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ORIGINAL ARTICLE

Comparative Analysis of Rhamnolipids from Novel Environmental Isolates of *Pseudomonas aeruginosa*

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Abstract A comparative analysis of rhamnolipids from environmental isolates of *Pseudomonas aeruginosa* was undertaken to evaluate strain-specific rhamnolipid fingerprints obtained under different growth conditions. Environmental isolates of *P. aeruginosa* produced rhamnolipids on different types of substrates, including cheap and renewable sources like sunflower oil from deep fryers and sunflower oil mill effluent. Rhamnolipids were monitored by high-performance liquid chromatography–electrospray ionization interface mass spectrometry, which allowed fast and reliable identification and quantification of the congeners present. The highest concentration of total rhamnolipids of 3.33 g/l was obtained by the strain *P. aeruginosa* 67, recovered from petroleum contaminated soil, and strains D1 (1.73 g/l) and D2 (1.70 g/l), recovered from natural microbial consortia originated from mazut-contaminated soil, grown on sunflower oil as a carbon source. Di- to mono-rhamnolipids ratios were in the range of 0.90–5.39 for different media composition and from 1.12 to 4.17 for different producing strains. Rhamnolipid profiles of purified mixtures of all tested strains are similar with chain length from C_8–C_{12}, pronounced abundance of Rha–C_{10}–C_{10} and Rha–Rha–C_{10}–C_{10} congeners, and a low content of 3-(3-hydroxyalkanoyloxy)-alkanoic acids. Concentrations of major congeners of RLs were found to slightly vary, depending on strain and growth conditions, while variations in minor congeners were more pronounced. Statistically significant increase of critical micelle concentration values was observed with lowering the ratio of total mono- to di-rhamnolipids ratio indicating that mono-rhamnolipids start to form micelles at lower concentration than di-rhamnolipids.

Keywords Rhamnolipids · HPLC–ESI–MS–MS · CMC · Environment · *Pseudomonas aeruginosa*

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Introduction

Rhamnolipids (RLs) are glycosides composed of rhamnose and lipid moieties. The variations in the lipid structure contribute largely to the biodiversity of RLs [1]. Some authors have divided the rhamnolipids into four different homologues: RL1 (mono-rhamno-di-lipidic), RL2 (mono-rhamno-mono-lipidic), RL3 (di-rhamno-di-lipidic) and RL4 (di-rhamno-mono-lipidic). RL1 and RL3 are described as the principal homologues, whereas RL2 and RL4 are described as the uncommon homologues being biosynthesized only under certain cultivation conditions [2]. In addition to RL, a family of 3-(3-hydroxyalkanoyloxy)-alkanoic acids (HAAs) with tensioactive properties, the precursors of rhamnolipids, have been found in culture media of *Pseudomonas aeruginosa* [3]. Previously reported studies showed that rhamnolipids play a major role in the architecture of biofilms of *P. aeruginosa* [4–6], whereas HAAs display potent surface-active properties and can act as modulators of swarming motility [7].

The development of sensitive, high throughput analytical techniques, such as soft ionization mass spectrometry has led to the discovery of a wide diversity of RL congeners, theirs homologues, as well as HAAs [3], produced by various *Pseudomonas* species [1]. Today, about 60 different RL congeners are known [1]. Variations in the nature and distribution of the different RL congeners identified in various RL mixtures were attributed to diverse cultivation conditions [8–10], as well as being *Pseudomonas* strain specific [1, 11]. Nevertheless, to date there are only a few reports where qualitative and quantitative rhamnolipid production of strains grown under the same conditions has been compared; whether in clinical [11] or environmental isolates [12–14]. Also, the effect of carbon sources on the nature and distribution of rhamnolipid homologues produced by the same bacterial strain has been sporadically studied [10, 15–17]. Thus, there is a particular lack of studies that describe comparatively the composition of RL mixtures and abundances of identified congeners in RL mixtures from environmental isolates.

Rhamnolipids are secondary metabolites and their production seems possible from most carbon sources supporting bacterial growth [2]. In addition to simple carbon sources, many strains of *Pseudomonas* are able to utilize a variety of unusual chemicals, including industrial waste and a wide range of hydrocarbons and their derivatives [18–21]. Being able to grow on a large number of hydrocarbons as the carbon and energy source, these strains are considered efficient hydrocarbon degraders [21]. At the same time, these strains are good producers of biosurfactants in hydrocarbon-rich culture medium [22]. It has long been speculated that release of RL is a part of naturally developed mechanisms for improved substrate uptake and it was concluded that the environmental conditions in the microorganisms' natural habitat impact their specific carbon source uptake [23]. Degradation of hydrocarbons by rhamnolipid-producing organisms is stimulated to a greater extent by autogenous rhamnolipids than by other biosurfactants [24] or synthetic surfactants [25], suggesting that interaction between rhamnolipid and rhamnolipid-producing strains is highly specific [26]. Furthermore, Gram-negative bacteria, including *Pseudomonas*, are often members of the natural microbial consortia, which collectively degrade complex mixtures of hydrocarbons such as petroleum products. However, there are a few reports regarding the specific biosurfactant profiles of *Pseudomonas* species isolated from such consortia.

