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# Structural and physiochemical characterization of rhamnolipids produced by *Acinetobacter calcoaceticus*, *Enterobacter asburiae* and *Pseudomonas aeruginosa* in single strain and mixed cultures

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ABSTRACT

Rhamnolipids are naturally occurring biosurfactants with a wide range of potential commercial applications. As naturally derived products they present an ecological alternative to synthetic surfactants. The majority of described rhamnolipid productions are single strain *Pseudomonas* spp. cultivations. Here we report rhamnolipids producing bacteria *Acinetobacter calcoaceticus*, *Enterobacter asburiae* and *Pseudomonas aeruginosa* that were cultivated separately and as mixed populations. The ratio and composition of rhamnolipid congeners was determined by tandem mass spectrometry with negative electrospray ionization. Mono-rhamnolipid and di-rhamnolipid homologues containing one or two saturated or monounsaturated 3-hydroxy fatty acids were found in all strains.

Physiochemical characterization of rhamnolipids was evaluated by the critical micelle concentration determination, the emulsification test, oil displacement test and phenanthrene solubilization. Critical micelle concentrations of rhamnolipids produced by both single strain and mixed cultures were found to be very low (10–63 mg/l) and to correspond with saturated/unsaturated fatty acid content of rhamnolipid homologues. The rhamnolipids produced by all strains effectively emulsified crude petroleum in comparison with synthetic surfactants Tween 80 and sodium dodecyl sulfate (SDS). Good performance of phenanthrene solubilization was exhibited by rhamnolipids from *E. asburiae*.

The single strain and co-cultures cultivations were proposed as a possible way to produce rhamnolipid mixtures with a specific composition and different physiochemical properties, which could be exploited in bioremediation of various hydrophobic contaminants.



## 1. Introduction

Biosurfactants are amphipathic secondary metabolites produced by various microorganisms. These structurally diverse substances include glycolipids (rhamnolipids and sophorolipids), phospholipids (phosphatidylethanolamine), lipopeptides (surfactin) and polymeric macromolecules (emulsan and alasan) (Abdel-Mawgoud et al., 2010).

Biosurfactants and their most studied representatives rhamnolipids are the focus of many studies because they are ecological alternatives to synthetic surfactants: they display lower toxicity, high surface activity and stability in a broad range of temperatures, pH and salinity. Most importantly, they are biodegradable and thus can be exploited in environmentally friendly technologies (Abdel-Mawgoud et al., 2010).

The glycolipid biosurfactants comprise a hydrophilic carbohydrate section and hydrophobic fatty acid chain. In rhamnolipids, the carbohydrate part consists of one or two rhamnose (Rha) moieties and the hydrophobic part is usually represented by one or more

*Abbreviations:* AHL, acylhomoserine lactone; C10, decanoic acid; C10:1, decenoic acid; CID, collision-induced dissociation; CMC, critical micelle concentration; PAH, polyaromatic hydrocarbon; Rha, rhamnose; MS, mass spectrometry; $MS^2$, tandem mass spectrometry; SDS, sodium dodecyl sulfate; *A.c.*, *A. calcoaceticus*; *E.a.*, *E. asburiae*; *P.a.*, *P. aeruginosa*.

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saturated or unsaturated 3-hydroxy fatty with C8–C14 carbon atoms (Soberon-Chavez, 2011). Rhamnolipid homologues RhaC10C10 and RhaRhaC10C10 are usually predominant components of rhamnolipid mixtures produced by *Pseudomonas aeruginosa*. Minor rhamnolipid congeners presence involving C8, C10, C12, and C14 3-hydroxy fatty acids (both saturated and unsaturated) were also reported (Abalos et al., 2001; Deziel et al., 2000).

The resultant proportion of rhamnolipid congeners is dependent on many parameters: the production microorganism, medium composition and cultivation conditions. The ratio of homologues determines the properties of the biosurfactant, and it was reported that even small differences in the mixture composition can have great influence on its physiochemical properties (Guo et al., 2009; Monteiro et al., 2007).

Several physiochemical properties of biosurfactant are crucial to ascertain its applicability. The critical micelle concentration (CMC) is an important primary characteristic of a surfactant, defined as the concentration of surfactants above which micelles form and surfactant monomers and micelles exist in dynamic equilibrium. Formation micelles causes the (aqueous surfactant) solution behaves as a microheterogeneous medium (solution is heterogeneous on a microscopic scale, even though is often homogeneous macroscopically) (Dominguez et al., 1997). In rhamnolipid biosurfactants, high CMC (230 mg/l) was reported for rhamnolipid mixtures with higher proportion of congeners with unsaturated fatty acids and low CMC values (11–20 mg/l) was observed in mixtures containing mainly mono-rhamnolipid with C10 fatty acids (Guo et al., 2009). The ability of surfactants to disperse specific hydrophobic compounds is evaluated by the emulsification and oil displacement test. In work by Pornsunthorntawee et al. (2008) rhamnolipids produced by *P. aeruginosa* SP4 were found to exhibit excellent emulsification properties for vegetable oils (palm oil, soybean oil, coconut oil, and olive oil) with comparison to synthetic surfactants SDS and Pluronic F-68 (triblock nonionic surfactant), but failed to emulsify short chain hydrocarbons (pentane, hexane, heptane, toluene, and 1-chlorobutane).

