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Original Research

Effect of External Addition of Rhamnolipids Biosurfactant on the Modification of Gram Positive and Gram Negative Bacteria Cell Surfaces during Biodegradation of Hydrocarbon Fuel Contamination

Ewa Kaczorek*

Institute of Chemical Technology and Engineering, Poznan University of Technology,
Pl. M. Skłodowskiej-Curie 2, 60-965 Poznań, Poland

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Abstract

The bacterial strains from the genera: *Bacillus*, *Pseudomonas*, *Aeromonas*, *Achromobacter*, and *Flavimonas* isolated from soil contaminated with crude oil were the subject of studies. The effect of the addition of rhamnolipids on cell surface properties and the removal efficiency of diesel oil were investigated. Rhamnolipids caused the modification of cell surface properties of tested strains, which depended on the amount of external additions of biosurfactant. Additionally, the decrease of Zeta potential was observed after the introduction of rhamnolipids to the diesel oil system. Particle size distribution provides information about system homogeneity and the tendency of particles toward agglomeration. Cell surface hydrophobicity during hydrocarbon biodegradation is a dynamic parameter. There were no different effects, after the addition of rhamnolipids, on the Gram positive and Gram negative bacterial strains. Moreover, the addition of rhamnolipids does not always increase the efficiency of diesel oil biodegradation. The most effective bacterial strain in diesel oil removal was the genus *Pseudomonas*.

Keywords: biodegradation, cell surface, hydrocarbon, hydrophobocity, rhamnolipids

Introduction

Petroleum hydrocarbons are widely used as a source of energy and hence are important environmental pollutants. In general, accidental contaminations are particularly dangerous to ecosystems. Petroleum contains non-aqueous and hydrophobic compounds like alkanes, aromatics, and asphaltenes. Low water insolubility of hydrophobic hydrocarbons pose an important ecological problem [1]. The ongoing pollution and toxicity of petroleum hydrocarbons to human and other organisms caused the development of different remediation strategies [2, 3]. Many of the bacterial strains are able to metabolize hydrophobic compounds using them as carbon and energy sources [4]. Bioremediation is an environmentally friendly technology and economical. Therefore, the development of an effective bioremediation process is also the subject of the study.

Surfactants play a crucial role in the biodegradation process, especially biosurfactants produced by microorganisms [5]. Many surfactants are tested for their potential application in hydrocarbon bioremediation. Surfactants enhance the remediation of hydrophobic compounds by

*e-mail: ewa.kaczorek@put.poznan.pl

increasing hydrocarbon solubilization [6, 7] or by the mobilization of hydrocarbons from the soil into aqueous phase [8]. According to Yuan et al. [9], the surfactant micelles can provide a barrier between bacteria and contamination. Many prokaryotic and eukaryotic microorganisms are able to synthesize different surface active compounds. Biosurfactants represent ecological alternatives to their synthetic counterparts: they exhibit lower toxicity, potential high activities, and stability at extreme temperatures, pH, and salinity [10]. They produce biosurfactants that can be either extracellular or part of the cell membrane [11, 12] from various substances, especially wastes. Biosurfactants represent various chemical types such as glycolipids, phospholipids, lipopeptides, and fatty acids [13]. Due to their properties, they have several industrial and environmental applications. Biosurfactants are used in the bioremediation of hydrocarbons as well as of toxic heavy metals like uranium, cadmium, and lead [14, 15].

Rhamnolipids are biosurfactants produced especially by the *Pseudomonas aeruginosa* strains. However, some isolates of the Pseudomonas genera *P. putida* and *P. chlororaphis* produce a variety of rhamnolipids [16, 17]. They have a wide range of potential commercial applications. Chemically they consist of rhamnose sugar molecules and β-hydroxyalkanoic acids. They have been studied extensively, but their natural function is still speculative [18]. Production starts with the exhaustion of nitrogen [19]. Exogenously added rhamnolipids are safe for the environment, because they are biodegradable. The activity of rhamnolipids makes them excellent assisting compounds in the breakdown and removal of oil spills [20]. Rhamnolipids are effective as enhancers in the biodegradation of alkanes in petroleum sludge [21], but the increase of solubilization of hydrophobic organic compounds after the addition of rhamnolipids does not guarantee enhanced biodegradation [12]. Rhamnolipids have sometimes antimicrobial properties against bacterial as well as fungal species [22, 23].

The aim of our research was to investigate the effect of rhamnolipids of Gram positive and Gram negative bacteria strains. The influence of different amounts of rhamnolipids on cell surface properties was studied. Moreover, the adsorption parameters of rhamnolipids in a mineral salt solution and in biological systems was tested, as well as the ability of using bacterial strains in diesel oil biodegradation. All bacterial strains used in these experiments were isolated from contaminated soil in Poland.

Experimental Procedures

Chemicals

Hydrocarbons and other fine chemicals employed in this study were of the highest purity grade, produced by Merck (Germany).

Surface active agents used in the experiments were: rhamnolipids – anionic biosurfactant (Jeneil Biosurfactant Company, USA, JBR 425 – content 25% of rhamnolipids).

