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Production and characterisation of a biosurfactant isolated from *Pseudomonas aeruginosa* UW-1

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Pseudomonas aeruginosa **UW-1 produced 17–24 g L^{-1} rhamnolipid in vegetable oil-containing media in shake flask cultures in 13 days. In time course studies of growth and rhamnolipid production in a salts medium containing 6% canola oil, total bacterial count reached 2.6 × 10^{10} CFU ml^{-1} after 48 h and a maximum rhamnolipid yield of 24.3 g L^{-1} was obtained after 9 days. Rhamnolipid components were purified and separated by chloroform-methanol extraction and TLC chromatography. The major rhamnolipid components were characterised as L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate by nuclear magnetic resonance and mass spectrometry. The components were separated preparatively by silica gel column chromatography. The recovered monorhamnosyl fraction contained no dirhamnosyl moiety while the recovered dirhamnosyl fraction contained 5% of the monorhamnosyl moiety when analyzed by HPLC. The ratio of mono- to dirhamnosyl components produced by *P. aeruginosa* UW-1 was determined by HPLC to be 4 : 1 by weight. Purified mono- and dirhamnosyl components had the same CMC value of 40 μg ml^{-1} and decreased the surface tension of water to 27.7 and 30.4 dynes cm^{-1}, respectively.**

Keywords: *Pseudomonas aeruginosa*; rhamnolipid; biosurfactant; fermentation; purification

Introduction

Biosurfactants are biological molecules which manifest properties similar to the well-known synthetic surfactant family. They include microbial compounds which exhibit surfactant properties and have been reported as being produced both on the microbial cell surface [17] and excreted extracellularly. Biosurfactants can be as effective as some widely-used synthetics [22].

Microbially-derived surfactants offer several advantages and interest in these biosurfactants continues to grow. Microorganisms, having diverse synthetic capabilities, offer potential in expanding the range of surfactants with novel compounds, thereby providing new possible applications. The most important advantage of biosurfactants over chemical surfactants is probably their ecological acceptability. Biosurfactants are biodegradable and thus problems of toxicity and accumulation in natural ecosystems are avoided [20,23]. In the environmental sector, biosurfactants have potential applications in bioremediation and waste treatment because of their inherent degradability. Indeed, microbial populations degrading petroleum hydrocarbons produce biosurfactants to increase substrate bioavailability [15].

Glycolipids, consisting of hydrophilic carbohydrates and long chain aliphatic acids or hydroxy-aliphatic acids, are the most common class of the microbially-produced surface active compounds. One group, the rhamnolipids, possess strong surfactant [21], antibacterial and antiviral activities [9], and spreading abilities, particularly on alkanes [3]. Four different rhamnolipid homologues, produced by *Pseudomonas aeruginosa*, have been identified and characterised [3,5,6,8,9,19,20]. These homologues contain one or two molecules of rhamnose as the carbohydrate component and one or two residues of β-hydroxydecanoate as the lipid moiety.

A biosynthetic pathway for synthesis of rhamnolipids, by sequential glycosyl transfer, has been proposed by Burger *et al* [1,2]. Ochsner *et al* [18] isolated and analysed a gene involved in regulation of rhamnolipid biosynthesis. In this paper we describe production and properties of rhamnolipids by a very high producing strain, *P. aeruginosa* UW-1.

Materials and methods

Microorganisms and cultivation conditions

Pseudomonas aeruginosa UW-1, which was isolated from hydrocarbon-contaminated soil, was a gift from the Biotechnology Department, Shanghai Institute of Organic Chemistry, Shanghai, China. Other *P. aeruginosa* strains were obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA. Cultures were maintained on the mineral salts medium set out below in agar supplemented with 2% corn oil. Inoculated slants were incubated at 30°C for 48–72 h. Cultures were stored at 4°C for up to one month.

