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Characterization of rhamnolipids produced by non-pathogenic *Acinetobacter* and *Enterobacter* bacteria

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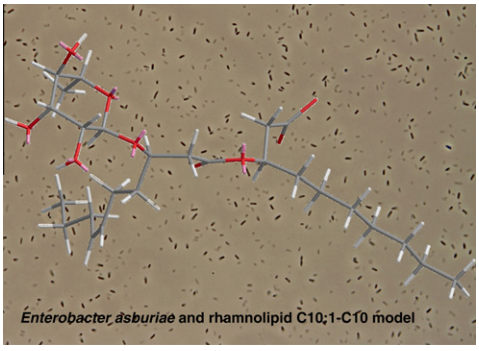
HIGHLIGHTS

- Rhamnolipids were produced by non-pathogenic bacteria *E. asburiae* and *A. calcoaceticus*.
- These rhamnolipids have mild impact on cell properties of soil bacteria.
- Growth media differing in carbon, nitrogen and phosphorus source were tested.
- Correlation of rhamnolipid fatty acid structure and its properties is indicated.

GRAPHICAL ABSTRACT



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ABSTRACT

Rhamnolipid production by two non-pathogenic bacterial strains *Acinetobacter calcoaceticus* and *Enterobacter asburiae*, and established rhamnolipid producer *Pseudomonas aeruginosa* was investigated.

Rhamnolipids were separated from supernatant and further purified by thin-layer chromatography. Mass spectrometry with negative electrospray ionization revealed rhamnolipid homologues varying in chain length and unsaturation. Tandem mass spectrometry identified mono-rhamnolipid and di-rhamnolipid homologues containing one or two 3-hydroxy fatty acids. Several media differing in carbon (sunflower oil, glycerol and sodium citrate), nitrogen (ammonium ions, nitrate) and phosphorus (total content) source, respectively, were tested to obtain enhanced rhamnolipid production. The best production (0.56 g/l) was obtained when nitrate was used as a nitrogen source. Both strains produced rhamnolipids that exhibited excellent emulsification activity with aromatic and aliphatic hydrocarbons and several plant oils. Unlike *P. aeruginosa* the two strains, i.e. *Acinetobacter* and *Enterobacter*, are not pathogenic to humans.

1. Introduction

Biosurfactants are a large heterogeneous group of microbial secondary metabolites. These amphipathic surface-active molecules reduce interfacial tensions on liquid–liquid or liquid–solid phase boundaries. Biological surfactants possess several advantages over synthetic surfactants including high biodegradability, high emulsifying abilities, low toxicity and good general environmental compatibility (Pacwa-Plociniczak et al., 2011). Biosurfactants are therefore products with a broad potential of industrial (bioremediations, cosmetics, food and beverage manufacture) and pharmaceutical applications (Rikalovic et al., 2012).

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Glycolipids, the most common biosurfactants, are composed of a long chain fatty acid hydrophobic moiety and a hydrophilic carbohydrate component (Müller and Hausmann, 2011). Rhamnolipids are the most studied glycolipid biosurfactants (Henkel et al., 2012). The hydrophilic part consists of rhamnose (Rha) molecule(s) while the hydrophobic part is usually represented by one or more 3-hydroxy fatty acids (3-OH-FAs) (also called β-hydroxy, i.e. β-OH-FA). If two rhamnose molecules are present, they are linked by 1,2-glykosidic bond. The rhamnose was documented to be in the L-form (Řezanka et al., 2011) and anomeric configuration is α, as determined by NMR spectroscopy (Price et al., 2009). 3-OH-FAs are mostly saturated or, less often, mono- or dienoic, usually with 8–16 carbon atoms (most often 8 or 10; (Abdel-Mawgoud et al., 2010)). The stereochemistry of 3-OH-FAs is *R* and the position of double bonds in monoenoic acids is *cis* ω-9 (Řezanka et al., 2011). An odd number of carbon atoms (C9, C17) has exceptionally been found and so were very-long-chain FAs (Nie et al., 2010). More than 60 different molecular species are known to date (Abdel-Mawgoud et al., 2010; Soberon-Chavez, 2011).

Virtually the only method applicable for both qualitative and quantitative determination is LC–MS (liquid chromatography–mass spectrometry) or tandem MS with electrospray ionization (ESI–MS/MS), preferably in negative mode. However, only tandem MS allows the identification of individual molecular species including positional isomers and their separation, e.g. Rha–C8–C10 from the isomer Rha–C10–C8 (Haba et al., 2003).

The most important producer of these glycolipids is *Pseudomonas aeruginosa*, a known human pathogen. However, many bacteria have been found to produce also rhamnolipids (Abdel-Mawgoud et al., 2010; Soberon-Chavez, 2011). These include other *Pseudomonas* species or species that are taxonomically more distant from *Pseudomonas*, e.g. *Acinetobacter calcoaceticus*, *Pseudoxanthomonas* sp., *Enterobacter* sp., *Pantoea* sp., *Renibacterium salmoninarum*, *Nocardioides* sp., *Tetragenococcus koreensis* or *Burkholderia* sp. (Abdel-Mawgoud et al., 2010; Rooney et al., 2009; Soberon-Chavez, 2011).

