Evaluation of Different Carbon Sources for Growth and Biosurfactant Production by *Pseudomonas fluorescens* Isolated from Wastewaters

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The indigenous strain *Pseudomonas fluorescens*, isolated from industrial wastewater, was able to produce glycolipid biosurfactants from a variety of carbon sources, including hydrophilic compounds, hydrocarbons, mineral oils, and vegetable oils. Hexadecane, mineral oils, vegetable oils, and glycerol were preferred carbon sources for growth and biosurfactant production by the strain. Biosurfactant production was detected by measuring the surface and interfacial tension, rhamnose concentration and emulsifying activity. The surface tension of supernatants varied from 28.4 mN m^{-1} with phenanthrene to 49.6 mN m^{-1} with naphthalene and heptane as carbon sources. The interfacial tension has changed in a narrow interval between 6.4 and 7.6 mN m^{-1}. The emulsifying activity was determined to be highest in media with vegetable oils as substrates. The biosurfactant production on insoluble carbon sources contributed to a significant increase of cell hydrophobicity and correlated with an increased growth of the strain on these substrates. Based on these results, a mechanism of biosurfactant-enhanced interfacial uptake of hydrophobic substrates could be proposed as predominant for the strain. With hexadecane as a carbon source, the pH value of 7.0–7.2 and temperature of (28 ± 2) °C were optimum for growth and biosurfactant production by *P. fluorescens* cells. The increased specific protein and biosurfactant release during growth of the strain on hexadecane in the presence of NaCl at contents up to 2% could be due to increased cell permeability. The capability of *P. fluorescens* strain HW-6 to adapt its own metabolism to use different nutrients as energy sources and to keep up relatively high biosurfactant levels in the medium during the stationary phase is a promising feature for its possible application in biological treatments.

Key words: Biosurfactants, Carbon Sources, *Pseudomonas fluorescens*

Introduction

Many microorganisms can produce biosurfactants that play an important ecological role in growth and survival strategy to microbial cells in different environments (Ron and Rosenberg, 2001). Biosurfactants have several advantages over chemical surfactants like low toxicity, high biodegradability, better environmental compatibility, high selectivity, specific activity at extreme temperature, pH value and salinity (Desai and Banat, 1997; Cameotra and Makkar, 1998).

To date, few biosurfactants have been used on an industrial scale because of the low yield and high production cost (Mukherjee *et al.*, 2006). Glycoside-containing biosurfactants are one of the most promising known ones due to production yields higher than those of other types of biosurfactants, and the possibility to use renewable resources for their production (Kitamoto *et al.*, 2002; Makkar and Cameotra, 2002).

Several bacterial strains have been previously isolated from industrial wastewater samples and screened for growth on hydrocarbons and biosurfactant production (Vasileva-Tonkova and Galabova, 2003; Vasileva-Tonkova *et al.*, 2006, 2008). In the present study, various carbon sources were assessed for their effectiveness in growth of and biosurfactant production by *Pseudomonas fluorescens* strain HW-6. Cell surface hydrophobicity, surface and interfacial tension lowering capacity, and emulsifying activity were followed dur-

ing growth of the strain with each of the carbon sources used. The most probable mechanisms for utilizing hydrophobic substrates have been explored.

Materials and Methods

Bacterial strain

Pseudomonas fluorescens strain HW-6, previously isolated in the Institute of Microbiology (Sofia, Bulgaria) from wastewater samples, was used throughout this work. The strain formed halos on blue agar plates which detect the production of anionic glycolipids (Vasileva-Tonkova and Galabova, 2003). The isolate was maintained on meat peptone agar slants at 4 °C and subcultured monthly.

Growth conditions

The experiments were carried out in 300-ml Erlenmeyer flasks containing 40 ml of mineral salts medium (MSM) with a composition as described earlier (Vasileva-Tonkova and Galabova, 2003). The medium was supplemented with one of the following hydrocarbons as the carbon source (20 g l^{-1}): *n*-alkanes (heptane, hexadecane, paraffin), monoaromatic compounds (benzene, toluene), mineral oils (lubricant, kerosene), vegetable oils (linseed, sunflower, olive oils), and 10 g l^{-1} polyaromatic compounds (naphthalene, phenanthrene). Glucose, maltose and glycerol at 20 g l^{-1} were used as water-soluble carbon sources. A control flask without any carbon source was also prepared. The pH value was adjusted to 7.0 ± 0.2 and the media were autoclaved at 121 °C for 15 min. The flasks were inoculated with 2% (v/v) starting culture growing exponentially in meat peptone broth medium. A parallel set of non-inoculated control flaks was also prepared with each carbon source for comparison purpose. The liquid cultures were incubated in the dark at (28 ± 2) °C in an orbital shaker (130 rpm).

