Use of Liquid Chromatography–Mass Spectroscopy for Studying the Composition and Properties of Rhamnolipids Produced by Different Strains of *Pseudomonas aeruginosa*

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ABSTRACT: Biosurfactants are produced as a mixture of different homologs. The application of liquid chromatography-electrospray mass spectrometry in negative mode for the analysis of rhamnolipid mixtures AT10 and 47T2 has been studied. Working at low (up to –35 V) extraction voltages, the $[M - H]^-$ for each compound was obtained. Increasing this potential to –75 V produced an increase in the fragmentation of compounds and enabled the co-eluting isomers of rhamnolipids to be distinguished and their proportions in the sample to be calculated. In this work, the physicochemical and biological properties of two different rhamnolipid mixtures produced by two *Pseudomonas* strains RL_{AT10} (Rha-Rha-C_{10}-C_{10}; Rha-Rha-C_{10}-$C_{12:1}$; Rha-Rha-C_{10}-C_{12}; Rha-C_{10}-$C_{12:1}$; Rha-C_{10}-C_{10}; RhaC_{10}-$C_{12:1}$; Rha-C_{10}-C_{12}; Rha-$C_{8:1}$; Rha-$C_{12:2}$) and RL_{47T2} (Rha-Rha-C_8-C_{10}; Rha-Rha-C_8-$C_{12.1}$; Rha-Rha-C_{10}-C_{10}; Rha-Rha-C_{10}-$C_{12.1}$; Rha-Rha-C_{10}-C_{12}; Rha-Rha-C_{12}-C_{10}; Rha-Rha-$C_{12:1}$-C_{12}; Rha-Rha-C_{10}-$C_{14:1}$; Rha-C_8-C_{10}; Rha-C_{10}-C_8; Rha-C_{10}-C_{10}; Rha-C_{10}-$C_{12:1}$; Rha-C_{10}-C_{12}; Rha-C_{12}-C_{10}, where Rha = rhamnose moiety) are compared. The surface tensions found were 26.8 and 32.8 mN/m for RL_{AT10} and RL_{47T2}, respectively. These two products differ in their antimicrobial properties, as based on their minimal inhibition concentrations (MIC). RL_{AT10} was effective against the fungal species (MIC) *Aspergillus niger* (16 μg/mL); *Gliocadium virens* (16 μg/mL); *Penicillium chrysogenum* (32 μg/mL); *Botrytis cinerea* (18 μg/mL); and *Rhizoctonia solani* (18 μg/mL), whereas RL_{47T2} was more effective against the bacteria (MIC) *Enterobacter aerogenes* (4 μg/mL); *Serratia marcescens* (8 μg/mL); *Bacillus subtilis* (16 μg/mL); and *Staphylococcus aureus* (32 μg/mL).

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KEY WORDS: Mass spectrometry, properties, *Pseudomonas*, rhamnolipids, structure.

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Abbreviations: CID, collision-induced dissociation; ES, electrospray; FFA, free fatty acids; LC, liquid chromatography; MIC, minimal inhibition concentration; MS, mass spectrometry; M.W., molecular weight; Rha, rhamnose moiety; RL, rhamnolipid.

Biosurfactants are a structurally diverse group of surface-active molecules synthesized by microorganisms. Being amphiphilic molecules, they generally absorb at interfaces and reduce the surface tension between two liquid phases, enabling the uptake of hydrophobic substrates by the microorganism. Bioemulsifiers have very different chemical structures and are produced by a wide variety of microorganisms living in different habitats, thus providing the cell with different physiological and therefore ecological advantages.

Rhamnolipids (RL), the most well-known bioemulsifiers, are produced by different strains of *Pseudomonas aeruginosa*, and their production process and recovery have been studied extensively. Up to four different molecules have been identified, depending on the strain and on culture conditions (1,2). RL mixtures traditionally have been analyzed by colorimetric determination of total hexoses (3) or by specific assay for rhamnose (4). The main advantage of these methods has been simplicity, and the main drawback has been that neither provides composition information.