Pantazaki et al. (2010) and Řezanka et al. (2011) identified rhamnolipids in thermophilic bacteria such as *Thermus* sp., *T. thermophiles*, *T. aquaticus*, and *Meiothermus ruber*. However, the most studied are still strains of *P. aeruginosa* (Abdel-Mawgoud et al., 2010).

Although many studies have demonstrated the advantages of biosurfactants over synthetic surfactants, large-scale production and application of these compounds is still not widespread due to the relatively high costs. Utilization of renewable raw substrates has been described to enhance the production (Soberon-Chavez, 2011). Improvement of rhamnolipid production by optimization of medium composition and use of inexpensive substrates are considered as best approaches to addressing this problem (Soberon-Chavez, 2011). The dependence of rhamnolipid production on cultivation medium composition has been acknowledged in many studies (Henkel et al., 2012; Mehdi et al., 2011; Rikalovic et al., 2012). The carbon, nitrogen and phosphorus source type and C/N and C/P ratios have been found to have a significant impact on the amount of produced rhamnolipid (Li et al., 2011). Also the presence and concentration of trace elements, especially divalent cations, have been found to have a great influence (Mehdi et al., 2011).

The aim of this work was to compare rhamnolipid production by a novel, non-pathogenic strain with that in an established rhamnolipid producer *P. aeruginosa*, a known human pathogen. The analysis was extended from rhamnolipids produced by thermophilic bacteria to further strains, i.e. *A. calcoaceticus* and *Enterobacter asburiae*. Further goals were to improve rhamnolipid production by optimizing medium composition with the emphasis on carbon, nitrogen and phosphorus sources. The structure of rhamnolipids was confirmed by ESI–MS/MS. The strains under study produced rhamnolipids with very good emulsification activity for aromatic and aliphatic hydrocarbons and several plant oils.

2. Methods

2.1. Microorganisms

Rhamnolipid-producing bacteria *P. aeruginosa* strain B-59188, *A. calcoaceticus* B-59190 and *E. asburiae* B-59189 were obtained from ARS Culture Collection, Bacterial Foodborne Pathogens and Mycology Research Unit, National Center for Agricultural Utilization Research, Peoria, Illinois, USA.

2.2. Cultivation conditions

The bacterial strains were cultivated in 500 ml Erlenmeyer flasks at 30 °C stirred at 100 rpm in 200 ml basic mineral medium (g/l): KH_2PO_4 0.17; K_2HPO_4 0.13; $(NH_4)_2SO_4$ 0.71; $MgCl_2{\cdot}6\ H_2O$ 0.34; (g/l): $MnCl_2{\cdot}4\ H_2O$ 1.0; $CaCl_2$ 0.196; $FeSO_4{\cdot}7\ H_2O$ 0.6; $Na_2MoO_4{\cdot}2\ H_2O$ 2.0) containing sodium citrate, glycerol or sunflower oil (20 g/l) as carbon source. For preculture, the cultivation was carried out in 500 ml Erlenmeyer flasks with nutrient broth at 30 °C for 48 h. The effect of different nitrogen and phosphorus sources was investigated by replacing $(NH_4)_2SO_4$ with $NaNO_3$ (0.5 g/l) and by testing total phosphorus concentrations in the range from 0.06 to 1.95 g/l. Biomass concentration was monitored by measuring optical density of cultivation media at 600 nm (OD_{600}). In the cultivation experiments utilizing sunflower oil the oil was extracted according to (Muller et al., 2011) prior to biomass quantification. The biomass growth and rhamnolipid production was monitored in the course of 200 h, with maximum rhamnolipid production in late stationary phase.

2.3. Rhamnolipid determination and isolation

Rhamnolipid concentration in cultivation medium was determined spectrophotometrically as rhamnose content by the phenol–sulphuric method according to (Dubois et al., 1956) using rhamnose as standard. After determining the rhamnolipid composition, correlation factors between rhamnose and rhamnolipid concentration were calculated. Rhamnolipid concentration is displayed in the text and figures instead of rhamnose equivalents. The respective correlation factors for *P. aeruginosa*, *E. asburiae* and *A. calcoaceticus* are 2.29, 2.22 and 2.12.

Rhamnolipids were isolated as described by (Abdel-Mawgoud et al., 2010). Biomass was removed by centrifugation (9000 rpm, 15 min) and the supernatants (culture volumes 2 l) were subjected to acidic precipitation (1 M HCl, 4 °C, 24 h). The precipitate was centrifuged (9000 rpm, 15 min) and dissolved in a chloroform:methanol mixture (2:1).

Rhamnolipids were separated by TLC with a mixture of dichloromethane-methanol-glacial acetic acid (65:15:2, v/v/v). Band visualization was carried out with orcinol followed by heating. Quantification was performed by means of a TLC scanner and calculated using Statistica 9 software.