From a practical aspect, RL biosurfactants are alternatives to the commonly used synthetic surfactants, because they are nontoxic to environment, showing comparable physicochemical properties to synthetic surfactants and can be produced by using renewable or waste substrates [20]. Important characteristics of RL surfactants, related to their amphiphilic structure, are surface activity, wetting ability, detergency, and other amphiphilic-related properties [1]. Reported data showed that rhamnolipids can lower surface tension from 72.75 to 30 mN/m [27, 28]. Recent study showed that application of rhamnolipid in the formulation of a detergent was effective in oil removal from the samples and that this formulation comparable with commercial powders in terms of stain removal [29]. However, there are obstacles standing against economic rhamnolipid production caused largely by lack of understanding of the environmental factors regulating rhamnolipid production in both quantitative and qualitative terms [11].

The present study reports the isolation and characterization of four novel rhamnolipid producing bacterial strains, production of RL on some low cost carbon sources and characterization of RL mixtures. This comparative research is the first to extensively examine the production and properties of rhamnolipids produced by five different bacterial environmental isolates of *P. aeruginosa*. Two of the examined strains were isolated as a single culture originated from metal cutting oil and soil contaminated with petroleum, while three strains were recovered from natural microbial consortia originated from soil contaminated with mazut. High-performance liquid chromatography–electrospray ionization interface (HPLC–ESI) mass spectrometry was used to characterize their specific RL fingerprints and to quantify the congeners present. Further characterization was performed by determination of critical micelle concentration (CMC) and surface activity of these natural rhamnolipid mixtures.

Experimental Procedures

Microorganisms

In the present study five strains of *P. aeruginosa* isolated from three different hydrocarbon-rich or hydrocarbon

derivatives-containing environments were used. Strains: 67, D1, D2 and D3 were isolated from soil samples collected for process monitoring during bioremediation. Strain 67 was isolated from a petroleum polluted soil sample with 7.7 g TPH (total petroleum hydrocarbon)/kg of dry soil originating from Pancevo Oil Refinery during transformation of saturated hydrocarbons in an ex-situ study of polluted soil bioremediation [30]. Strains D1–D3 were isolated from a soil sample (5.2 g TPH/kg dry soil) from a mazut bioremediation pile constructed in Dobanovci, Serbia, and represent a part of a natural consortium [31]. It might be interesting to mention that the consortia was used in an ex-situ field-scale bioremediation study where TPH content of the contaminated soil was reduced to 6 % of the initial value [31]. Such an efficient biodegradation might be related to biosurfactant induced solubilization of hydrocarbons. Strain *P. aeruginosa* NCAIM (P) B 001380, previously designated as *P. aeruginosa* san-ai was isolated from mineral cutting oil [32] and deposited in the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary. Strain *P. aeruginosa* ATCC 27853 was used as a reference being described as the prototype in different kinds of experiments, including RL investigations [33].

Isolation of Microorganisms

The bacterial strains: 67, D1, D2, and D3 were isolated from hydrocarbon rich environments in Serbia using selective agar media. Briefly, serial dilutions of soil samples in saline were prepared and used to inoculate mineral base agar [34] with 2 g/l diesel fuel as the sole carbon source, afterwards single oxidase positive colonies were transferred to *Pseudomonas* isolation agar (Difco Laboratories, USA) in order to obtain pure cultures using the streak plate method.

Detection of Surface Active Compounds Production

Among isolated strains, biosurfactant producers were selected using cetyltrimethyl ammonium bromide (CTAB) methylene blue agar (Liofilchem, Italy) [35]. The plates were inspected for the presence of dark blue halos around the bacterial colonies after 48 h of incubation at 37 °C. The isolated bacterial cultures were maintained on nutrient agar slants and stored at 4 °C.

Identification of Environmental Isolates of *P. aeruginosa* Strains by Biochemical and 16S rRNA Sequence Analysis

Strains were identified by means of 16S rRNA sequencing and biochemical tests, which appear to be the predominant strategy for characterization of new isolated *P. aeruginosa* strains in the literature [8, 10, 12].

For biochemical characterization of *P. aeruginosa* strains D1, D2, D3 and 67, commercial API 20 NE test and apiweb^{TM} software (www.biomerieux.com) were used.

For genomic DNA isolation, DNeasy Blood & Tissue Kit (Qiagen, USA) was used. The 16S rRNA sequences were determined by Macrogen service using 518F and 800R primers. Taxonomic analysis was conducted by the Genbank basic local alignment search tool (BLAST) program.