In the first step, aliquots were taken periodically to monitor the cell growth. In the second step, aliquots were taken at time-defined intervals, selected previously from the first step: the exponential and the stationary phase, and analyzed. The growth was monitored by measuring the optical density at 570 nm (OD_{570}). The experiments were done in triplicate and the results reported are the average from three independent assays.

Biosurfactant isolation

The cells were removed by centrifugation (6000 × *g* for 10 min) and the pH value of the obtained culture supernatants was adjusted to 2.0. Biosurfactants were extracted from the supernatant fluids by a mixture of chloroform and methanol (2:1 v/v). The combined organic extracts were dried with anhydrous Na_2SO_4 and concentrated. The remaining pellets were dissolved in a small volume of methanol, and aliquots were analyzed by thin layer chromatography (TLC) on silica gel plates (G60; Merck, Germany). The chromatograms were developed with chloroform/methanol/water (85:15:2 v/v) and visualized with TLC reagents. Glycolipid spots were detected by the orcinol reagent using purified rhamnolipids from *Pseudomonas* sp. PS-17 as a standard (Shulga *et al.*, 2000).

Surface and interfacial tension measurements

The surface tension/time dependence was measured in a Teflon-coated Microtrough X (Kibron Inc., Helsinki, Finland): area, 111.1 cm^2; width, 55 mm; length, 202 mm; sub-phase volume, 22 ml. The surface tension γ (mN m^{-1}) was measured with an accuracy of ± 0.01 mN m^{-1} by a single-channel Delta Pi tensiometer (Kibron Inc.). The method of du Nouy was used with a platinum wire probe attached to the microbalance sensor head. Each measurement was repeated at least three times with every sample preparation.

The interfacial tension of cell-free culture broths was determined against kerosene as oil phase using the method of drop spinning. The shape of a rotating drop of a lighter oil phase surrounded by an aqueous solution is determined by the balance of centrifugal and capillary forces giving the possibility to determine the interfacial tension by the equation: $\gamma = (1/32) \Delta\rho\ \omega^2\ d^3\ f(l/d)$, where $\Delta\rho$ denotes the density difference, ω is the rotational velocity, d and l denote, respectively, the height and length of the rotational ellipsoid, and $f(l/d)$ is a tabulated function. $f(l/d) = 1$ for drops which are large enough ($l/d > 4$), simplifying significantly the experimental procedure.

Cell surface hydrophobicity test

The cell surface hydrophobicity was measured by the bacterial adherence to hexadecane (BATH) (Rosenberg *et al.*, 1980). The hydrophobicity was

expressed as the percentage decrease in absorbance at 550 nm of the lower aqueous phase, following the mixing procedure, compared with that of the cell suspension prior to mixing.

Emulsifying activity assay

The emulsifying activity (EU) of samples was determined according to the test of Berg *et al.* (1990) with slight modification. One unit of emulsifying activity per ml (EU ml^{-1}) was considered as an absorbance of 1.0 at 550 nm (multiplied by dilution factor).

Effect of pH value, temperature and ionic strength

The effect of the pH value was studied by growing the strain in hexadecane-containing MSM at 30 °C using either 1 M HCl or NaOH solution to adjust the pH value to 5.0, 7.0 or 8.7. To evaluate the effect of the temperature, the isolate was grown at 20, 30 and 37 °C in MSM with hexadecane as carbon source, pH 7.0. In order to evaluate the effect of ionic strength, the strain was grown in hexadecane-containing MSM supplemented with different contents of NaCl (0.5, 1, 2, 4, 6, and 8%), at pH 7.0 and 30 °C. The experiments were carried out in triplicate.

Analytical methods

The protein content was determined by the method of Bradford (1976). The orcinol assay was used for quantification of glycolipids in the samples (Chandrasekaran and Bemiller, 1980). The glycolipid concentration was expressed in terms of rhamnose (g l^{-1}) using a standard curve prepared with L-rhamnose.

Results and Discussion

Growth and biosurfactant production

The strain *P. fluorescens* HW-6 was cultivated in MSM with different carbon sources, and growth curves were prepared for each substrate (not shown). Values for growth (expressed as OD$_{570}$) in the stationary phase are presented in Fig. 1. Hexadecane, mineral oils, vegetable oils, and glycerol were observed to be preferred carbon sources for the growth of *P. fluorescens* HW-6 cells. A possible reason for the inability of the strain to grow on benzene, toluene, heptane, and naphthalene could be the potential toxicity of these hydrocarbons (Perry, 1984).