Frozen stock cultures were also maintained at –70°C. These cultures were prepared by transferring a 5% (v/v) amount of seed culture to a 250-ml Erlenmeyer flask containing 50 ml of mineral salts medium and 2% (v/v) corn oil as carbon source. The culture was grown at 30°C, 200 rpm, on an orbital incubator shaker for 3 days after

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which 30% (v/v) sterile glycerol was added and mixed thoroughly. Aliquots, 1.5 ml, were then dispensed into sterile glass vials and flash frozen in a dry ice–acetone bath before storage at −70°C. Frozen cultures were recovered by transferring whole vials of thawed culture to 25 ml of sterile minerals salts medium containing 25% (v/v) corn oil as carbon source and incubated on the orbital shaker for 3 days at 30°C, 200 rpm.

Production of rhamnolipid was conducted by growing cultures in 4-L Erlenmeyer flasks containing 500 ml sterile mineral salts medium and a 6% carbon source at 30°C and 200 rpm. The mineral salts medium used throughout this study was taken from Lindhardt *et al* [11], and contained (g L^{-1}): $NaNO_3$, 15; KCl, 1.1; NaCl, 1.1; $FeSO_4 7H_2O$, 0.00028; KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4 7H_2O$, 0.5; yeast extract 0.5; carbon source, 60 and 5 ml of a trace element solution containing (g L^{-1}): $ZnSO_4 \cdot 7H_2O$, 0.29; $CaCl_2 \cdot 4H_2O$, 0.24; $CuSO_4 5H_2O$, 0.25; $MnSO_4 H_2O$, 0.17. The trace element solution was filter-sterilised through a 0.2-μm acrylic sterile syringe filter (Nalgene, Rochester, NY, USA) and then added to the medium, which had been autoclaved and allowed to cool. The final pH of the medium was adjusted to 6.5 with HCl. Preliminary studies indicated that the medium of Lindhardt *et al* [11] was optimised for rhamnolipid production with respect to nitrogen and phosphate content.

Properties of crude rhamnolipid extract

Rhamnolipid was extracted from cultures using a modification of the method of Hisatsuka *et al* [6]. The pH of cultures was adjusted to approximately 2.0 with 1 N HCl and the rhamnolipid extracted for 15 min with an equal volume of chloroform : methanol (2 : 1 v/v). The separated solvent layer was removed and the upper aqueous phase re-extracted as before. The combined extracts were concentrated under vacuum using a rotoevaporator to give the crude extract (Evapotec Rotary Evaporator, Haake Buchler Product, Berlin, Germany) at 40°C.

Table 1 Effect of carbon source on rhamnolipid production by *P. aeruginosa* UW-1

Carbon source	Incubation time (days)	Crude extract weight (g L^{-1} culture)	Rhamnolipid content (Anthrone method)	
			g L^{-1} culture	% (w/w) of crude extract
Ethanol	7	1.0	0.3	30.0
	13	0.0	0.0	—
Glycerol	7	0.0	0.0	—
	13	38.2	0.9	2.3
Glucose	7	2.0	1.2	60.0
	13	0.3	0.2	66.6
Olive oil	7	29.0	11.5	39.7
	13	31.8	17.2	54.1
Soya oil	7	27.0	14.5	53.7
	13	44.8	22.4	50.0
Canola oil	7	28.0	11.0	39.3
	13	47.0	23.9	50.9
Corn oil	7	34.0	15.0	44.1
	13	42.4	22.1	52.1
Paraffin	7	15.0	0.5	3.3
	13	0.4	0.4	100.0

