Physico-chemical characterization and antimicrobial properties of the rhamnolipids produced by *Pseudomonas aeruginosa* 47T2 NCBIM 40044.

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Running title: Characterization of new rhamnolipid product from waste frying oil by *Pseudomonas aeruginosa* 47T2

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ABSTRACT

Pseudomonas aeruginosa 47T2, grown in submerged culture with waste frying oil as a carbon source, produced a mixture of rhamnolipids with surface activity.

Up to eleven rhamnolipid homologues (Rha-Rha-C_8-C_{10}; Rha-C_{10}-C_8/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-C_{10}-C_{10}; Rha-Rha-C_{10}-C_{12}/Rha-Rha-C_{12}-C_{10}; Rha-C_{10}-$C_{12:1}$/Rha-$C_{12:1}$-C_{10}; Rha-Rha-$C_{12:1}$-C_{12}; Rha-Rha-C_{10}-$C_{14:1}$; Rha-C_{10}-C_{12}/Rha-C_{12}-C_{10}) were isolated from cultures of *Pseudomonas aeruginosa* 47T2 from waste frying oil and identified by HPLC-MS analysis.

This paper deals with the production, isolation and the chemical characterization of the rhamnolipid mixture, RL_{47T2}. The physicochemical and biological properties of RL_{47T2} as a new product were also studied. Its surface tension decreased to 32.8 mN/m; and the interfacial tension against kerosene to 1 mN/m. The Critical Micellar Concentration for RL_{47T2} was 108.8 mg/mL. The product showed excellent antimicrobial properties. Antimicrobial activity was evaluated according the minimum inhibitory concentration (MIC), the lowest concentration of antimicrobial agent that inhibits development of visible microbial growth. Low MIC values were found for bacteria *Serratia marcescens* (4 μg/mL), *Enterobacter aerogenes* (8 μg/mL), *Klebsiella pneumoniae* (0.5 μg/mL), *Staphylococcus aureus* and *Staphylococcus epidermidis* (32 μg/mL), *Bacillus subtilis* (16 μg/mL) and phytopathogenic fungal species: *Chaetonium globosum* (64 μg/mL), *Penicillium funiculosum* (16 μg/mL), *Gliocadium virens* (32 μg/mL) and *Fusarium solani* (75 μg/mL).

Keywords: Rhamnolipid, *Pseudomonas*, physicochemical properties, biological properties, chemical structure, waste oil.

INTRODUCTION

Several structural homologues of rhamnolipids produced by different strains of *Pseudomonas aeruginosa* have been identified so far. The type of rhamnolipid produced depends on the bacterial strain, the carbon source used and the process strategy (Lang and Wullbrandt, 1999, Déziel et al., 2000, Itoh et al.,1971). Rhamnolipids are formed by one or two rhamnose molecules linked to one or two fatty acids of saturated or unsaturated alkyl chain between C_8 and C_{12} (Rendell et al., 1990). Up to now, four homologues (Rha-Rha-C_{10}-C_{10}; Rha-C_{10}-C_{10}; Rha-Rha-C_{10}; Rha-Rha-C_{10}) from hydrophobic or hydrophilic substrates have been described (Syldatk et al., 1985; Parra et al., 1989; Arino et al., 1996, Lang and Wullbrandt, 1999).

Pseudomonas aeruginosa 47T2 produced two main rhamnolipid homologues, L-α-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-C_{10}-C_{10}) and 2-O-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-Rha-C_{10}-C_{10}), when grown on olive oil waste water (Mercadé et al., 1993) or on waste frying oils (Haba et al., 2000). However, new analytical methods of high-performance liquid chromatography and Mass spectrometry (HPLC-MS) have identified numerous homologues which contain one or two rhamnose molecules and one or two residues of 3-hydroxydecanoic acid groups. Even 3-hydroxydodecenoic and 3-hydroxytetradecenoic acid residues have been reported (Déziel et al., 1999, Déziel et al., 2000). In this study, HPLC-ES-MS in negative mode was used to identify rhamnolipids produced by *P. aeruginosa* 47T2.

Reports published on individual characterization of several rhamnolipid molecules show that these can decrease surface tension in the range 25-30 mN/m and have a critical micelle concentration from 5-200 mg/L (Lang and Wagner, 1987). To our knowledge, there are few reports in the literature on the physicochemical characterization of rhamnolipid mixtures to be used as a single

product. The properties of such a product depend on the proportion of homologues proportion of homologues (Abalos et al., 2001).

Due to their ability to disperse hydrophobic compounds in water, biosurfactants have extensive industrial use: bioremediation, crop protection, and in the cosmetic and pharmaceutical industries (Klekner and Kosaric 1993, Banat, 1995). These products are particularly highly valued for their antimicrobial activity and lack of toxicity (Lang and Wullbrandt, 1999, Stanghellini and Miller, 1997).

In the general context of characterizing the rhamnolipid mixture (RL_{47T2} throughout the text) produced by *P. aeruginosa* from waste-frying oils as new surface active product, this study specifically deals with: 1) the production and purification of the rhamnolipids, 2) the chemical identification of the components of RL_{47T2}, 3) the physicochemical properties of RL_{47T2}, 4) the evaluation of its biological properties.