The carbon sources were examined for their effectiveness in biosurfactant production by *P. fluorescens* HW-6 cells. TLC assays of organic extracts showed that the strain produced rhamnose-containing glycolipid biosurfactants during growth on
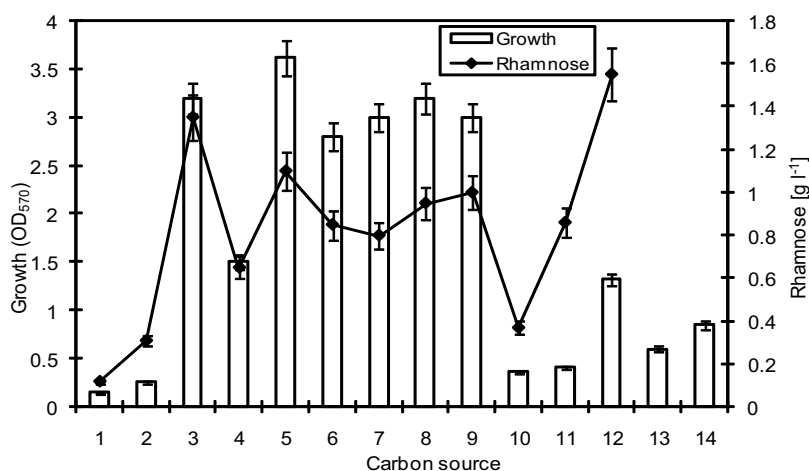

Fig. 1. Growth and glycolipid production (expressed as rhamnose content) by *P. fluorescens* HW-6 cells in mineral salts medium with different carbon sources: 2–11, hydrocarbons; 12–14, water-soluble compounds. 1, Mineral salts medium (MSM, control); 2, heptane; 3, hexadecane; 4, paraffin oil; 5, lubricant oil; 6, linseed oil; 7, sunflower oil; 8, olive oil; 9, kerosene oil; 10, naphthalene; 11, phenanthrene; 12, glycerol; 13, maltose; 14, glucose. Values are the mean of three separate experiments ± S.D. within 5–10%.
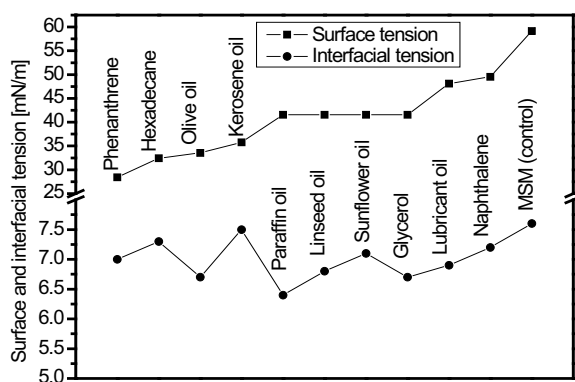
Fig. 2. Surface tension (at air/water interface) and interfacial tension (at kerosene drop/culture medium interface) in culture media of *P. fluorescens* HW-6 cells with different carbon sources. Averages from three independent experiments are given ± S.D. within 5–10%.

The supernatants of eight of the carbon sources reduced the surface tension from 59.2 mN m^{-1} in MSM (control) to below 42.0 mN m^{-1} (Fig. 2). Although the growth of the strain was poor on phenanthrene, a low surface tension of 28.4 mN m^{-1} was measured. The interfacial tension of cell-free culture broths, measured at the kerosene drop/medium interface, changed in a narrow interval between 6.4 and 7.6 mN m^{-1} being lowest for paraffin oil as carbon source (Fig. 2).

Results for the extracellular emulsifying activity of *P. fluorescens* HW-6 cells grown on different carbon sources are shown in Fig. 3. Relatively high values of the emulsifying activity were determined with lubricant and vegetable oils as substrates, in the range of 5.6–7.5 EU ml^{-1}. Values of the emulsifying activity with hexadecane, paraffin and kerosene oil as carbon sources were comparable to those with water-soluble substrates (Fig. 3).

each of the carbon sources used (data not shown). Biosurfactant production was detected indirectly by determining the surface and interfacial tension lowering, rhamnose concentration and emulsifying activity. The best rhamnolipids yield was obtained with hexadecane and glycerol as water-insoluble and water-soluble carbon source, respectively (Fig. 1).

