al. (1979). The culture broth was then used to streak on BMM agar plates coated with diesel oil, and the fastest-growing colonies were selected for further characterization. Crude oil and untreated diesel were supplied locally by Idrabel-Italia (Genoa, Italy).

Optimum growth temperature and range were determined on BHI (Biolife) at 30, 37, 45 and 50°C while monitoring the optical density at 600 nm (OD_{600}). Motility was investigated by swimming, swarming and twitching assays according to Dèziel et al. (2001). Electron microscopy was also carried out to determine the presence and the position of flagella as described later. Pigment production was also examined by streaking on King's A and King's B media (Oxoid) that promote pyocyanin and pyoverdine production, respectively (King et al. 1954).

Taxonomic identification

Preliminary identification was carried out using the Biolog GN2 plates system (Biolog Inc., Hayward, CA). Confirmation was obtained through determining the 16S rRNA gene sequence. DNA was extracted from a pure culture using a BIO 101 Inc. Soil DNA extraction kit. Polymerase chain reaction (PCR) was carried out using standard methods with the universal eubacterial forward primer 27F and a bacterial reverse primer 1942R for the 16S rRNA gene, both described by Bond et al. (2000). PCR amplification was carried out as described in Banat et al. (2004), and DNA sequencing was carried out directly on purified PCR products as described in Marchant et al. (2002).

Microbial growth on hydrocarbons and biodegradation

The ability of *P. aeruginosa* AP02-1 to utilize a number of hydrocarbon growth substrates was investigated by the Biolog system using MT2 plates. Each well was inoculated with 15 μl of a substrate-containing solution (0.2% v/v) and 135 μl of bacterial cells grown on BMM with 2% (v/v) diesel oil and then suspended in a saline solution. The plate was incubated at 45°C and read at 590 nm as the carbon source utilization was indicated by the colour development of a redox dye indicator. Duplicate readings were taken at 24, 48 and 72 h of incubation.

The ability of AP02-1 to degrade hydrocarbons was also investigated by gas chromatography. The bacterium was inoculated into 250-ml flasks containing 100 ml of BMM with 1%(v/v) of crude oil or 2%(v/v) of diesel oil as sole carbon source and was incubated at 45°C for 7 days. Samples were collected at intervals, centrifuged at 10,000×*g* for 15 min and the supernatants used to extract total petroleum hydrocarbons (TPHs) according to the EPA 3510C method using dichloromethane, which was injected into a gas chromatograph equipped with a DB-5MS capillary column (30 m×0.25 mm×ID 0.25 μm) and with a 40–320°C temperature gradient to detect the TPHs.

Bacterial adhesion to hydrocarbons

Bacterial adhesion to hydrocarbons (BATH) was measured as described by Rosenberg et al. (1980). Cells were grown on BMM with 2%(v/v) diesel oil as carbon source and harvested at the early stationary phase, washed twice with phosphate buffer and resuspended in the same buffer to reach OD_{600} of about 0.6. Seven test tubes were set up introducing 3 ml of the cell suspension and 0.15 ml of hexadecane. The mixture was vortexed for seven different times (0, 10, 20, 30, 40, 50, 60 s) and allowed to separate for 10 min. The OD_{600} of the aqueous phase was then measured spectrophotometrically. The difference between the OD of the aqueous phase before and after mixing time was used to calculate the adhesion as a percentage: $100\times[1-(OD_{600}$ after mixing/OD_{600} before mixing)].

The increase in adhesion (%) at increasing mixing times indicated the level of hydrophobicity of cell surfaces.

Determination of biosurfactant production

Preliminary simple and quick methods were used to detect biosurfactants production. (1) Haemolytic activity was

tested by spotting 10 µl of microbial culture grown on BMM with added 2% diesel oil on a blood agar plate. After 24 h of incubation at 45°C, the development of a haemolysis halo around the bacterial colony was measured. (2) Drop-collapsing test was also used as described by Trevors et al. (1991). (3) Oil-spreading technique was carried out as described by Morikawa et al. (2000). Sodium dodecylsulphate was used as positive control, whereas uninoculated BMMs served as negative controls.