Bacterial Strains and Growth Conditions

Bacterial strains were isolated from contaminated soil with crude oil. A soil sample was introduced into the culture medium solution containing appropriate amounts of nitrogen and phosphorus and trace elements. In order to amplify microorganisms in the initial phase, glucose as a source of carbon was introduced. After 24 hours of cultivation, microorganisms were transferred into the new culture medium system containing diesel oil as a carbon source. Then the cultivation was carried out for a month with microorganisms transferred into new medium with increasing amounts of diesel oil every 48 hours. This allowed for the isolation of Gram negative and Gram positive bacterial strains with the potential ability of biodegradation.

Four strains of *Bacillus* were used: *Bacillus subtilis* KE, *Bacillus cereus* KE, *Bacillus licheniformis* KE, and *Bacillus laterosporus* KE. Gram negative bacteria also were tested: *Achromobacter denitrificans* SM, *Psudomonas stutzeri* SM, *Aeromonas hydrophila* SM, *Pseudomonas alcaligenes* SM, *Pseudomonas fluorescens* SM, and *Flavomonas oryzihabitns* P1. The identification was performed using biochemical tests and molecular techniques. The culture medium used throughout these studies consisted of ($g \cdot l^{-1}$): $Na_2HPO_4 \cdot 2H_2O$ 7.0, KH_2PO_4 2.8, NaCl 0.5, NH_4Cl 1.0, $MgSO_4 \cdot 7H_2O$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.001, $MnSO_4 \cdot 4H_2O$ 0.0005, $ZnCl_2$ 0.00064, $CaCl_2 \cdot 6H_2O$ 0.0001, $BaCl_2$ 0.00006, $CoSO_4 \cdot 7H_2O$ 0.000036, $CuSO_4 \cdot 5H_2O$ 0.000036, H_3BO_3 0.00065, EDTA 0.001, and HCl 37% 0.0146 $ml \cdot l^{-1}$. The pH of the medium was 7.2. For bacteria, stock cultures yeast extract (0.3 $g \cdot l^{-1}$) was added. Stock cultures were prepared in a 250 ml Erlenmeyer flask containing 50 ml of medium. Next, a loop full of cells from an agar plate was added to the flask with medium. After approximately 24 hrs a few ml (3-5 ml) of this liquid culture was used for the inoculation of the final culture to reach an OD of ca. 0.1 (this corresponds to $1 \cdot 10^8$ cells per ml).

Microbial growth was monitored through culture densities, measuring absorption spectrophotometrically at 600 nm (data not given).

Microbial Adhesion to Hydrocarbons

Microbial surface hydrophobicity was assessed with microbial adhesion to the hydrocarbon method (MATH) described by Rosenberg et al. [24]. The culture was grown on different carbon sources: diesel oil, glucose, and hexadecane, as well as rhamnolipid biosurfactants at different concentrations (6, 30, 60, 120, 240, and 360 $mg \cdot l^{-1}$) and its mixture with diesel oil. Cells in exponential phase were centrifuged at 8,000 g for 5 min and washed twice with a PUM buffer to remove residual hydrocarbons. The PUM buffer contained: 19.7 $g \cdot l^{-1}$ K_2HPO_4, 7.26 $g \cdot l^{-1}$ KH_2PO_4, 1.8 $g \cdot l^{-1}$ $H_2NC(O)NH_2$, and 0.2 $g \cdot l^{-1}$ $MgSO_4 \cdot 7H_2O$. Washed cells were re-suspended in the PUM buffer to fit an optical density of ca. 1.0 (A_o). Optical density was measured at 600 nm on a Shimadzu UV-Visible Spectrophometer. Next, 500 µl of hydrocarbon was added to 5 ml of microbial suspension

and vortexed for 2 minutes. After 10 minutes the optical density of the aqueous phase was measured (A_1). Bacterial adhesion to hydrocarbon is calculated as [1 – $(A_0–A_1)/A_0$]·100% [%].

The determination of means and standard deviations were by means of Microsoft Excel. Each experiment was repeated three times to attain accuracy of ±2.1%.

Zeta Potential

The electrophoresis mobility was automatically measured, and the Zeta potential was calculated from electrophoresis mobility using the Smoluchowski equation [25]. The measurements were performed at 21ºC with a ZetaPlus instrument (Brookhaven Instruments Co., USA).

Adsorption parameters of Rhamnolipids

The equilibrium surface tension was performed using the du Nouy ring technique with the Kruss K12 tensiometer with a platinum ring. Experiments were done at 21±1ºC. The surface tension data can be fitted by adsorption equations.

From a physicochemical point of view it is suitable to use the Szyszkowski equation [26]:

$$\gamma^{Sz} = \gamma_0 \left[1 - B \ \ln\left(\frac{C}{A} + 1 \right) \right] \tag{1}$$

...where γ_0 is the surface tension for the distilled water, and A and B are the adsorption coefficients. By using Eq. (1) and introducing the term into the Gibbs isotherm:

$$\Gamma = -\frac{1}{RT} \cdot \frac{d\sigma}{d \ln C} \tag{2}$$

...where Γ is the surface excess, R – gas constant, and T – temperature, Eq. (3) is obtained for the surface excess in the case of the nonionic system:

$$\Gamma^{Sz} = \frac{\gamma_0 BC}{RT(C + A)} \tag{3}$$

The adsorption coefficients of Szyszkowski isotherm A and B can be used to estimate the Gibbs free energy of adsorption (ΔG_{ads}) and the surface excess at the saturated interface (Γ^{∞}).