A further advance in the quantification of RL was the application of LC coupled to a diode array detector (5), enabling up to four species of molecules to be quantified separately. More recent studies have used liquid chromatography (LC) coupled with MS (LC–MS) for determining RL or their precursors (6–9). The main advantage of this method is that it provides information that enables identification of the composition of the mixture. Soft ionization in atmospheric pressure ionization techniques, e.g., electrospray (ES), produces little structural information, but a proper adjustment of the extraction voltage in single quadrupole instruments readily produces this information from fragment ion signals. This mode of operation is known as collision-induced dissociation (CID).

In this paper we present: (i) the use of high-flow LC–ES–MS for characterizing RL mixtures (RL_{AT10} and RL_{47T1}) produced by two *Pseudomonas* strains; (ii) the application of LC–ES–MS to the kinetics of the different species of RL accumulated in the culture; and (iii) the physicochemical and biological properties of RL_{AT10} and RL_{47T1} with respect to their composition.

MATERIAL AND METHODS

Obtaining RL mixtures. The RL mixtures were obtained from the supernatant of the culture medium of two pseudomonad strains: *P. aeruginosa* AT10 and *P. aeruginosa* 47T2. The strains were cultured in a mineral medium using waste free fatty acids (FFA) from soybean oil production ($C_{18:2}$, 43.4%; $C_{18:1}$, 21.1%) and used cooking oils ($C_{18:1}$, 41.6%; $C_{18:2}$, 27.9%), respectively, as the carbon source.

RL purification and quantification. Cells were removed from the culture medium by centrifugation (12,000 × *g*) for 30 min. Purification was achieved by adsorption chromatography on a polystyrene resin, Amberlite XAD2 (Supelco, Bellefonte, PA). The resin (60 g) was placed in a glass column (60 × 3 cm), yielding a bed volume of 200 mL. The column was equilibrated with 0.1 M phosphate buffer, pH 6.1. The culture supernatant was applied through a sieve placed on top of the resin to prevent resuspension. Adsorption of the active compounds on the resin was monitored by measuring the surface tension (γ_{ST}) at the column outlet. The saturation of the resin was terminated when γ_{ST} of the effluent dropped below 40 mN/m. The column was then washed with 3 vol of distilled water. Biosurfactants were eluted with methanol and, finally, the solvent was evaporated to dryness under vacuum (Büchi, Flawil, Switzerland).

Samples were prepared by diluting 10 mg/mL of each rhamnolipid mixture, RL_{47T2} and RL_{AT10}, in methanol.

LC–MS. RL mixtures were separated and identified by LC coupled to MS using a Waters 2690 separation module (Milford, MA). The samples (100 µL) were injected into a Hypersil C8 WP-300 (5 µm) 150 × 4.6 mm column (Teknokroma, Sant Cugat, Spain). The LC flow rate was 1 $mL \cdot min^{-1}$. An acetonitrile/water gradient was used, starting with 30% acetonitrile for 4 min, followed by a ramp of 30–100% acetonitrile for 40 min, standby for 5 min, and then a return to initial conditions. Postcolumn addition of acetone at 200 $\mu L \cdot min^{-1}$ was injected using a Phoenix 20 (Carlo Erba, Milano, Italy) syringe. The LC effluent and acetone were mixed in a tee (Valco) and split (1:50) before entering the mass spectrometer. MS was performed with a single quadrupole mass spectrometer, VG Platform II (Micromass, Manchester, United Kingdom), equipped with a pneumatically assisted ES source. The negative ion mode was used. Full scan data were obtained by scanning from *m/z* 100 to 750 in the centroid mode using a scan duration of 2.0 s and an inter-scan time of 0.2 s. The working conditions for ES were as follows: Drying nitrogen was heated at 80°C and introduced into the capillary region at a flow rate of 400 $L \cdot h^{-1}$. The capillary was held at a potential of –3.5 kV. The extraction voltage was held at –75 V.

