

Cell surface properties of *Pseudomonas stutzeri* in the process of diesel oil biodegradation

Ewa Kaczorek · Teofil Jesionowski · Anna Giec · Andrzej Olszanowski

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Abstract *Pseudomonas stutzeri*, isolated from crude oil-contaminated soil, was used to degrade diesel oil. Of three surfactants, 120 mg rhamnolipids l^{-1} significantly increased degradation of diesel oil giving 88% loss after 14 days compared to 54% loss without the surfactant. The system with rhamnolipids was characterised by relatively high particle homogeneity. However, the addition of saponins to diesel oil caused the cells to aggregate (the polydispersity index: 0.542) and the biodegradation of diesel oil was only 46%. The cell yield was $0.22\text{ g }l^{-1}$.

Keywords Biodegradation · Cell surface hydrophobicity · Particle size distribution · *Pseudomonas stutzeri* · Sedimentation profile

Introduction

Surface properties of microorganisms are vital for effective biodegradation of hydrophobic sources of carbon. The initial high hydrophobicity of the surface of bacteria cells favours the interactions between the cell surface and the carbon source (Deziel et al. 1999; Stenstrom 1989) which leads to a faster and more efficient utilisation of hydrocarbons. Other factors favouring the biodegradation rate of hydrophobic carbon sources are microbial surfactants. They increase the dispersion of hydrocarbons in water and enlarge the area of contact with bacteria cells. Moreover, biosurfactants can modify the surface of bacteria cells (Lai et al. 2009). Zhang and Miller (1994) suggested that the bioavailability of octadecane in the presence of rhamnolipids is controlled by dispersion of these compounds in water and bacteria cell hydrophobicity. The presence of biosurfactant increases the cell hydrophobicity of slow degraders, but has no effect on the hydrophobicity of fast degraders. Rhamnolipids adsorbed on the cell surface of bacteria can extract membrane lipopolysaccharides leading to an increase in hydrophobicity (Al-Tahhan et al. 2000). However, Obuekwe et al. (2009) have described *Williamsia muralis* as lacking a hydrophobic character but still having a good ability to degrade hydrocarbons. In this case, mechanisms other than hydrophobic interaction allow hydrocarbon uptake by microorganisms.

The aim of the study was to check the applicability of measurements of sedimentation profile and particle

E. Kaczorek (✉) · T. Jesionowski · A. Giec · A. Olszanowski
Institute of Chemical Technology and Engineering,
Poznan University of Technology,
M. Skłodowskiej-Curie 2, 60-965 Poznan, Poland
e-mail: ewa.kaczorek@put.poznan.pl

T. Jesionowski
e-mail: teofil.jesionowski@put.poznan.pl

A. Giec
e-mail: anna.giec@wp.pl

A. Olszanowski
e-mail: andrzej.olszanowski@put.poznan.pl

size distribution as an effective tool for observation of changes in the properties of *Pseudomonas stutzeri* cell surface. Their cell surface adhesion to hydrocarbon was assessed using the microbial adhesion to hydrocarbon (MATH) method described by Rosenberg et al. (1980). The influence of different stress factors on the surface properties of the bacteria cells was assessed and the effect of the presence of surfactants (rhamnolipids, Triton X-100, saponins) on efficiency of diesel oil biodegradation was indicated.

Materials and methods

Chemicals

Hydrocarbon and other fine chemicals employed high grade were purchased from Merck (Germany). Surfactants used were: rhamnolipids (Jeneil Biosurfactant Company, USA, JBR 425; content 25% of rhamnolipids), saponins (Sigma) and Triton X-100.

Bacterial strain and growth conditions

Pseudomonas stutzeri AG 22 (biochemical profile: 04573043222) was identified using ID 32 GN biochemical tests (bio-Merieux, France) and molecular techniques. It had been isolated from soil contaminated with crude oil. The culture medium used throughout these studies was described previously (Kaczorek et al. 2010).

Biodegradation test

Cultures were grown in Erlenmeyer flasks (500 ml) containing 100 ml culture medium (see Table 3) with 2% (w/v) diesel oil. When used, 120 mg surfactant l⁻¹ was added to the medium. Each experiment was repeated five times to attain the accuracy of $\pm 3.7\%$. Diesel oil content was determined as described in detail by Kaczorek et al. (2010). The final results were calculated with respect to blank samples (hydrocarbon with a medium, without microorganisms).