Culture Conditions

The strains were subcultured in nutrient agar (Torlak, Serbia) at 30 °C for 24 h, growing colonies were used to inoculate 500-ml Erlenmeyer flasks, containing 100 ml Kay's mineral medium (3 g/l $NH_4H_2PO_4$, 2 g/l K_2HPO_4, 20 g/l glucose, 0.5 mg/l $FeSO_4$, 1 g/l $MgSO_4$), [36]. Flasks were incubated at 30 °C for 20 h and agitated at 250 cycles/min on a horizontal shaker (Kühner, Switzerland) to prepare the seed culture.

One milliliter of 10^8 cfu of the seed culture was dispensed, into 500-ml Erlenmeyer flasks containing 100 ml of phosphate-limited proteose peptone-ammonium salt (PPAS) medium and incubated at 30 °C. PPAS is a modification of PPGAS (phosphate-limited peptone glucose ammonium salt) medium [36] with the following composition: 1.07 g/l NH_4Cl, 1.49 g/l KCl, 14.54 g/l Tris–HCl, 0.20 g/l $MgSO_4$, 10 g/l Proteose Peptone I and 20 g/l carbon source. As carbon sources: glucose (carbon source in original PPGAS medium), sunflower oil (Plima M, Serbia), sunflower oil from a deep fryer, and sunflower oil mill effluent (SOME) (Plima M, Serbia) were used. Two SOME fractions were tested: SOME-1, a residue after oil degumming (composed of water, oil and phosphatides), and SOME-2, obtained after fatty acids neutralization and saponification (composed of neutral oil, fatty acids and wax). Time course samples of the culture medium were drawn at appropriate time intervals and monitored for biosurfactant production, growth level, and surface tension.

Determination of RL Concentration in Fermentation Broth

The rhamnolipid was quantified in triplicate by the colorimetric determination of sugars with orcinol [37]. The rhamnolipid was purified from the supernatant obtained after removal of cells by centrifugation at 10,000 rpm for 20 min (mini centrifuge Denver Instruments, SAD). In 250 µl of supernatant, 250 µl of 500 mM glycine buffer (pH 2) was added, and after centrifugation (at 5,000 rpm for 10 min), the biosurfactant was extracted from the precipitate using 500 µl of a chloroform and methanol mixture (2:1, v/v), [38, 39]. The organic phase was evaporated to

dryness and 150 µl of H_2O was added. The concentration of the rhamnolipid was determined with 0.19 % orcinol (in 53 % H_2SO_4). After heating for 30 min at 80 °C the samples were cooled at room temperature and the OD_{421} was measured. The rhamnolipid concentration was calculated from standard curves prepared with L-rhamnose and expressed as a rhamnose equivalent.

Extraction of RL

Total RL was extracted from fermentation supernatants after separation of the bacterial cells by centrifugation at 10,000 rpm for 20 min (Sorvall, UK, rotor SS-1). A crude preparation of RL was obtained by acidic precipitation, followed by extraction with a mixture of chloroform and methanol as previously described [38]. Dried RL extracts were resuspended in methanol, before analysis by HPLC–ESI mass spectrometry.

CMC–Surface Tension Assay

Surface tension and the CMC were measured using a Krüss K12 tensiometer (Tensiometer K9, Krüss ETS-S) tensiometer using the du Nouy ring method [12, 40]. Surface activity was determined for supernatants, after separation of the bacterial cells by centrifugation of the culture from the mid stationary phase of growth, when maximum biosurfactant production was detected. The CMC was determined for extracted RL mixtures dissolved in distilled water at concentrations ranging from 200 to 5 mg/l [14]. The CMC value, expressed in mg/l, was estimated from the breakpoint of the surface tension versus the concentration. Analyses were performed in four replicates at room temperature (25 ± 1 °C). Distilled water was used for instrument calibration.

Surface activities of in-silico generated RL and HAA were calculated by ACD ChemSketch for mono-RLs (chain lengths form C_8 to C_{12}), di-RL (chain lengths C_8 to C_{12}) and HAA (chain lengths from C_6 to C_{12}) (ACD/ChemSketch Freeware, version 10.00 Advanced Chemistry Development, Inc. Toronto, Canada, www.acdlabs.com, 2012).

Structural Characterization and Quantification of Rhamnolipids

LC/MS analysis was performed according to Déziel et al. [41] and Dubeau et al. [16], with some modifications. To prepare samples for LC/MS analysis, dried RL extracts were dissolved in 1 ml methanol and diluted 200 times in water: acetonitrile (30:70) containing 5 mg/l deuterium-labeled 4-hydroxy-2-heptylquinoline (HHQ-D4) as an internal standard. The analyses were performed on a high-performance liquid chromatograph (HPLC; Waters 2795, Alliance HT) equipped with a C_8 reversed phase column (Eclipse XDB-C8, particle size of 5 µm, and of 4.6 × 150 mm dimensions, Agilent, USA). The detector was a triple tandem quadrupole mass spectrometer (qMS/MS, Quattro Premier XE, Micromass) equipped with an electrospray ionization interface (ESI; UPLC-ESI-qMS/MS). All rhamnolipids were detected and quantified in the negative ion mode generating $[M–H]^-$ pseudomolecular ions. Data were processed by MassLynx™ software with its QuanLynx™ function (version 4.1, Waters).