Zhang et al. (1997) reported that solubilization of phenanthrene in absence of biosurfactant was 0.69 mg/l whereas in the presence of purified rhamnolipids from *P. aeruginosa* ATCC 9027 (0.30–0.35 mmol/l) the solubilization of phenanthrene increased up to 35 mg/l. Study by Noordman et al. (2000) reported that rhamnolipids concentration below its CMC did not display solubilization activity for PAHs.

Most studies concerning bioreactor rhamnolipids production are focused on *P. aeruginosa*, a known human opportune pathogen. Although other rhamnolipid-producing bacteria have been reported, very little is yet known about the biotechnological potential of these species (Soberon-Chavez, 2011). The aim of this work was to examine, compare and characterize rhamnolipids production in bioreactor by strains *Acinetobacter calcoaceticus* B-59190 and *Enterobacter asburiae* B-59189 with an established rhamnolipid producer *P. aeruginosa* B-59188, with the emphasis on determination of composition and physiochemical characterization of rhamnolipid mixtures focused on their exploitability in bioremediation techniques. These strains were reported to produce rhamnolipid mixtures differing in the relative composition of rhamnolipid congeners (Hošková et al., 2013). Another aim was to investigate the possibility of co-culturing *A. calcoaceticus*, *E. asburiae* and *P. aeruginosa* in terms of overall production rhamnolipids and their qualitative composition. Resulting rhamnolipid mixtures characteristics were compared with surfactants obtained in single strain cultures. The structure of rhamnolipids and congener composition and relative ratio was investigated by ESI-MS/MS. The characterization of surfactants properties was done by emulsification test, phenanthrene solubilization test and oil displacement test.

## 2. Materials and 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, IL, USA.

### 2.2. Cultivation in Erlenmeyer flasks

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): $KH_2PO_4$ 3.4; $K_2HPO_4$ 4.4; $NaNO_3$ 15; NaCl 1.1; KCl 1.1; $MgSO_4 \cdot 7H_2O$ 0.5; $CaCl_2$ $9 \times 10^{-4}$; $FeSO_4 \cdot 7H_2O$ $2.8 \times 10^{-4}$; yeast extract 0.5; $ZnSO_4 \cdot 7H_2O$ $1.45 \times 10^{-3}$; $MnSO_4 \cdot 7H_2O$ $8.4 \times 10^{-4}$; $CuSO_4 \cdot 5H_2O$ $1.25 \times 10^{-3}$ containing sodium citrate (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. Biomass concentration was monitored by measuring optical density of cultivation media at 600 nm ($OD_{600}$).

### 2.3. Cultivation in a bioreactor

The cultivation was carried out in a 2.5 l Biostat A bioreactor (B. Braun Biotech International) with a 1.7 l working volume, equipped with foam recycling system, oxygen and pH electrodes. Temperature was maintained at 30.0 °C and aeration at 0.25 VVM. The initial pH of mineral medium was adjusted to 7.0 without regulation during cultivation. Mineral medium and preculture procedure were the same as in Erlenmeyer flask experiments. The foam recycling system consisted of a foam outlet in the headspace of the bioreactor, peristaltic pump that broke the foam into liquid state and an inlet into bioreactor.

### 2.4. Rhamnolipid determination and isolation

Rhamnolipids concentration in cultivation medium was determined spectrophotometrically as rhamnose content by the phenol-sulfuric method according to (Dubois et al., 1956) with rhamnose as standard. After determining the rhamnolipids composition, correlation factors between rhamnose and rhamnolipids content were calculated. Rhamnolipids concentration is displayed in the text and figures instead of rhamnose equivalents.

Rhamnolipids were isolated as described by Abdel-Mawgoud et al. (2010). Briefly, biomass was removed by centrifugation (9000 rpm, 15 min) and the supernatant (culture volume 1.7 l) was 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) and analyzed by MS.

### 2.5. 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 instrument 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 tandem MS ($MS^2$) 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.

### 2.6. 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).

For critical micelle concentration determination the crude isolates of rhamnolipids were diluted in mineral medium (concentrations 1–50 mg/l). A 5 μl drop of rhamnolipids solution was placed on a Teflon plate (constant temperature 30 °C) and a 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 rhamnolipids (expressed as rhamnose) concentration. The break point observed in the plot corresponded to the value of CMC.

