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High-performance liquid chromatographic determination of the rhamnolipids produced by *Pseudomonas aeruginosa*

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Abstract

The bacterial biosurfactants 3-[3'-(L-rhamnopyranosyloxy)decanoyloxy]decanoic acid (RL-1) and 3-[3'-(2''-O-α-L-rhamnopyranosyl-α-L-rhamnopyranosyloxy)decanoyloxy]decanoic acid (RL-2) were isolated from *Pseudomonas aeruginosa* DSM 2659 cultures. An HPLC method was developed for the *p*-bromophenacyl esters of the rhamnolipids. Separation was obtained within 25 min on a RP C_{18} column using a linear gradient of water–acetonitrile (30:70 to 0:100) and UV detection (265 and 320 nm). Linearity of response existed for 1.2–25 μg of the RL-1- and 1.8–25 μg of the RL-2-*p*-bromophenacyl ester (0.9–19.2 μg RL-1 and 1.3–18.0 μg RL-2). The reproducibility of the entire analytical method (extraction, derivatisation) was tested.

1. Introduction

In recent years, interest in microbial surfactants has been increasing, because these natural products are considered both an alternative and addition to synthetic surfactants. The advantages of biosurfactants compared to synthetic products are their biodegradability, low toxicity, and simple production by microbial fermentation processes [1]. Biosurfactants show a wide range of applications, such as clean-up of oil spills, secondary and tertiary oil recovery. It is also possible to use biosurfactants as additives in cosmetics, foodstuffs, beverages and pharmaceutical products [2].

Aerobic bacteria of the species of *Pseudomonas aeruginosa* are known to produce as biosurfactants four chemically closely related glycolipids, when grown on glucose, glycerol or *n*-alkanes. These compounds are rhamnolipids composed of rhamnose and β-hydroxycarboxylic acid subunits, and are excreted into the culture medium [3–6]. Structural characterization of the compounds has been achieved [6,7]. The *P. aeruginosa* glycolipids play an important and crucial role during cultivation of the bacteria, especially when *n*-alkanes are used as sole carbon source [8]. Consequently, the glycolipids are also known to enhance the biodegradation of some hydrocarbons in soil [9]. The strain *P. aeruginosa* DSM 2659 primarily produces two of the four rhamnolipids. These are 3-[3'-(α-L-rhamnopyranosyloxy)decanoyloxy]decanoic acid (RL-1) and 3-[3'- (2''-O-α-L-rhamnopyranosyl-α-L-rhamnopyranosyloxy)decanoyloxy]decanoic acid (RL-2; Fig. 1). Rhamnolipids RL-3 [3-(α-L-rhamnopyranosyloxy)decanoic acid] and RL-4 [3-(2'-O-α-L-rhamnopyranosyl-α-L-rhamnopyra-

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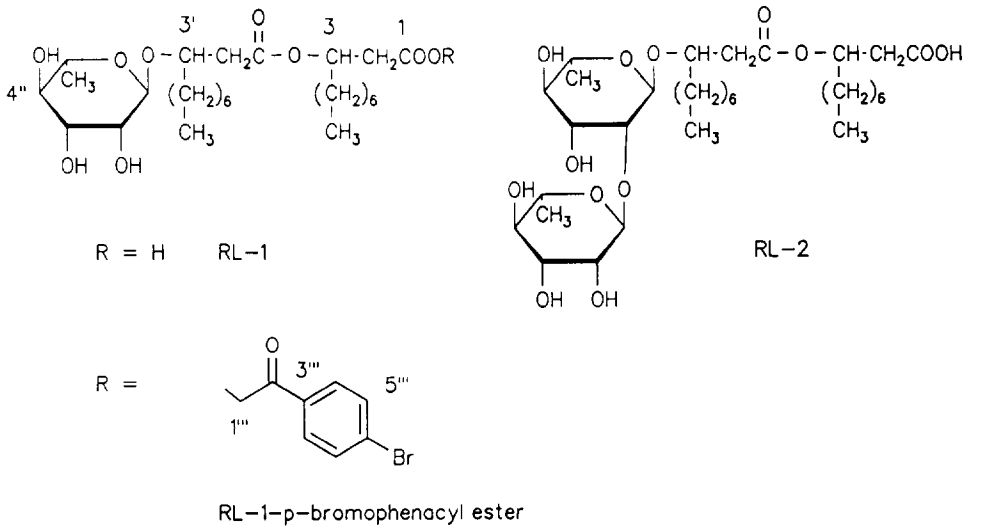

Fig. 1. Chemical structures of the rhamnolipids RL-1 and RL-2.

nosyloxy)decanoic acid] are biosynthesized under certain cultivation conditions only.