HPLC separation of rhamnolipids was performed with a sample injection volume of 20 µL, mobile phase at a flow rate of 0.4 ml/min with a gradient of water (C): acetonitrile (D), both containing 2 mM ammonium acetate, pH 8. The 20-min gradient was set according to the following: 0 min, 30 % C + 70 % D; 1 min, 25 % C + 75 % D; 10 min, 100 % D; 16 min, 100 % D; 17 min, 30 % C + 70 % D; 20 min, 30 % C + 70 % D. The mobile phase flow was split through a Valco Tee with 10 % of the flow introduced into the mass spectrometer. The mass spectrometer was in the negative ESI mode with capillary voltage set at 3.0 kV, cone voltage at 20 V, source and desolvation temperatures of 120 and 150 °C respectively, cone and desolvation gas (N_2) flow of 15 and 100 l/h, respectively. The scanning mass range was from 130 to 800 Da. Quantification was performed by comparing integration data of the pseudomolecular ion $[M–H]^-$ peak areas of different rhamnolipids homologues with those of the internal standard (HHQ-D4) using response factors previously determined through calibration curves made with a mono-RL and di-RL standards. Collision-induced dissociation (CID) experiments for fragmentation of the parent molecular ion were performed using argon as the collision gas at a pressure of 3.28×10^{-3} mBar, corresponding to a flow of 0.35 ml/min. The scanning mass range for daughter ions was from 100 to 530 Da for mono-RL and from 100 to 650 Da for di-RL. The collision energy was adjusted at 20 eV for both molecules.

Statistical Analysis

Statistical analysis was performed by SPSS (Version, 11.5) Pearson's correlation was used for determining any significant correlation between CMC values and the ratio of: total mono-RL/di-RL and the major congeners Rha–C_{10}–C_{10}/Rha–Rha–C_{10}–C_{10}. Significance was determined at the 0.05 level of significance.

Results and Discussion

Characterization of Environmental Isolates of *P. aeruginosa*

Environmental strains of *P. aeruginosa* were isolated from three different hydrocarbon-rich environments by an

enrichment technique. Bacterial isolates were assayed by CTAB-Methylene Blue agar method to verify their ability to produce biosurfactant. The bacterial strains with the highest biosurfactant production according to the diameter of the blue halo on CTAB agar were selected for further examination. Strain 67 was isolated from a petroleum-polluted soil sample while strains D1–D3 were isolated from a mazut bioremediation pile, representing a part of a natural consortium. The consortium was used in an ex-situ field-scale bioremediation study of mazut- and mazut-sediment-polluted soil with the high efficacy of TPH reduction (94 % of the initial value). Such an efficient biodegradation might be related to the biosurfactant induced solubilization of hydrocarbons. Previous studies unambiguously confirmed the potential for application in bioremediation processes of *P. aeruginosa* consortium D1–D3 and strain 67 [30, 31]. Strain *P. aeruginosa* NCAIM (P) B 001380, formerly named as san-ai, isolated from mineral cutting oil was chosen for this investigation as a well-characterized producer of several potentially applicable substances [32, 42, 43]. Results of biochemical, taxonomic and physico-chemical characterization of environmental strains of *P. aeruginosa* and reference strain ATCC 27853 are listed in Table 1.

BLAST search of partial 16S rRNA sequences of bacterial isolates revealed similarity to *P. aeruginosa* (Table 1).

The API-based biochemical characterization of four environmental isolates of *P. aeruginosa* D1, D2, D3 and 67 confirmed that all strains were *P. aeruginosa* species (Table 1, Supplemental material Table 1). Taxonomic characterization of *P. aeruginosa* NCAIM (P) B 001380 was previously reported [32].

Fermentation broths of tested strains obtained in the PPAS medium with sunflower oil as a carbon source were collected in the mid stationary phase of growth, when the maximum of RL production occurred and were subjected to measurement of surface activities. The minimum values to which the surface tension was reduced were found to be in the range of 36.3–39.9 mN/m (with standard deviations below 5 %), (Table 1). The range of values obtained for the surface activity of the fermentation broth was in agreement with previous studies (the surface activity was in the range from 22 to 52 mN/m), indicating that surface activity depended on medium composition, time of cultivation and strain [10, 12].

