Production and Properties of Biosurfactants from a Newly Isolated *Pseudomonas fluorescens* HW-6 Growing on Hexadecane

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The newly isolated from industrial wastewater *Pseudomonas fluorescens* strain HW-6 produced glycolipid biosurfactants at high concentrations (1.4–2.0 g l^{-1}) when grown on hexadecane as a sole carbon source. Biosurfactants decreased the surface tension of the air/water interface by 35 mN m^{-1} and possessed a low critical micelle concentration value of 20 mg l^{-1}, which indicated high surface activity. They efficiently emulsified aromatic hydrocarbons, kerosene, *n*-paraffins and mineral oils. Biosurfactant production contributed to a significant increase in cell hydrophobicity correlated with an increased growth of the strain on hexadecane. The results suggested that the newly isolated strain of *Ps. fluorescens* and produced glycolipid biosurfactants with effective surface and emulsifying properties are very promising and could find application for bioremediation of hydrocarbon-polluted sites.

Key words: Biosurfactants, Glycolipids, Hydrophobicity, *Pseudomonas fluorescens*

Introduction

Petroleum hydrocarbons are serious environmental pollutants due to their persistence and high toxicity to all biological systems. Bacteria are the most active agents in petroleum biodegradation (Leahy and Colwell, 1990; Geerdink *et al*., 1996). The efficiency of biodegradation of oil pollutants has been limited by their low water solubility (Desai and Banat, 1997; Noordman *et al*., 2002). A promising approach to increasing the biodegradation rates of hydrocarbons was to use biosurfactants. Biosurfactants are amphiphilic compounds that reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids or of a fluid and a solid and increase the surface areas of insoluble compounds leading to increased mobility, bioavailability and subsequent biodegradation (Desai and Banat, 1997; Rosenberg and Ron, 1999; Ron and Rosenberg, 2002; Rahman *et al*., 2003). A large variety of microbial surface active compounds are produced by bacteria, yeasts, and fungi. Biosurfactants have recently received much more attention due to their potential to become an environment-friendly alternative to conventional chemically synthetic surfactants because of their biodegradability, low toxicity, and ability to be produced from renewable resources (Lang and Wullbtandt, 1999; Banat *et al*., 2000; Makkar and Cameotra, 2002; Rahman *et al*., 2002; Ron and Rosenberg, 2002; Mulligan, 2005). The structural diversity of biosurfactants determines their more or less specific role in different ecological niches (Banat *et al*., 2000; Ron and Rosenberg, 2001). It is the three-way interaction among the biosurfactant, substrate, and cell that is crucial to achieve enhanced biodegradation rates. It is therefore promising to isolate indigenous microorganisms from contaminated sites with high abilities to grow on hydrocarbons and producing effective surfactants. Co-treatment of polluted sites with adapted isolates and produced surface active complexes would be more effective for bioremediation. Biosurfactants are mainly classified into four categories, based on the structure of their hydrophilic part: glycolipid type (Kitamoto, 2001), fatty acid type (Fujii, 1998), lipopeptide type (Peypoux *et al*., 1999), and polymer type (Rosenberg and Ron, 1999). Among these, glycolipid biosurfactants are of great interest because of their high surface activity, and they are also the most promising for

commercial production and utilization (Kitamoto *et al.*, 2002).

In a previous work, fifteen bacterial strains were isolated from lubricant-polluted water samples and were screened for growth on solid media with different hydrocarbons, and in liquid media with hexadecane as a sole carbon source (Vasileva-Tonkova and Galabova, 2003). Isolate marked as HW-6 showed high growth on hydrocarbons and was selected for more detailed investigations. In this study, glycolipid biosurfactants produced by the selected strain were isolated and their surface and emulsifying properties were determined. In addition correlations between biosurfactant production, changes in cell surface hydrophobicity and growth of the strain on hexadecane have been assessed.

Materials and Methods

Microorganism

Bacterial strain HW-6 was isolated in the Institute of Microbiology from lubricant-polluted water samples (Vasileva-Tonkova and Galabova, 2003). It was identified as *Pseudomonas fluorescens* by routine morphological, microbiological and biochemical methods according to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). The isolate was maintained by monthly transfers onto slants containing meat-peptone broth (MPB), and, after growth, stored at 4 °C. A stock culture was also prepared as cells were suspended in a cryoprotective agent (20% sterile glycerol, final content, 10% v/v) and stored at – 18 °C.