During our investigations concerning the biodegradation of *n*-alkanes, and the enhancing effects exerted by glycolipids, the need for a rapid and sensitive analytical method to determine the individual rhamnolipid concentrations has emerged. So far, rhamnolipids in the culture medium of bacteria have been determined quantitatively either indirectly by determination of F_{cmc} values [10], or directly by determination of rhamnose concentrations after hydrolysis of the rhamnolipids according to Hodge and Holreiter [11], and Chandrasekaran and Bemiller [12]. Both methods have the disadvantage that only the total amount of the glycolipids, but not the concentrations of the individual rhamnolipids can be determined quantitatively. In addition, interfering substances present in the culture broth of the bacteria may affect the determination of the F_{cmc} values. The objectives of the present study were to (1) isolate the individual rhamnolipids of *P. aeruginosa* DSM 2659, (2) develop an HPLC method for the rhamnolipids including derivatisation, and (3) apply the method for the examination of the rhamnolipid concentrations in authentic bacterial culture media.

2. Experimental

2.1. Microorganism and cultivation conditions

P. aeruginosa DSM 2659 (Rsan-ver strain) was obtained from DSM (Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany), and used throughout this work. Originally, the strain was isolated from soil samples in the vicinity of an oil refinery located in Mexico [10]. The freeze-dried microorganisms were rehydrated with 1 ml of standard I medium (Merck, Darmstadt, Germany) and inoculated onto standard I agar plates. The optimized medium 3M (18.2 g l^{-1} glucose) for the liquid cultivation of *P. aeruginosa* and production of the rhamnolipids was as described by Guerra-Santos et al. [10], except that $FeSO_4$ was omitted, and H_3PO_4 was replaced by KH_2PO_4 and K_2HPO_4 (4 and 5 g l^{-1}, respectively). The pH of the medium was adjusted to 6.8. A liquid culture was started by adding a loopful of cells from a standard I agar plate to a 250-ml Erlenmeyer flask containing 100 ml medium. *P. aeruginosa* was grown at 37°C with gyratory shaking at 133 rpm. After 18 h, 8 ml of this culture were inoculated into a 1000-ml fernbach flask, containing 400 ml medium and

cultivated as described for another 7 days. The latter culture was used for the preparative isolation of the rhamnolipids RL-1 and RL-2. In order to follow analytically the production of the glycolipids during the growth of the bacterial culture, *P. aeruginosa* was cultivated in 100 ml medium contained in a 250-ml Erlenmeyer flask as described above for 7 days.

2.2. Isolation of the rhamnolipids

For preparative isolation, bacteria and medium were separated by centrifugation (10 000 *g* for 10 min at 4°C). An 8-g amount of $Al_2(SO_4)_3$ was added to the supernatant, and the rhamnolipids were precipitated by stirring for 1 h. The precipitate was collected by centrifugation at 5000 *g* for 10 min, and resuspended in water. Then, the slurry was acidified with 1 *M* HCl (pH 2), extracted 3 × with 50 ml EtOAc, and the combined extracts were dried over anhydrous Na_2SO_4. The resulting yellowish, oily rhamnolipids were analysed by TLC on silica gel plates (Macherey–Nagel, Düren, Germany) using the solvent system $CHCl_3$–MeOH–AcOH (65:15:2, v/v/v). Localisation of the separated zones was performed with a thymol spray reagent {0.5 g thymol in EtOH–conc. H_2SO_4 (95:5, v/v); after spraying, plates were heated to 120°C for 20 min [13] }; R_F values of RL-1 and RL-2 were 0.74 and 0.36, respectively. The rhamnolipids were purified by ion-exchange chromatography on a 100-ml DEAE-Sepharose CL 6B (Fluka, Buchs, Switzerland) column (50 × 2.5 cm) equilibrated with a mixture of 10 m*M* Tris · HCl (pH 8; Sigma, Deisenhofen, Germany) and EtOH (8:2, v/v). After application to the column, the biosurfactants were washed (until the yellow pigment was totally removed) and eluted with 0.4 *M* NaCl in 10 m*M* Tris · HCl (fractions 1–65, 12 ml each), and then 0.6 *M* NaCl in the same buffer (66–120). Fractions containing the purified rhamnolipids (50–85) were combined, acidified with 1 *M* HCl (pH 2), and the glycolipids were recovered as described. Subsequently, the rhamnolipids were separated chromatographically, or were subjected to the preparative derivatisation procedure.