Detection of rhamnolipid biosurfactants

The synthesis of glycolipid-type biosurfactants by strain AP02-1 was detected using the Siegmund and Wagner (1991) technique. A spot of microbial culture grown on BMM with added 2% (v/v) diesel oil was deposited on the surface of a cetyltrimethylammonium bromide (CTAB)–methylene blue agar plate. After 24 h of incubation at 45°C, the plate was inspected for the formation of a dark blue halo around the culture spot which indicated the occurrence of a specific reaction between the cationic CTAB–methylene blue complex and the anionic glycolipid biosurfactants. CTAB plates were prepared adding 0.2 g of CTAB and 0.005 g of methylene blue to 1 l of mineral medium containing (in g/l) 20 glucose, 0.7 of KH_2PO4, 0.9 Na_2PO_4, 2 $NaNO_3$, 0.4 Mg_2SO_4 H_2O, 0.1 $CaCl_2{\cdot}H_2O$ and 2 ml mineral solution as described earlier.

Mass spectrometry (MS) was used to characterize the rhamnolipid biosurfactants produced by AP02-1 using a cell-free supernatant that was extracted with an equal volume of 2:1 chloroform/methanol solvent mixture and mixing thoroughly. The organic layer was separated using a separating funnel, air-dried and dissolved in methanol. MS characterization and detection of the rhamnolipid fractions under investigation were carried out using an LCQ quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) utilizing electrospray ionisation (ESI). Standard solutions and samples under investigation were infused into the mass spectrometer at a flow rate of 10 µl/min. In the ESI source nitrogen sheath and auxiliary gas flows were maintained at 50 and 5, respectively, and refer to arbitrary values set by the software. The heated capillary temperature was 250°C, and the spray voltage was set to 5 kV. Negative ion mode was used, and scans run over the 50–2,000 *m*/*z* range.

Biosurfactant production using different carbon sources

A series of 250-ml flasks containing 100 ml of BMM and a 2 g/l carbon using glucose, glycerol, soybean oil, safflower oil and hexadecane were prepared and inoculated with AP02-1. The flasks were incubated in a shaker at 200 rpm at 45°C, and samples were withdrawn at intervals for the analyses of OD_{600}, surface tension and MS of rhamnolipid extract.

Surface tension and emulsification measurements

Surface tensions of the culture broths were measured using a digital tensiometer (model K10ST, Krüss, Hamburg, Germany) equipped with a De Nouy platinum ring. An average of triplicates was used for the study.

Emulsification index was also determined as described by Cooper and Goldenberg (1987). A variety of hydrocarbons including diesel and crude oil were used as test substrates.

Thermostability of biosurfactants was also evaluated by measuring the emulsification index (EI) after sterilizing the culture broth.

Electron microscopy

Scanning electron microscope (SEM) observation was carried out by sticking suspended cells from culture broth to cover slips coated with poly-L-lysine, fixing in 2.5% buffered glutaraldehyde followed by dehydration in ethanol series, critical point drying and sputter-coating with gold.

Transmission electron microscopy for the examination of flagella was carried out by placing drops of cell suspension (10 µl) on collodion-carbon-coated grids and allowing to adsorb for 60 s. Excess liquid was removed gently touching the filter paper. The adsorbed specimen was then fixed for 5 min at room temperature, floating on a drop of 4% paraformaldehyde plus 0.05% glutaraldehyde in phosphate-buffered saline, pH 7.2. Negative staining was carried out by washing the specimen grid on a drop of negative stain (2% uranyl acetate in distilled water), blotting and repeating this step once more, this time leaving the specimen grid for 60 s on a new drop of negative-stain solution. Samples were observed in a JEOL 1200 EX II electron microscope.