Characterization of Rhamnolipids from *P. aeruginosa* Environmental Isolates

HPLC–ESI MS Analysis of RL of P. aeruginosa

Production Profile of RL from P. aeruginosa NCAIM (P) B 001380 Grown on PPAS with Different Sources of Carbon The great potential for the use of RL as environmentally compatible agents has resulted in a demand for their improved production based on: high yield, low-cost and ecologic processes. There is a wide range of natural and petrochemical carbon sources that have been investigated for use in an economic production of RL [44]. An adequate carbon source for industrial application should be low cost but effective. In general, crude and waste materials are particularly promising carbon sources.

Strain *P. aeruginosa* NCAIM (P) B 001380 was chosen for this investigation of the carbon source effect on RL production [32, 42, 43]. In order to reveal the effect of the carbon source on the RL production profile of *P. aeruginosa* NCAIM with regard to composition, relative abundance and concentration of congeners, RL were monitored on PPAS medium supplemented with various carbon sources including pure commercial renewable (glucose and sunflower oil) and complex resources such as wastes from the oil industry (sunflower oil from deep fryers and two fractions of sunflower oil mill effluent—SOME-1 and SOME-2). RL were extracted from culture broths at the stationary phase of growth when highest RL production was detected as determined using an orcinol assay (data not shown). Under our experimental conditions, oil from a deep fryer, sunflower oil and oil waste SOME-1, were found to be the best carbon sources for rhamnolipid production with total rhamnolipids yields of: 1.30, 0.43, and 0.40 g/l, respectively (Table 2). Different compositions and complexity of sunflower oil and oil waste (oil from a deep fryer and SOME-1) resulted in different RL yields. Namely, commercial sunflower oil is mainly triglycerides of palmitic, stearic, oleic and linoleic acids, while oil waste, apart from triglycerides, comprises phosphatides (SOME-1) and a variety of short chain products which are produced with subjection of sunflower oil to high temperature and oxygen during the deep frying process.

Confirmation of the identity of rhamnolipids was made by detection of the masses of different RL homologues followed by fragmentation analysis of the identified homologues using HPLC coupled with tandem MS/MS. Identified RL congeners with their production and relative abundances are shown in Tables 2 and 3.

The highest concentrations of the major Rha–C_{10}–C_{10} and Rha–Rha–C_{10}–C_{10} were found to be 850.00 and 314.02 mg/l for sunflower oil from a deep fryer. In RL mixtures of *P. aeruginosa* NCAIM strain, 3-hydroxy fatty acid moieties were also detected and were comprised of chain lengths varying from C_8–C_8 to C_{10}–$C_{12:1}$ (Table 2). Using all tested carbon sources, Rha–Rha–C_{10}–C_{10} was found to be the most abundant di-rhamnolipid congener (relative abundance values ranging from 24 to 72 % of the total rhamnolipids), while Rha–C_{10}–C_{10} dominates as the mono-rhamnolipid congeners (relative abundance of 11–66 %), (Table 2). Rha–C_{10}, Rha–$C_{12:2}$ and Rha–C_{10}–

Table 1 Taxonomical, biochemical and physico-chemical characteristics of five environmental *P. aeruginosa* strain and clinical reference strain ATCC 27853: API numbers, 16S RNA GenBank accession numbers, surface activity of fermentation broth and CMC values with surface activity of isolated rhamnolipid mixtures

Characterization	*P. aeruginosa* strain					
	NCAIM (P) B 001380	D1	D2	D3	67	ATCC 27853
Taxonomical						
Shape	Rod	Rod	Rod	Rod	Rod	Rod
Size (μm)	0.5 × 1.0	0.5 × 1.0	0.5 × 1.0	0.5 × 1.0	0.5 × 1.0	0.5 × 1.0
Motility	+	+	+	+	+	+
Flagella arrangement	−	−	−	−	−	−
Gram stain	−	−	−	−	−	−
Spore formation	−	−	−	−	−	−
Growth temperature (°C)	30–45	30–45	30–45	30–45	30–45	30–45
16S RNA GenBank accession number	JQ012798	JN995662	JN995663	JN995664	JN995661	AY268175
Production of pigment pyocyanin	+	+	+	+	+	+
Production of pigment pyorubin	−	−	−	+	−	−
Biochemical						
API profile	[a]	1 354 475	1 354 475	1 354 575	1 354 475	1 154 575
Surface activity of fermentation broth (mN/m)[b]	38.9	39.9	39.1	36.3	38.1	37.3
Surface activity of isolated rhamnolipid mixture (mN/m)[b]	40.1	39.6	39.8	38.8	40.5	39.1
CMC (mg/l)	131	162	143	135	131	88

[a] The taxonomic characterization of *P. aeruginosa* NCAIM (P) B 001380 was previously reported [32]

[b] Standard deviations below 5 %

C_{12} represented the minor mono-RL congeners (below 1 %), while in di-RL fraction, Rha–Rha–C_8–C_{10} was the low abundant congener (range 1–4.5 %). Rha–Rha–C_{12}–C_{12} was the minor congener (below 1 %), for all tested carbon sources (Table 2).