### 2.7. Determination of emulsification activity

The surfactant emulsification activity was determined according to Pornsunthorntawee et al. (2008). Shortly, a mixture of 3 ml of the studied hydrocarbon or oil (hexane, crude petroleum, sunflower oil, and toluene) and 2 ml of 1 g/l of the rhamnolipids, Tween 80 or SDS was vortexed at a high speed for 2 min. The emulsion activity was investigated after 24 h and the emulsification index was calculated by dividing the measured height of the emulsion layer by the total height of the mixture and multiplying by 100.

### 2.8. Oil displacement test

The oil displacement test was done by adding 100 ml of distilled water to a petri dish with a diameter of 25 cm. After that, 40 μl of crude petroleum was dropped onto the surface of the water, followed by the addition of 20 μl of an aqueous solution of rhamnolipids, Tween or SDS (concentration 1 g/l) onto the surface of the oil. The diameter of the clear zone indicates the ability of surface tension reduction efficiency of a given biosurfactant. The experiment was replicated at least seven times for each surfactant. The binomial diameter allows an evaluation of the surface (Pornsunthorntawee et al., 2008).

### 2.9. Phenanthrene solubilization

The solubility of phenanthrene in the presence of biosurfactant solution was determined using batch test according to (Shin et al., 2004). An excess amount (0.2 g/l) of phenanthrene above its solubility was added to 10 ml deionized water with various concentrations of biosurfactant. Biosurfactant concentrations spanned over a wide range of values below and above CMC (0–250 mg/l). Samples were placed on an orbital shaker for 48 h, 100 rpm, at 20 °C. Afterwards samples were sedimented for 24 h, and supernatant was extracted by hexane. Extraction rate supernatant–hexane were 2:1. Hexane extract was analyzed for phenanthrene by gas chromatography (Focus GC, Thermo Scientific) with TR-V1 column (30 m, ID 0.25 mm, 1,4 μm) coupled with Dual Stage Quadrupole mass detector.

## 3. Results and discussion

Compared to their chemically synthesized counterparts, microbial surfactants have many properties exploitable in industry with less environmental risk and therefore are a subject of many studies (Abdel-Mawgoud et al., 2010; Banat et al., 2010; Soberon-Chavez, 2011). In the present research, the possibility of using selected microorganisms for the production of rhamnolipids suitable for commercial applications and the physiochemical properties of produced mixtures were examined. The bacterial strains *A. calcoaceticus* B-59190, *E. asburiae* B-59189 and *P. aeruginosa* B-59188 were employed. The ability of these strains to produce diverse rhamnolipid homologues was established in our previous work, in which also the medium composition (with emphasis on carbon, nitrogen and phosphorus sources) was investigated (Hošková et al., 2013).

Biosynthesis of rhamnolipids in *P. aeruginosa* is regulated through quorum sensing, where signaling molecules are acyl-homoserine lactones (AHLs). *A. calcoaceticus* and *E. asburiae* are also known to use AHLs in the quorum sensing control mechanism (Gonzalez et al., 2001; Lau et al., 2013). There was the question whether and to what extent these similar regulatory mechanisms influence the composition and quantity of rhamnolipid produced under cultivation conditions of mixed populations and whether this would be the appropriate way to prepare rhamnolipids with desirable properties for various applications. This paper proves the possibility of bioreactor co-culturing of these microorganisms and evaluates the composition and properties of produced rhamnolipid mixtures.

### 3.1. Rhamnolipids production and cell growth in Erlenmeyer flask and bioreactor

*A. calcoaceticus*, *E. asburiae* and *P. aeruginosa* and their mixed populations were cultivated in Erlenmeyer flasks and a bioreactor; biomass growth (determined as optical density at 600 nm) and rhamnolipids production were measured during the cultivation (see Fig. 1). The kinetics of growth had a similar progress in both cases, slower in the bioreactor for all tested strains and their combinations but with higher biomass yield. Rhamnolipids production was determined at three cultivation time points: end of lag-phase (5 h); end of exponential phase (50 h); late stationary phase (100 h) and in the late stationary phase were achieved highest rhamnolipids productions.

