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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; $CaCl_2$ 9×10^{-4}; $FeSO_4 \cdot 7H_2O$ 2.8×10^{-4}; yeast extract 0.5; $ZnSO_4 \cdot 7H_2O$ 1.45×10^{-3}; $MnSO_4 \cdot 7H_2O$ 8.4×10^{-4}; $CuSO_4 \cdot 5H_2O$ 1.25×10^{-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.