$$\Delta G_{ads} = - \text{RTln} A \tag{4}$$

$$\Gamma^{\infty} = \frac{\gamma_0 B}{RT} \tag{5}$$

Particle Size Distribution

The respective particle size distribution was determined using the Zetasizer Nano ZS Apparatus (Malvern Instruments Ltd.) using the Non-Invasive Back Light Scattering Method (NIBS). Particle size distribution enabled a polydispersity index. A cumulants analysis gave a width parameter known as polydispersity, or the polydispersity index (PdI). The cumulants analysis is actually the fit of a polynomial to the log of the G1 correlation function: $Ln[G1] = a + bt + ct^2 + dt^3 + et^4 + ...$. The value of b is known as the second order cumulant, or the z-average diffusion coefficient. The coefficient of the squared term, c, when scaled as $2c \cdot b^{-2}$ is known as polydispersity.

Biodegradation Test

Diesel oil was used as a carbon source for microorganisms, the concentration in the experiments was 2% (w/v). The influence of rhamnolipids biosurfactant on diesel oil biodegradation was also tested. Surfactant was used at 120 $mg \cdot l^{-1}$ concentration. The laboratory tests with surfactants showed that hydrocarbon biodegradation in the presence of such an amount of surfactant was the most effective. Experiments were performed in Erlenmeyer flasks containing 100 ml of culture medium of the composition described in the section "Bacterial strains and growth conditions." Experiment samples contained diesel oil, a culture medium, and a few ml of bacteria stock cultures (to reach an OD of ca. 0.1).

In experiments with emulsified hydrocarbons an appropriate amount of surfactant was added to such prepared samples. The determination of means and standard deviations were done using Microsoft Excel. Each experiment was repeated five times to attain accuracy of ±2.7%. The total mass of hydrocarbon residues was determined using the "standard method for gravimetric determination of hydrocarbon" [27]. Samples were incubated at 25ºC and shaken at 120 rpm for 7 days. After the biodegradation process, the whole cultivation broth was centrifuged to separate biomass. To the residual aqueous phase saturated salt solution and acids were added to a pH of 1.0. The aqueous phase was then double extracted with diethyl ether. The organic phase after extraction was dried and then evaporated. The dried residue was measured as the amount of hydrocarbon after biodegradation. Biodegradation was calculated as (X_o – X_1)/X_o·100% [%], where X_o is the initial amount of hydrocarbon and X_1 is the amount of hydrocarbon after biodegradation. The final results are calculated with respect to blank samples (hydrocarbon with medium without microorganisms).

Results and Discussion

Effect of Glucose and Diesel Oil on Cell Surface Properties

The cell surface hydrophobicity (CSH) in the glucose system for Gram negative and Gram positive bacterial strains showed that most of the tested microorganisms had hydrophilic properties (Fig. 1). This is especially visible for *Pseudomonas* strains and *A. dentrificans* SM and *A. hydrophila* SM, where the hydrophilic cells greatly out-

Table 1. Adsorption parameters of rhamnolipids in a mineral salt solution and in a biological system.

Parameter	Unit	Rhamnolipids (medium solution)	Rhamnolipids (biological system, *B. subtilis* KE)	Rhamnolipids (biological system, *A. denitrificans* SM)
Γ^{∞}	mol/m^2	$5.829 \cdot 10^{-7}$	$2.7 \cdot 10^{-6}$	$2.79 \cdot 10^{-7}$
A_{min}	m^2	$28.5 \cdot 10^{-19}$	$6.19 \cdot 10^{-19}$	$5.94 \cdot 10^{-19}$
$-\Delta G_{ads}$	kJ/mol	45.89	17.70	19.60

Γ^{∞} – surface excess at the saturated interface, A_{min} – minimum surface occupied by statistical molecule, $-\Delta G_{ads}$ – Gibbs free energy of adsorption

numbered those with hydrophobic surface properties (they were present in less than 6% of the population). The highest result of CSH – 65 was observed for *F. oryzihabitans* P1 strain. Moreover, a significant increase in CSH was noticed during the growth of all tested strains, except for *F. oryzihabitans* P1 strain. The highest cell surface modification was observed for *P. fluorescens* SM (CSH = 46%). For all studied Gram positive bacterial strains, cell surface hydrophobicity in a diesel oil system was about twice higher than in the glucose system (Fig. 1b). The highest was observed for *B. cereus* KE strain. However, for Gram negative bacteria strains, such correlation was not observed. The results have shown that the kind of carbon source used for microorganism cultivation causes changes in cell surface properties. Bos et al. [28] observed that cell surface properties play an important role in the adherence of different substrates. Interactions between microorganisms and oil dispersions may cause direct adhesion and therefore facilitate oil biodegradation [29].