For separation, the purified rhamnolipids were chromatographed on a column (40 × 3.00 cm) of 110 g silica gel 60 (Merck) with a gradient of $CHCl_3$–MeOH (85:15 to 65:35, v/v). After evaporation of the solvents of the respective fractions and lyophylisation, pure RL-1 and RL-2 were obtained in crystalline form. RL-1: ^{1}H NMR (Varian VXR 300; 300 MHz, $C^2H_3O^2H$): δ 0.9 [t, 6H, $J = 7$ Hz, H–C (10, 10′)], 1.22 [d, 3H, $J = 6$ Hz, H–C (6″)], 1.27–1.36 [m, 20H, H–C (5, 6, 7, 8, 9, 5′, 6′, 7′, 8′, 9′)], 1.5–1.65 [m, 4H, H–C (4, 4′)], 2.4–3.9 [m, 7H, H–C (2″, 3″, 4″, 5″), HO–C (2″, 3″, 4″)], 3.97 [m, 1H, H–C (3′)], 4.05 [m, 1H, H–C (3)], 4.89 [d, 1H, $J = 2$ Hz, H–C (1″)]. ^{13}C NMR (Varian VXR 300; 75 MHz, $C^2H_3O^2H$): δ 14.5 (C-10, C-10′), 18.0 (C-6″), 23.7 (C-9, C-9′), 25.8 and 26.2 (C-5, C-5′), 30.3 and 30.4 (C-6, C- 6′), 30.7 (C-7, C-7′), 32.9 and 33.0 (C-8, C-8′), 39.9 and 41.2 (C-2, C-2′), 70.1 (C-5″), 71.9 (C-3″), 72.2 (C-2″), 73.8 (C-3, C-3′), 74.3 (C-4″), 99.1 (C-1″), 172.4 and 174.2 (C-1, C-1′). IR (Perkin-Elmer Fourier transform IR 1700 S; KBr): 3368 (COOH, OH), 2956 (CH), 2926 (CH), 2858 (CH), 1733 (CO) cm^{-1}. Identical spectra were obtained for RL-2, excepting the integrals of the ^{1}H NMR spectrum.

For analytical purposes, 2 ml were aseptically withdrawn from the bacterial culture (100 ml medium in 250-ml Erlenmeyer flask). After centrifugation in order to remove the microorganisms, the supernatant was acidified with 1 *M* HCl (pH 2) and extracted 3 × with 5 ml EtOAc. The extract was dried over Na_2SO_4. After evaporation of the solvent, the partly purified rhamnolipids were obtained, which were directly subjected to the analytical derivatisation procedure.

2.3. Preparative derivatisation of the rhamnolipids

Generally, the synthesis was performed as described by Jupille [14], and modified by Syldatk [5]. A 100-mg amount of the purified rhamnolipid mixture, 125 μl Et_3N and 75 mg *p*-bromoacetophenone (Aldrich, Steinheim, Ger-