Biological assays. Antimicrobial activity was determined on the basis of minimal inhibition concentration (MIC) values, defined as the lowest concentration of antimicrobial agent needed to inhibit the development of visible growth after incubation for the required time. A twofold serial RL dilution technique was used to measure antimicrobial activity. Antibacterial activity was determined on liquid medium, which was incubated for 24 h at 37°C (10). Antimicrobial activity against yeast and fungi was determined on solid Sabouraud medium agar plates, which were incubated for 72 h at 25°C (11). Numerous Gram-positive and Gram-negative bacteria were tested: *Alcaligenes faecalis* ATCC 8750, *Bordetella bronchiseptica* ATCC 461, *Citrobacter freundii* ATCC 22636, *Enterobacter aerogenes* CECT 689, *Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* var. pneumoniae CECT 178, *Proteus mirabilis* CECT 170, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhimurium* ATCC 16028, *Serratia marcescens* CECT 274, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* var. mycoide ATCC 11778, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 11228, *Micrococcus luteus* ATCC 9631, *Arthrobacter oxydans* ATCC 8010, *Mycobacterium phlei* ATCC 41423, *Clostridium perfringens* ATCC 486. Fungal strains tested were as follows: *Aspergillus niger* ATCC 14604, *Aureobasidium pullulans* ATCC 9348, *Chaetonium globosum* ATCC 6205, *Gliocadium virens* ATCC 4645, *Penicillium chrysogenum* CECT 2802, *Penicillium funiculosum* CECE 2914, *Botrytis cinerea, Colletotrichum gloeosporioides, Rhizoctonia solani,* and *Fusarium solani.*

RESULTS AND DISCUSSION

Different RL production strategies have been described for several strains of *Pseudomonas* (12–14), and new applications have been reported (15). RL are accumulated in the culture medium as a mixture of different molecules. The physicochemical properties of each mixture (or product), and hence the applications, will depend on the proportion and type of monomers. The application of analytical tools, such as LC–ES–MS, to the kinetics of the accumulation process is a further advance in the routine monitoring of RL production and in understanding the properties of the different RL mixtures.

Optimization of LC–MS conditions. The sample containing the RL mixture (100 µL) was injected into the LC–MS system to identify the main ions in the mass spectra for each RL. The extraction voltage was varied from –30 to –100 V, and the mass spectra were recorded in full scan mode. The spectra generated for RL by ES under the negative ion mode at an extraction voltage up to –35 V gave the pseudomolecular ion $[M - H]^-$, which could be attributed to deprotonation of the carboxylic group, as described by Deziel *et al.* (6). The proportions of the different congeners of RL were obtained from the relative intensities of their $[M - H]^-$ ions. However, given that isomers of RL were not chromatographically resolved, the $[M - H]^-$ ion could not be used for this purpose as both congeners have the same molecular weight (M.W.) Thus, a more extensive fragmentation was needed to discriminate between congeners. By using a single quadrupole mass spectrometer, this was achieved by increasing the extraction voltage to –75 V in CID conditions. The increase in potential produced a fragmentation of the molecules in the source of the mass spectrometer and enabled isomers to be distinguished, because the *m/z* of fragments varied according to their 3-hydroxy fatty acid side