Growth conditions

Ps. fluorescens HW-6 was cultivated in 300-ml Erlenmeyer flasks containing 50 ml mineral salts (MS) medium with the following composition (g l^{-1}): $(NH_4)_2SO_4$ (2.0), KH_2PO_4 (6.0), sodium citrate (1.0), $MgSO_4$ (0.1), and $CaCl_2$ (0.002 M) (Vasileva-Tonkova and Galabova, 2003). The medium was supplemented with 1.5% hexadecane or glucose as a sole carbon source. Hexadecane was sterilized through 0.2 μm Millipore membrane filters. The flasks were inoculated with 2% log phase culture (grown in MPB) and incubated in the dark at (28 ± 2) °C on a rotary shaker at 130 rpm. An abiotic control was prepared for each series of flasks for comparison purpose. Samples were taken aseptically at time-defined intervals and analyzed. Growth was monitored by measuring the optical density at 570 nm (OD_{570}). The experiments were carried out with three independent replicates.

Partial purification and quantification of glycolipids

The glycolipids were concentrated from the culture supernatants (at pH 6.5) by addition of $ZnCl_2$ as described by Koch *et al.* (1991). The precipitates were dissolved in sodium phosphate buffer, pH 6.5, and extracted twice with equal volumes of chloroform/methanol (2 : 1 v/v). The pooled organic extracts were evaporated and the pellets dissolved in a small volume of methanol. They were analyzed by three tests: hemolysis of erythrocytes (Johnson and Boese-Marrazzo, 1980), growth inhibition of *Bacillus subtilis* (Itoch *et al.*, 1971), and thin-layer chromatography (TLC) (Kates, 1972). Concentrated glycolipids (10 μl) were spotted on filter paper discs and then put onto blood agar plates or onto MPB agar plates with freshly grown *B. subtilis* cells. The blood agar plates were incubated at 28 °C for 2 d, and the *B. subtilis* plates at 28 °C overnight, and then the zones of hemolysis and growth inhibition were measured. TLC was performed on silica gel plates (G60; Merck, Germany) with a solvent system of chloroform/methanol/acetic acid (85 : 15 : 2 v/v) (Kates, 1972). Purified rhamnolipid from *Pseudomonas* sp. PS-17 was used as a standard (Shulga *et al.*, 2000).

The orcinol assay was used for quantification of glycolipids in samples (Koch *et al.*, 1991). As it was assumed that the secreted glycolipids are rhamnolipids, a standard curve was made with L-rhamnose, and glycolipid concentration was expressed as rhamnose equivalents, RE (g l^{-1}).

Surface activity assay

The surface tension (γ) of the samples at the air/water interface was used as an indirect measure of surfactant concentration. It was measured in a Teflon trough with an area of 706 mm^2 (volume 7 ml). The kinetics of adsorption and the equilibrium surface tension values (γ_{eq}) were monitored using an automatic Wilhelmy tensiometer (Biegler Electronic, Mauerbach, Austria) and platinum float (1 × 1.6 cm). The dependence γ/time was followed by the Wilhelmy plate method (with accuracy of ± 0.5 mN m^{-1}) as previously described (Lalchev, 1997; Christova *et al.*, 2004). The trough

temperature was controlled with a precision of ± 0.5 °C, and each experiment was repeated at least three times.

For determination of the critical micelle concentration (CMC) of the samples the organic extracts were dried and redissolved in 0.14 M NaCl. Appropriate dilutions of samples were prepared with 0.14 M NaCl and γ/time and γ_{eq}/c dependences were recorded. At least three experiments were performed for each concentration c. The CMC value was that point at which the biosurfactant no longer aggregated to form micelles during dilutions, and was determined graphically from γ_{eq}/c plots.

Cell surface hydrophobicity test

The cell surface hydrophobicity was measured by bacterial adherence to hydrocarbons (BATH) as described by Rosenberg *et al.* (1980). Cell hydrophobicity was expressed as the percentage decrease in OD_{550} of the lower aqueous phase following the mixing procedure, compared with that of the cell suspension prior to mixing.

Emulsifying activity

Emulsifying activity of samples was determined at 25 °C according to the test of Berg *et al.* (1990) with modification. An absorbance of 1.0 at 550 nm (multiplied by dilution factor, if any) was considered as one unit of emulsifying activity per ml (EU ml^{-1}). Effects of pH (from 2.2 to 11.0) and NaCl (from 1 to 15% w/v) on emulsifying activity were tested by incubating the sample with equal volumes of 0.1 m buffers (or with required quantities of NaCl) at 25 °C for 15 h and then the standard emulsification assay was carried out.