MATERIAL AND METHODS

Micro-organism and growth culture

Pseudomonas aeruginosa 47T2 NCIB 40044, isolated from an oil-contaminated soil sample, was selected due to its capacity to accumulate surface-active rhamnolipids from hydrophobic substrates (Robert et al., 1989, Mercadé et al., 1996, Mercadé et al., 1997). After being grown on TSA (Tripticase Soy Agar, Pronadisa, Barcelona, Spain), the bacterial strain was maintained at 4ºC and was also preserved in cryobilles (AEB 400100 EAS Laboratoire, France) at -20ºC.

Experiments were carried out in 2L baffled Erlenmeyer flasks containing 400 mL of medium with the following composition (g/L): $NaNO_3$ 5, KH_2PO_4 2.0, K_2HPO_4 1.0, KCl 0.1, $MgSO_47H_2O$ 0.5, $CaCl_2$ 0.01, $FeSO_47H_2O$ 0.012, yeast extract 0.01. 0.05 mL of a trace elements solution containing (g/L): H_3BO_3 0.26, $CuSO_4{\cdot}5H_2O$ 0.5, $MnSO_4{\cdot}H_2O$ 0.5, $MoNa_2O_4{\cdot}2H_2O$ 0.06, ZnSO4.7H2O 0.7, was added. Finally 40 g/L of olive/sunflower (50:50; v/v) waste frying oil was used as carbon source. Medium components were sterilized separately at 120 ºC, 1 atm for 20 min. The initial pH of the medium was adjusted to 7.2. A 2% (v/v) cell suspension on saline serum of an overnight culture on TSA (Pronadisa, Barcelona, Spain) was used as inoculum. Cultures were incubated at 30 ºC on a reciprocal rotary shaker at 150 rpm.

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Rhamnolipid production and purification

Microbial growth was calculated by measuring the protein content of the cultures, following the method described by Lowry (Lowry et al., 1951). Total rhamnolipid production was measured as rhamnose by a specific colorimetric method (Chandrasekaran and Bemiller, 1980). The RL_{47T2} content was calculated by multiplying the rhamnose concentration by a factor of 3.0, which represents the rhamnolipid/rhamnose calculated using the purified product.

Rhamnolipids were isolated using a modification of the chromatographic method proposed by Reiling (Reiling et al., 1986). Cells were removed from the culture by centrifugation (12,000 x g) for 30 min. Purification was achieved by adsorption chromatography on a polystyrene resin Amberlite XAD2 (Supelco, USA). The resin (60 g) was placed in a glass column (60 x 3 cm), yielding a bed volume of 200 mL. The column was equilibrated with 0.1M phosphate buffer, pH 6.1. The culture supernatant was applied through a sieve placed on top of the resin to prevent whirling up. The adsorption of the active compounds on the resin was monitored by measuring the surface tension (γ_{ST}) of the column outlet. The saturation of the resin was terminated when γ_{ST} of the effluent dropped below 40 mN/m. Then the column was washed with three volumes of distilled

water. Biosurfactants were eluted with methanol and finally the solvent was evaporated to dryness under vacuum (Büchi, Flawil, Switzerland).

Chemical characterization

Individual rhamnolipids were separated and identified by HPLC-ES-MS using a Waters 2690 Separation Module (Waters, Midford, USA). Samples (20 µL) dissolved in methanol were analysed by HPLC using a HYPERSIL C8 WP-300 of 150 x 4.6 mm column (Teknokroma, Sant Cugat, Spain). Acetone was added post-column at 200 µL/min with a Phoenix 20 syringe pump (C. E. Instruments, Milan, Italy). MS was performed using a VG Platform II quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a pneumatically-assisted electrospray (ES) ion source. Negative ion mode was used.

An acetonitrile-water gradient (both eluents contained 0.1% acetic acid) was used: 30% acetonitrile for 2 min, followed by a ramp 30-100% acetonitrile for 30 min, standby for 5 min and then a return to initial conditions. The HPLC flow rate was 1 mL/min. The mobile phase and acetone were mixed in a tee (Valco) and a 1/20 split system was used to introduce the effluent into the ES source. Full scan data were obtained by scanning from *m/z* 100 to 750 in centroid mode with a cycle time of 1 sec. and an inter-scan time of 0.1 sec. The working conditions for the ES source were as follows: drying nitrogen was introduced into the source body at a flow-rate of 400 NL/h, nebulizing nitrogen was set at 20 NL/h and the counter electrode block was heated at 100ºC. The capillary was held at a potential of -3.5 kV. Different cone voltages (-15, -20, -35, -50, -75, -100 V) were tested to study the fragmentation of RL_{47T2}. All chemicals and solvents were ACS grade and used as received.

Physicochemical characterization

Equilibrium surface tension (γ_{ST}) and interfacial tension (γ_{IT}) were measured at 25ºC with a Krüss K12 (Krüss, Helsinki, Finland) tensiometer by the Wilhelmy plate method. The instrument was

calibrated against Mili-Q-4 ultrapure distilled water (Millipore, Illinois, USA). The platinum plate and all glassware used were cleaned in chromic mixture.