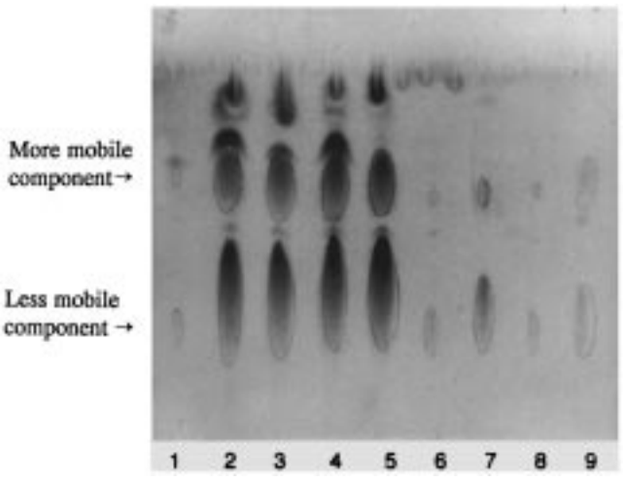


Figure 1 TLC plate of crude extracts produced on various carbon sources. Lane 1, 100 μg ml^{-1} pure rhamnolipid standard (Shanghai Institute of Organic Chemistry, China); 2, corn oil; 3, olive oil; 4, soybean oil; 5, canola oil; 6, glycerol; 7, paraffin; 8, ethanol; 9, glucose. Lanes 2–9 represent the extracts taken from cultures of *P. aeruginosa* UW-1 grown on the respective carbon sources. Supernatants (500 ml) from each carbon source condition were extracted twice with an equal volume of chloroform : methanol (2 : v/v). Five-microlitre samples from each combined chloroform : methanol extract (ie the rhamnolipid recovered from 500 ml of culture) or 5 μl of standard were applied at each spot. Plates were developed in a chloroform : methanol : water (65 : 15 : 2 v/v/v) solvent system for approximately 15 min and sprayed with 4-methoxy-benzaldehyde, in concentrated acetic and sulfuric acids (0.5 : 50 : 1 v/v/v).

Partial purification of rhamnolipids

For analytical purposes, a 10-g sample of crude rhamnolipid extract was purified on silica gel 100 (particle size 0.063–0.200 mm, mesh size 70–230, Merck, Darmstadt, Germany). Ten grams of rhamnolipid extract and 100 g heat-activated silica gel were mixed with enough chloroform to make a liquid slurry. This slurry was then poured into a Buchner funnel, lined with filter paper (15 cm, 1 PS, Whatman Ltd, Maidstone, UK), and rinsed with 500 ml chloroform while stirring. Volumes of chloroform (200 ml) were then added under vacuum and fractions were collected until neutral lipids were no longer detected by thin layer chromatography (TLC). Rhamnolipids (polar lipids) were then recovered from the silica gel by rinsing with 200 ml volumes of chloroform : methanol (1 : 1 v/v). Rinsing was continued with 200 ml chloroform : methanol (1 : 2 v/v) until TLC analysis indicated recovery of all rhamnolipids.

Separation of mono- and dirhamnosyl components

The component mono- and dirhamnosyl lipids were separated from the partially purified rhamnolipid extract by TLC on precoated silica gel (250 μm) glass plates (Baker Si250 TLC Plates, JT Baker, Phillipsburg, NJ, USA). Partially purified rhamnolipid (0.3 g) was solubilised in 1 ml of chloroform and approximately 100 μl applied to the 20 × 20 cm glass plates with a 25-μl micropipet tube (Drummond microcaps, Acadian Instruments, Etobicoke, ON, Canada). The plates were developed in a chloroform : methanol : water solvent system (65 : 15 : 2