Rhamnolipids are frequently the main biosurfactants produced by *P. aeruginosa* strains, able to lower the surface tension of water from 72 mN m^{-1} to 25–30 mN m^{-1} (Maier and Soberon-Chavez, 2000). The major types of carbon sources used for rhamnolipid production are carbohydrates, hydrocarbons, and vegetable oils (Desai and Banat, 1997; Lang and Wullbtandt, 1999).
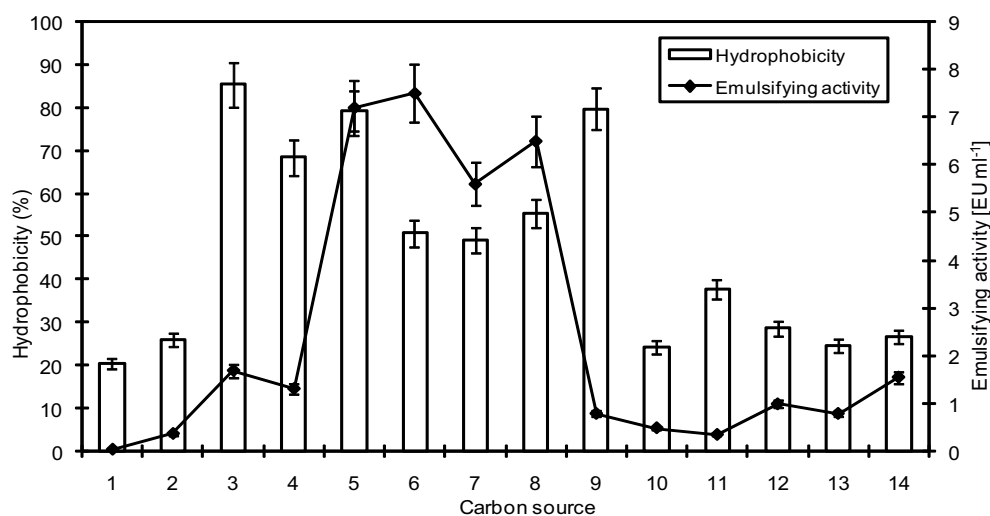

Fig. 3. Cell surface hydrophobicity and emulsifying activity of *P. fluorescens* HW-6 cells grown in mineral salts medium with different carbon sources. Carbon sources as in Fig. 1. Values are the mean of three separate experiments ± S.D. within 5–10%.

Cell hydrophobicity

During growth on most of the water-insoluble carbon sources, *P. fluorescens* cells became gradually more hydrophobic reaching high values at the stationary growth phase (Fig. 3). The hydrophobicity of cells grown on hexadecane, paraffin, lubricant and kerosene oils was highest, in the range of 68–85%, followed by that of cells grown on vegetable oils (50–55%). The development of higher hydrophobicity correlated with an increased growth rate on the hydrophobic substrates. The cells poorly growing on heptane, naphthalene, and phenanthrene had low (heptane, naphthalene) or medium (phenanthrene) hydrophobicity. With water-soluble substrates, the cell hydrophobicity was in the range of 25–29% and remained unchanged during the growth of the strain.

The cell hydrophobicity is an important factor in microbial adhesion to surfaces including hydrophobic compounds. An increase in hydrophobicity promotes the attachment of microbial cells to hydrophobic substrates thus stimulating their degradation (Zhang and Miller, 1994). This process is often mediated by biosurfactants produced by many microorganisms. The mode of action of biosurfactants is to modify the cell surface hydrophobicity and/or to promote emulsification and/or solubilization of substrates (Beal and Betts, 2000). Two modes of hydrocarbon uptake are generally considered (Hommel, 1994): (i) interfacial uptake (direct contact of cells with hydrocarbon droplets), and (ii) biosurfactant-mediated hydrocarbon transfer (cell contact with emulsified or solubilized hydrocarbons). Usually both mechanisms take place but the dominance of one or the other depends on the strain.