In the preparative mode, the appropriate four bands were scraped off from the preparative plates, eluted by the mixture dichloromethane-methanol-glacial acetic acid (65:15:2) and evaporated. The composition of distinct rhamnolipid groups in the four bands was further identified by mass spectrometry and fatty acids by gas chromatography–mass spectrometry (GC–MS).

2.4. Acid hydrolysis of rhamnolipids

A solution of Rha-FAs, Rha-Rha-FAs, Rha-FA-FAs and Rha-Rha-FA-FAs (each 1 mg) was refluxed in 2 N HCl (0.5 ml) for 2 h. The

3-OH-FAs were extracted three times with EtOAc (10 ml). The free 3-OH-FAs were derivatized by diazomethane and resulting methyl esters were analyzed by GC–MS.

2.5. GC–MS

MS of the corresponding methyl ester mixture was done using a Finnigan 1020 B single-state quadrupole GC–MS instrument in the electron ionization mode. The temperature program was as follows: 100 °C for 1 min with a subsequent increase to 185 °C at a gradient of 20 °C/min and to 330 °C at 2 °C/min, the last temperature being maintained for 1 min. A fused silica capillary column (60 m × 0.32 mm, ID/SPB-1 Supelco), using splitless injection and hydrogen as the carrier gas (a linear velocity of 600 mm/s) was used. The temperature program was 100–320 °C at a gradient of 4 °C/min, ionization energy was 70 eV, and electron multiplier voltage was 2.5 kV. All spectra were scanned within the range of m/z 50–600 (Dembitsky et al., 1991, 1992).

2.6. Mass spectrometry

LTQ Orbitrap Velos (Thermo Scientific), a high resolution hybrid mass spectrometer was used. ESI–MS analysis was performed in the negative ion mode. MS spectra were obtained in the FT mode. MS spectra were acquired with target mass resolution of R = 30,000 at m/z 400. The ion spray voltage was set at −2500 V and the scan range of the instruments was set at m/z 150–2000. Nitrogen was used as a nebulizer gas – set at 18 arbitrary units (sheath gas) and 7 arbitrary units (auxiliary gas). Helium was used as a collision gas for CID experiments. The CID normalization energy of 35% was used for the fragmentation of the parent ions. The MS/MS product ions were detected by the high resolution FT mode.

Flow Injection Analysis (FIA) was used for sample introduction into the heated ESI (H-ESI) ion source. Acetonitrile/water (50/50 v/v) was used at the flow rate of 150 μl/min. The H-ESI temperature was set to 250 °C.

2.7. Determination of critical micelle concentration

Critical micelle concentration was determined by contact angle measurement, based on inverse relation of surface tension and the contact angle of solution drop on solid material (Kwok and Neumann, 2000). The comparison of contact angle measurement method and the ring method is given in Table S5.

As the critical micelle concentration is dependent on ionic strength and pH of the solution (Özdemir et al., 2004), the crude isolates of rhamnolipids were diluted in basic mineral medium used in cultivation experiments (concentrations 1–50 mg/l). A 5 μl drop was placed on a Teflon plate (constant temperature 30 °C) and picture of the drop was taken. Each measurement was repeated 10 times. The pictures were evaluated and contact angle was determined by FTA 32 software. CMC was obtained from a plot of contact angle as a function of rhamnolipid (expressed as rhamnose) concentration. The break point observed in the plot corresponded to the value of CMC.

2.8. Determination of solubilization activity

The effect of rhamnolipids on cell integrity was determined as solubilization activity. Cells of the bacteria *Rhodococcus erythropolis* and *Pseudomonas fluorescens* and the yeast *Trichosporon cutaneum* were harvested in exponential and stationary phase (basic mineral medium, composition see Section 2.2) containing glucose (10 g/l) as carbon source, washed twice (9000 g, 10 min) and placed in cultivation mineral medium with rhamnolipids (concentration: CMC). After five hours the total protein content was determined by the method according to Bradford (Bradford, 1976). Cells in blank experiment (mineral medium without rhamnolipids) under the same conditions did not release any proteins.

2.9. Determination of cell envelope hydrophobicity

The effect of rhamnolipids on cell hydrophobicity was determined by the MATH method (Rosenberg et al., 1980). Cells of the bacteria *R. erythropolis* and *P. fluorescens* and the yeast *T. cutaneum* were cultivated in mineral medium (composition see Section 2.2) with rhamnolipids (concentration: CMC). After 24 h (exponential phase) the cells were centrifuged and washed twice (9000*g*, 10 min) and the cell hydrophobicity was determined. The harvested cells were resuspended to optical density $OD_{600}(1)$ (approximately 0.3) and 2.5 ml of this suspension was mixed thoroughly with 0.25 ml of hexane. After 5 min the water phase was separated and $OD_{600}(2)$ ascertained. Cell surface hydrophobicity was determined as $H = [100 \times (OD_{600}(1) - OD_{600}(2)]/OD_{600}(1)$.