many) in 2 ml acetone (freshly distilled, kept over molecular sieve 4 Å) were stirred for 16 h at 20°C. After filtration and evaporation of the solvent, the resulting residue was chromatographed on silica gel 60 (85 g; 40 × 3.00 cm) with $CHCl_3$–MeOH (94:6, v/v). After evaporation of the solvents of the respective fractions, pure RL-1 and RL-2 phenacyl ester derivatives were obtained. RL-1-*p*-bromophenacyl ester: ^1H NMR (300 MHz, C^2HCl_3): δ 0.87 [t, 6H, $J = 7$ Hz, H–C (10, 10′)], 1.25–1.29 [m, 20H, H–C (5, 6, 7, 8, 9, 5′, 6′, 7′, 8′, 9′)], 1.26 [m, 3H, H–C (6″)], 1.5–1.65 [m, 4H, H–C (4, 4′)], 2.4–3.9 [m, 7H, H–C (2″, 3″, 4″, 5″), HO–C (2″, 3″, 4″)], 3.82 [m, 1H, H–C (3′)], 4.14 [m, 1H, H–C (3)], 4.88 [br. s, 1H, H–C (1″)], 5.31 [d, 2H, $J = 2.7$ Hz, H–C (1‴)], 7.63 [d, 2H, $J = 9$ Hz, H–C (5‴, 7‴)], 7.78 [d, 2H, $J = 9$ Hz, H–C (4‴, 8‴)]. ^{13}C NMR (75 MHz, C^2HCl_3): 14.0 and 14.1 (C-10, C-10′), 17.4 (C-6″), 22.6 and 24.7 (C-9, C-9′), 25.1 and 29.1 (C-5, C-5′), 29.2 and 29.3 (C-6, C-6′), 29.5 and 29.6 (C-7, C-7′), 31.7 and 31.8 (C-4, C-4′), 32.7 and 34.0 (C-8, C-8′), 38.7 and 39.9 (C-2, C-2′), 66.3 (C-1‴), 66.2 (C-1‴), 68.3 (C-5″), 70.6 (C-3, C-3′), 71.3 (C-2″), 71.8 (C-3″), 73.5 (C-4″), 129.3 (C-6‴, C-4‴, C-8‴), 132.3 (C-5‴, C-7‴), 132.7 (C-3‴), 170.4 and 171.1 (C-1, C-1′), 191.1 (C-2‴). IR ($CHCl_3$): 3419 (COOH and OH), 2955 (CH), 2929 (CH), 2858 (CH), 1738 (CO), 1708 (CO), 1588 (ArC) cm^{-1}. The substance gave a single peak when analysed by HPLC. The RL-2-*p*-bromophenacyl ester was similarly analysed spectroscopically, and showed identical spectra, excepting the ^1H NMR integrals. Both RL-1 and RL-2 derivatives were used as reference compounds in the analytical experiments.

2.4. Analytical derivatisation of the rhamnolipids

For routine analysis of the rhamnolipid concentrations in the culture broth, the partly purified rhamnolipids (usually 0–5 mg/ml) were dissolved in 1 ml CH_3CN in a screw cap vial. Molar ratios of approximately 1:4:2 (glycolipid:*p*-bromoacetophenone:Et_3N) were used for maximal derivatisation rates of ca. 95% [14,15]; the reaction was carried out at 60°C for 1 h. The product was directly used for the HPLC analysis, or was otherwise stored at −20°C.

2.5. Reagents and solvents

CH_3CN (distilled successively over P_2O_5 and CaH_2) and water (double distilled) were used exclusively as HPLC eluents. Both were filtered (0.45 μm; Schleicher & Schuell, Dassel, Germany) and degassed before use. All other solvents and reagents were commercially available and used without further purification.

2.6. HPLC apparatus and chromatographic conditions

The HPLC instrument consisted of a System Gold Beckman chromatograph, equipped with an Altex 210A valve injector joined to a 100-μl loop, a Beckman System Gold diode array detector 168 and a Beckman System Gold programmable solvent module 126. The chromatography was carried out on an ET 250/8/4 Nucleosil 5 C_{18} (5 μm; Macherey–Nagel) column (250 mm × 4.6 mm). Water (solvent A) and CH_3CN (solvent B) were used as mobile phase: 4 min, A:B, 3:7 (v:v); 10 min, gradient from 3:7 to 0:10; 9 min 0:10; 2 min, return to 3:7. Chromatography was performed at 25°C with a flow-rate of 0.8 ml/min and an injection volume of 100 μl. The column effluent was monitored at 265 and 320 nm.

3. Results and discussion

3.1. Extraction of rhamnolipids

Different extraction methods for the isolation of the rhamnolipids from aqueous media have been reported. These include solvent systems such as EtOAc [6], $CHCl_3$–MeOH (2:1, v/v) [7] and Et_2O [9]. Preliminary experiments (see below) demonstrated that EtOAc was most efficacious. An almost quantitative extraction of the glycolipids was achieved, which was regarded as crucial for the subsequent HPLC analysis.

Generally, the procedure used in the present study was a modification of the method described by Pang [16]. In the preparative experiment, the rhamnolipids were precipitated from the medium by addition of 2% $Al_2(SO_4)_3$ and were separated from the supernatant, in order to reduce the large volume of the aqueous phase. In the case of the analytical assays (2-ml samples), the glycolipids were directly extracted with EtOAc after acidification of the aqueous phase.