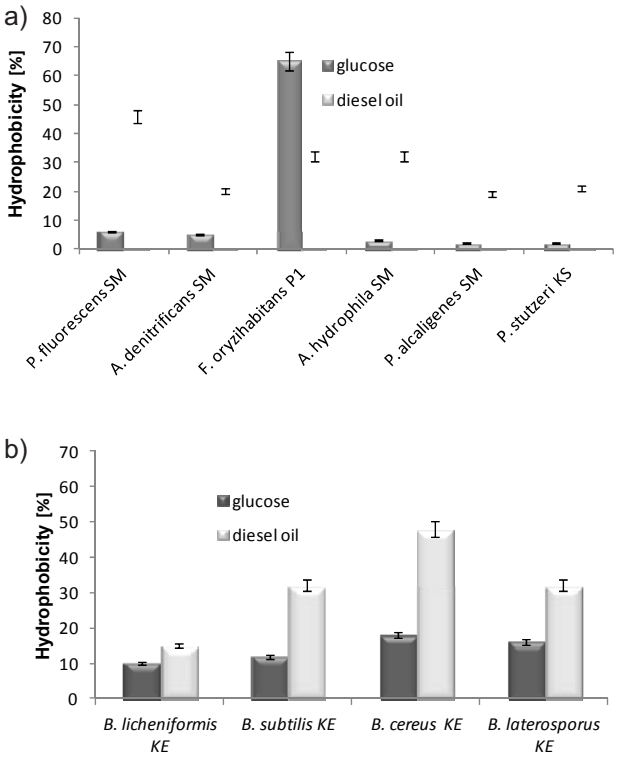

Fig. 1. Cell surface hydrophobicity after bacterial growth on glucose and diesel oil; 7 days of experiments; hydrophobicity of Gram negative bacterial strains (a) hydrophobicity of Gram positive bacterial strains (b).

Adsorption Parameters of Rhamnolipids

The adsorption parameters of rhamnolipids in two systems was calculated using Szyszkowski's equation (Table 1): in both the mineral salts solution and biological systems (*A. denitrificans* SM and *B. subtilis* KE). The obtained results indicated that the adsorption parameter rhamnolipids were different for the two tested systems. The surface excess at the saturated interface is smaller in the medium solution ($\Gamma^{\infty}=5.83 \cdot 10^{-7}$ mol·m^{-2}) than in the biological system. For *B. subtilis* KE system the surface excess was higher ($\Gamma^{\infty}=2.7 \cdot 10^{-6}$ mol·m^{-2}) than for *A. denitrificans* SM system. Results indicated that the structure of monolayer adsorption was different for the two tested systems. Moreover, the minimum surface occupied by the statistical molecule in the adsorption layer is higher for rhamnolipids in mineral salt solution ($A_{min}=28.5 \cdot 10^{-19} \cdot$m^2) than for two biological systems. This means that rhamnolipids have a higher efficiency to adsorption in mineral salt solution system.

Molecules are more densely arranged at the saturated water/air interface. However, free energy of adsorption was the smallest ($-\Delta G_{ads}= 45.89$ kJ·mol^{-1}). This fact suggests that in this system the surfactant had the biggest tendency of adsorption, than in biological systems. The free energy of adsorption for *A. denitrificans* SM ($-\Delta G_{ads}= 19.60$ kJ·mol^{-1}) and for *B. subtilis* KE ($-\Delta G_{ads}= 17.70$ kJ·mol^{-1}) was higher. Previous research indicated that the difference free energy depended on not only the kind of a system, but also on the kind of biological system [30]. Adsorption of rhamnolipids on bacterial cell surface could cause differences in critical micellar concentration (CMC). Zhang and Miller [31] estimated rhamnolipids CMC at level 40 mg·l^{-1}. Chlifford et al. [32] reported that the CMC of rhamnolipids produced by *P. aeruginosa* ATCC 9027 was about 30 mg·l^{-1}. However, Rahnam et al. [33] noted that CMC of rhamnolipids was approximately 22 mg·l^{-1}. Differences can arise from various rhamnolipid structures and purity. Moreover, the critical micellar concentration in the biological system could be higher than in the water solution and different for various bacterial strains. My previous study [30] indicated that for *Aeromonas hydrophila* it is three times higher.

Effect of Rhamnolipids Concentration on Cell Surface Properties

The addition of different rhamnolipid concentrations has various effects on the cell surface properties of studied

Table 2. Changes in the surface charge values measured for the *A. denitrificans* SM, *P. stutzeri* KS, and *B. subtilis* KE strains measured for the parent sample and after 7 days of growth on glucose, diesel oil, and hexadecane; pH 7; DO – diesel oil, DO-Rha – diesel oil, and rhamnolipids, Rha-rhamnolipids; rhamnolipids concentration 120 mg·l^{-1}.

Bacterial strain	Surface charge (ζ potential) [mV]					
	Parent sample	Glucose	Rha	DO-Rha	Diesel oil	Hexadecane
A. denitrificans SM	-11.6	-9.2	-8.5	-9.4	-10.7	-18.0
P. stutzeri KS	-24.8	-23.4	-16.2	-18.7	-19.2	-21.8
B. subtilis KE	-19.2	-17.1	-12.1	-15.4	-9.1	-20.1

bacterial strains. This is especially observed for *A. hydrophila* SM, *P. fluorescens* SM (Fig. 2a), and *P. fluorescens* SM. For other strains, the changes were not so significant. Among *Bacillus* strains, the addition of rhamnolipids led to higher hydrophobicity of *B. cereus* KE strain (Fig. 3a) than for the other strains. The rhamnolipids concentration did not have any influence on cell surface modifications.