3. Results and discussion

Rhamnolipids used for industrial purposes are currently almost exclusively produced by the bacterial strains of *P. aeruginosa*. The unpleasant fact is that many strains of this organism belong to human pathogens. For this reason, the possibility was tested of using selected non-pathogenic microorganisms for the production of rhamnolipids suitable for commercial applications. The bacterial strains *A. calcoaceticus* B-59190 and *E. asburiae* B-59189 were studied for this purpose. Rhamnolipid producing pathogenic strain *P. aeruginosa* B-59188 was used for comparison. All these strains were reported to produce rhamnolipids (Rooney et al., 2009). Also other *Acinetobacter* strains have been reported to produce biosurfactants (Chen et al., 2012). Rhamnolipid biosynthetic pathways in the case of *P. aeruginosa* are well described, but their regulatory systems are quite complicated. This makes it difficult to improve the technological properties of the strains (Müller and Hausmann, 2011; Reis et al., 2011). Most of the works devoted to enhancing production of rhamnolipids therefore focused on the modulation of environmental conditions.

The ability to produce rhamnolipids is generally recognized as highly dependent on medium composition, especially with regard to carbon sources (Henkel et al., 2012; Rikalovic et al., 2012). Based on results published by (Rikalovic et al., 2012), sodium citrate, glycerol and sunflower oil (20 g/l) in mineral medium were selected for testing the influence of carbon source on rhamnolipid production. Sodium citrate showed in preliminary experiments very good properties with regard to growth of the microbial strains and rhamnolipids production. Glycerol and sunflower oil were chosen as carbon sources enhancing rhamnolipid production according to the literature (Mehdi et al., 2011; Rikalovic et al., 2012).

3.1. Rhamnolipid production and characterization

The comparison of rhamnolipid production (expressed as rhamnose concentration) in mineral medium with various carbon sources by the referent strain *P. aeruginosa* and the two non-pathogenic strains *E. asburiae* and *A. calcoaceticus* is shown in Fig. 1. The rhamnolipid production is depicted in three growth phases – at the end of lag-phase, at the end of exponential phase of growth and at the late stationary phase when the rhamnolipid production was maximal. Times of sampling for *P. aeruginosa* and *A. calcoaceticus* (all substrates) were 8, 50 and 100 h, for *E. asburiae* 8, 30 and 100 h. The growth (expressed as optical density of cultivation media – OD_{600}) on sodium citrate, sunflower oil and glycerol in mineral medium is shown in Fig. 2. The rhamnolipid

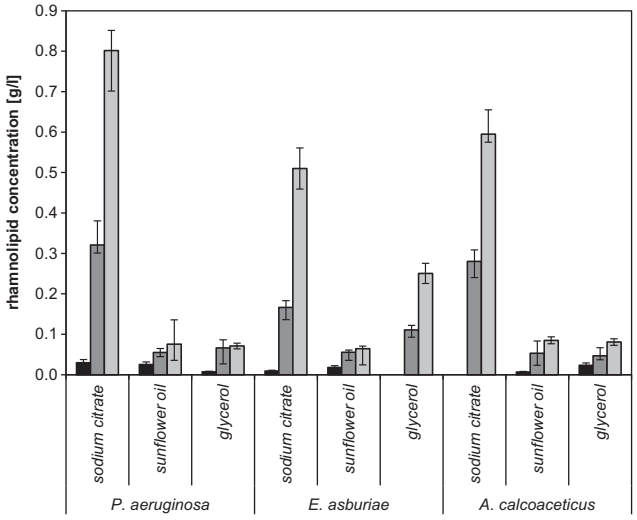

Fig. 1. Rhamnolipid production by *P. aeruginosa*, *E. asburiae* and *A. calcoaceticus* in mineral medium with sodium citrate, glycerol and sunflower oil as carbon source (■ end of lag-phase; ▩ end of exponential phase; □ late stationary phase).

concentration in the medium was the highest on sodium citrate (0.80 g/l; 0.51 g/l and 0.59 g/l by *P. aeruginosa*, *E. asburiae* and *A. calcoaceticus*, respectively) compared to significantly lower rhamnolipid concentrations obtained on glycerol and sunflower oil. Glycerol (unlike sunflower oil) was found to be an unsuitable substrate also with respect to growth, as all tested strains reached only low optical densities when grown solely on glycerol (see Fig. 2). Under these culture conditions, the positive effect of both these carbon sources on the productivity of rhamnolipids reported by other authors (Henkel et al., 2012; Rikalovic et al., 2012) was not confirmed. The highest reported productions by non-pathogenic microorganisms *Pseudomonas chlororaphis* and *Pseudomonas nitroreducens* are 5,46 g/l and 1,0 g/l respectively (Gunther et al., 2005; Müller and Hausmann, 2011).

The influence of modification of nitrogen and phosphorus source was investigated (see Fig. 3) by observing the rhamnolipid production in mineral media with sodium citrate as carbon source. The replacement of nitrogen source ($NaNO_3$ instead of $(NH_4)_2SO_4$) had the most significant effect on rhamnolipid production, increasing the total concentration by 36% and 50% (by *P. aeruginosa* and *A. calcoaceticus*, respectively). In contrast, none of the media modification had influence on rhamnolipid production by *E. asburiae*. An increase of phosphorus source concentration in the culture medium, reported by other authors (Abdel-Mawgoud et al., 2010) as a positive factor in increasing the production of rhamnolipids was not confirmed (only data for the highest phosphorus concentration are shown in Fig. 3). Other authors reported limitation by phosphorus source as a means to enhance rhamnolipid production (Mulligan et al., 1989) but this was again not confirmed by the results of this study (data not shown).