Microbial adhesion to hydrocarbons

Microbial surface hydrophobicity was assessed with the MATH method described by Rosenberg et al. (1980). The culture was grown on different sources of

carbon: hexane, hexadecane, tridecane, diesel oil, glucose, cumene, *t*-butylbenzene, and three surfactants (rhamnolipids, saponins and Triton X-100) at 120 mg l⁻¹ used alone or in a mixture with diesel oil. Each experiment was repeated three times to attain the accuracy of $\pm 1.2\%$.

Sedimentation profile evaluation

The sedimentation profile was measured on the K-100 tensiometer (Krüss, Germany) by a suspended probe to directly measure the mass sediment versus time. After 7 days, cells in the late growth phase were centrifuged and the sedimentation profile was determined as described by Kaczorek et al. (2011).

Particle size distribution

Particle size distribution of the tested systems was determined using a Zetasizer Nano ZS apparatus (Malvern Instruments Ltd., UK) applying the non-invasive back light scattering method. On the basis of particle size distribution, the polydispersity index (PdI) was calculated.

Results and discussion

The effect of carbon source on bacterial adhesion to the hydrocarbon used

Bacterial adhesion to hydrocarbon depends on the type of carbon source used. During growth on hexadecane the cell surface had hydrophilic properties comparable to those of the parent strain (Table 1). The highest cell surface hydrophobicity (CSH) was noted when the bacterial strain had contact with cumene. Moreover, the effect of surfactants and their concentration was studied and only the effect of Triton X-100 was irrespective of its concentration (Fig. 1a). Different cell surface properties of the tested strain were observed in the systems made of a surfactant and diesel oil. These changes were particularly pronounced in the diesel oil-Triton X-100 system (Fig. 1b), which indicated that surfactants can interact with the microbial cell surface. Moreover, modification of the cell surface was dependent on the time of contact with the carbon source during the experiments (Table 2). Neu (2005) and Zeng et al. (2005) described

Table 1 Microbial adhesion to hydrocarbon (MATH) for *Pseudomonas stutzeri* AG 22 after growth on 2% (w/v) carbon source (glucose, hexane, diesel oil, cumene, *t*-butylbenzene, tridecane, hexadecane)

MATH (%) for the <i>Pseudomonas stutzeri</i> AG 22							
Parent strain	Glucose	Hexane	DO	Cumene	<i>t</i> -Butyl-benzene	Tridecane	Hexadecane
10 ± 0.8	25 ± 1.1	29 ± 0.7	38 ± 0.9*	67 ± 0.9	41 ± 1.5	48 ± 1.2	9 ± 0.8

Process was carried out 7 days at 25°C. Initial cell concentration was 10^8 cells per ml ($OD_{600} \sim 0.1$). Results have absolute (100%) quantitative value

DO diesel oil; * after 14 days adhesion increased to $54\% \pm 0.9$

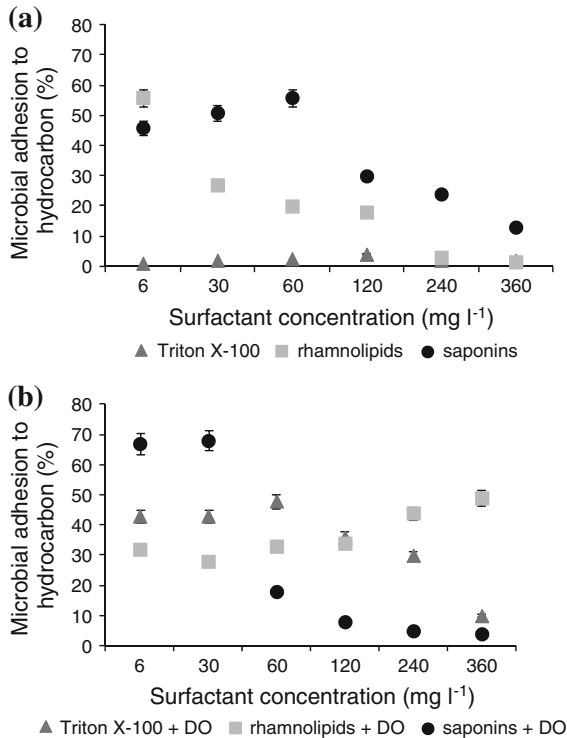


Fig. 1 Bacterial adhesion to hydrocarbon determined for *Pseudomonas stutzeri* AG 22 after 7 days in a surfactant system (a) and surfactant-diesel oil system (b), for different surfactant (Triton X-100, rhamnolipids, saponins) concentrations ($g\ l^{-1}$; 6, 30, 60, 120, 240, 360). Cells were centrifuged at $8,000\times g$ for 5 min and washed twice to remove residual hydrocarbons with a PUM buffer ($g\ l^{-1}$; 19.7 K_2HPO_4 , 7.26 KH_2PO_4 , 1.8 urea and 0.2 $MgSO_4\cdot 7H_2O$). Results have absolute (100%) quantitative value

that contact with surfactants can cause changes in CSH. However, Zhong et al. (2008) have demonstrated that cell surface hydrophobicity is a consequence of surfactant adsorption and that changes in the cell surface depend on the surfactant structure and cell type.