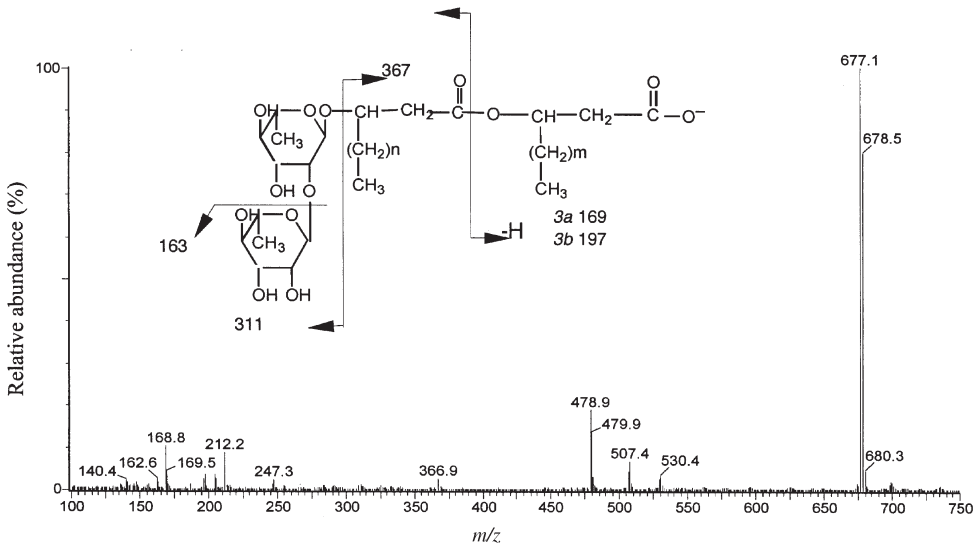

FIG. 1. Molecular structure showing fragmentation pathways and mass spectra of the two co-eluting congeners, Rha-Rha-C_{12}-C_{10} and Rha-Rha-C_{10}-C_{12}, at an extraction voltage of –75 V in the liquid chromatography-electrospray mass spectrometry conditions described in the text. **3a**; 3-hydroxy fatty acid moiety for Rha-Rha-C_{12}-C_{10}; **3b**, 3-hydroxy fatty acid moiety for Rha-Rha-C_{10}-C_{12}; for details see text.

chains. Figure 1 shows the molecular structure and mass spectra of the two co-eluting congeners, Rha-Rha-C_{12}-C_{10} and Rha-Rha-C_{10}-C_{12}, at –75 V. The $[M - H]^-$ ion was the most intense ion in the mass spectrum (cone voltage –75 V). When the extraction voltage was increased, two main ions, in addition to the pseudomolecular ion $[M - H]^-$, were observed in the mass spectra of rhamnolipids: These ions correspond to the cleavage at the 3 carbon-oxygen bond of the 3-hydroxy fatty acid moiety (*m/z* 507 and 169 for Rha-Rha-C_{12}-C_{10} and *m/z* 479 and 197 for Rha-Rha-C_{10}-C_{12}) and the cleavage at the *O*-glycosidic bond in the rhamnose unit (*m/z* 367). In some cases, the ions corresponding to two rhamnose units (*m/z* 311) could also be seen in mass spectra.

Table 1 presents the RL found in the mixture produced by AT10 and 47T2 strains and the main ions at two extraction voltages with their relative abundance. In general, the $[M - H]^-$ ion was found to be the most abundant for di-RL, whereas its abundance for mono-RL was between 50 and 80%. From the results presented in this table, it can be seen that the composition of the RL mixture depends on the strain of *Pseudomonas* used. Thus, 47T2 produces several classes of RL with M.W. ranging from 704 to 532, whereas AT10 produces RL with M.W. between 678 and 302. These results illustrate how MS can be used to describe a complex mixture of compounds, such as the RL produced by *Pseudomonas*. Figure 2 shows the total ion chromatogram and the ion chromatogram at different *m/z* values for all the RL identified in the 47T2 mixture at an extraction voltage of –75 V; good chromatographic separation was obtained for all the RL in 20 min.

RL production. LC–MS enables the accumulation of the different RL homologs to be followed throughout the process; as an example, the time course for the production of the different species of RL in *P. aeruginosa* AT10 on waste FFA is presented in Figure 3. The total amount of RL produced from soybean residual fatty acids was 16.5 g/L at 96 h of incubation. The most abundant pair of homologs of the mixture was Rha-C_{10}- C_{10}/Rha-Rha-C_{10}-C_{10}, which accounted for up to 50.5%; the mono-RL homolog appeared first (23.8%), at 24 h of culture, whereas the di-RL (Rha-Rha-C_{10}-C_{10}) was detected at 48 h of incubation, most of it (Fig. 3) being accumulated between 72 and 96 h of incubation. Similarly, production of this RL was reported to be the most abundant when the carbon sources were corn oil (16), by *P. aeruginosa* UG2, or mannitol (6), by *P. aeruginosa* 57RP. In the case of the Rha-C_{10}-$C_{12:1}$/Rha-Rha-C_{10}-$C_{12:1}$ pair, which accounted for 19.3% of the final mixture, both homologs were detected at 24 h of incubation. At the end of the process, 14% of the total amount was found in the pair Rha-Rha-C_{10}-C_{12}/Rha-C_{10}-C_{12} and in this case the di-RL homolog was the most abundant RL after 24 h (47.6%), the percentage decreasing along with the culture; the mono-RL was detected in low amounts (3.4%) after 48 h of culture and remained fairly constant until the end of the process. Two single unsaturated molecules were detected: 11.6% of Rha-$C_{8:2}$, which appeared at 72 h of incubation, and Rha-$C_{12:2}$, which was detected at 24 h of the culture. Similar results were obtained with *P. aeruginosa* 47T1 (results not shown).