Results and Discussion

Biosurfactant production

Ps. fluorescens HW-6 produced glycolipid biosurfactant(s) when grown on MS medium with 1.5% hexadecane as a sole carbon source. Biosurfactant production was estimated using the decrease in surface tension, increase in emulsifying activity, and in terms of rhamnose concentration.

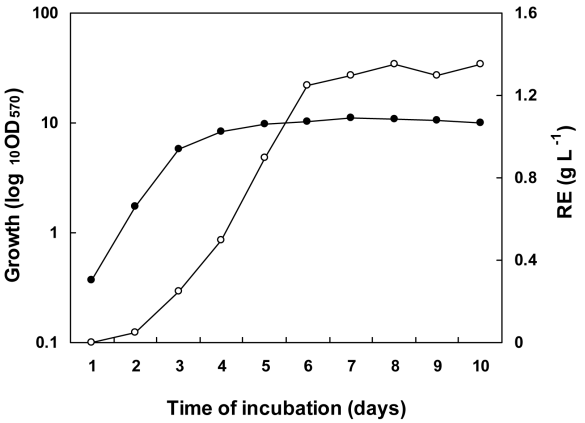

Fig. 1. Time-course of biosurfactant production by *Ps. fluorescens* HW-6 grown in a batch culture with 1.5% hexadecane as a sole carbon source. Glycolipid levels are expressed as rhamnose equivalents (RE). Mean values from three separate experiments are given. (○), rhamnose concentration, RE; (●), OD_{570}.

As can be seen in Fig. 1, secretion of biosurfactants started in the late-exponential growth phase reaching values of (1.4 ± 0.22) g l^{-1} in the stationary growth phase. Simultaneously, an increase in emulsifying activity was registered (up to 1.69 U ml^{-1} with kerosene). Disappearance of hexadecane droplets was observed and biosurfactant production was accompanied by appearance of white turbidity of the culture broth at the third day of growth.

As biosurfactants are secondary metabolites, maximal production occurs in the stationary growth phase (Bodour and Maier, 2002). Limiting addition of inorganic nutrients, including phosphate, nitrogen, iron, and carbon excess, has been reported to increase the biosynthesis of lipids, polysaccharides or secondary metabolites (Boulton and Ratledge, 1987; Bodour and Maier, 2002; Ron and Rosenberg, 2002).

Detection of surface active compounds

Cell-free supernatants of *Ps. fluorescens* HW-6 grown on hexadecane lowered the surface tension of the medium below 37 mN m^{-1}. The organic extracts showed high hemolytic activity (34-mm in diameter zone of hemolysis on blood agar) and inhibition of the growth of *B. subtilis* (18-mm in diameter zone of inhibition), tests usually used for detection of anionic glycolipids, mainly rhamnolipids (Koch *et al.*, 1991). In the thin-layer chroma-

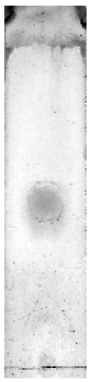

Fig. 2. Thin-layer chromatogram of surfactants produced by hexadecane-grown *Ps. fluorescens* HW-6. The organic extract (concentrated glycolipids) was spotted onto silica gel plates and stained for carbohydrates as described in the text.

togram one spot was revealed after orcinol/sulfuric acid staining at R_f of 0.46 (Fig. 2), corresponding to the spot of the standard dirhamnolipid. With iodine vapors one yellowish spot at the same R_f value was revealed indicating a lipid moiety of the compound. No spots were visualized after staining with ninhydrin indicating lack of free amino groups. The results suggested that *Ps. fluorescens* HW-6 produced rhamnose-containing glycolipid(s) when grown on hexadecane as a sole carbon source. Rhamnolipids have been identified predominantly from *Pseudomonas aeruginosa* strains (up to 28 diverse homologues are reported), and usually greatly decreased the surface and interfacial tensions of the medium (Deziel *et al.*, 1999; Lang and Wullbtandt, 1999; Maier and Soberon-Chavez, 2000; Wei *et al.*, 2005).