The production of rhamnolipids in individual growth phases is shown in Fig. 2 (Erlenmeyer flasks) and Fig. 3 (bioreactor). In Erlenmeyer flasks, the highest rhamnolipids production was observed in *A. calcoaceticus* cultivation (1.54 g/l). The highest productions in Erlenmeyer flasks reported so far for non-recombinant non-pathogenic microorganisms *Pseudomonas chlororaphis* and *Pseudomonas nitroreducens* are 1.0 g/l and 5.46 g/l, respectively (Gunther et al., 2005; Onwosi and Odibo, 2012). Although the growth rate was not substantially influenced by the experiment setup, the rhamnolipids concentrations obtained in the bioreactor were higher. Müller et al. (2010) reported a significant increase of rhamnolipids production by *P. aeruginosa* PAO1 when cultivation was transferred from shaken flasks to a large scale bioreactor. The same phenomenon was observed in this work; the transition from small scale cultivations (Erlenmeyer flasks) to a 1.7 l working volume bioreactor led to up to five times higher production in the bioreactor – in single strain cultures the rhamnolipids production was the highest in *P. aeruginosa* cultivation (4.8 g/l) and second highest (3.8 g/l) in *E. asburiae* cultivation.

The co-culturing of all tested strains did not negatively influence the growth and the resulting final concentrations of rhamnolipids was not significantly diminished. On the other hand, analysis of the qualitative composition of rhamnolipids derived from these mixed cultivations showed a clear shift in the distribution of particular groups of rhamnolipids. When compared with the

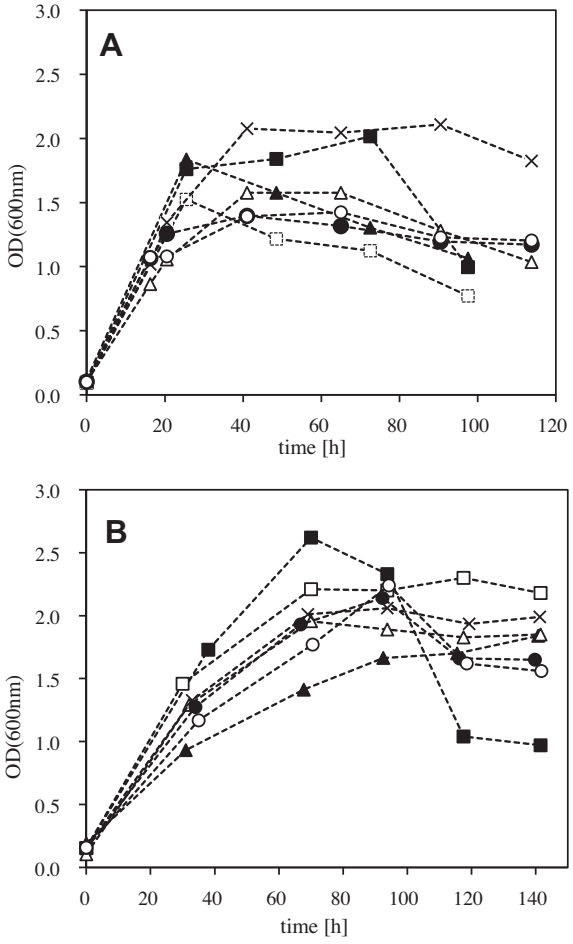


**Fig. 1.** Growth of *A. calcoaceticus* (*A.c.*), *E. asburiae* (*E.a.*) and *P. aeruginosa* (*P.a.*) and their mixed populations in Erlenmeyer flasks (A) and bioreactor (B) (■ *A.c.*; □ *E.a.*; ▲ *P.a.*; △ *A.c.* + *E.a.*; × *A.c.* + *P.a.*; ● *P.a.* + *E.a.*; ○ *A.c.* + *E.a.* + *P.a.*).

single strain cultivation, in all cultivation combinations of these strains was observed an increase in the content of almost all rhamnolipids congeners in the group RhaRhaFAFA and RhaFA and decrease of congeners in groups RhaFAFA and RhaRhaFA. As expected, rhamnolipids obtained by this process had different physico-chemical properties, as evidenced by the subsequent results.

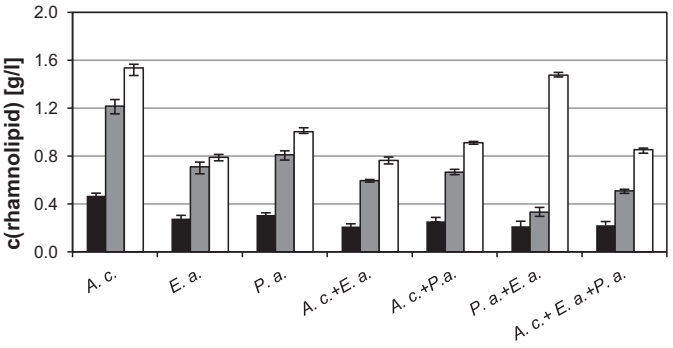


**Fig. 2.** Rhamnolipid production of *A. calcoaceticus* (*A.c.*), *E. asburiae* (*E.a.*) and *P. aeruginosa* (*P.a.*) and their mixed populations in Erlenmeyer flasks (■) end of lag-phase; (▩) end of exponential phase; □ late stationary phase).