3.2. Chromatography

Complete separation of the RL-1- and RL-2-*p*-bromophenacyl esters was achieved in 25 min using a RP C_{18} column. Typical HPLC runs for the reference compounds are shown in Fig. 2. Additional peaks appeared in the chromatograms, but according to their respective UV spectra, were not identified with other rhamnolipids. Presumably, they were produced by other microbial products present in the culture medium, which could not removed completely by a single run on a silica gel column. In the authentic nutrient solutions, the derivatised RL-1 and RL-2 were identified by cochromatography (retention times) with the reference compounds. A typical run (Fig. 3) shows that the RL-1- and RL-2-*p*-bromophenacyl esters could be sufficiently separated from other compounds present in the bacterial media. Additional proof for the identity of RL-1 and RL-2 was derived from the UV spectra of their respective *p*-bromophenacyl ester derivatives (Fig. 4). The detection limits were 1.2 μg for the RL-1- and 1.8 μg for the RL-2-*p*-bromophenacyl ester (0.9 μg and 1.3 μg RL-1 and RL-2, respectively).

The underivatised rhamnolipids RL-1 and RL-2 could also be chromatographed and separated by ion chromatography on a ET 250/8/4 Nucleosil 5 C_{18} (5 μm) column (250 mm × 4.6 mm I.D.). The mobile phase was CH_3CN–phosphate buffer [0.02 *M*, containing $5 \cdot 10^{-3}$ *M* tetrabutylammonium hydrogensulfate, TBA (Sigma); pH 7.5] (65:35, v/v). Chromatography was performed as described, and the rhamnolipids were detected at 215 and 254 nm. The detection limit of the underivatised RL-1 and RL-2 was 200 mg.

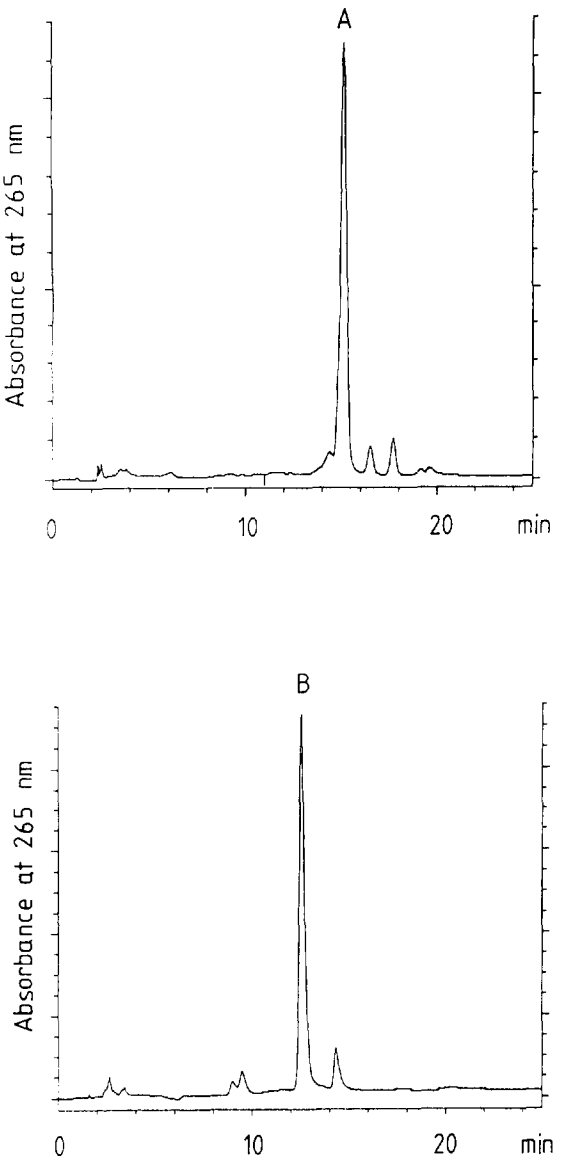

Fig. 2. HPLC chromatograms of the reference compounds RL-1-*p*-bromophenacyl ester (A) and RL-2-*p*-bromophenacyl ester (B).