Surface tension and CMC

The surface tension lowering capacity is an indirect measure of biosurfactant production. For practical purposes it is important also to distinguish between an efficient surfactant and an effective surfactant. Efficiency is measured by the surfactant concentration required to produce a significant reduction in the surface tension of water, whereas effectiveness is measured by the minimum value, to which the surface tension can be reduced (Kim *et al.*, 2000). Therefore, important properties of surfactants are their abilities to lower the surface tension in aqueous solutions and to possess a low CMC (Sheppard and Mulligan, 1987). The individual rhamnolipids are able to lower the surface tension of water from 72 mN m^{-1} to 25–30 mN m^{-1}, at concentrations of 10–200 mg l^{-1} (Lang and Wullbtandt, 1999; Bodour and Maier, 2002).The results obtained on the surface tension lowering capacity of glycolipids produced by *Ps. fluorescens* HW-6 revealed their fast kinetics of adsorption to the interface and that they were able to reduce strongly the surface tension of water at very low concentrations (Fig. 3). The increase in concentration up to 18 mg l^{-1} caused a decrease of the sample surface tension to 37 mN m^{-1}, which is with 35 mN m^{-1} lower than the surface tension of pure water. At concentrations above 20 mg l^{-1}, the surface tension remained practically unchanged thus the CMC value of approx. 20 mg l^{-1} was determined. The results indicated that glycolipids produced by *Ps. fluorescens* possess high surface tension lowering capacity and are both efficient and effective surfactants.

Cell surface hydrophobicity

Hydrophobicity of hexadecane or glucose-grown cells of *Ps. fluorescens* HW-6 at the stationary growth phase was tested towards different hydrocarbons. Hexadecane-grown cells were more hydrophobic than glucose-grown cells for all the

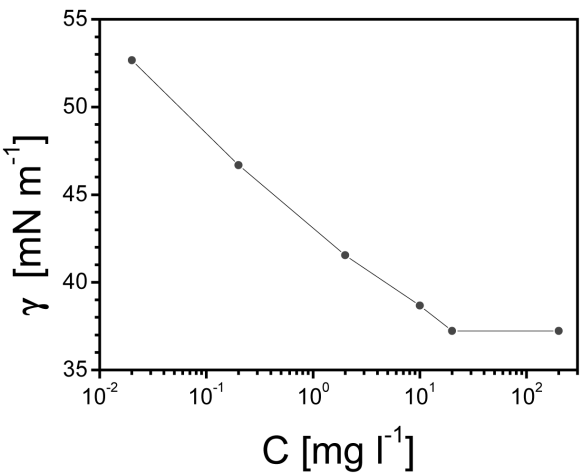

Fig. 3. Dependence of the surface tension γ (mN m^{-1}) on the glycolipid concentration c (mg l^{-1}). Surface tension of the organic extract was measured after dilutions with 0.14 M NaCl, and the CMC was determined graphically.

Table I. Adherence to some hydrocarbons of *Ps. fluorescens* HW-6 cells grown on 1.5% hexadecane or glucose.

Hydrocarbon	Hydrophobicity* (%)	Hydrophobicity** (%)
n-Hexane	89.8 ± 0.6	15.1 ± 0.6
n-Heptane	85.5 ± 1.0	15.3 ± 1.2
n-Hexadecane	89.1 ± 1.4	26.2 ± 3.1
Kerosene	85.1 ± 1.2	16.2 ± 0.4
Benzene	81.5 ± 1.7	26.1 ± 1.2

* Cells grown on hexadecane. ** Cells grown on glucose. Results are averages of three determinations ± s.d.

hydrocarbons tested (Table I). During growth on glucose, cell hydrophobicity to hexadecane was in the range 21–29% and remained unchanged during the growth. During growth on hexadecane, the cells became gradually more hydrophobic reaching to a value of approx. (87 ± 2.6) % at the stationary growth phase. In the earlier stages of growth on hexadecane, biosurfactant is cell-associated that makes the cell surface hydrophobic and enhance the attachment of cells to the large alkane droplets. At the stationary growth phase (day 10), return of a small part of the cells to the hydrophilic state was observed. This could be explained by the release of cell-bound biosurfactant into the medium thus recovering the initial cell hydrophobicity of 28%. The larger part of the cells remained with high hydrophobicity (to 90%). At the stationary growth phase high levels of glycolipids in the culture broth were measured (Fig. 1). Simultaneously, an increase in the emulsifying activity was observed (up to 1.69 U ml^{-1} with kerosene) indicating that emulsification and solubilization of the alkane occur. Secreted biosurfactants form a polymeric film on the oil droplets with hydrophilic outer surfaces, so-called micelles, and the detached starved cells can move to these micelles.