The addition of rhamnolipids to diesel oil system caused changes in the cell surface of studied Gram negative bacterial strains. This was especially noticeable for the *P. fluorescens* SM strain. In this case, the tested strain exhibited a significant increase in its hydrophobicity. A similar effect was observed for *A. hydrophila* SM, but only when a lower concentration of rhamnolipids was added to diesel oil system (Fig. 2b). However, for *Bacillus* strains the addition of rhamnolipids to diesel oil system caused an increase in the hydrophobicity for *B. laterosporus* KE and *B. subtilis* KE strains in comparison to the rhamnolipids system (Fig. 3b). The most significant increase in CSH occurred for *B. cereus* KE strain. This strain had the highest CSH among studied Gram negative and Gram positive bacterial strains. The changes in the CSH for *B. laterosporus* KE and *B. subtilis* KE strains were moderate.

According to Zhong et al. [34] rhamnolipid adsorption plays an important role in the modification of cell surface hydrophobicity. They observed that monomer adsorption at low levels of rhamnolipid concentration is more significant than in that of micelle adsorption. Al-Tahhan et al. [35] showed that the loss of lipopolysacchride from outer membrane due to interaction with rhamnolipids caused an increase in cell surface hydrophobicity. Obtained results indicate that cell surface modification is dependent on rhamnolipid concentration, as well as species of bacterial strains.

Zeta Potential (ζ) Effect

Surface charge of bacterial strains is usually negative due to the presence of amines, carboxylic acids, phosphates, and sometimes sulfate moieties on the outer surface.

The measurement of Zeta potential showed changes induced by growth of *A. denitrificans* SM, *P. stutzeri* KS, and *B. subtilis* KE strains on different carbon sources (Table. 2), as well as on different amounts of rhamnolipids in the two systems (with and without diesel oil). Among the samples of the parent population strain, *P. stutzeri* KS had lower Zeta potential (-24.8 mV), than *B. subtilis* KE (-19.2 mV), and *A. denitrificans* SM strain (-11.6 mV). During growth on different carbon sources, the surfaces of both tested Gram negative and a one Gram positive bacterial strains characterized by significant differences of surface charge values. *P. stutzeri* KS reached a lower value of Zeta potential in all tested systems in comparison to *A. denitrificans* SM and *B. subtilis* KE strains. The highest Zeta potential value (-8.5 mV) *A. denitrificans* SM exhibited after growth on rhamnolipids, while the lowest was observed for parent strain *P. stutzeri* KS (-24.8 mV).

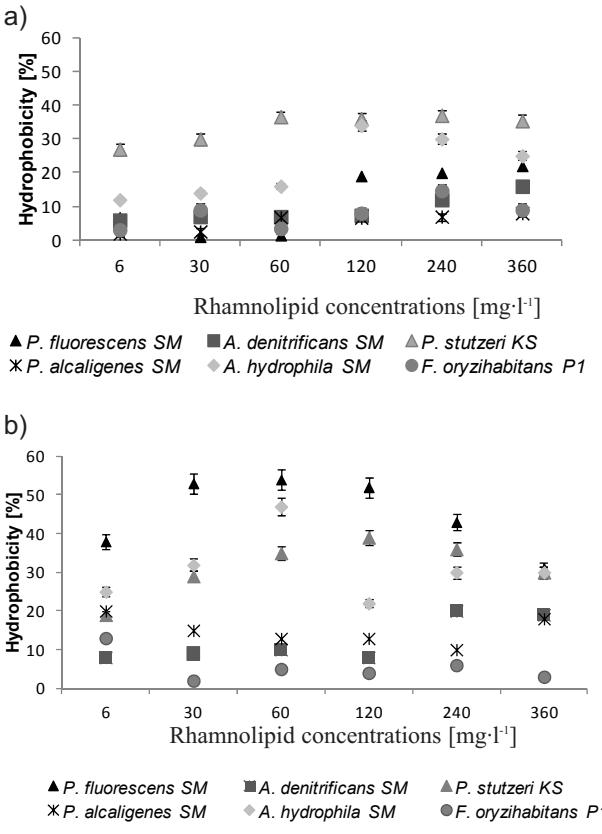

Fig. 2. Influence of rhamnolipid concentrations on Gram negative bacterial cell surface (process carried out in a mineral salt medium over 7 days); cell surface hydrophobicity in rhamnolipids system (a) cell surface hydrophobicity in rhamnolipids-diesel oil system (b).