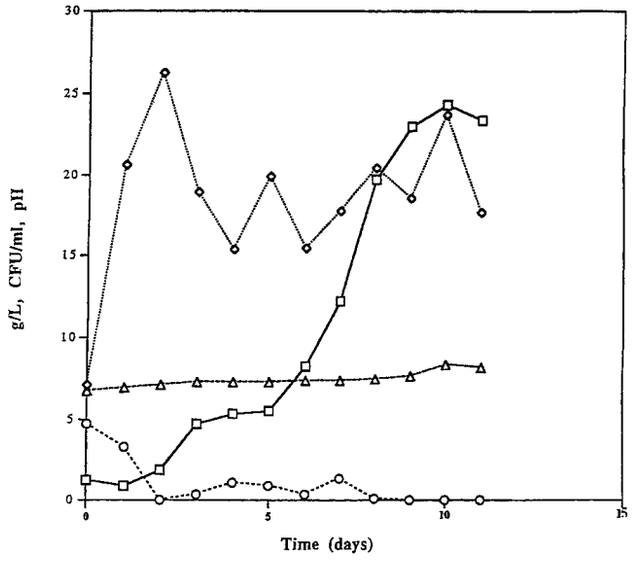


Figure 2 Time course of growth and rhamnolipid production by *Pseudomonas* UW-1. Cultures were incubated in the MSM medium containing 6% canola oil. Rhamnolipid was measured by the anthrone method, using rhamnolipid from the Shanghai Institute of Organic Chemistry, China as standard. Growth of *Pseudomonas* UW-1 was monitored by the plate count method and nitrogen levels were determined by a photometric assay. Rhamnolipid (g L^{-1}) —□—; E9 (CFU ml^{-1}) —◇—; NO_3–N (g L^{-1}) ---○---; pH —△—.

v/v/v) and a small portion of the plate, 3 cm from the edge, was sprayed with 4-methoxy-benzaldehyde in concentrated acetic and sulphuric acids (0.5 : 50 : 1 v/v/v) spraying reagent. The portions of the plates not sprayed were scraped, corresponding to the bands visualised in the sprayed area. The silica gel scrapings of the two bands were collected and the rhamnolipids extracted twice with 8 ml of chloroform : methanol (1 : 2 v/v). The extraction involved vortexing the solvent-scrapings mixture for 1 min, centrifuging down the silica gel for 10 min and then pipetting off the solvent. The extracts were dried under nitrogen and analyzed by TLC. The separated rhamnolipids were identified at the Shanghai Institute of Organic Chemistry by nuclear magnetic resonance (NMR) and mass spectrometry (MS). Analytical equipment used in the identification were as follows: Varian XL-200 (Varian, Sugarland, TX, USA) for ^{1}H NMR; JEOL FX-90Q (JEOL, Tokyo, Japan) for ^{13}C NMR; Finnigan 4021 GC/MS and FABMS MAT 711 (Finnigan, Austin, TX, USA).

Table 3 ^{13}C NMR chemical shift data for rhamnolipid components

Carbon aglycon	More mobile component	Less mobile component	Carbon sugar	More mobile component	Less mobile component
1–C	13.4	13.6	1′–C	97.9	97.8
2	22.2	22.1	2′	72.1	70.3
3	25.1	23.9	3′	72.3	70.6
4	28.8	28.4	4′	73.5	72.1
5	28.9	28.9	5′	69.5	69.8
6	29.2	31.2	6′	17.3	16.8
7	31.4	32.3	7′		102.4
8	72.1	73.3	8′		77.1
9	41.0	40.2	9′		70.6
10	171.8	170.7	10′		72.1
11	13.4	13.6	11′		69.3
12	22.2	22.1	12′		16.9
13	24.3	24.8			
14	28.8	28.4			
15	28.9	28.9			
16	29.2	31.2			
17	31.4	33.1			
18	72.3	72.1			
19	40.2	42.3			
20	178.7	176.1			

Column chromatography

For separation of large volumes of rhamnolipid, liquid column chromatography was utilised. A column 26 cm × 3.5 cm (diam) was prepared with a 50-g activated silica gel (230–400 mesh) chloroform slurry. A 5-g sample of crude rhamnolipid extract was prepared in 10 ml of chloroform and loaded with a Pasteur pipette. The column was washed with chloroform until neutral lipids were completely eluted. Chloroform : methanol mobile phases were then applied in sequence: 50 : 3 v/v (1000 ml); 50 : 5 v/v (200 ml); and 50 : 50 v/v (100 ml) at a flow rate of 1 ml min^{-1} and 20-ml fractions were collected. 1-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate was eluted with 50 : 3 v/v chloroform : methanol. A final wash with 50 : 50 chloroform : methanol, removed any remaining 1-rhamnosyl-1-rhamnosyl-β-hydroxydecanoyl-β-hydroxy-decanoate from the column. Fractions were combined and dried under vacuum with a rotoevaporator (Evapotec Rotary Evaporator) at 40°C. The monorhamnosyl component was recovered at 100% purity and the dirhamnosyl component at 95% purity.