Aqueous solutions of purified RL_{47T2} in the concentration range of 200–5 mg/L were obtained by successive dilutions of a concentrated sample prepared by weight in Millipore ultrapure water. To reach equilibrium, all sample solutions were aged in appropriate cells at room temperature (25ºC).

The emulsification behavior of purified RL_{47T2} was studied with various oil phases: linseed oil, almond oil, i-propyl-palmitate, crude oil, kerosene, toluene, n-alkanes (C_{12-14}) and mineral oil. Emulsions were prepared as follows: A stock solution of 1% (W/W) of RL_{47T2} in water was prepared. Aliquots of this stock solution were diluted in the required amount of water and the emulsion was formed by slow addition of the oil phase into the aqueous phase. After adding the oil, emulsions were vortexed for 1 min. The shaking time and vortex speed were the same in all cases. Emulsions were allowed to settle at a constant temperature then the two emulsion heights were measured, one after 24 hours ans another after 1 week.

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 two-fold serial rhamnolipid dilution technique was used to measure antimicrobial activity. Antibacterial activity was determined on liquid medium which was incubated for 24h at 37 ºC (Woods and Washington, 1995). Anti-microbial activity against yeast and fungi was determined on solid medium Sabouraud agar plates which were incubated for 72 hours at 25 ºC (Espinel-Ingroff and Pfaller, 1995). A wide range of 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 mirabillis* CECT 170, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella thyphimurium* 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 perfringes* ATCC 486. Yeast strains: *Candida albicans* ATCC 10231, *Rhodotorula rubra* CECT 1158, *Saccharomyces cerevisiae* ATCC 9763. Fungal strains: *Aspergillus niger* ATCC 14604, *Aureobasidium pullulans* ATCC 9348, *Chaetonium globosum* ATCC 6205, *Gliocadium virens* ATCC 4645, *Penicillium chrysogeum* CECT 2802, *Penicillium funiculosum* CECE 2914, *Botrytis cinerea, Colletotrichum gloeosporioides, Rhizoctonia solani, Fusarium solani*.

Ocular and skin toxicity test were evaluated in male albino rabbits. A total of 0.1 ml of product was instilled into the conjunctival sac of the right eye of 2 animals and the lids were held together for one second. The contra lateral eye served as a control. The eyes were examined each day for a week and the ocular damage was evaluated in accordance with the criteria of Draize (Draize et al., 1944, Draize and Kecley, 1952; Draize, 1959). Fluorescein was used as an aid in reading damaged eyes (Annonymous, 1977).

The skin test was performed by applying 0.5 ml of product with a gauze holding it on the shaved area (approx. 6 cm^2) with no irritant plaster for 24 hours. 60 minutes after eliminating the remaining product with water, skin damage was also evaluated by Draize's technique.

RESULTS AND DISCUSSION

Time course of rhamnolipid production and recovery

We reported previously that *Pseudomonas aeruginosa* 47T2 NCIB 400044 grown in a mineral salt medium with frying oil as carbon substrate accumulated surface active rhamnolipids (Haba et al., 2000). Cell population increased for 24 hours, reaching 8.7 g/L of biomass. Nitrogen was exhausted during the first 24 hours of culture. Rhamnolipid production started soon after inoculation; most of the rhamnolipids were produced after growth ceased (2.02 g/L in 24 hours of culture) and increased throughout the stationary phase, reaching 8.1 g/L of rhamnolipids at 96 hours incubation. The overall cell production yield (g rhamnolipids/g cell protein) was 0.93.

The most commonly used method for biosurfactant recovery in rhamnolipid production is acid precipitation prior to purification. However, when a complex carbon source is used in the process, the down-stream recovery of biosurfactants is a delicate step because of the similar polarity of the product and the substrate components. In the case under study, other fatty acid components from the culture supernatant contaminated the desired product (Haba et al., 2000). Adsorption column chromatography proved to be a simple technique for obtaining isolated rhamnolipid mixtures from the culture. The γ_{ST} of the culture supernatant before purification was 37 mN/m. Rhamnolipid adsorption on the resin column was monitored by γ_{ST} measurements of collected fractions. Resin saturation was detected when γ_{ST} was higher than 40mN/m. The increase in γ_{ST} was due to the adsorption of rhamnolipids at the air/water interface. After resin saturation, rhamnolipids were eluted from the column with methanol. The recovery yield was 61%. The degree of purification was monitored by GC and LC. No free fatty acids were detected in the isolated product.

Chemical characterization

To identify the chemical structure of the product, an aliquot of the purified rhamnolipids produced by *P. aeruginosa* 47T2 was analysed by HPLC-MS-ES at –35 V. This analysis revealed that RL_{47T2} identified 11 homologues, with the pseudomolecular ions being between *m/z* 475 and 703.

