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

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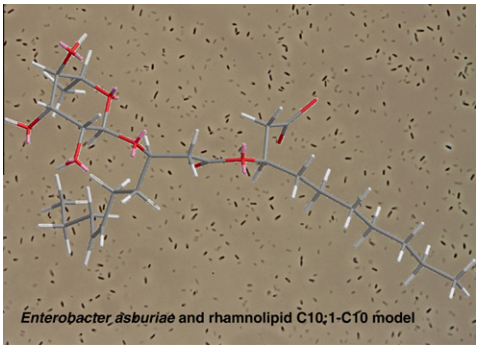
HIGHLIGHTS

- 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