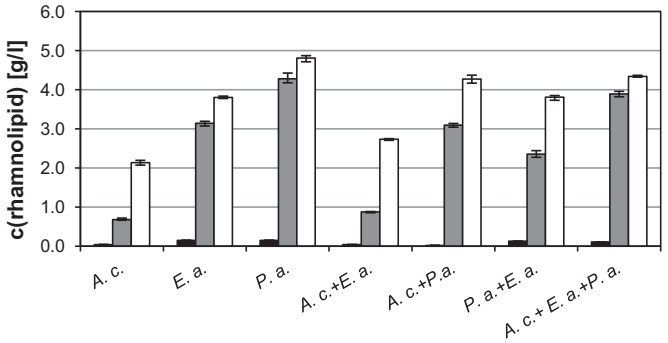


**Fig. 3.** Rhamnolipid production of *A. calcoaceticus* (*A.c.*), *E. asburiae* (*E.a.*) and *P. aeruginosa* (*P.a.*) and their mixed populations in bioreactor (■) end of lag-phase; (▩) end of exponential phase; □ late stationary phase).

### 3.2. Rhamnolipids characterization

#### 3.2.1. Critical micelle concentration

One of the most important physicochemical characteristic of a surfactant is its critical micelle concentration, which is the lowest concentration at which the given compound forms micelles (Pacwa-Plociniczak et al., 2011). Biosurfactants, especially rhamnolipids, are notable and easily exploitable by their very low CMCs compared to synthetic surfactants. The published results state a wide range of CMCs, from 5 to 200 mg/l depending on the rhamnolipid origin (Pornsunthorntawee et al., 2008; Samadi et al., 2012). Critical micelle concentration values (Table 1) revealed a strong dependence on the producing microorganism. In single strain cultures, *P. aeruginosa*-produced rhamnolipid mixture had the highest CMC – 56 mg/l, while both *A. alcoaceticus* and *E. asburiae* displayed CMC more than two-times lower (15 and 21 mg/l, respectively). Interestingly, rhamnolipid mixtures produced by co-culturing of *P. aeruginosa* and *E. asburiae* retained higher CMC (63 mg/l), while combination with *A. calcoaceticus* resulted in lowering of CMC to 10 mg/l, corresponding with the lowest CMC of *A. calcoaceticus* pure culture. This effect could be correlated with rhamnolipids composition (see Table 2) – rhamnolipids with more unsaturated fatty acids formed micelles at a higher concentration of the surfactant. The total content of rhamnolipids containing an unsaturated fatty acid was 17% in *P. aeruginosa* culture, 11% and 6% in *A. calcoaceticus* and *E. asburiae*, respectively. This finding is in agreement with the results presented by Guo et al. (2009) where *P. aeruginosa* produced rhamnolipids with a high content of unsaturated congeners (21% of total rhamnolipids) and the CMC was accordingly higher – 120 mg/l.

#### 3.2.2. Structural characterization of rhamnolipids by tandem mass spectrometry

The rhamnolipids obtained from bioreactor single strain and mixed cultures were examined to ascertain their properties and composition (see Table 2). The procedure of structural identification of individual rhamnolipids by tandem mass spectrometry is

**Table 1**
Critical micelle concentrations of rhamnolipids produced by *A. calcoaceticus*, *E. asburiae* and *P. aeruginosa* and their mixed populations.

| Microorganism | CMC c(rhamnolipid) [mg/l] |
|---|---|
| *A. calcoaceticus* | 15 |
| *E. asburiae* | 21 |
| *P. aeruginosa* | 56 |
| *A. calcoaceticus* + *E. asburiae* | 19 |
| *A. calcoaceticus* + *P. aeruginosa* | 10 |
| *P. aeruginosa* + *E. asburiae* | 63 |
| *A. calcoaceticus* + *E. asburiae* + *P. aeruginosa* | 11 |

**Table 2**
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.*) and their mixed populations cultivated in a bioreactor.