Composition vs. properties. Rosen (17) established the close relationship between the surface-active properties of a surfactant and its chemical structure. As they are proportionally different from the molecular species, RL mixtures considered as a single product (RL_{AT10} or RL_{47T2}) are expected to have different properties because of differences in their molecular composition. Table 2 shows their physicochemical characteristics. However, it is difficult to understand the amphipathic behavior of RL due to the presence of one or two hydrophilic moieties and one or two hydrophobic groups with different alkyl chain lengths. As shown in Table 2, the effectiveness, that is, the minimum value of γ_{st}

TABLE 1
Ion Fragmentation and the Pseudomolecular Ion $[M - H]^-$ of the Rhamnolipid (RL) Mixtures RL_{AT10} and RL_{47T2} Produced in Submerged *Pseudomonas* Cultures

Compounds	RL_{AT10}	RL_{47T2}	M.W.	Pseudomolecular ion (*m/z*)	Relative intensity (%) –35 V	Relative intensity (%) –75 V
Rha-Rha-C_8-C_{10}	—	+	622	621	100	33
				452	—	8
				311	—	100
				310	—	40
				169	—	12
				141	—	10
Rha-Rha-C_8-$C_{12:1}$	—	+	648	647	100	100
				452	—	15
Rha-Rha-C_{10}-C_{10}	+	+	650	649	100	100
				479	—	24
				339	—	16
				311	—	22
				169	—	40
				163	—	12
Rha-Rha-C_{10}-$C_{12:1}$	+	+	676	675	100	100
				479	—	28
				195	—	8
				169	—	63
				311	—	15
				119	—	5
				103	—	15
Rha-Rha-C_{10}-C_{12}	+	+	678	677	100	100
				311	—	3
				479	—	26
				169	—	14
Rha-Rha-C_{12}-C_{10}	—	+	678	677	100	100
				507	—	88
Rha-Rha-$C_{12:1}$-C_{12}	—	+	704	703	100	100
				506	—	38
Rha-Rha-C_{10}-$C_{14:1}$	—	+	704	703	100	100
				480	—	17
Rha-C_8-C_{10}	—	+	476	475	100	100
				305	—	64
				169	—	79
Rha-C_{10}-C_8	—	+	476	475	100	41
				333	—	12
				311	—	100
Rha-C_{10}-C_{10}	+	+	504	503	100	78
				333	—	20
				339	—	8
				169	—	100
				163	—	15
				119	—	13
				103	—	28
Rha-C_{10}-$C_{12:1}$	+	+	530	529	100	47
				365	—	6
				333	—	30
				169	—	100
				195	—	6
				163	—	25
				119	—	17
				103	—	38
Rha-C_{10}-C_{12}	+	+	532	531	100	100
				333	—	80
				197	—	25
Rha-C_{12}-C_{10}	—	+	532	531	100	79
				361	—	7
				169	—	100
Rha-$C_{8:2}$	+	—	302	301	100	—
Rha-$C_{12:2}$	+	—	358	357	100	—