The spectra generated by ES at cone voltage fragmentation (CVF) or collision-induced dissociation (CID) below –50 V caused little fragmentation, giving only the pseudomolecular $[M-H]^-$ ion due to the deprotonation of the carboxylic acid group. Whereas higher extraction voltages (-100 V) led to higher fragmentation, a decrease in the intensity of the $[M-H]^-$ ion also occurs. In the case of rhamnolipid characterization of *Pseudomonas* 47T2, where the isomers are not resolved chromatographically, some fragments of the $[M-H]^-$ ion are needed to discriminate between congeners. Therefore, to select the extraction voltage, a compromise should be reached between sensitivity and fragmentation. In the case under study, -75 V was selected. The injection of the sample at –75 V allowed the identification of fragments for 11 rhamnolipid congeners, 3 of which were isomeric pairs.

The retention time, the main pseudo-ions and the ion fragments for the RL_{47T2} found in the sample are shown in Table I. Study of the mass spectra of the different rhamnolipids leads to the conclusion that some fragments are common to all the rhamnolipid structures: the *m/z* 163 ion corresponds to the cleavage of the rhamnose moiety (Rha^-). Also, the ions *m/z* 119 and 103 are produced by the cleavage of the rhamnose moiety, as described by Déziel and co-workers (Déziel, et al., 2000). In this way Rha-Rha-C_{10}-C_{10}, Rha-Rha-C_{10}-$C_{12:1}$ and Rha-Rha-C_{10} structures could be identified. However, there are other ions that allow discrimination between congeners. For instance, Figures 1 and 2 show the mass spectra of Rha-Rha-C_{12}-C_{10} (Fig. 1, 1a), Rha-Rha-C_{10}-C_{12} (Fig. 1, 1b), Rha-C_{12}-C_{10} (Fig. 2, 2a) and Rha-C_{10}-C_{12} (Fig. 2, 2b). The rhamnolipids in Figure 1 have a mass spectrum with a *m/z* 677 pseudomolecular ion for both compounds and differ by the presence

of a *m/z* 479 ion for the former and a *m/z* 507 ion for the latter. These two ions arise from cleavage at the 3 carbon-oxygen bond in both compounds. In addition to the *m/z* 479 and 507 ions, the corresponding *m/z* 197 (C_{12}^-) and 169 (C_{10}^-) ions represent the fatty acid moiety with loss of additional hydrogen. Similarly, the rhamnolipids in Figure 2 have a mass spectrum with a *m/z* 531 pseudomolecular ion for both compounds and differ in the presence of a *m/z* 333 ion for the former and a *m/z* 333 ion for the latter. The corresponding *m/z* 197 (C_{12}^-) and 169 (C_{10}^-) ions are also seen. For other rhamnolipids, *m/z* 141 (C_8^-) and *m/z* 195 ($C_{12:1}^-$) were found. Recently, Déziel reported a wide range of rhamnolipid homologues, found in cultures of *P. aeruginosa* 57RP grown in mannitol or naphthalene, using a triple quadrupole mass spectrometer (Déziel et al., 1999).

Rhamnolipids with some unsaturated fatty acid, Rha-C_{10}-$C_{12:1}$/Rha-$C_{12:1}$-C_{10} (*m/z* 529), Rha-Rha-C_{10}-$C_{12:1}$ (*m/z* 675), Rha-Rha-C_8-$C_{12:1}$ (*m/z* 647), Rha-Rha-$C_{12:1}$-C_{12} and Rha-Rha-C_{10}-$C_{14:1}$ (*m/z* 703), were present in the mixture. Therefore, applying LC-MS to rhamnolipid mixtures is an efficient technique, because it enables compounds to be identified and chromatographically unresolved pairs of congeners to be quantified.

Equilibrium Surface parameters

One of the main characteristics of surfactants is their tendency to adsorb at interfaces in an oriented fashion as a consequence of their amphipathic structure. As surfactant concentration increases, the surface tension of the solution initially decreases and then becomes almost constant due to the interface saturation with surfactant. The concentration at which this phenomenon occurs is known as the critical micelle concentration (CMC) and is determined from the break point of the surface tension *versus* concentration curve (Rosen, M. 1989). The CMC is the minimum surfactant concentration at which the free monomer form micelle aggregates. To confirm that RL_{47T2} is a new surfactant product, its surface active behavior was studied. The measurements of surface tension as a function of concentration showed that the tension decreased gradually until a minimum constant

value of 32.8 mN/m was reached. The break point of the experimental curve, yielded a CMC of 108 mg/L. This value is consistent with those reported in the literature (Abalos et al., 2001), but differs from the CMC values reported for other mixtures of unsaturated rhamnolipid compounds (Syldatk et al., 1985, Mata- Sandoval et al., 1999). The chemical characterization carried out helps to define some relationships between molecular structure components and surface active behavior. 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. The CMC values shown by the unsaturated compounds are greater than those of the corresponding saturated. This could be attributed to the steric factor in micelle formation, (Mata- Sandoval et al., 1999, Yakimov, 1996). In our case, the behaviour agrees with the fact that the mixture RL_{47T2} contained 18.95% of unsaturated hydrophobic chains. In (Abalos et al., 2001), a similar behaviour is reported for a rhamnolipid mixture derived from *P. aeruginosa* AT10 with a 27% of unsaturated components with a CMC value of 150 mN/m.