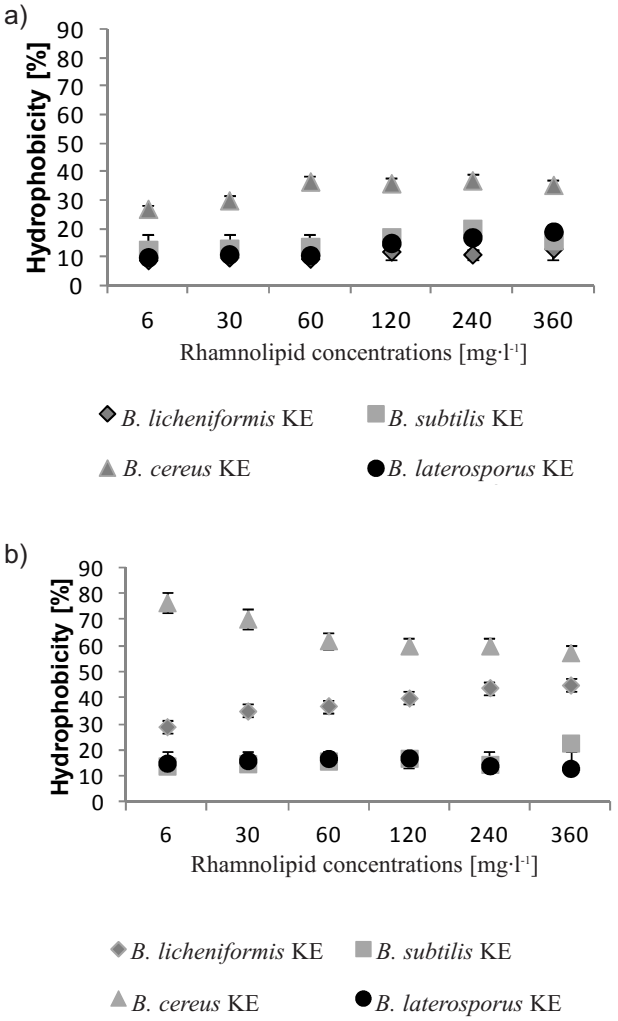

Fig. 3. Influence of rhamnolipid concentrations on Gram positive bacteria cell surface (process carried out in a mineral salt medium over 7 days); cell surface hydrophobicity in rhamnolipids system (a) cell surface hydrophobicity in rhamnolipids-diesel oil system (b).

In the case of *B. subtilis* KE, significant differences in surface charge of the cells in the two systems were observed: diesel oil-rhamnolipids (-15.4 mV) and diesel oil (-9.1 mV), whereas for Gram negative strains *A. denitrificans* SM and *P. stutzeri* KS changes were not that significant. However, surface charges for Gram negative strains in the system with diesel oil-rhamnolipids were lower than in the rhamnolipid system.

In addition, it was observed that with an increasing amount of rhamnolipids added to the system there follow an increase in the Zeta potencial of *A. denitrificans* SM as well as for *B. subtilis* KE (Fig. 4). The values for *A. denitrificans* SM ranged from -11.4 mV (concentration of rhamnolipids 6 mg·l^{-1}) to -8.4 mV (concentration of rhamnolipids 360 mg·l^{-1}). A similar tendency was observed for the diesel oil-rhamnolipids system, but the obtained results were slightly lower. The values ranged from -13.5 mV to -7.6 mV. And for *B. subtilis* KE strain, in the rhamnolipids system the values ranged from -18.2 mV to -6.4 mV and in the diesel oil-rhamnolipids system from -23.5 mV to -7.6 mV. It was also observed that lower concentrations of rhamnolipids reduced the surface charge of *B. subtilis* KE strain comparing to *A. denitrificans* SM. Acording to Zeng et al. [36] the Zeta potential of *Candida tropicalis* cell surface related closely to the biosurfactant concentration. They noticed that when the concentrations of monorhamnolipids were below the CMC of 38 mg·l^{-1}, the Zeta potential increased slightly, while it increased significantly with monorhamnolipids at an initial concentration of 114 mg·l^{-1}. Ly et al. [37] demonstrated that the Zeta potential of cell surface depended upon the strains and pH.

The surface charge on bacteria is different even for the bacterial strains that belong to the same species. The changes could be induced by growth on different carbon sources. Chakrabrty et al. [38] noted that adherence to hexadecane was significantly impacted by cell surface charge. They observed that adherence was highest at near zero and for positive Zeta potential. However, the cell surface charge of bacteria affected the stability of emulsions [37]. They observed that interaction between bacteria and surfactant-coated droplets are mainly due to electrostatic forces. Nonetheless, Zeng et al. [36] showed that changes of Zeta potential of cell surface was closely related with biosurfactant concentration. Similar results were also obtained by Hua et al. [39].

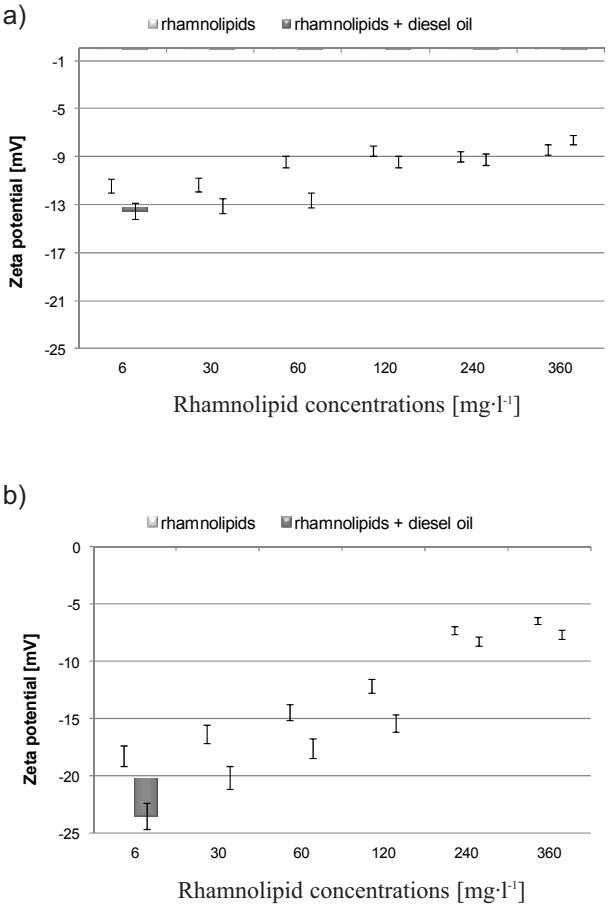

Fig. 4. Changes in the surface charge values measured for the *A. denitrificans* SM (a) and *B. subtilis* KE (b) after the addition different concentrations of rhamnolipids; pH 7.