| | *A.c.* | *E.a.* | *P.a.* | *A.c.* + *E.a.* | *A.c.* + *P.a.* | *P.a.* + *E.a.* | *A.c.* + *E.a.* + *P.a.* |
|---|---|---|---|---|---|---|---|
| *RhaRhaFAFA* | | | | | | | |
| RhaRhaC8C8 | 0.4 | 0.3 | 0.4 | 0.4 | 0.6 | 0.5 | 0.6 |
| RhaRhaC8C10 | 1.5 | 2.2 | 2.4 | 2.3 | 3.3 | 3.5 | 3.0 |
| RhaRhaC10C8 | 0.5 | 0.6 | 0.3 | 0.7 | 0.6 | 0.6 | 0.7 |
| RhaRhaC10C10:1 | 0.0 | 0.0 | 0.9 | 0.0 | 0.8 | 0.8 | 0.6 |
| RhaRhaC10:1C10 | 0.0 | 0.0 | 0.2 | 0.0 | 0.2 | 0.2 | 0.2 |
| RhaRhaC10C10 | 10.3 | 14.9 | 6.0 | 16.2 | 13.1 | 14.5 | 13.7 |
| RhaRhaC10C12:1 | 2.3 | 1.2 | 0.2 | 2.3 | 1.8 | 0.9 | 1.7 |
| RhaRhaC12:1C10 | 0.6 | 0.1 | 0.1 | 0.4 | 0.4 | 0.0 | 0.5 |
| RhaRhaC10C12 | 1.3 | 1.6 | 2.0 | 1.8 | 2.7 | 2.8 | 2.4 |
| RhaRhaC12C10 | 0.7 | 0.9 | 0.9 | 1.0 | 1.3 | 1.3 | 1.3 |
| RhaRhaC12C12:1 | 0.2 | 0.0 | 0.2 | 0.0 | 0.2 | 0.1 | 0.3 |
| *RhaFAFA* | | | | | | | |
| RhaC8C8 | 0.2 | 0.1 | 0.4 | 0.0 | 0.1 | 0.1 | 0.2 |
| RhaC8C10 | 13.0 | 3.5 | 7.0 | 4.9 | 5.4 | 3.5 | 4.5 |
| RhaC10C8 | 1.4 | 1.4 | 1.3 | 0.9 | 0.7 | 0.9 | 0.9 |
| RhaC10C10 | 23.1 | 20.9 | 20.9 | 15.1 | 12.2 | 15.2 | 13.4 |
| RhaC10C10:1 | 1.0 | 0.0 | 5.1 | 0.2 | 1.7 | 1.4 | 1.2 |
| RhaC10:1C10 | 0.0 | 0.1 | 4.3 | 0.1 | 1.2 | 1.2 | 0.9 |
| RhaC10C12:1 | 3.5 | 2.0 | 3.1 | 1.7 | 1.8 | 1.7 | 1.7 |
| RhaC12:1C10 | 2.6 | 0.4 | 0.4 | 0.8 | 0.7 | 0.3 | 0.7 |
| RhaC10C12 | 3.2 | 0.9 | 1.0 | 1.2 | 1.1 | 0.6 | 1.1 |
| RhaC12C10 | 0.4 | 0.3 | 0.3 | 0.2 | 0.1 | 0.2 | 0.3 |
| RhaC12C12 | 0.2 | 0.1 | 0.4 | 0.0 | 0.1 | 0.1 | 0.2 |
| *RhaFA* | | | | | | | |
| RhaC8 | 1.7 | 1.3 | 0.4 | 2.6 | 2.3 | 1.6 | 2.3 |
| RhaC10:1 | 0.0 | 0.0 | 0.7 | 0.0 | 1.1 | 1.1 | 0.9 |
| RhaC10 | 9.6 | 14.9 | 5.4 | 20.2 | 18.9 | 20.5 | 19.2 |
| RhaC12 | 1.5 | 0.7 | 0.4 | 1.9 | 2.2 | 1.3 | 1.9 |
| RhaC12:1 | 0.1 | 0.2 | 0.2 | 0.2 | 0.4 | 0.5 | 0.5 |
| *RhaRhaFA* | | | | | | | |
| RhaRhaC8 | 2.7 | 2.1 | 1.6 | 2.4 | 2.2 | 1.4 | 2.1 |
| RhaRhaC10:1 | 0.0 | 0.0 | 4.1 | 0.0 | 1.4 | 1.4 | 1.1 |
| RhaRhaC10 | 13.0 | 26.7 | 25.5 | 18.5 | 17.1 | 19.4 | 18.1 |
| RhaRhaC12:1 | 3.2 | 1.4 | 2.1 | 2.5 | 2.6 | 1.3 | 2.2 |
| RhaRhaC12 | 1.8 | 1.2 | 1.8 | 1.5 | 1.7 | 1.1 | 1.6 |