The physico-chemical properties of isolated rhamnolipids were determined. Critical micelle concentration (CMC) is an important physico-chemical property of surfactants, indicating their emulsifying properties. The CMC value of 60 mg/l of rhamnolipids produced by *P. aeruginosa* was found to be consistent with results published by other authors, as CMCs were reported in a wide range of values from 5 to 200 mg/l (Mata-Sandoval et al., 1999; Pornsunthorntawee et al., 2008; Samadi et al., 2012). Rhamnolipids produced by *A. calcoaceticus* and *E. asburiae* formed micelles at considerably lower concentrations (CMC 15 mg/l and 22 mg/l, respectively) than rhamnolipids produced by referent *P. aeruginosa* strain (60 mg/l).

The potential rhamnolipid applications in *in situ* soil remediation present a need for predicting possible negative interactions with indigenous microbial communities. For this purpose, the effect of rhamnolipids on cell integrity of three ubiquitous microorganisms, two bacteria (Gram-positive *R. erythropolis* and Gram-negative *P. fluorescens*) and the yeast *T. cutaneum* was evaluated by monitoring the release of cell proteins after exposure to rhamnolipids (see Section 2). These microorganisms were chosen as representatives of the three groups and as ubiquitous, well-known degraders, such as might be present in contaminated soil in potential rhamnolipid-mediated remediation applications.

The exponential phase and stationary-phase cells of *R. erythropolis, P. fluorescens* and *T. cutaneum* were subjected to the five-hour presence of critical micelle concentrations of all three studied rhamnolipids. The released proteins were monitored and compared to blank (see Section 2). The results in Fig. 4 indicate that rhamnolipids from *P. aeruginosa* bring about the highest protein release from all tested microorganisms in both exponential and stationary phase. Lowest solubilization activity under the experimental conditions was displayed by rhamnolipids isolated from *E. asburiae*, which did not release any significant amount of proteins from *R. erythropolis* and *P. fluorescens*. Vasileva-Tonkova et al. (2011) reported solubilization activities of rhamnolipids produced by *P. aeruginosa strains* on *Bacillus subtilis* cells.

The hydrophobicity of exponential-phase cells of the Gram-positive bacterium *R. erythropolis*, Gram-negative bacterium *P. fluorescens* and the yeast *T. cutaneum* cultivated in the presence of rhamnolipids was evaluated by the MATH test. Rhamnolipids produced by different strains displayed a significantly diverse influence. Rhamnolipids isolated from *P. aeruginosa* lowered the

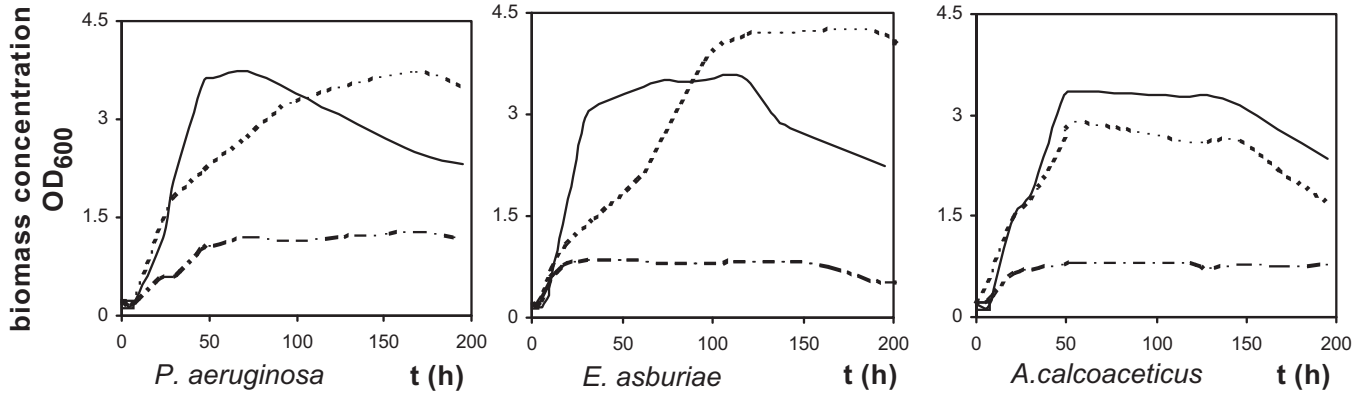

Fig. 2. Growth of *P. aeruginosa*, *E. asburiae* and *A. calcoaceticus* in mineral medium with sodium citrate (——), sunflower oil (- - -) and glycerol (-·-·-) as carbon source.