Results

Isolation and characterization of *P. aeruginosa* strain AP02-1

Six thermophilic bacterial strains were able to grow on mineral medium (BMM) containing hydrocarbons as sole carbon source. Strain AP02-1 grew best on BMM containing 2%(v/v) diesel oil reaching an OD_{600} of 1.0 after 72–96 h, gave clear colonies within 48 h when streaked on BMM agar plates with diesel oil and was therefore selected for further investigation. AP02-1 was an aerobic Gram-negative motile rod and was able to grow at temperatures between 30 and 50°C with an optimum at 45°C. Electron microscopy examination showed clear peritrichous flagellated rod-shaped cells.

On the basis of the metabolic pattern obtained by the Biolog method, the strain showed a high similarity to *P. aeruginosa* (99%). When the partial 16S rRNA gene sequence (1,007 bp, EBI accession number AM087130)

was used in a Blast search, seven of the top ten matches were to *P. aeruginosa* strains, and the remaining three were to unidentified pseudomonads. Since the sequence similarities to *P. aeruginosa* were uniformly 100%, the identity of strain AP02-1 was confirmed.

Since the success of *P. aeruginosa* in various habitats, both environmental and clinical, is attributed to a large variety of competition/virulence factors, some of them have been investigated. The bacterium was able to carry out all three known modes of motility (swimming, swarming and twitching). A wide concentric ring (4-cm diameter) of swimming zone was displayed within 48–72 h, whereas on swarm agar plates, the cells formed the expanding and irregular branching pattern that is characteristic of swarming motility in *P. aeruginosa* as described by Rashid and Kornberg (2000). While examining the twitching motility capability, we observed the development of an elliptical-shaped area from the inoculation point.

The analysis of secondary metabolite production demonstrated that pyocyanin, the blue phenazine pigment of *P. aeruginosa* with antibiotic activity, and pyoverdine, the green fluorescent pigment which is a primary siderophore, were abundantly produced by AP02-1 on appropriate media.

Hydrocarbon utilization

As shown in Table 1, AP02-1 grew well on C5–C19 alkanes, aromatic hydrocarbons (benzene, toluene, *o*-xylene), PHAs (anthracene, naphthalene), heterocyclic sulphorate hydrocarbons (thianaphtene, dibenzothiophene) and metyl-*tert*-butyl-ether.

Table 1 Growth characteristics of *Pseudomonas aeruginosa* AP02-1 on various hydrocarbons

Hydrocarbons	C source utilization[a]
Pentane	+++
Hexane	+++
Cyclohexane	+++
Octane	+++
Dodecane	+++
Hexadecane	+++
Pristane	+++
Benzene	+
Toluene	+
o-Xylene	++
Naphthalene	+
Anthracene	+
Thianaphtene	++
Dibenzothiophene	++
Metyl-*tert*-butyl-ether	+++

[a]MT2 plate (Biolog) was carried out with test hydrocarbons, and the utilization as carbon source was indicated by the colour development of a redox indicator dye. Plus signs were assigned on the basis of the absorbance values measured at 590 nm (A_{590}) by the automated plate reader

The ability of this strain to utilize the main classes of hydrocarbons was further supported through gas chromatographic analyses for cultures grown on both 2%(v/v) diesel oil and 1%(v/v) crude oil, which showed 99% degradation of the TPHs after only 7 days of incubation (Fig. 1).

Detection of biosurfactant production

The three simple methods used to detect biosurfactant production all gave positive results.

AP02-1 grew forming colonies surrounded by halos (>2-cm diameter) of haemolysis on blood agar, and the culture drop caused oil to spread, thus forming a wide clear zone on the oil–water surface and completely collapsed over an oil-covered slide surface.

The isolate grew well (OD >1.0 within 6 h) on all four substrates tested (glucose, glycerol soybean oil and hexadecane) with a slight lag phase on hexadecane only. Surface tension measurements of the culture broth showed significant reduction depending on the carbon source used. Glucose resulted in the least reduction from 70 (water) to 42 mN/m, followed by hexadecane resulting in surface tension of 38 mN/m. Glycerol and soybean oil however were good substrates as the culture surface tensions were reduced to 28–30 mN/m.