Table 2 ^{1}H NMR chemical shift data for rhamnolipid components

Aglycon	$–CH_3$	$–CH_2–CH_5$	$–CH_2–CH_2–CH_2–$	$–CH_2–COO–$	$–COO–CH_2–$	–O–C–H
More mobile component	0.88	1.21–1.33	1.60	2.63	5.38	4.50
Less mobile component	0.88	1.21–1.40	1.67	2.46	5.32	4.21
Sugar	1′–H	CH_5	2′–,3′–,5′–H	4′–H		
More mobile component	4.92	1.28	3.68–3.82	3.40		
Less mobile component	4.90	1.26	3.68–3.88	3.45		
	4.75			3.42		

High pressure liquid chromatography (HPLC)

Isolated components were tested for purity by HPLC (Waters, Millipore Corp, MA, USA) using a Hi-Pore™ reversed-phase column (C_{18}, 5 μm, 4.6 × 250 mm). The HPLC unit consisted of a Model 600E multisolvent delivery system, and a Model 745 data integrator. The mobile phase was acetone-acetonitrile (30 : 70 v/v) at a flow rate of 0.4 ml min^{-1}.

Quantitative determination of rhamnolipids

Rhamnolipids were quantified by weight and by the colorimetric determination of sugars with anthrone sulphate [7]. In determining rhamnolipid yields by weight, round-bottom flasks were preweighed and then used to evaporate solvent from rhamnolipid extracts.

A modified anthrone sulphate method was developed which involved overlaying 1.25 ml of sample with 2.5 ml of reagent (0.20 g, 9,10,dihydro-9-oxoanthracene per 100 ml concentrated sulfuric acid) in a test tube immersed in a cold water bath (10–15°C). Tubes were vortexed, heated for 15 min in a boiling water bath and then cooled to room temperature within 1 h. Absorbance was measured in a spectrophotometer at 625 nm against distilled water. Standards of rhamnose and pure rhamnolipid (provided by the Shanghai Institute of Organic Chemistry, Shanghai, China) were prepared over a range of concentrations. Standards, reagent blanks and unknowns were analyzed in triplicate and the linear correlation was demonstrated between the quantity of rhamnolipid and optical density.

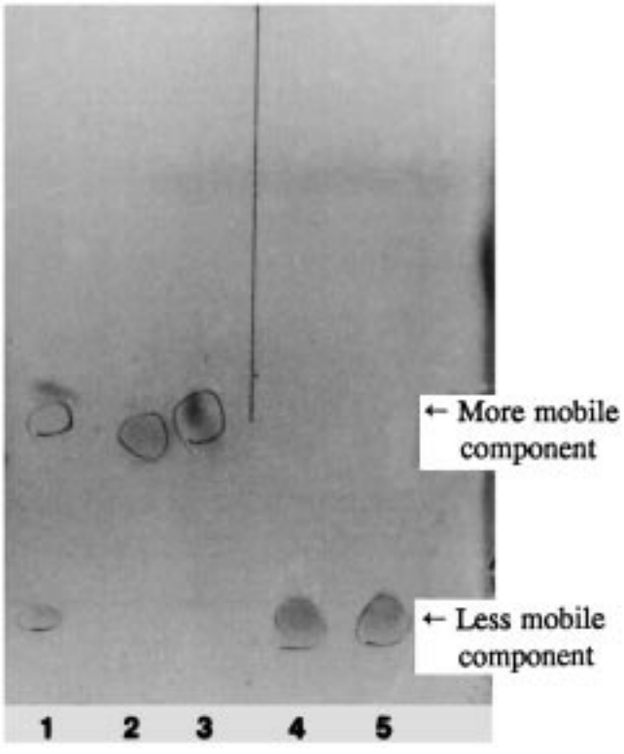


Figure 3 Thin layer chromatograph of isolated rhamnolipids produced by *Pseudomonas* UW-1. Lane 1, 100 μg ml^{-1} pure rhamnolipid standard (Shanghai Institute of Organic Chemistry, China); 2 and 3, the more mobile rhamnolipid component; 4 and 5, the less mobile rhamnolipid component. Rhamnolipid components were separated by thin layer chromatography on silica gel-coated plates and recovered by chloroform : methanol (1 : 2 v/v) extraction. Common fractions were combined and the standard and samples were applied in 5.0-μl volumes onto the shown TLC plate and developed in a chloroform : methanol : water (65 : 15 : 2 v/v/v) solvent system.