The reduction of the tension at an interface by a surfactant in aqueous solution when a second liquid phase is present is known as liquid-liquid interfacial tension. If the second liquid phase is a non-polar saturated hydrocarbon, the interfacial tension reduction by the surfactant at the aqueous solution-hydrocarbon interface is greater than at the aqueous solution-air interface. Low liquid-liquid interfacial tension is important in promoting emulsification and enhanced oil recovery by use of surfactant solutions. Interfacial tension for RL_{47T2} at the hexadecane - surfactant solution interface was 1mN/m. Similar values were found for pure rhamnolipids (Lang and Wagner, 1987).

Rhamnolipids mixtures are commonly used as emulsifiers, (Bruheim et al., 1997). Since our interest is to applied RL_{47T2} in the cosmetic, agro-chemical or bioremediation industries, its emulsifying properties were studied. Table II shows measures of the stability of the oil-in water emulsions

when pure product (RL_{47T2}) is used. A strong and stable emulsion of RL_{47T2} was formed with linseed oil. As shown, no emulsion was formed with almond oil and toluene, and weak emulsions, which collapsed within a week, were formed with i-propyl-palmitate. Unstable emulsions were formed with C_{12}-C_{14} n-alkanes and mineral oil, whereas fairly stable emulsions were obtained with crude oil (Table II).

Biological assay

The biocompatibility of purified rhamnolipids RL_{47T2} was assayed by the ocular and skin tests. The examination of the eyes included cornea, iris and conjunctiva. No superficial damage of the cornea was observed, corneal opacity; from the results obtained, rhamnolipids are included in category IV of the EPA classification which includes non-irritant products. Similarly, the mixture of rhamnolipids caused no dermal irritation or oedema.

The antimicrobial activity of RL_{47T2}, determined by performing the minimal inhibitory concentration (MIC), i.e. the minimum concentration of product to lower the growth of a microorganism, is shown in Table III.

RL_{47T2} was highly active against *K. pneumoniae* (0.5 μg/mL), *E. aerogenes* (4 μg/mL) and *S. marcescens* (8 μg/mL). Good activity was detected against *Alcaligenes* and *Citrobacter* (64 μg/mL); and weak activity against *Salmonella* and *Bordetella* (128 μg/mL*)* and *Pseudomonas* (256 μg/mL). Among the Gram-positive bacteria assayed, good activity values were found against *Bacillus* (16 and 64 μg/mL), *Staphylococcus* (32 μg/mL), *Micrococcus* (64 μg/mL); and weak activity, against *Arthrobacter, Mycobacterium* and *Clostridium* (128 μg/mL).

The point of attack of surface-active agents is thought to be the biological membrane (Lang and Wagner, 1993). It has been reported that Rha-C_{10}-C_{10}/Rha-C_{10} prevents the growth of *B. subtilis* at 35 µg/mL and *S. epidermidis* at 350 µg/mL (Lang and Wagner, 1993). Glycolipids produced by *Tsukamurella* spec were active against *E. coli*, *Bacillus megaterium* and *Ustilago violacea* (Vollbrecht et al., 1999). Manoerythrol lipids produced by *Candida antarctica* were active against *B. subtilis* (6.2 µg/mL), *M. luteus* (12.5 µg/mL) and *S. aureus* (12.5 µg/mL) Kitamoto, et al., 1993).

Purified RL_{47T2} were active against fungal strains. The phytopathogenic fungi assayed were of particular interest. Key pathogens in the local agricultural industry, they were isolated from diseased plants. Rhamnolipids also improve the wettability of leaf surfaces (Bunster et al., 1989), a property that makes them relevant to agricultural pest control.

No activity was found against *Aspergillus niger*, *Penicillium chrysogenum* and *Aureobasidium pullulans* at the levels assayed (MIC >256 µg/mL). High activity against *Penicillium funiculosum* and *Gliocadium virens* was observed, with MIC values of 16 µg/mL and 32 µg/mL, respectively; and *Chaetonium globosum* and *Fusarium solani*, with MIC values of 64 µg/mL and 75 µg/mL, respectively.

Antimicrobial activity against *Botrytis cinerea* was observed (MIC, 170 µg/mL). This genus is the pathogenic agent of gray rot on grape skins and of dry eye rot (blossom end rot) on apples. Growth of *Rhizoctonia solani*, which is the causal agent of eye spot at the base of the stems and roots of wheat and other cereals, was inhibited at levels of 109 µg/mL. The sensitivity of *Fusarium solani*, the causal agent of foot, root, stem and fruit rot of cucurbits and green bean root rot, was high (75 µg/mL). Weak activity (276 µg/mL) against *Colletotrichum gloeosporioides*, responsible for anthracnose in strawberries, was found.

In conclusion, RL_{47T2} is a new product formed by 11 rhamnolipid homologues produced by *P. aeruginosa* 47T2 from waste cooking oils. Due to its physicochemical and antimicrobial properties, RL_{47T2} could be used in bioremediation in the food and agrochemical industries.