Diesel oil biodegradation

Pseudomonas stutzeri degraded diesel oil (see Table 3) and surfactants increased its efficiency. From among the surfactants used, rhamnolipids were the best for the acceleration of diesel oil degradation. After 14 days, 88% of diesel oil was successfully removed from the system. In the presence of saponins, the biodegradation of diesel oil was comparable to that in the system without a surfactant, though in the diesel oil/saponins system the cell surface was more hydrophilic than that in the system with diesel oil only.

Results of our earlier work have shown that saponins could be good surfactants for hydrocarbon biodegradation in the presence of the *Pseudomonas alcaligenes* bacteria strain (Kaczorek et al. 2011). CSH of *P. alcaligenes* was similar to that of *P. stutzeri* AG 22, which indicated that a mechanism, different than modification of the cell surface, determined the uptake of diesel oil biodegradation. The efficiency of cell surface modification was dependent on the duration of biodegradation. Longer contact time between diesel oil, surfactant and bacteria facilitated surfactant incorporation into the cell membrane and favoured increase in cell hydrophobicity.

Sedimentation profile and particle size distribution

Measurements of sedimentation profile and particle size distribution were tested for their use in evaluating bacteria cell surface modifications. The highest sedimentation of biomass amongst glucose, diesel oil and hexadecane was when glucose was metabolised by *P. stutzeri* (Fig. 2a). The polydispersity index (PdI), as well as particle size distribution (Fig. 3a) indicated that this system is not homogenous and contained large particles. The sedimentation profiles, PdIs and particle size distribution determined for diesel oil and

Table 2 Microbial adhesion to hydrocarbon for *Pseudomonas stutzeri* AG22 after growth on 120 surfactants mg l⁻¹: Triton X-100, rhamnolipids, saponins added to 2% (w/v) diesel oil system

Surfactant	Microbial adhesion to hydrocarbon (%)	
	Diesel oil and 120 mg surfactant l ⁻¹ ; 7 days	Diesel oil and 120 mg surfactant l ⁻¹ ; 14 days
Triton X-100	36 ± 1.3	54 ± 1.3
Rhamnolipids	34 ± 1.2	68 ± 2.5
Saponins	8 ± 0.8	19 ± 1.4

Process was carried out 7 and 14 days at 25°C. Initial cell concentration was 10⁸ cells per ml (OD₆₀₀ ~ 0.1). Results have absolute (100%) quantitative value

Table 3 Influence of 120 surfactants mg l⁻¹ (rhamnolipids, saponins, Triton X-100) on the biodegradation of 2% (w/v) diesel oil by *Pseudomonas stutzeri*

Day	Diesel oil (DO) biodegradation (%)			
	DO	DO + Triton X-100	DO + rhamnolipids	DO + saponins
7	25 ± 0.9	36 ± 2.1	42 ± 2.4	29 ± 1.3
14	44 ± 1.8	59 ± 2.6	88 ± 3.7	46 ± 1.5

Experiments were carried out in Erlenmeyer flasks containing 100 ml of culture medium, pH 7.2 (in g l⁻¹: Na₂HPO₄·2H₂O 7.0, KH₂PO₄·2.8, NaCl 0.5, NH₄Cl 1.0, MgSO₄·7H₂O 0.01, FeSO₄·7H₂O 0.001, MnSO₄·4H₂O 0.0005, ZnCl₂ 0.00064, CaCl₂·6H₂O 0.0001, BaCl₂ 0.00006, CoSO₄·7H₂O 0.000036, CuSO₄·5H₂O 0.000036, H₃BO₃·0.00065, EDTA 0.001 and 37% HCl 0.0146 ml l⁻¹) at 25°C and 250 rpm for 7 or 14 days. For bacteria stock cultures, yeast extract (0.3 g l⁻¹) was added. Initial cell concentration was 10⁸ cells per ml (OD₆₀₀ ~ 0.1)

hexadecane were comparable. The use of surfactants resulted in the formation of cell aggregates, especially when natural surfactants (rhamnolipids and saponins) were used (Fig. 3b). For these surfactants, a high PdI was obtained and the values of the sediment mass were comparable as a result of a higher rate of sedimentation of large particles (Fig. 2b).