The cell surface hydrophobicity has been suggested to be a significant factor in microbial adhesion on surfaces including hydrophobic substrates (Van Loosdrecht *et al.*, 1987; Neu, 1996; Ron and Rosenberg, 2002). It was shown that an increase in cell hydrophobicity promoted the attachment of cells to hydrocarbon droplets thus stimulating alkane degradation (Al-Tahhan *et al.*, 2000). The increase in cell hydrophobicity can be induced in the presence of a combination of both biosurfactant and slightly soluble substrate (Zhang and Miller, 1994).

Emulsifying activity

The ability of biosurfactants to emulsify various hydrocarbons was tested. As can be seen by Table II, the sample of hexadecane-grown cells efficiently emulsified aromatic hydrocarbons, hydrocarbon mixtures, *n*-paraffins and mineral oils. The emulsifying activity of the sample of glucose-grown cells was much lower than that of hexadecane-grown cells.

The emulsifying activity to kerosene of the supernatant after growth of the strain on hexadecane was maximal at pH values in the range from 7.0 to 11.0. At acidic pH of 2.2, 3.5 and 5.5, 24%, 38% and 75% of activity was retained, respectively. The sample retained 68% of emulsifying activity after heating at 100 °C for 15 min, and NaCl from 1 to 15% had no effect on the activity.Alkane transport into microbial cells occurs by two mechanisms (Hommel, 1990). The first is direct contact with larger alkane droplets, which involves little or no emulsification. The second is pseudosolubilization (emulsification) and cellular assimilation of emulsified small hydrocarbon droplets. In either case, bacterial growth is restricted to the hydrocarbon interface. The cell contact with hydrophobic compounds is a requirement as the first step in hydrocarbon degradation is the introduction of molecular oxygen into the molecules by membrane-bound oxygenases (Rosenberg, 1993). This process is often mediated by surfactant molecules produced by many microorganisms. Biosurfactants facilitate hydrocarbon biodegradation by increasing solubilization and dispersion of hydrocarbon and

Table II. Emulsifying activity of biosurfactants with some hydrocarbons and oils after growth of *Ps. fluorescens* HW-6 on 1.5% hexadecane or glucose.

Hydrocarbon	Emulsifying activity* [EU ml^{-1}]	Emulsifying activity** [EU ml^{-1}]
n-Hexane	0.81	0.02
n-Heptane	0.39	0.02
n-Hexadecane	0.36	0.14
Kerosene	1.69	1.18
Benzene	8.71	1.10
Toluene	7.10	1.28
Xylene	4.26	0.98
n-Paraffins	2.74	n.d.
Mineral oils	6.76	3.10
Sunflower oil	0.98	0.40

* Cells were grown on hexadecane; maximal values are given. ** Cells were grown on glucose; maximal values are given. n.d., not determined.

by inducing increases in cell surface hydrophobicity (Zhang and Miller, 1992, 1994; Rosenberg and Ron, 1999; Beal and Betts, 2000).

If taken together the obtained for increased cell hydrophobicity of hexadecane-grown *Ps. fluorescens* HW-6, low CMC and enhanced both surface tension lowering capacity and emulsifying potential of the produced biosurfactants, it could be proposed the occurrence of both modes of biosurfactant-enhanced growth on hexadecane (Beal and Betts, 2000): (i) direct contact with large alkane droplets even at the earlier stage of growth and low concentration of biosurfactant in the culture broth, and (ii) alkane transfer mechanism which involves both solubilization and emulsification of the alkane at higher biosurfactant concentration in the culture broth.

Pseudomonads are known for their broad nutritional versatility which enables them to use many naturally-occuring and synthetic wastes as sources of carbon and energy. *Ps. fluorescens* encompasses a group of common, nonpathogenic saprophytes that colonize soil, water and plant surface environment. Moreover, fluorescent pseudomonads have often been used as biological control agents, and as biosensors in wastewater bioremediation (Matrubutham *et al.*, 1997; Bagnasco *et al.*, 1998; Nivens *et al.*, 2004). The results in this paper showed that when grown on hexadecane, *Ps. fluorescens* HW-6 produced glycolipid biosurfactants with effective surface and emulsifying properties. In view of this complex of possibilities, the newly isolated strain is very promising and could find application for bioremediation of hydrocarbon-polluted environment.

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