FIGURES

Con formato: Fuente: Negrita

Con formato: Fuente: Negrita

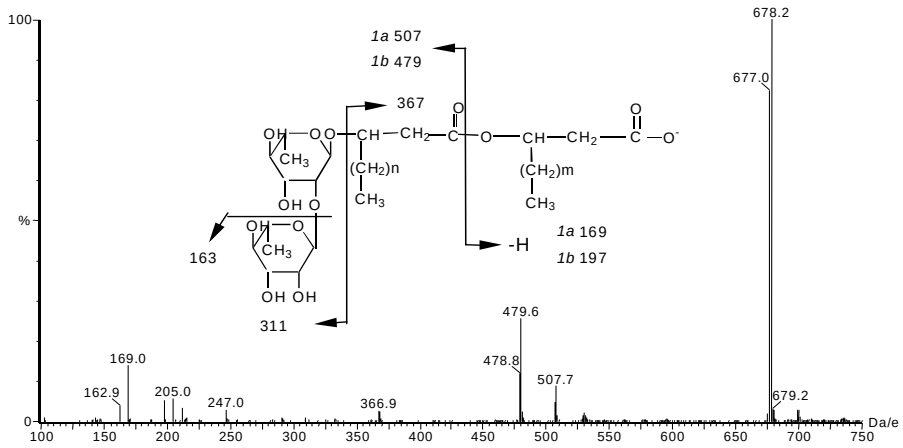

Figure 1.- Mass spectra and structure of rhamnolipid isomers with *m/z* 677. *1a)* Rha-Rha-C12-C10: m = 6, n = 8; *1b)* Rha-Rha-C10-C12: m = 8, n = 6.

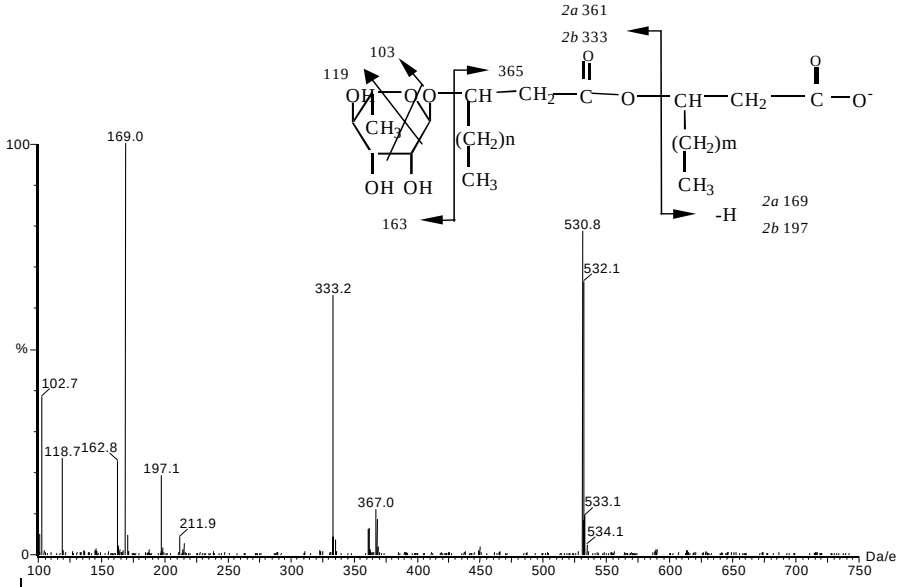

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Figure 2.- Mass spectra and structure of rhamnolipid isomers with m/z 531 *2a)* Rha-C12-C10: m = 6, n = 8; 2*b)* Rha-C10-C12: m = 8, n = 6.

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TABLES

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Table I.- Components of *Pseudomonas aeruginosa* 47T2 rhamnolipid mixture found by HPLC-MS-ES at extraction voltage of –75 V .

Rhamnolipid structure	Retention time (min)	Pseudomolecular ion (m/z)	Relative rhamnolipid Abundance (%)	Ion fragments (m/z)
Rha-Rha-C_8-C_{10}	8.91	621	2.43	141,169,311, 452
Rha-C_{10}-C_8 Rha-C_8-C_{10}*g	10.70	475	0.37	163,168,311
Rha-Rha-C_8-$C_{12:1}$	10.72	647	0.69	195,452
Rha-Rha-C_{10}-C_{10}	12.26	649	34.12	163,169,311, 339,479
Rha-Rha-C_{10}-$C_{12:1}$	14.00	675	12.22	103,119,195, 311,479
Rha-C_{10}-C_{10}	14.11	503	18.62	103,119,163, 169,333,339
Rha-Rha-C_{10}-C_{12} Rha-Rha-C_{12}-C_{10}*	15.29	677	19.93	163,169,197, 311,479,507
Rha-C_{10}-$C_{12:1}$ Rha-$C_{12:1}$-C_{10}	16.09	529	5.08	103,119,163, 169,311,333, 365
Rha-Rha-$C_{12:1}$-C_{12}	16.42	703	**	197, 507
Rha-Rha-C_{10}-$C_{14:1}$	17.04	703	**	480
Rha-C_{10}-C_{12} Rha-C_{12}-C_{10}	17.49	531	5.59	103,119,163, 169,197,311, 333,361,367

(*) majority isomeric form.
(**) Proportion of both pseudomolecular ion *m/z* 703 is 0.96.