Based on the results obtained for growth, cell hydrophobicity, surface and interfacial tension lowering capacity and emulsifying potential of *P. fluorescens* HW-6 cells, most probable mechanisms of utilizing hydrophobic substrates by the
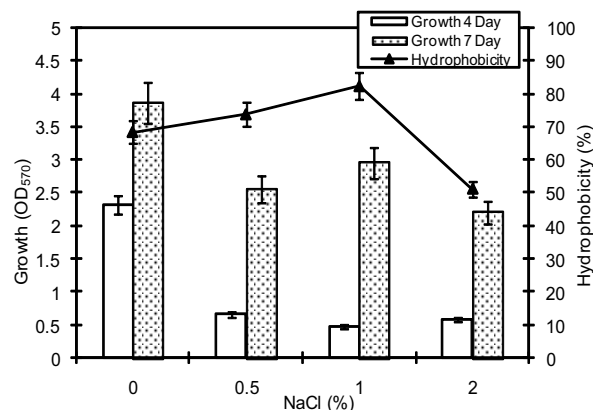

Fig. 4. Cell surface hydrophobicity during growth of *P. fluorescens* HW-6 cells on hexadecane as a carbon source in the presence of NaCl. Values are the mean of three separate experiments ± S.D. within 5–10%.

strain could be proposed. The mechanism of biosurfactant-enhanced interfacial uptake of hexadecane, paraffin and kerosene oil by the strain could be suggested as predominant based on data of increased cell hydrophobicity and relatively low emulsifying activity during growth on these substrates. Such a mechanism could be proposed also with phenanthrene as carbon source although the delayed growth of the strain on this substrate. Relatively high or medium values for cell hydrophobicity and emulsifying activity during growth of the strain on lubricant and vegetable oils suggested the existence of both mechanisms of biosurfactant action, to promote both direct interfacial uptake and substrate emulsification.

Effect of pH value, temperature and ionic strength

Hexadecane was selected for further evaluation of the effect of pH value, temperature and ionic strength on the growth, hydrophobicity and biosurfactant production by *P. fluorescens* HW-6 cells.

Table I. Effect of pH value and temperature on growth, rhamnose release and hydrophobicity of *P. fluorescens* HW-6 cells cultivated in mineral salts medium with hexadecane as a carbon source.

Parameter	pH			Temperature [°C]		
	5.4	7.0	8.7	20	28	37
Growth (OD$_{570}$)	1.75 ± 0.20	2.50 ± 0.25	0.18 ± 0.02	2.20 ± 0.15	2.80 ± 0.20	0.80 ± 0.10
Rhamnose [g l^{-1}]	0.39 ± 0.02	0.70 ± 0.10	0.10 ± 0.02	0.50 ± 0.10	0.84 ± 0.15	0.50 ± 0.05
Hydrophobicity (%)	61.60 ± 2.60	77.10 ± 4.20	22.1 ± 1.10	71.70 ± 1.85	77.10 ± 4.10	37.3 ± 1.50

The levels of the parameters tested were found to be highest at pH 7.0–7.2 and (28 ± 2) °C (Table I). Although the growth of the strain was poor at pH 8.7 and 37 °C, enhanced specific biosurfactant production (expressed as a ratio rhamnose content/growth) was determined under these conditions. Perhaps the increase in rhamnose secretion is one of the ways of the strain to adapt to these conditions.

A delay in the growth (adaptation lag) and decrease in the growth rate of P. fluorescens HW-6 cells on hexadecane was observed in the presence of up to 2% NaCl in the medium (Fig. 4). Contents of NaCl of 4, 6 and 8% completely inhibited the growth of the strain on hexadecane. An increase in the specific protein and biosurfactant production (expressed as the ratio protein or rhamnose content/growth) was determined in the presence of NaCl in the medium (Table II). Increased protein and rhamnose release during growth of the strain on hexadecane in the presence of NaCl up to 2% could be due to destabilization of the cell membrane by Na cations leading to increased cell permeability (Homma and Nakae, 1982).

Fluorescent pseudomonads encompass a group of non-pathogenic saprophytes that colonize soil, water and plant surface environments. A number of strains of P. fluorescens are used for the biocontrol of pathogens in agriculture and also as green fluorescent protein-based bacterial biosensors to measure environmental contaminants (Nivens et al., 2004; Tran et al., 2007). The capability of P. fluorescens strain HW-6 to adapt its own metabolism to use different nutrients as energy sources and to keep up relatively high biosurfactant levels in the medium during the stationary phase is a promising feature for its possible application in biological treatments.

Table II. Specific rhamnose and protein release by P. fluorescens HW-6 cells grown on hexadecane as carbon source in the presence of NaCl.

NaCl (%)	Rhamnose [g $(l \cdot OD_{570})^{-1}$]	Protein [mg $(OD_{570})^{-1}$]
0.0	0.168 ± 0.02	3.00 ± 0.15
0.5	0.312 ± 0.02	6.25 ± 0.50
1.0	0.472 ± 0.05	6.89 ± 0.58
2.0	0.545 ± 0.10	11.45 ± 1.10

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