3.3. Linearity and reproducibility of the method

External standardization by peak area was used for the quantitative determination of the rhamnolipids. The calibration graphs were linear over the ranges 1.2–25 μg for the RL-1- and 1.8–25 μg for the RL-2-*p*-bromophenacyl ester

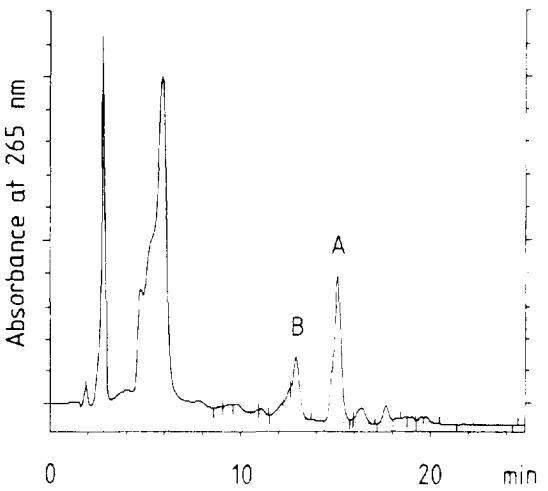

Fig. 3. Typical chromatogram of the derivatised rhamnolipids (A = RL-1; B = RL-2) from the culture medium of *Pseudomonas aeruginosa* DSM 2659.

(correlation coefficients, r, 0.998 and 0.999, respectively). In order to determine the recovery of the entire method, 50 μg, 500 μg and 5 mg RL-1 were dissolved in 10 ml culture medium, extracted and derivatised as described. The results shown in Table 1 demonstrate that ca. 74% of RL-1 were recovered; this was regarded as adequate with regard to the physico-chemical properties of the biosurfactants. Similar experiments using Et_2O for extraction gave considerably poorer recoveries of about 13%.

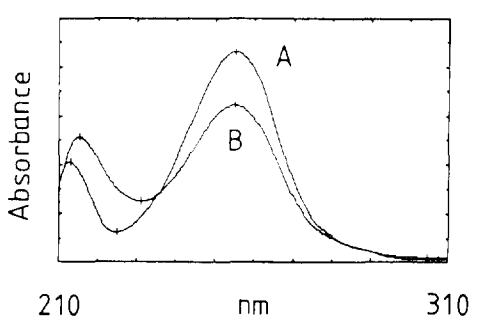

Fig. 4. UV spectra of the RL-1 (A) and RL-2 (B) derivatives in CH_3CN (77 μg/ml).

Table 1
Recovery of the reference compound RL-1 added to the autoclaved culture medium

RL-1 added (μg)	RL-1 derivative recovered	
	μg	%
50	44.6	64.6
500	548.7	78.9
5000	5362.3	77.1

Extraction and derivatisation were performed as outlined in the Experimental section.

3.4. Application of the method to the rhamnolipid analysis in authentic culture media

The production of the rhamnolipids RL-1 and RL-2 in the course of a cultivation cycle of *P. aeruginosa* as monitored by the HPLC method described above is depicted in Fig. 5. The concentrations determined in the culture medium ranged from 0.050 to 1.689, and 0.015 to 0.869 g l^{-1} in the case of RL-1 and RL-2, respectively. Fig. 5 shows that after a lag phase of about 24 h, an intense production of the biosurfactants occurred up to 60 h of cultivation.

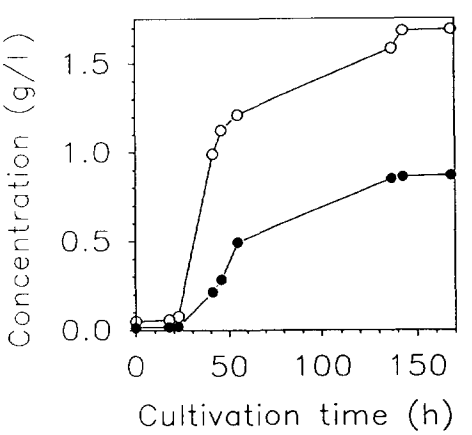

Fig. 5. Concentrations of the rhamnolipids RL-1 (○) and RL-2 (●) in the culture medium of *Pseudomonas aeruginosa* DSM 2659 during one growth cycle.

Then, the cells entered the stationary phase and production ceased. On the whole, these results are in agreement with previously published data on the rhamnolipid production by *P. aeruginosa* DSM 2659 (2.25 g l^{-1} total rhamolipids at the end of the cultivation period [17]) which were determined using F_{cmc} values. The advantages of the present method have been discussed above. In addition, it may be assumed that the rapid and precise determination of the individual rhamnolipids will help getting a better insight into the processes occurring in *P. aeruginosa* cultures, especially when *n*-alkanes are used as carbon source. Future experiments will demonstrate whether the procedure is suitable for the quantitative analysis of the rhamnolipids in soils, in order to evaluate the effects of the compounds in a natural environment. It is hoped that analogous methods will be developed for other classes of (glycolipid) biosurfactants.

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