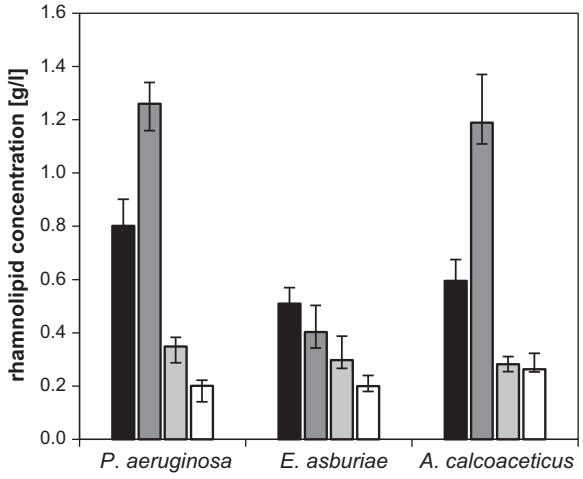

Fig. 3. The effect of nitrogen and phosphorus source modification on rhamnolipid production by *P. aeruginosa*, *E. asburiae* and *A. calcoaceticus* (■ basic medium (control); ▩ N-source: $NaNO_3$; ▩ P-source 4x higher; □ N-source: $NaNO_3$ and P-source 4x higher).

hydrophobicity of *R. erythropolis* and *T. cutaneum*, both of which have under normal conditions highly hydrophobic cell envelopes. Rhamnolipids produced by *E. asburiae* and *A. calcoaceticus* did not influence the hydrophobicity of the tested strains. The Gram-negative *P. fluorescens* with hydrophilic cell surface was not affected by the addition of any tested rhamnolipids. These data are in agreement with published results suggesting that the influence of rhamnolipids on cell surface hydrophobicity is dependent on both rhamnolipid composition (Zhong et al., 2008) and the original cell surface hydrophobicity of tested strain (Zhao et al., 2011).

Isolated rhamnolipids were further characterized by TLC, FAME analysis and tandem mass spectrometry. Quantification of rhamnolipids separated by TLC scanner and calculated by Statistica 9 software is shown in Figs. 1S and 2S(Supplement) and Table 1. Neutral lipids that were not consumed by the bacteria showed a TLC band at Rf 0.86. Four additional bands were detected with Rf values 0.24, 0.32, 0.50 and 0.68. The bands having Rf 0.24 and Rf 0.32 consisted of dirhamnolipids, while the bands at Rf 0.50 and 0.68 consisted of monorhamnolipids. These data fully comply with the previously reported results of TLC of rhamnolipids from different strains of bacteria, see (Silva et al., 2010) and studies cited therein.

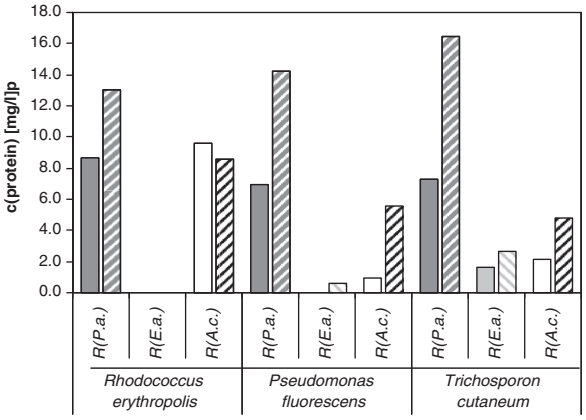

Fig. 4. The solubilization activity (expressed as total content of liberated cell proteins) of rhamnolipids isolated from ▩ *P. aeruginosa* R(P.a.), ▩ *E. asburiae* R(E.a.) and □ *A. calcoaceticus* R(A.c.) on bacteria *R. erythropolis*, *P. fluorescens* and yeast *T. cutaneum* (cells from exponential phase – full columns, cells from stationary phase – striped columns).

Table 1
TLC analysis of rhamnolipids extracted from culture broth of *A. calcoaceticus*, *E. asburiae*, and *P. aeruginosa*.

R_f	Rhamnolipid	*A. calcoaceticus*	*E. asburiae*	*P. aeruginosa*
0.68	Rha-Rha-FA	17.8[a]	21.8	13.7
0.50	Rha-Rha-FA-FA	48.6	29.7	44.3
0.32	Rha-FA	12.9	17.1	7.1
0.24	Rha-FA-FA	20.7	31.4	34.9

[a] The values in the table are in relative percent.

3.2. Fatty acid analysis

Table 2 shows the composition of FAME from the three strains after GC–MS analysis. Only fatty acids with abundances above 0.1% of total fatty acids are included.

The content of fatty acids, with decanoic acid as the major component, is similar in all three strains. The results thus indicate that both *A. calcoaceticus* and *E. asburiae* can be used as full-fledged replacements of *P. aeruginosa*. Similar results, at least on a qualitative level, were reported by, e.g. Monteiro et al. (2007) who found decanoic acid in an amount of 68% total fatty acids.

3.3. Tandem mass spectrometry

Table 3 show the proportions of rhamnolipids identified in the three strains of bacteria. Structural similarity of the rhamnolipids produced by all three strains was demonstrated by MS^2. Partial differences were found in the quantitative proportions; these differences were reflected in the particular properties of the rhamnolipids.