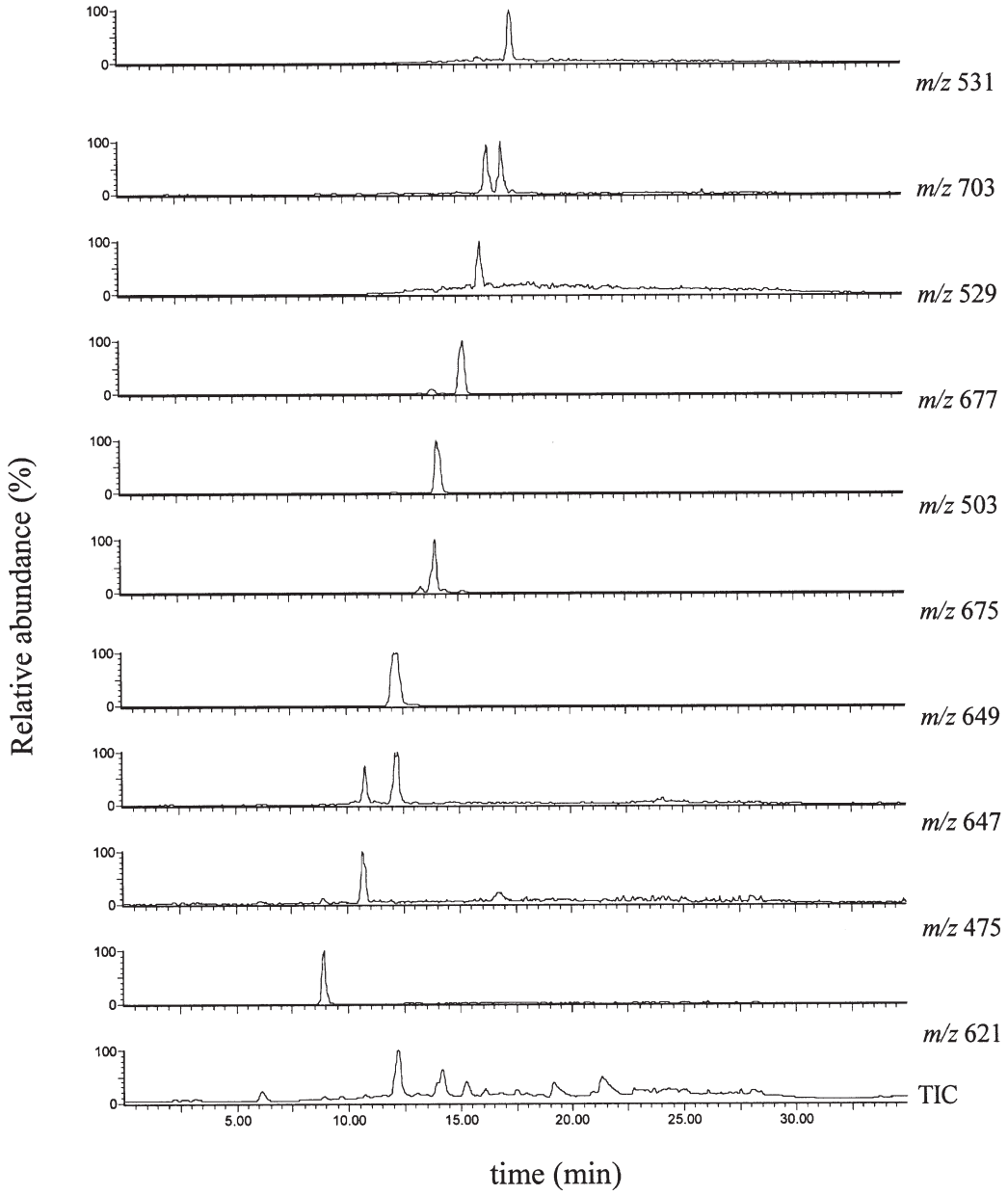

FIG. 2. Total ion chromatogram (TIC) and the ion chromatogram at different *m/z* values for all the rhamnolipids (RL) identified in the RL_{47T2} product at an extraction voltage of –75 V, as stated in the text.

produced by the surface-active compound in water, was higher for RL_{AT10} (γ_{ST}, 26.8 mN/m) than for RL_{47T2}; however, the critical micelle concentration of the latter was lower than that of RL_{AT10}, 108.8 and 150 mg/L, respectively. This effect may be due to the higher hydrophobicity of the components: In the case of RL_{AT10}, the proportion of di-RL is lower (37.8%) than that found in RL_{47T2} (32.8 mN/m). A similar effect was reported by Mata-Sandoval *et al.* (7). Alternatively, the effect may be due to unsaturation in the hydrophobic group; RL_{AT10} contained 35.7% unsaturated fatty acids whereas RL_{47T2} contained 17.9%.