The ratio of di-/mono-RL was highest for SOME-1 and SOME-2 (5.39 and 3.82, respectively), while a lower ratio was found when using sunflower oil as the carbon source (1.37), indicating that these carbon sources favorably produce di-rhamnolipids (Table 2). On the other hand, glucose and sunflower oil from a deep fryer had a higher % of mono-rhamnolipids, where the di-/mono-RL ratio was found to be 0.90 and 0.43, respectively (Table 2).

The present study showed that there were variations in distribution and concentrations of the different RL congeners using different carbon sources, which is consistent with previous studies [9, 10, 16]. It was shown that for different carbon sources, overall production was characterized with the same major congeners and variations of the minor congeners [16]. A higher ratio of di-RLs/mono-RLs of about four was connected with genetic aspect of rhamnolipid biosynthesis [16]. Comparing the composition of rhamnolipid mixtures produced by our strain NCAIM and strain NY3 obtained with glucose as a carbon source (2 %) on PPAS medium [10], a difference in the yield of rhamnolipids (strain NCAIM: 0.27 and strain NY3: 0.20 g/l) and in the total number of detected RL congeners (strain NCAIM-9, and strain NY3-25) was noticed implying that the strain origin affects rhamnolipid production.

Production Profile of RL from Different Environmental Isolates of P. aeruginosa Grown on PPAS Medium with Sunflower Oil as a Carbon Source Although, current evidence confirms that RLs primarily play a role in surface-associated modes of bacterial motility and are involved in biofilm development, the reason and mechanics behind their production still remain unclear [23]. On the other hand, since the low solubility of petroleum hydrocarbons limits their bioavailability to biodegraders, it was expected that surfactant-induced solubilization would result in an enhanced biodegradation process [45]. Therefore, the exact role or function of RL for the producing strain is still unknown. We wanted to study the variations in RL production profiles by strains obtained from different environmental niches to tentatively guess their potential function.

In the light of reported data, the biosurfactant production profile (composition, abundance and concentration of congeners) of five different environmental isolates of *P. aeruginosa*: NCAIM (from mineral cutting oil), strains from consortium: D1, D2 and D3 (soil contaminated with

Table 2 Rhamnolipid congeners of *P. aeruginosa* NCAIM (P) B 001380 present in a PPAS medium with different carbon sources, with their production and relative abundance detected by HPLC-ESI MS analysis

Carbon sources	Production (mg/l) and relative abundances (%) of HAAs and rhamnolipid congeners of *P. aeruginosa* NCAIM (P) B 001380 obtained on PPAS medium with different carbon sources[a]									
	Glucose		SOME-1		SOME2		Sunflower oil from deep fryer		Sunflower oil	
HAA/rhamnolipid	mg/l	%	mg/l	%	mg/l	%	mg/l	%	mg/l	%
C_8–C_8/C_6–C_{10}	0.01	–	0.54	–	0.04	–	0.30	–	0.13	–
C_8–C_{10}	0.06	–	0.89	–	0.07	–	0.67	–	0.25	–
C_{10}–C_{10}	0.06	–	1.14	–	0.15	–	4.49	–	0.97	–
C_{10}–C_{12}	0.01	–	0.11	–	0.02	–	0.68	–	0.13	–
C_{12}–C_{12}	0.16	–	5.68	–	0.07	–	40.61	–	13.76	–
C_8–$C_{12:1}$	<0.01	–	0.27	–	0.02	–	1.06	–	1.10	–
C_{10}–$C_{12:1}$	<0.01	–	0.13	–	0.04	–	0.56	–	0.11	–
Total HAA	0.30	–	8.77	–	0.40	–	48.37	–	16.45	–
Rha–C_{10}	0.29	0.11	0.44	0.11	0.35	0.26	1.19	0.09	0.34	0.08
Rha–$C_{12:2}$	0.04	0.02	0.90	0.23	0.16	0.12	9.76	0.75	2.79	0.65
Rha–C_8–C_{10}	10.10	3.73	11.18	2.82	1.85	1.41	18.05	1.39	6.56	1.54
Rha–C_{10}–C_{10}	124.69	46.13	44.64	11.27	23.13	17.57	850.00	65.59	162.69	38.05
Rha–C_{10}–$C_{12:1}$	7.05	2.61	2.27	0.57	1.70	1.29	25.87	2.00	7.65	1.79
Rha–C_{10}–C_{12}	0.07	0.02	2.50	0.63	0.14	0.11	0.08	0.01	0.73	0.17
Rha–Rha–C_8–C_{10}	7.10	2.63	18.10	4.57	5.59	4.24	15.43	1.19	7.78	1.82
Rha–Rha–C_{10}–C_{10}	92.77	34.32	288.39	72.82	78.16	59.35	314.02	24.23	208.66	48.80
Rha–Rha–C_{10}–C_{12}	26.99	9.98	26.97	6.81	19.80	15.04	58.95	4.55	29.10	6.81
Rha–Rha–C_{12}–C_{12}	1.27	0.47	0.69	0.17	0.80	0.61	2.62	0.20	1.31	0.31
Total RLs	270.36	100	396.08	100	131.69	100	1,296.97	100	427.62	100
Total mono-RLs	142.23	52.61	6.94	15.64	27.33	20.75	904.95	69.83	180.77	42.27
Total di-RL	128.13	47.39	334.14	84.36	104.36	79.25	391.02	30.17	246.85	57.73
Ratio total di-/mono-RL	0.90		5.39		3.82		0.43		1.37	