The structure of individual rhamnolipids was determined as described in a preceding study (Řezanka et al., 2011); only the structure determination of two rhamnolipids, i.e. Rha-Rha-10:0-12:1 and Rha-Rha-12:1-10:0 is therefore described here. The $[M-H]^-$ ion at m/z 677.41 was identified in the mass spectrum. A pair of main ions (at m/z 479.25 and at m/z 507.28) was observed in the mass spectrum of the mixture; these ions correspond to the cleavage at the carbon–oxygen bond, see Figs. 3S and 4S (Supplement). The cleavage at the *O*-glycosidic bond in the fatty units gives further ions at m/z 197.15 and at m/z 169.12. Similarly, the ion corresponding to two rhamnose units at m/z 309.21 was also seen in the mass spectrum. The mass spectra of negative ESI of all four classes of rhamnolipids can be used to reflect the qualitative and quantitative content of individual rhamnolipids (Table 3).

As mentioned above, total rhamnolipids contain only five acids, with decanoic acid as the major component. This corresponds also with the proportions of the molecular species of rhamnolipids in the two bacterial strains, in which a half or more of molecular species given in Table 3 only decanoic acid.

Rhamnolipids from *Pseudomonas* exhibit low variability and all four classes of rhamnolipids have been identified by only a few authors (Shen et al., 2005; Silva et al., 2010). The number of molecular species identified by different authors is also low: Price et al. (2009) identified twelve molecular species, Monteiro et al. (2007)

Table 2
The composition of FAME of rhamnolipids determined by GC–MS.

Fatty acid	*A. calcoaceticus*	*E. asburiae*	*P. aeruginosa*
Octanoic	12.3[a]	7.7	8.6
Decanoic	70.3	83.9	70.6
Decenoic	0.4	0.1	10.1
Dodecanoic	8.2	4.3	6.5
Dodecenoic	8.8	4.0	4.2

[a] The values in the table are in relative percent.

Table 3
Qualitative and quantitative results of fraction Rha-Rha-FAs-FAs, Rha-FAs-FAs, Rha-FAs and Rha-Rha-FAs of strains *A. calcoaceticus* (*A.c.*) *E. asburiae* (*E.a.*) and *P. aeruginosa* (*P.a.*).

RhaRhaFAFA	*A.c*	*E.a.*	*P.a.*	Formula	$[M-H]^-$	$[M-H]^-$	Key fragments[a]
RhaRhaC8C8	2.3	1.2	3.2	C28H50O13	593.3173	593.32	451
RhaRhaC8C10	8.6	10.1	17.4	C30H54O13	621.3486	621.35	451
RhaRhaC10C8	3.0	2.9	2.1	C30H54O13	621.3486	621.35	479
RhaRhaC10C10:1	0.0	0.0	6.9	C32H56O13	647.3642	649.36	479
RhaRhaC10:1C10	0.0	0.0	1.8	C32H56O13	647.3642	649.36	477
RhaRhaC10C10	57.7	68.4	44.0	C32H58O13	649.3799	649.38	479
RhaRhaC10C12:1	12.8	5.5	1.8	C34H60O13	675.3956	675.40	479
RhaRhaC12:1C10	3.5	0.5	0.4	C34H60O13	675.3956	675.40	505
RhaRhaC10C12	7.2	7.3	14.6	C34H62O13	677.4112	677.41	479
RhaRhaC12C10	4.0	4.1	6.4	C34H62O13	677.4112	677.41	507
RhaRhaC12C12:1	0.9	0.0	1.4	C36H64O13	703.4268	703.43	507
RhaFAFA							
RhaC8C8	0.5	0.4	0.9	C22H39O9	447.2594	447.26	305
RhaC8C10	26.8	11.8	15.8	C24H44O9	475.2907	475.29	305
RhaC10C8	2.8	4.8	2.9	C24H44O9	475.2907	475.29	333
RhaC10C10	47.5	70.5	47.2	C26H48O9	503.3220	503.32	333
RhaC10C10:1	2.0	0.0	11.5	C26H46O9	501.3063	501.31	333
RhaC10:1C10	0.0	0.2	9.8	C26H46O9	501.3063	501.31	331
RhaC10C12:1	7.2	6.6	6.9	C28H50O9	529.3376	529.34	333
RhaC12:1C10	5.3	1.5	1.0	C28H50O9	529.3376	529.34	359
RhaC10C12	6.6	3.0	2.3	C28H52O9	531.3533	531.35	333
RhaC12C10	0.8	1.0	0.7	C28H52O9	531.3533	531.35	361
RhaC12C12	0.5	0.2	1.0	C30H56O9	559.3846	559.38	361
RhaFA							
RhaC8	13.1	7.6	5.7	C14H25O7	305.1601	305.16	143
RhaC10:1	0.0	0.0	9.2	C16H27O7	331.1756	333.18	169
RhaC10	74.6	86.9	75.8	C16H29O7	333.1913	333.19	171
RhaC12	11.4	4.3	6.3	C18H33O7	361.2226	361.22	199
RhaC12:1	0.9	1.2	3.0	C18H31O7	359.2070	359.21	197
RhaRhaFA							
RhaRhaC8	13.1	6.6	4.5	C20H35O11	451.2179	451.22	143
RhaRhaC10:1	0.0	0.0	11.7	C22H37O11	477.2335	479.23	169
RhaRhaC10	62.7	85.1	72.7	C22H39O11	479.2492	479.25	171
RhaRhaC12:1	15.4	4.6	5.9	C24H41O11	505.2649	505.26	199
RhaRhaC12	8.8	3.7	5.2	C24H43O11	507.2805	507.28	197

[a] Determine the sequence of rhamnosyl and hydroxyl fatty acid linkages.