Particle Size Distribution in Rhamnolipid System

Obtained results of particle size distribution for three selected bacterial strains: A. denitrificans SM (Fig. 5a), *P. stutzeri* KS (Fig. 5b), and *B. subtilis* KE (Fig. 5c) indicated that rhamnolipids interacted in a different way with the bacterial outlayer.

Results of particle size distribution during growth on rhamnolipids showed that the largest particles were observed in the system with *P. stutzeri* KS. The polydispersity index (PdI) was the highest with a PdI=0.67, which means that particles are not homogenous. In those systems particle size distribution presented two bands that fitted the diameter range of 342-5,560 nm with a maximum intensity of 14.5%, characterizing particles of 712 nm, and 7% characterizing particles of 5,560 nm. In case of *A. denitrificans* SM and *B. subtilis* KE strains, different diameter results were obtained. The system was more homogenous and the

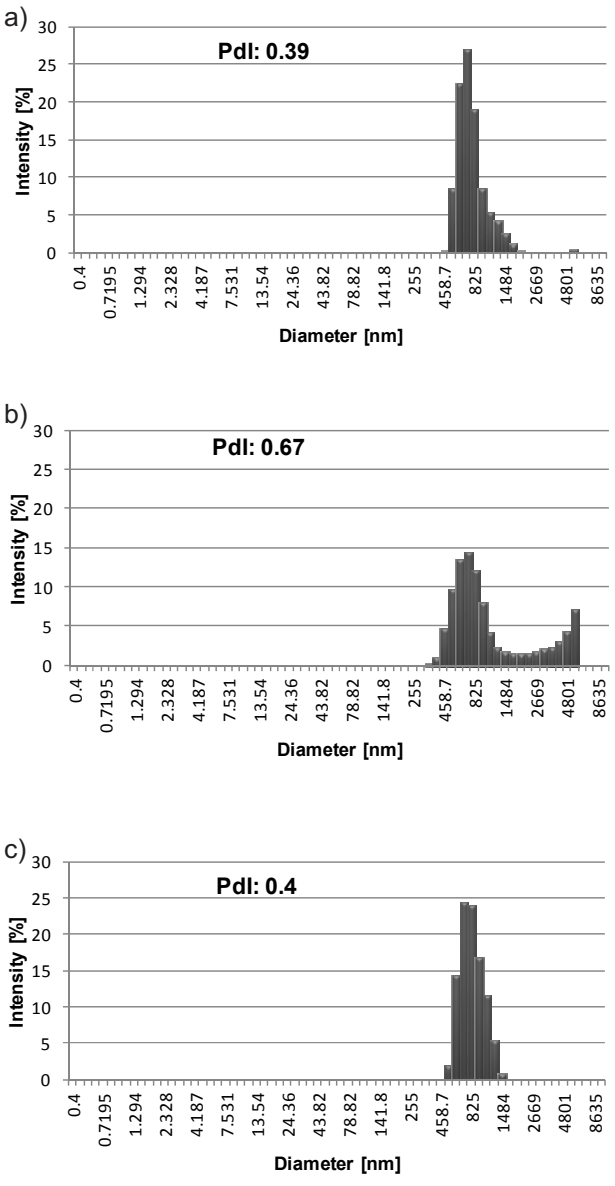

Fig. 5. Particle size distribution during *P. stutzeri* KS (a), *A. denitrificans* SM (b), and *B. subtilis* (c) growth on rhamnolipids at a concentration of 120 mg·l^{-1}.

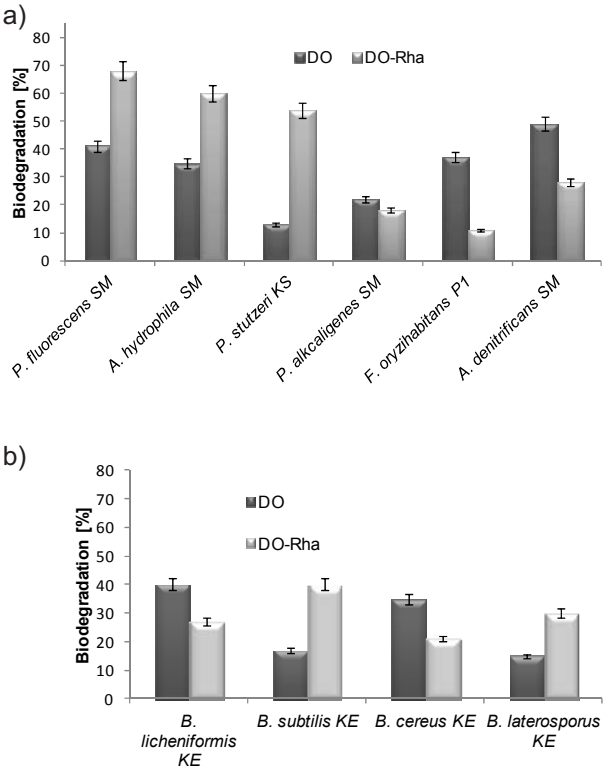

Fig. 6. The influence of rhamnolipids on diesel oil biodegradation by Gram negative bacterial strains (a) and Gram positive bacterial strains (b) after 7 days of process; DO – diesel oil, DO-Rha – diesel oil and rhamnolipids; concentration of rhamnolipids 120 mg·l^{-1}.