[a] All determinations of HAAs and RLs were performed in triplicate and data are given as average values

mazut), strain 67 (soil contaminated with petroleum) and one reference strain ATCC 27853, was monitored on the PPAS medium supplemented with sunflower oil at the stationary phase of growth when the highest RL production was detected as determined with an orcinol assay. The highest concentration of total rhamnolipids, obtained for strain 67, was 3.33 g/l (Table 3). Rha–C_{10}–C_{10} and Rha–Rha–C_{10}–C_{10} were found to be the major mono- and di-RL congeners while the minor ones were: Rha–C_{10} and Rha–C_{10}–C_{12} (abundance below 0.2 %) and for di-RL Rha–Rha–C_{12} (abundance below 0.5 %) for all strains (Table 3). The ratio of di-RLs/mono-RLs was larger than 1.00 for all *P. aeruginosa* strains showing a higher abundance of di-RLs, especially for strains D1 and D2 with the highest values for ratio of di- to mono-RLs (Table 3). HAA was also detected in RL extracts of all six *P. aeruginosa* strains. Their 3-hydroxy fatty acid moieties were comprised of chains varying from C_8–C_8 to C_{10}–$C_{12:1}$ (Table 3).

Being isolated from hydrocarbons rich environments, where the low solubility of hydrocarbons limits their bioavailability to biodegraders, it was expected that all environmental isolates of *P. aeruginosa* would show a significant potential for RL production. Indeed, all strains, especially strains 67, D1 and D2, were found to be good RL producers. The reference strain *P. aeruginosa* ATCC 27853, in comparison with environmental isolates, had lower RL production (with the exception of strain D3). Interestingly, although *P. aeruginosa* D1, D2 and D3, were recovered from the same microbial consortium (originated from mazut-contaminated soil) and with similar physiological-biochemical characteristics, they showed differences in their yields of total RL and the ratio di-RL/mono-RL. The reason for such differences in RL production could be an adaptation of the surfactant-producing bacterial consortium on living in this hydrocarbon-contaminated environment.

Critical Micelle Concentration

The critical micelle concentration was determined for RL mixtures extracted from the PPAS medium supplemented

Table 3 Rhamnolipid congeners of different *P. aeruginosa* strains present in a PPAS medium, with sunflower oil as a carbon source, with their production and abundances, detected by HPLC–ESI MS analysis