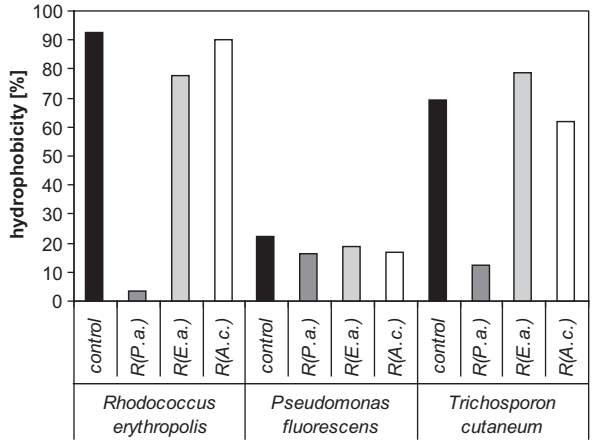

Fig. 5. The effect of rhamnolipids isolated from ■ *P. aeruginosa* R(P.a.), ▨ *E. asburiae* R(E.a.) and □ *A. calcoaceticus* R(A.c.) on cell surface hydrophobicity of bacteria *R. erythropolis, P. fluorescens* and yeast *T. cutaneum*. (■ control – cell surface hydrophobicity without rhamnolipid addition).

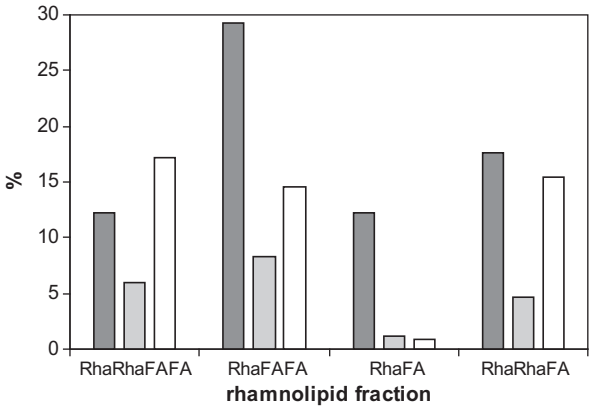

Fig. 6. The proportion of rhamnolipids containing unsaturated fatty acids (rhamnolipids isolated from ■ *P. aeruginosa*, ▨ *E. asburiae* and □ *A. calcoaceticus*).

only eight. These findings are fully in agreement with the results in Table 3. It was also found that among rhamnolipids containing two saturated FAs, the molecular species having saturated FAs with fewer carbon atoms bound by a glycosidic bond directly to rhamnose is always more abundant. This holds, e.g., for the 8.6:3.0 ratio of Rha-Rha-8:0-10:0 to Rha-Rha-10:0-8:0.

Fig. 6 depicts the percent content of rhamnolipids with unsaturated fatty acid in their structure in the four isolated rhamnolipid fractions. This content varies strongly depending on microbial producer. *P. aeruginosa* has in all fractions the largest proportion of rhamnolipids with unsaturated fatty acids (except Rha-Rha-FA-FA), while the proportion of such rhamnolipids produced by *E. asburiae* is very low.

The ratio of rhamnolipids with saturated and unsaturated fatty acids indicates a correlation with the CMC (see above). Rhamnolipids with more hydrophobic saturated fatty acids form micelles at a lower concentration of the surfactant. It therefore allows increasing the bioavailability of highly hydrophobic environmental

pollutants at concentrations which are more friendly towards the microflora present in the ecosystem. In other words, rhamnolipids with a lower proportion of unsaturated fatty acids significantly less affect, among others due to low CMC values, the envelope layers of microorganisms (see the change in hydrophobicity of the cell surface – Fig. 5), and the integrity of microbial cells (see cell solubilization – Fig. 4).

4. Conclusions

The biosurfactant-producing activity of the pathogenic bacterium *P. aeruginosa,* and the non-pathogenic *E. asburiae* and *A. calcoaceticus* was compared. Unlike the total phosphorus content, the carbon source sodium citrate and the replacement of nitrogen source ($NaNO_3$ instead of $(NH_4)_2SO_4$) had a significant effect on rhamnolipid production. Rhamnolipid-type surfactants were isolated, their structure identified by MS^2 and their interactions with bacterial and yeast cells were assessed. Solubilization activity towards target cells was found to correlate with saturation of fatty acids in rhamnolipid structure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.12.085.

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