With Triton X-100, the smallest sedimentation mass and particle size were observed. Different values of sediment mass were determined when surfactants were introduced to the systems with diesel oil (Fig. 2c), which meant different types of cell surface modification. Because of the presence of saponins and Triton X-100 the systems' homogeneity was reduced though they were characterised by a high polydispersity index (Fig. 3c).

However, a different situation was observed in the presence of rhamnolipids. The polydispersity index was smaller in the rhamnolipids/diesel oil system than in the rhamnolipids system. Introduction of rhamnolipids to diesel oil resulted in increasing the homogeneity of the

system. Generally, for almost all systems a higher gradient of sedimentation profile resulted in higher values of sediment mass.

A number of methods have been developed for determination of cell surface properties. However, a common problem is the poor correlation between the same parameters determined by different methods (Mozes and Rouxhet 1987). The methods that have been proposed hitherto for determination of the cell surface hydrophobicity either measure overall or localised cell surface and are characterised by sensitivities varying widely (Kjelleberg and Hermansson 1984). Relative hydrophobicity of a bacterial cell can be determined by using for example, microbial adhesion to hydrocarbons (MATH), salting out, hydrophobic interaction chromatography (HIC), adhesion to a nitrocellulose filter or water contact angle measurements. MATH is a simple and rapid method for determination of cell adhesion to hydrocarbon while, according to van Loosdrecht et al. (1987), the contact angle should be recommended as a good

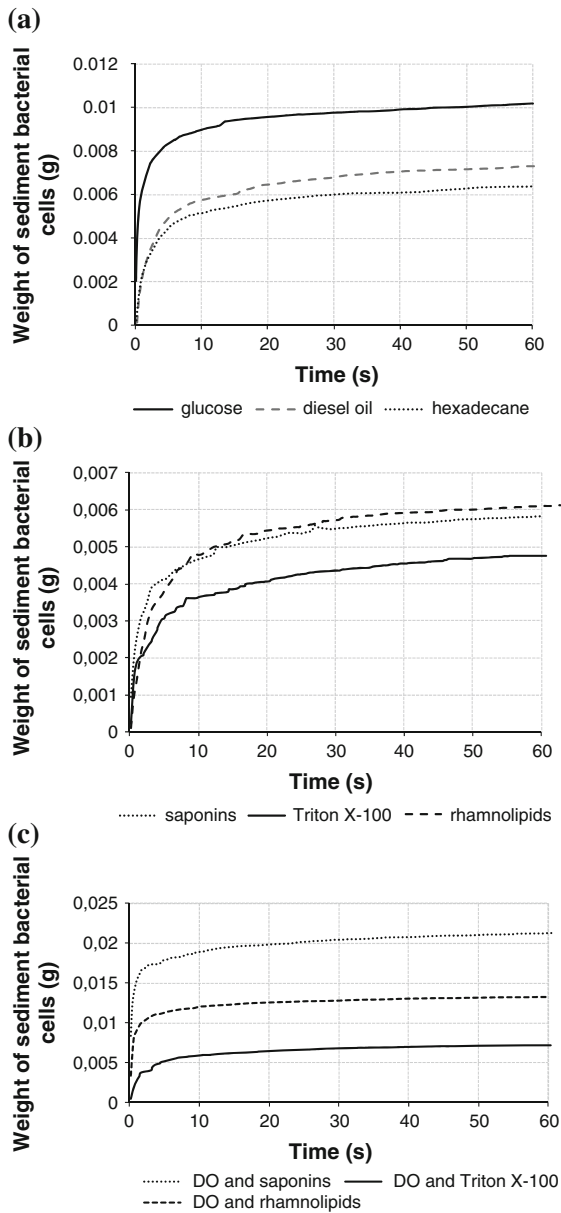


Fig. 2 Weight of sediment bacterial cells (*Pseudomonas stutzeri* AG 22) accumulated per 100 ml with time. Bacteria strain was inoculated with different sources of carbon: glucose, diesel oil, hexadecane (a); Triton X-100, rhamnolipids or saponins (b); Triton X-100 + DO, rhamnolipids + DO, saponins + DO (c); surfactants in concentration 120 mg l^{-1} ; DO—diesel oil. After 7 days cells were centrifuged at $8,000\times g$ for 5 min and washed twice with PUM buffer (g l^{-1} ; $19.7 \text{ K}_2\text{HPO}_4$, $7.26 \text{ KH}_2\text{PO}_4$, 1.8 urea and $0.2 \text{ MgSO}_4\cdot 7\text{H}_2\text{O}$). 0.1 g of bacterial cells were then suspended in 75 ml of PUM buffer and mixed 120 s directly before measurement