	Production (mg/l) and relative abundances (%) of HAAs and rhamnolipid congeners obtained on PPAS medium with sunflower oil as a carbon source[a]											
P. aeruginosa strain	D1		D2		D3		67		NCAIM (P) B 001380		ATCC 27853	
HAA/rhamnolipid	mg/l	%	mg/l	%	mg/l	%	mg/l	%	mg/l	%	mg/l	%
C_8–C_8/C_6–C_{10}	0.02	–	0.01	–	0.05	–	7.00	–	0.13	–	0.01	–
C_8–C_{10}	0.23	–	0.05	–	0.30	–	30.53	–	0.25	–	0.10	–
C_{10}–C_{10}	5.51	–	6.76	–	0.66	–	76.48	–	0.97	–	0.46	–
C_{10}–C_{12}	0.98	–	1.11	–	0.06	–	3.33	–	0.13	–	0.04	–
C_{12}–C_{12}	8.16	–	7.18	–	5.39	–	0.60	–	13.76	–	2.22	–
C_8–$C_{12:1}$	1.11	–	0.60	–	0.03	–	19.74	–	1.10	–	0.09	–
C_{10}–$C_{12:1}$	0.72	–	0.87	–	0.05	–	2.66	–	0.11	–	0.05	–
Total HAA	16.73	–	16.58	–	6.55	–	140.34	–	16.45	–	2.97	–
Rha–C_{10}	1.21	0.07	0.59	0.03	0.07	0.13	2.07	0.06	0.34	0.08	0.18	0.17
Rha–$C_{12:2}$	9.36	0.54	10.40	0.61	2.14	4.01	68.32	2.05	2.79	0.65	1.29	1.20
Rha–C_8–C_{10}	9.14	0.53	8.28	0.49	0.51	0.96	35.78	1.07	6.56	1.54	1.52	1.41
Rha–C_{10}–C_{10}	300.86	17.40	433.41	25.40	17.76	33.26	1,327.23	39.75	162.69	38.05	45.52	42.19
Rha–C_{10}–$C_{12:1}$	13.55	0.78	19.04	1.12	1.12	2.10	29.12	0.87	7.65	1.79	2.22	2.06
Rha–C_{10}–C_{12}	0.05	<0.01	0.07	<0.01	0.26	0.49	0.10	<0.01	0.73	0.17	0.07	0.07
Rha–Rha–C_8–C_{10}	38.25	2.21	28.91	1.69	0.77	1.44	71.83	2.15	7.78	1.82	1.36	1.26
Rha–Rha–C_{10}–C_{10}	1,197.78	69.27	1,052.12	61.67	26.88	50.33	1,685.65	50.48	208.66	48.80	46.96	43.50
Rha–Rha–C_{10}–C_{12}	152.95	8.85	147.85	8.67	3.79	7.10	113.76	3.41	29.10	6.81	8.51	7.88
Rha–Rha–C_{12}–C_{12}	5.97	0.35	5.36	0.31	0.09	0.16	5.13	0.15	1.31	0.31	0.28	0.26
Total RLs	1,729.16	100	1,706.04	100	53.38	100	3,339.00	100	427.62	100	107.92	100
Total mono-RLs	334.21	19.33	471.79	27.65	21.86	40.95	1,462.63	43.80	180.77	42.27	50.82	47.09
Total di-RL	1,394.95	80.67	1,234.25	72.35	31.52	59.05	1,876.37	56.20	246.85	57.73	57.10	52.91
Ratio total mono-/di-RL	0.24		0.38		0.68		0.78		0.73		0.88	
Ratio total di-/mono-RL	4.17		2.62		1.44		1.28		1.37		1.12	

[a] All determinations of HAAs and RLs were performed in triplicate and data are given as average values

with sunflower oil as a carbon source in the mid stationary phase of bacterial growth. CMC determination was performed to investigate the potential of rhamnolipid mixtures produced by different *P. aeruginosa* isolates that have different RL production profiles, to lower surface tension. It is interesting to note that surface activities of in-silico generated RL and HAA with chain lengths from C_8 to C_{12}, calculated by ACD ChemSketch were found to be 47–53 for mono-RLs, 53–57 for di-RLs and 37–40 mN/m for HAAs. CMC values with surface activities of isolated RL mixtures determined in our investigation are shown in Table 1 and are consistent with reported data for surface tension values of RL mixtures, which are around 30 mN/m, and for CMC values which are in the order of 20–200 mg/l [14, 27].

In light of the presented data, RL profiles of examined environmental isolates were further analyzed to explain the chemical basis of determined CMC values. It has been reported that the ratio and composition of the homologues, the presence of unsaturated bonds, the branching and length of the alkylic chain, or the size of the hydrophilic head group of the surfactant can all affect the CMC values [14, 16]. Beside this, HAAs detected in RL mixtures, also have a potential for lowering surface tension even to a greater extent than RL [16]. In the present case, isolated RL mixtures of all the tested strains are similar with chain lengths from C_8–C_{12}, with a pronounced abundance of Rha–C_{10}–C_{10} and Rha–Rha–C_{10}–C_{10} congeners, and with a low content of HAAs. Composition of RL mixtures, where only small concentrations of HAAs were detected, could indicate that RL congeners, rather than HAA, mainly contribute to surface activity and affect CMC values. Albeit having very similar RL profiles, the ratio of total di-RL/mono-RL differs between strains. Statistically significant correlations were found between CMC values and the ratio of total mono-/di-RL ($P = 0.033$) and between CMC values and the ratio of total Rha–C_{10}–C_{10}/Rha–Rha–C_{10}–C_{10} ($P = 0.015$) as well. A lower CMC arises from a

higher mono-/di-RL ratio indicating that mono-RL start to form micelles at lower concentrations than di-RL. On the other hand, it was reported that di-rhamnolipids have better bioavailability than mono-rhamnolipids [41, 46, 47]. Zhang et al. [47] found that bioremediation of phenanthrene was more effective in di-rhamnolipids solutions than in mono-rhamnolipids solution and suggested that di-rhamnolipids were more useful for remediation of contaminated sites. Moreover, di-rhamnolipids are also known to be useful in clinical application. In light of the favorable features of di-rhamnolipids, their improved production is desirable. This study successfully pointed out conditions for their improved production.

Comparative investigation of RL from environmental isolates of *P. aeruginosa* gave an insight into their physicochemical properties, as well as the composition and distribution of different congeners, which would facilitate a better selection of media and strains that efficiently produce rhamnolipids with improved characteristics for potential application.

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