described in our previous study (Hošková et al., 2013). Tandem mass spectrometry revealed partial differences in the quantitative proportions; these differences were reflected in the critical micelle concentrations of the rhamnolipids. The identified four classes of rhamnolipids contained only five fatty acids, with decanoic acid as the major component. This finding is in accordance with published data, where rhamnolipids produced by *P. aeruginosa* usually display low variability, with homologues containing decanoic acid being the most common (Abalos et al., 2001; Abdel-Mawgoud et al., 2010; Deziel et al., 2000). Also the first report of *A. calcoaceticus* and *E. asburiae* as rhamnolipid producers by Rooney et al. (2009) identified the same fatty acids (C8, C10, C12 and C12:1; decenoic acid was not reported) as rhamnolipids components and reported rhamnolipid homologues with C10 as the prevailing ones. As in these reports, also in our work, the observed differences were mainly in unsaturated minor rhamnolipid congeners. Particularly rhamnolipids containing C10:1 (decenoic acid) were found to be produced mainly by *P. aeruginosa*, the production by *A. calcoaceticus* and *E. asburiae* was observed only in insignificant quantities. The relative ratio of all rhamnolipid congeners containing decenoic acid was 12.5, 1.1 and 0.4% for *P. aeruginosa*, *A. calcoaceticus* and *E. asburiae*, respectively. In co-cultures, the presence of congeners with decenoic fatty acid was noted in cultivations with *P. aeruginosa* – 6.4% in co-culture with *A. calcoaceticus*, 6.1% in co-culture with *E. asburiae* and 4.9% in a mixed culture of all three strains. The lower relative content of these congeners in comparison with single strain *P. aeruginosa* cultivation corresponded with the trend in critical micelle concentration and indicated that the microorganisms in co-cultures did not significantly influence each other's metabolism, allowing the bacteria to produce rhamnolipid mixtures of specific characteristics.

### 3.2.3. Emulsification test

The *P. aeruginosa*-produced rhamnolipids emulsification abilities were reported to vary greatly depending on cultivation conditions, however, good stabilization and emulsification was reported for a wide range of oils and hydrocarbons (Pornsunthorntawee et al., 2008). Fig. 4 illustrates the emulsification index of the rhamnolipid mixtures in comparison to those of Tween 80 and SDS using model alkane (hexane), vegetable oil (sunflower oil), crude petroleum and aromatic compound (toluene). All rhamnolipid mixtures were able to provide equivalently high emulsification activities of crude petroleum and toluene in comparison with both Tween 80 and SDS. The hexane and sunflower oil emulsification was dependent on rhamnolipid mixture producer. *E. asburiae*-produced rhamnolipids failed to emulsify hexane as well as rhamnolipids produced by mixed population with *E. asburiae* as component. Similar results were observed with *P. aeruginosa* rhamnolipids and sunflower oil, although the mixed culture-produced rhamnolipids with *P. aeruginosa* displayed better emulsification abilities than the single strain rhamnolipids. When compared with synthetic surfactants, *A. calcoaceticus*, *P. aeruginosa* and their mixed populations were found to have higher emulsification index for hexane. The producer dependency was confirmed in comparison with study by Pornsunthorntawee et al. (2008) where rhamnolipids produced by *P. aeruginosa* displayed reverse properties compared to results in this work and emulsified vegetable oils but they failed to emulsify short-chain hydrocarbons. The excellent emulsification

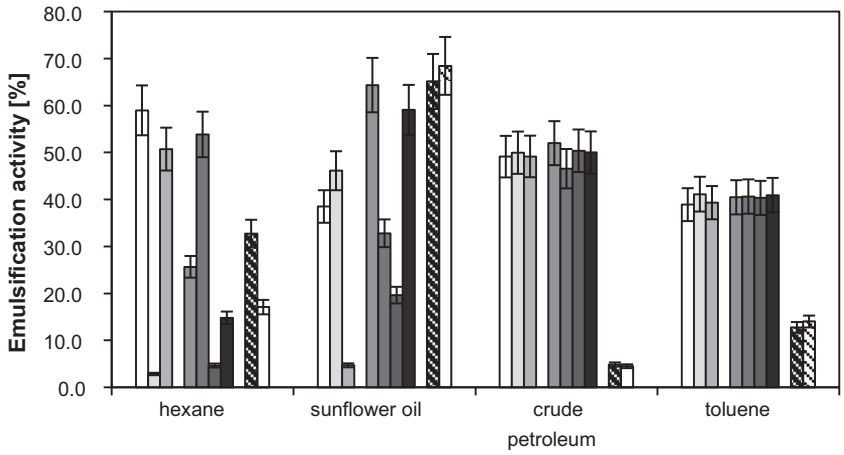


**Fig. 4.** Emulsification activity of the rhamnolipids isolated from *A. calcoaceticus* (*A.c.*), *E. asburiae* (*E.a.*) and *P. aeruginosa* (*P.a.*) and their mixed populations compared with synthetic surfactants (first bar group: single strain RLs □ *A.c.*; □ *E.a.*; ■ *P.a.*; second bar group: co-cultured RLs ■ *A.c.* + *E.a.*; ■ *A.c.* + *P.a.*; ■ *P.a.* + *E.a.*; ■ *A.c.* + *E.a.* + *P.a.*; third bar group: synthetic surfactants ▩ Tween 80; ▩ SDS).

properties of all rhamnolipids in comparison with synthetic surfactants toward aromatic hydrocarbons and crude petroleum may be advantageously exploited in bioremediation technologies.