Owing to their intrinsic properties, surface-active compounds interfere with cell surfaces and disrupt microbial membranes. The biological activity of the products was studied with respect to several bacterial and fungal species. The MIC of the product needed to inhibit microorganism growth is presented in Table 3. The biological activity of RL_{47T2} was higher than that of RL_{AT10} against Gram-negative bacteria. The MIC values found against *Serratia marcescens* are particularly interesting because this bacterium is multiply resistant to antibiotics and biocides. RL_{AT10} was more active against Gram-positive bacteria, such as *Staphylococcus epidermidis* and *Mycobacterium phlei*, but similar values have been found for selected RL against *B. subtilis* (18). The results clearly demonstrate the activity of RL_{AT10} against a wide range of fungal species. This is an important finding, as there is still little

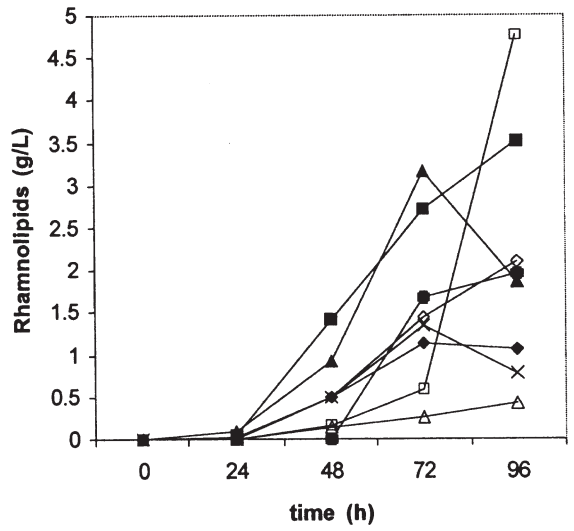

FIG. 3. Time course of rhamnolipid homolog accumulation in a submerged culture of *Pseudomonas aeruginosa* AT10 incubated in aerated mineral medium with free fatty acids from soybean at 30°C. Rha-$C_{8:2}$ (●); Rha-C_{10}-$C_{12:1}$ (◆); Rha-Rha-C_{10}-$C_{12:1}$ (◇); Rha-C_{10}-C_{10} (■); Rha-Rha-C_{10}-C_{10} (□); Rha-$C_{12:2}$ (X); Rha-C_{10}-C_{12} (△); Rha-Rha-C_{10}-C_{12} (▲).

information about antimicrobial activity of surfactants. Kitamoto (19) reported MIC ranging from 3.1 to 25 μg/mL against bacteria, but weak antifungal activity (MIC > 400 μg/mL), of the surface-active mannose-erythrol lipids produced by *C. antarctica*, whereas Lang and Wagner (18) claimed a MIC of 50 μg/mL for sophorolipids produced by *T. bombicola.*

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TABLE 2
Physicochemical Characterization of the Products[a]

Physicochemical parameters[b]	RL_{47T2}	RL_{AT10}
γ_{ST} (mN/m)	32.8	26.8
γ_{IT} (mN/m)	1.0	1.0
CMC (mg/L)	108.8	150
Γ_T (mol/cm^2)	0.89×10^{-10}	1.23×10^{-10}

[a]Obtained from cultures of *Pseudomonaaeruginosa* 47T2 and *P. aeruginosa* AT10 incubated at 30°C for 96 h at 150 rpm.
[b]γ_{ST}, surface tension; γ_{IT}, interfacial tension; CMC, critical micelle concentration; Γ_T, superficial excess. For other abbreviations see Table 1.

TABLE 3
Antimicrobial Activity of Rhamolipids RL_{AT10} and RL_{47T2}[a] Based on the Minimal Inhibitory Concentration (μg/mL) Against Different Microorganisms

Microorganism	RL_{AT10}	RL_{47T2}
Alcaligenes faecalis	32	64
Citrobacter freundii	>256	64
Enterobacter aerogenes	>256	4
Escherichia coli	32	64
Serratia marcescens	16	8
Arthrobacter oxydans	16	128
Bacillus subtilis	64	16
Micrococcus luteus	32	64
Mycobacterium phlei	16	128
Staphyloccocus aureus	128	32
Staphyloccocus epidermidis	8	32
Aureobasidium pullulans	32	>256
Aspergillus niger	16	>256
Chaetomium globosum	32	64
Gliocadium virens	16	32
Penicillium chrysogenum	32	>256
Penicillium funiculosum	128	16
Botrytis cinerea	18	170
Colletotrichum gloeosporioides	65	256
Rhizoctonia solani	18	109
Fusarium solani	65	75

[a]For abbreviation see Table 1.

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