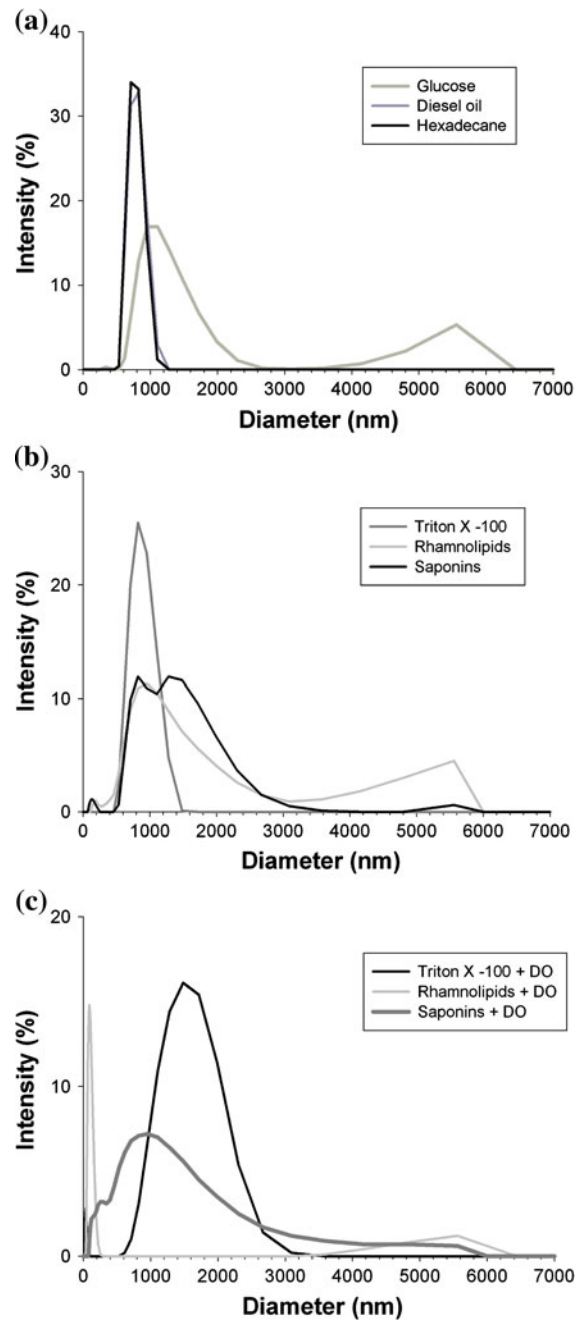


Fig. 3 Particle size distribution (PdI) during *Pseudomonas stutzeri* AG22 growth on a different carbon sources: glucose—PdI: 0.481, diesel oil—PdI: 0.145, hexadecane—PdI: 0.152 (a); surfactants in concentration 120 mg l^{-1} : Triton \times 100—PdI: 0.061, rhamnolipids—PdI: 0.421, saponines—PdI: 0.386 (b); Triton X-100 + DO—PdI: 0.285, rhamnolipids + DO—PdI: 0.183, saponines + DO—PdI: 0.542 (c); DO—diesel oil, hexadecane and glucose were added in a concentration of 2% (w/v)

measurement of bacterial hydrophobicity. Results obtained using the hydrophobic interaction chromatography (HIC) assay, however, differ completely from those obtained from the MATH assay (Gannon et al. 1991). In our opinion, there is no universal method for hydrophobicity determination and moreover results differ considerably.

Conclusion

Measurements of sedimentation profile and particle size distribution provide information on changes in the bacteria cell surface properties of taking place in the process of diesel oil biodegradation. From among the tested surfactants, rhamnolipids have been found most effective in increasing the rate of diesel oil biodegradation. In general, the efficiency of diesel oil biodegradation (rate of removal of hydrophobic sources of carbon from the environment) is determined by the homogeneity of the system after introduction of surfactants. The type of modification of surface cells of *P. stutzeri* AG 22 strain is found to depend on the type and concentration of surfactants present.

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