Type of biosurfactants produced

The Siegmund and Wagner technique results indicated that the biosurfactants produced were rhamnolipid in nature, which was confirmed by MS. This also showed a variation in the composition of the rhamnolipid mixture depending

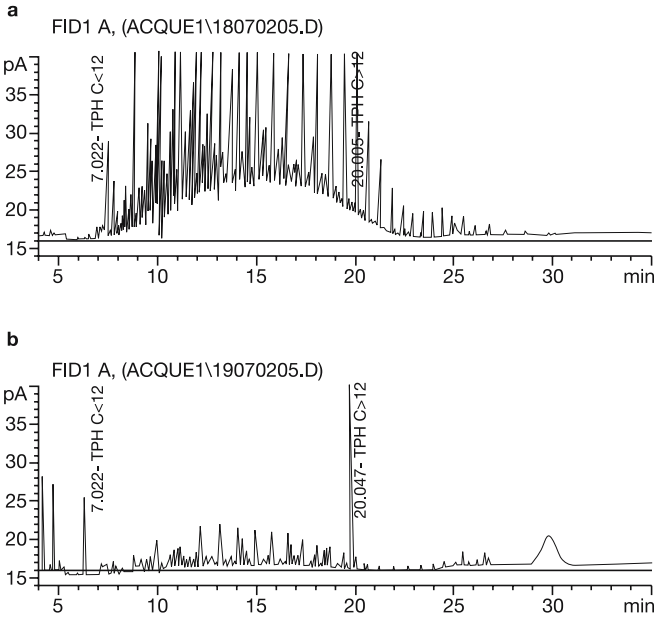

Fig. 1 GC profiles of crude oil. Abiotic control (**a**) and after degradation by *P. aeruginosa* AP02-1 (**b**)

on the carbon source, but the relative amount of each mixture component could not be quantified by the method we used. In the glucose- and soybean oil-containing cultures, the main rhamnolipid produced was the Rha-C_{12}. In comparison, in both the hexadecane- and glycerol-containing cultures, there were two main rhamnolipid peaks at 503.1 and 649.1 *m*/*z*, corresponding to the two main rhamnolipids produced by most *P. aeruginosa* strains, L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rha-C_{10}-C_{10}) and L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rha-Rha-C_{10}-C_{10}). The di-rhamnolipid was the dominant product. In the glycerol culture broth however, ten rhamnolipid homologues were identified (Table 2). The di-rhamnolipid Rha-Rha-C_{10}-C_{10} was the predominant component, followed by rhamnolipid Rha-C_{10}-C_{10}. Both mono- and di-rhamnolipids with C_{12} chain were also detected; however, because isomeric rhamnolipids have the same pseudomolecular ions, it was not possible by this method to separate Rha-C_{12}-C_{10} from Rha-C_{10}-C_{12} at 531.2 *m*/*z*, as well as Rha-Rha-C_{12}-C_{10} from Rha-Rha-C_{10}-C_{12} at 677.1 *m*/*z*. The peak at 621.8 *m*/*z* corresponded to di-rhamnolipids with the C_8 chain (Rha-Rha-C_8-C_{10}, Rha-Rha-C_{10}-C_8). As previously reported (Déziel et al. 2000), peaks at 333.0 *m*/*z* (Rha-C10) and 479.1 *m*/*z* (Rha-Rha-C_{10}) indicated fragments produced by cleavage of rhamnolipid molecules. Some other less-common rhamnolipid congeners most likely with the C_{14} chain were also detected but not identified above 677.1 *m*/*z*. The chemical compositions of the ten different rhamnolipid mixture produced by the AP02-1 culture on glycerol are shown in Table 2.