Table II.- Stability of the o/w emulsions for rhamnolipids with some organic compounds.

Substrat	RL: Subst: H_2O	% E_{24}	% E_{168}
Linseed oil	0.10: 0.56: 0.34	90	90
Almond oil	0.10: 0.56: 0.34	-	-
i- Propilpalmitate	0.10: 0.56: 0.34	30	-
	0.05: 0.75: 0.20	30	-
Crude oil	0.10: 0.50: 0.40	66.6	40
	0.10: 0.15: 0.75	77.7	70
	0.15: 0.31: 0.54	62.5	40
Kerosene	0.15: 0.31: 0.54	80	-
	0.05: 0.45: 0.50	50	-
Toluene	0.15: 0.31: 0.54	-	-
n- Alkanes (C_{12-14})	0.15: 0.31: 0.54	60	-
Mineral oil	0.15: 0.31: 0.54	50	-

A solution of 1% of RL_{47T2} was used to prepare the emulsions. Aliquots of this stock solution were used and emulsions were preapred by slow addition of the hydrophobic phase (see text).

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Table III.- Antimicrobial properties of RL_{47T2}.

Microorganism	MIC (µg/ml)
Gram Negative	
Salmonella thyphimurium ATCC 16028	128
Proteus mirabillis CECT 170	>256
Enterobacter aerogenes CECT 689	4
Escherichia coli ATCC 8739	64
Serratia marcescens CECT 274	8
Klebsiella pneumoniae CECT 17832	0.5
Alcaligenes faecalis ATCC 8750	64
Citrobacter freundii ATCC 22636	64
Pseudomonas aeruginosa ATCC 9027	256
Bordetella bronchiseptica ATCC 461	128
Gram Positive	
Arthrobacter oxydans ATCC 8010	128
Staphyloccocus epidermidis	32
Staphylococcus aureus ATCC 6538	32
Bacillus cereus var. mycoide ATCC 11778	64
Bacillus subtilis ATCC 6633	16
Micrococcus luteus ATCC 9631	64
Mycobacterium phlei ATCC 41423	128
Clostridium perfringens	128
Yeast	
Candida albicans ATCC 10231	>256
Rhodotorula rubra CECT 1158	>256
Saccharomyces cerevisiae ATCC 9763	>256
Fungi	
Aspergillus niger ATCC 14604	>256
Chaetonium globosum ATCC 6205	64
Penicillium chrysogeum CECE 2802	>256
Penicillium funiculosum CECE 2914	16
Aureobasidium pullulans ATCC 9348	>256
Gliocadium virens ATCC 4645	32
Botrytis cinerea	170
Colletotrichum gloeosporioides	276
Rhizoctonia solani	109
Fusarium solani	75

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REFERENCES

Abalos A, Pinazo A, Infante MR, Casals M, García F, Manresa A. (2001). Physicochemical and Antimicrobial Properties of New Rhamnolipids Produced by *Pseudomonas aeruginosa* AT10 from Soybean Oil Refinery Wastes. Langmuir 17: 1367-1371.

Annonymous. 1977. In: Principles and procedures for evaluating the toxicity of household substances. NAS Publication. vol. 1138.

Arino S, Marchal R and Vandecasteele JP. 1996. Identification and production of a rhamnolipidic biosurfactant by a Pseudomonas species. Appl Microbiol Biotechnol 45: 162-168.

Banat IM. 1995. Characterization of biosurfactants and their use in pollution removal- state of the art. Acta Biotechnol 15: 251-267.

Bruheim P, Bredholt H, Eimhjellen K. 1997. Bacterial degradation of emulsified crude oil and the effect of various surfactants. Can J Microbiol 43: 17-22.

Bunster L, Fokkema NJ, Schippers B. 1989. Effect of surface- active *Pseudomonad* spp. on leaf wettability. Appl Environ Microbiol 55: 1340-1345.

Chandrasekaran EV, Bemiller JN. 1980. In: Methods in Carbohydrate Chemistry. Wrhiste L, Wolfrom ML eds, New York: Academic Press. vol III: p. 89-97.

Déziel E, Lépine F, Dennie D, Boismenu D, Mamer O, Villemur R. 1999. Liquid chromatography/mass spectrometry analysis of mixture of rhamnolipids produced by

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Francés (Francia)

Pseudomonas aeruginosa strain 57RP grown on mannitol or napthalene. Biochim Biophys Acta 1440: 244-252.

Déziel E, Lepine F, Milot S, Villemur R. 2000. Mass spectrometry monitoring of rhamnolipids from a growing culture of *Pseudomonas aeruginosa* strain 57RP. Biochim Biophys Acta 1485: 145-152.

Draize JH, Woodard G, Calvely HO. 1944. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J Pharm Exptl Ther 82.

Draize JH, Kecley EA. 1952. Toxicity to eye mucosa of certain cosmetic preparation containing surface active agents. Proc Sci Sec T6A .

Draize JH. 1959**.** In: Appraisal of the safety of Chemicals in Foods, Drugs and Cosmetics. A. F. D. O. O. T. U.S., ed., Austin, Texas.