polydispersity index was 0.39 for *A. denitrificans* SM and 0.4 for *B. subtilis,* KE. For *A. denitrificans* SM, there was one band that fit the diameter range of 1,000-1,990 nm with a maximum intensity of 25%, characterizing particles of 1484 nm. However, for *B. subtilis* KE dominated a band, which fitted the diameter range of 458-1,718 nm with a maximum intensity of 27%, characterizing particles of 712 nm. In this system, smaller particles dominated. Analyzing the difference in the polydispersity index and the particle size distribution, one can conclude that rhamnolipid influence on cell surface is strongly dependent on the genus of bacteria.

Effect of Rhamnolipids on Diesel Oil Removal Efficiency

The addition of rhamnolipids to the system does not always increase the biodegradation of diesel oil. Those observations apply to both Gram positive and Gram negative bacteria. In the case of Gram positive bacteria strains, I observed that the addition of rhamnolipids led to significant increases in diesel oil removal efficiency only for *B. subtilis* KE and *B. laterosporus* KE strains. Diesel oil removal increased from 17% to 40%, and from 15% to 30%, respectively (Fig. 6b). Both of the tested strains indicated hydrophilic properties of cell surface in the diesel oil-rhamnolipids system (Fig. 3). For the other two strains, lower

diesel oil biodegradation rates in the system with rhamnolipids were observed. In those cases rhamnolipids were negative additive. The cell surface properties were more hydrophobic, in comparison to *B. subtilis* KE and *B. laterosporus* strains.

However, for Gram negative bacteria strains (*P. fluorescens* SM, *P. stutzeri* KS, and *A. hydrophila* SM) the greatest impact of rhamnolipids on the biodegradation of diesel oil were observed. After 7 days, diesel oil biodegradation increased from 41% (system without rhamnolipids) to 68% (system with rhamnolipids) for *P. fluorescens* SM, from 13% to 54% for *P. stutzeri* KS, and from 35% to 60% for *A. hydrophila* SM (Fig. 6a). For the other tested Gram negative bacteria strains, the negative effect of rhamnolipids on diesel oil biodegradation was observed. The strains were characterized by hydrophilic-hydrophobic or hydrophobic features after the addition of rhamnolipids to the diesel oil removal process. Changes in cell surface properties during the biodegradation process was observed. Higher biodegradation corresponded with the increase of CSH.

The addition of rhamnolipids could enhance the removal of petroleum hydrocarbon pollutants and therefore may have the potential for facilitating the bioremediation of soil contaminated by hydrocarbons [31]. However, biosurfactants sometimes exhibit antimicrobial activity [39]. Therefore, it is important for studies to be carried out before the application of biosurfactants into the bioremediation process. Rhamnolipids increase the solubilization of hydrophobic organic compounds, but this does not guarantee enhanced biodegradation [12]. My results indicated that better biodegradation was observed when hydrophilic-hydrophobic or hydrophobic strains dominated in a population of tested bacterial strains.

Conclusion

This study focused on the influence of rhamnolipids on CSH isolated from contaminated soil bacterial strains and the removal efficiency of diesel oil from the environment. A modification of cell surface depended on the amount of biosurfactants, inherency of other carbon sources in the system, and genera of microorganisms. Analysis of adsorption parameters indicated that the structure of monolayer adsorption is different for the tested strains. The amount of rhamnolipids in biological system change due to their adsorption on cell surface, biodegradation, and hydrocarbon emulsifications. This is the reason why CMC changes during hydrocarbon biodegradation and it is different than in either water or medium solutions. Particle size distribution provides information on changes in the bacteria cell surface properties taking place in the process of diesel oil biodegradation. Cell surface hydrophobicity is correlated with the kind of a carbon source used for inoculation. Moreover, the cell surface properties of different bacterial strains could be modified in different ways by the same carbon source. CSH is a dynamic parameter that depends on the composition of the system at the time of the process. There was no trend in the surface modification and diesel oil biodegradation after the addition of rhamnolipids, which can generalize different effects of this biosurfactant on Gram positive and Gram negative bacteria. The addition of rhamnolipids to the diesel oil system does not always result in an increase of biodegradation rates. This was observed among Gram positive and Gram negative bacteria. Due to the fact that rhamnolipis differently affect the efficiency of removal of diesel oil by microorganisms, the prior tests of their introduction into the system are very important. The most effective bacterial systems in diesel oil removal were *P. fluorescens* SM, *P. stutzeri* KS, and *A. hydrophila* SM.

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