Emulsification activity

Formation of emulsions during the growth of *P. aeruginosa* AP02-1 on diesel oil was attributed to biosurfactant production leading to lowering of the surface tension coupled to increasing of the emulsification activity (Fig. 2). When the OD_{600} reached 1.1, the culture had a surface tension of 40 mN/m and an EI (24%) with diesel oil of 60%. Moreover, at this stage of growth, the cell surface of the microorganism was highly hydrophobic, and the BATH test showed 71% of cells adhered to hydrocarbons. At the same time, the process of solubilization of diesel oil was evident by SEM, which showed diesel oil droplets breaking down to smaller sizes, and after 96 h of incubation, bacterial cells tightly adhered to the surface of the microdroplets of approximately 30-μm diameter. Analysis of a sterilized microbial culture showed a slight increase in surface activity, which might be due to the release of intracellular biosurfactants and/or cellular fragments having surfactant components (Kosaric et al. 1983).

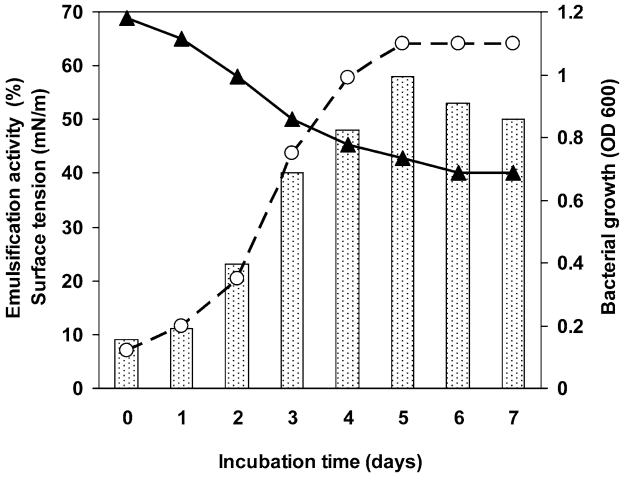

Fig. 2 Emulsification index (EI24) (*columns*) and surface tension (▲) measurements during the bacterial growth (○) of *P. aeruginosa* AP02-1 on mineral medium supplemented with 2% (v/v) diesel oil as carbon source

Table 2 Chemical compositions of rhamnolipid mixture produced by *Pseudomonas aeruginosa* AP02-1 culture with glycerol as carbon source determined by MS analysis

Rhamnolipid congeners	Pseudomolecular ion (*m*/*z*)
Rha-Rha-C_8-C_{10}	621.8
Rha-Rha-C_{10}-C_8	621.8
Rha-Rha-C_{10}-C_{10}	649.2
Rha-Rha-C_{10}-C_{12}	677.1
Rha-C_{10}-C_{12}	531.2
Rha-C_{10}	333.0
Rha-C_{10}-C_{10}	503.1
Rha-Rha-C_{12}-C_{10}	677.1
Rha-C_{12}-C_{10}	531.2
Rha-Rha-C_{10}	479.1

Discussion

Oil-degradation and biosurfactant-producing microbial investigations almost exclusively deal with mesophiles, while a few reports refer to thermophilic conditions as reviewed by Cameotra and Makkar (1998). Thermophiles are of great interest for biotechnological applications, because they can be used in most industrial processes that run at elevated temperatures (Niehaus et al. 1999). *P. aeruginosa* AP02-1 to our knowledge is the first thermophilic biosurfactant producing *Pseudomonas* strain reported in the literature. This isolate grew optimally at 45°C using a variety of hydrocarbons as carbon source while producing rhamnolipid biosurfactants. The involvement and role of rhamnolipids in the uptake of hydrocarbons have been widely documented mostly on pure compounds such as hexadecane (Beal and Betts 2000; Bouchez-Naïtali et al. 1999; Noordman and Janssen 2002), octadecane (Zhang and Miller 1992, 1994), naphthalene (Déziel et al. 1996) or phenanthrene (Prabhu and Phale 2003; Zhang et al. 1997). Our results showed that the same mechanisms, increase in the solubilization of hydrocarbons as well as in the hydrophobicity of degrading cells, occurred on *n*-alkanes, aromatics and more complex

substrate such as diesel oil. Such a multiphasic, biosurfactant-mediated process allowed *P. aeruginosa* AP02-1 to degrade almost completely crude oil and diesel oil in less than 1 week of incubation.