Espinel-Ingroff A, Pfaller MA. 1995. In: Manual of Clinical Microbiology. Murray PR ed. Washington: D.C.ASM Press. vol. X: p. 1405-1415.

Haba E, Espuny MJ, Busquets M and Manresa A. 2000. Screening and production of rhamnolipids by Pseudomonas aeruginosa 47t2 NCIB 40044 from waste frying oils. J Appl Microbiol 88: 379-387.

Itoh S, Honda H, Tomita F, Suzuki T. 1971. Rhamnolipids Produced by *Pseudomonas aeruginosa* Grown on n-Paraffin. J Antibiotics 24: 855-859.

Kitamoto D, Yanagishita H, Shinbo T, Nakane T, Kamisawa C, Nakahara T. 1993. Surface active properties antimicrobial activities of mannosylerythritol lipids as biosurfactants produced by *Candida antarctica*. J Biotechnol 29: 91-96.

Klekner V, Kosaric N.1993. In: Biosurfactants, Kosaric N editor, New York.: Dekker. vol. 48: p. 373-384.

Lang S, Wagner F. 1987. In: Biosurfactants and Biotechnology, Kosaric N, Cairns WL, Gray NCC eds. NewYork.: Marcel Dekker. vol. 25: p 21-45.

Con formato: Italiano (Italia)

Con formato: Inglés (Reino Unido)

Con formato: Portugués (Brasil)

Lang S, Wagner F. 1993. In: Biosurfactant properties and applications. Kosaric N ed. New York: Marcel Dekker. vol. 48: p. 251-269.

Lang S and Wullbrandt D. 1999. Rhamnose lipids- biosynthesis, microbial production and application potential. Appl Microbiol Biotechnol 51: 22-32.

Lowry OH, Rosebrought NJ, Farr A, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 139: 265-274.

Mata- Sandoval J, Karns J, Torrents A. 1999. High-performance liquid chromatography method for the characterization of rhamnolipids mixture produce by *Pseudomonas aeruginosa* UG2 on corn oil. J Chromat 864: 211-220.

Mercadé ME, Manresa A, Robert M, Espuny MJ, de Andrés C, Guinea J. 1993. Olive Oil Mill Effluent (OOME). New substrate for biosurfactant production. Bioresource Technol. 43: 1-6.

Mercadé ME, Monleón L, de Andrés C, Rodón I, Espuny MJ, Manresa A. 1996. Screening and selection of surfactant-producing bacteria from waste lube oil. J Appl Bacteriol 81: 161-166.

Mercadé ME, Espuny MJ, Manresa A. 1997. The use of oil substrate for biosurfactant production. In: Recent Research Developments in oil chemistry, Pandalai SG editor. Trivandurum:Tansw. res. Net. p177-185.

Parra JL, Guinea J, Manresa A, Mercadé ME, Robert M, Comelles F and Bosch P. 1989. Chemical characterization and physicochemical behavior of biosurfactants. JAOCS 66: 141-145.

Reiling HE, Thanei-wyss U, Guerra-Santos LH, Hirt R, Käppeli O, Fiechter A. 1986. Pilot Plant Production of Rhamnolipid Biosurfactant by *Pseudomonas aeruginosa*. Appl Environ Microbiol 51: 985-989.

Rendell NB, Taylor GW, Somerville M, Todd H, Wilson R, Cole J. 1990. Characterization of *Pseudomonas* rhamnolipids. Biochim Biophys Acta 1045: 189-193.

Con formato: Inglés (Reino Unido)

Con formato: Portugués (Brasil)

Con formato: Inglés (Reino Unido)

Robert M, Mercadé ME, Bosch P, Parra JL, Espuny MJ, Manresa A, Guinea J. 1989. Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T1. Biotechnol Letters 11: 871-874.

Rosen, M. 1989. Surfactants and Interfacial Phenomena. 2nd ed. Wiley Interscience. N.Y.

Stanghellini ME, Miller RM. 1997. Their Identity and potencial Efficacy in the Biological Control of Zoosporic Plant Pathogens. Plant Disease 81: 4-12.

Syldatk C, Lang S, Matulovic V and Wagner F. 1985. Production of four interfacial active rhamnolipids from *n* -alkanes or glycerol by resting cells of *Pseudomonas species* DSM 2847.*Z.* Naturforsch 40c: 61-67.

Vollbrecht E, Rau U, Lang S. 1999. Microbial conversion of vegetable oils into surface-active di-, tri-, and tetrasaccharide lipids (biosurfactants) by the bacterial strain *Tsukamurella* spec. Fett Lipid 101: 389-394.

Woods GL, Washington JA. 1995. In: Manual of Clinical Microbiology. Murray PR ed. Washington: D.C.ASM Press. vol. X: p. 1327-1341.

Yakimov M. 1996. Effect of heterogenety of hydrophpbic moieties on surface activity of lichenysin A, a lipopeptide biosurfactant from *Bacillus licheniformis* BAS50. Biotechnol Appl. Biochem. 23: 13-18.

Con formato: Portugués (Brasil)

Con formato: Inglés (Reino Unido)

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