### 3.2.4. Oil displacement test

The oil displacement test is an indirect indicator of the surface activity of a surfactant against specific oil. The larger the diameter of the clear zone, the higher the surface activity of tested surfactant solution (Rodrigues et al., 2006). In this study, the surface activities of rhamnolipids produced by single strain and mixed cultures were investigated in comparison with Tween 80 and SDS with crude petroleum as model oil. As shown in Fig. 5, rhamnolipids produced by all mixed cultures had the highest surface activity, while SDS displayed the lowest activity among tested surfactants. When compared to the synthetic surfactants, all tested rhamnolipid mixtures displayed higher surface activity, the rhamnolipids produced by mixed cultures displaying slightly higher activities.

### 3.2.5. Phenanthrene solubilization

The enhancement of phenanthrene solubilization by rhamnolipids in comparison with Tween 80 is shown in Fig. 6. All tested surfactants had high effect on solubilization of phenanthrene. The solubilization effects were dependent on surfactant concentration and producer, while rhamnolipids produced by *P. aeruginosa, E. asburiae* and their co-cultures displayed the highest activity. The highest solubilization of phenanthrene in biosurfactant solution was 23 mg/l (250 mg/l of biosurfactant, the highest tested concentration). The works by Shin et al. (2004) and Yin et al. (2009) report comparable achieved values of phenanthrene solubilization by biosurfactants in the same concentration.

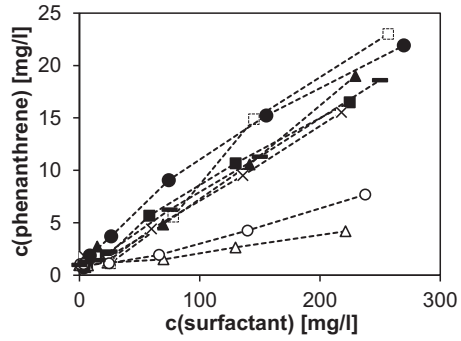


**Fig. 6.** Phenanthrene solubilization with rhamnolipids isolated from *A. calcoaceticus* (*A.c.*), *E. asburiae* (*E.a.*) and *P. aeruginosa* (*P.a.*) and their mixed populations compared with Tween 80 (■ *A.c.*; □ *E.a.*; ▲ *P.a.*; △ *A.c.* + *E.a.*; × *A.c.* + *P.a.*; ● *P.a.* + *E.a.*; ○ *A.c.* + *E.a.*+*P.a.*; —Tween 80).

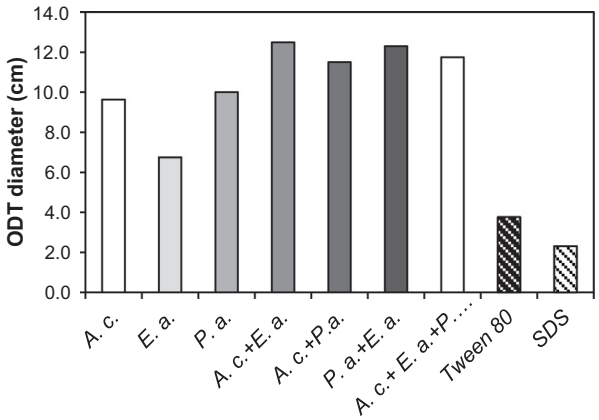


**Fig. 5.** Diameters of the clear zones on the oil surface obtained from oil displacement testing with the rhamnolipids isolated from *A. calcoaceticus* (*A.c.*), *E. asburiae* (*E.a.*) and *P. aeruginosa* (*P.a.*) and their mixed populations compared with Tween 80 and SDS.

## 4. Conclusion

The production and physio-chemical properties of rhamnolipids produced by single strain and mixed cultures of *A. calcoaceticus*, *E. asburiae* and *P. aeruginosa* were studied in this work. Structure and composition of rhamnolipids was determined by $MS^2$ and correlated with critical micelle concentration. Rhamnolipids with higher proportion of unsaturated fatty acids were found to form micelles at higher concentrations and therefore displayed accordingly higher critical micelle concentrations. Emulsification test, oil displacement test and phenanthrene solubilization were studied to elucidate and compare the applicability of all rhamnolipid mixtures. The physio-chemical properties were found to be producer-dependent with the rhamnolipids produced by mixed population displaying different behavior in comparison with their single strain culture-produced counterparts. Mixed populations thus represent a promising way for the relatively easy preparation of rhamnolipids possessing the desired properties for specific applications. In this regard, it will be necessary to further study the

relations and regulatory mechanisms between these microorganisms clarifying proven shifts in the composition of rhamnolipids.

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