The effect of the nutrient medium and particularly the carbon source on the synthesis of rhamnolipids is not well understood. *Pseudomonas* strains have been reported to produce biosurfactants on hydrocarbons, water-immiscible substrates (Benincasa et al. 2004; Rahman et al. 2002) and on readily available carbon such as glucose (Bodour et al. 2003), mannitol (Déziel et al. 1999) or glycerol (Arino et al. 1996). Strain AP02-1 when grown on glycerol, soybean oil, safflower oil and hexadecane produced culture broth with low surface tension (30, 28, 29 and 38 mN/m, respectively), which meant that both water-immiscible and miscible compounds stimulated the synthesis of significant amounts of biosurfactants in the bacterium.

Furthermore, chemical analysis by MS clearly demonstrated that the carbon source not only influenced the quantity but also had an effect on the structures of biosurfactants.

Rhamnolipids are produced as a mixture of different congeners, and up to 28 different structural homologues are currently known (Déziel et al. 1999). When cultivated on glycerol, AP02-1 produced a complex mixture of ten congeners with one or two rhamnose groups linked to 3-hydroxy fatty acids at C_8, C_{10} and C_{12}. Rha-Rha-C_{10}-C_{10} was predominant, followed by Rha-C_{10}-C_{10}. Growth on hexadecane allowed the synthesis of only low concentrations of the two main congeners, Rha-Rha-C_{10}-C_{10} and Rha-C_{10}-C_{10}. These results showed that (1) despite the key role of biosurfactants in the hydrocarbon uptake, hexadecane dramatically decreased the variety of rhamnolipids compared with those produced on water-soluble glycerol; (2) in all the mixtures, di-rhamnolipids were more abundant than mono-rhamnolipid homologues; (3) in the Rha-C_{10}-C_{10}/Rha-Rha-C_{10}-C_{10} ratio, the proportion of Rha-Rha-C_{10}-C_{10} relative to the Rha-C_{10}-C_{10} was higher on hexadecane that on glycerol. Even slight differences in the structure of a surfactant can have great consequences on the physico-chemical properties. Mono-rhamnolipids are less soluble, sorb to surfaces more strongly, require higher critical micelle concentration for the hydrocarbon solubilization and bind cationic metals more strongly than the homologue di-rhamnolipids (Zhang and Miller 1995; Zhang et al. 1997).

Thus, the activity of a rhamnolipid mixture and hence its functional potential can vary depending on the individual congeners and their ratios. Such information may be essential for designing specific biosurfactants for particular industrial applications; however, there is little information in the literature.

When cultivated with hydrocarbon substrate as a sole carbon source, *P. aeruginosa* AP02-1 synthesised biosurfactants with high emulsifying activity, and the effectiveness was higher against the same hydrocarbon (data not shown). Additionally, the stability of the emulsions was relatively strong and mainly due to the bacterial cell attachment to the oil–water interface which may hinder the coalescence of oil droplets. The role of hydrophobic microorganisms as emulsion-stabilizing agents has been reported earlier (Dorobantu et al. 2004) for *Rhodococcus* and *Acinetobacter* strains, both of which are well known for the ability to produce bioemulsifiers that also give high hydrophobicity to the cell surface.

Such a strong emulsifying activity however may be counter-productive for enhanced oil recovery applications of biosurfactant in oil tank cleaning. We carried out a small-scale preliminary enhanced oil recovery pilot trial as described by Banat et al. (1991), in which equal volumes (50 ml) of AP02-1 microbial product and oil sludge were mixed until achieving a 100% emulsion. After leaving overnight, only 25ml of free water phase was released, indicating that the emulsion was highly stable. Treatment with an emulsion breaker did not lead to further separation due to the undesirable high emulsion stability of this product.

Finally, this study showed that *P. aeruginosa* AP02-1 has an advantage because of its ability to grow at higher temperatures, which may be useful for industrial processes for the obvious advantages of lower cooling energy cost in large-scale fermentation runs and the reduction of cross-contamination when operating at higher temperatures.

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