Determination of surfactant properties

Surfactant properties of the purified rhamnolipids were determined by the drop volume method [10]. A 1-ml pipette having a 0.1-ml incremental scale was modified such that the tip was polished level and the mouth connected to a glass syringe so that drops could be slowly released in a controlled manner. The critical micelle concentration and the ability of the rhamnolipids to reduce the surface tension of water were calculated as described by Lando and Oakley [10].

Rhamnolipid extracts, purified mono- and dirhamnosyl components, and the mixed form of the rhamnolipids were diluted with distilled water and the critical micelle concentration was taken as the point in the range of concentrations at which the surface tension displayed an abrupt change in value [4]. The ability of the rhamnolipid(s) to reduce the surface tension of water was taken as the concentration at which the lowest surface tension was reached before stabilising.

Nitrate determination

Nitrate was measured using the CHEMetrics (Calverton, VA, USA) nitrate photometric assay kit. Samples were diluted with distilled water to 0–1.5 ppm nitrate.

Bacterial counts

The spread plate technique was used by serially diluting well-mixed samples in sterile 0.025 M potassium phosphate buffer, pH 7.0, and spreading on nutrient agar plates using a sterile glass spreader. Plates were counted after a 48-h incubation at 30°C. Only plates giving counts of 30–300 were used to calculate the colony-forming units per ml (CFU ml^{-1}) for a given sample.

Experimentation and analyses

Rhamnolipid determinations were carried out on samples in triplicate. Results are the average of three determinations. Typical variations between triplicates were less than 5%. Experiments were reproducible with typical variations in data from experiment to experiment of less than 10%.

Results

In preliminary experiments, three biosurfactant-producing strains of *Pseudomonas aeruginosa*, strains ATCC 10145, ATCC 9027 and UW-1, were compared for possible rhamnolipid-producing ability by cultivation in the mineral salts medium, containing corn oil as the carbon source, at 30°C for 10 days. Entire culture flasks were extracted twice with chloroform and methanol to recover lipid-containing material including any rhamnolipid. Weights of recovered lipid material were 15.6, 6.0 and 3.4 g L^{-1} for strains UW-1, ATCC 10145 and ATCC 9027, respectively. A qualitative comparison of the crude extracts was made by TLC, followed by spraying with acidified 4-methoxy-benzal-

dehyde and also by weight determination of the crude extract. The standard exhibited two spots corresponding to a more mobile and less mobile component. Strain UW-1 manifested the largest, most distinct spots, while strain ATCC 10145 exhibited a lower concentration of rhamnolipids by TLC, while no spot corresponding to these standards was shown by strain ATCC 9027. From this preliminary study, *P. aeruginosa* UW-1 was selected for further work on rhamnolipid production.

The effect of a variety of carbon sources on rhamnolipid production by *P. aeruginosa* UW-1 in Erlenmeyer flasks after 7 and 13 days is illustrated in Table 1. Content of rhamnolipid was determined in crude extracts by the anthrone method. While the organism could grow on substrates such as glycerol and glucose, rhamnolipid, as detected by the anthrone method in culture supernatants, was negligible. It was concluded from this result and from carbohydrate-reducing sugar measurements, that *P. aeruginosa* UW-1 does not normally secrete large amounts of carbohydrate into the medium and that the anthrone procedure could be used as a measure of rhamnolipid production. The extraction procedure is designed to recover lipid components and, thus, use of anthrone tests on the lipid extracts is a valid approach to estimating glycolipids in the extract.

Plant oils promoted highest production of rhamnolipids ranging from 11–15 g L^{-1} to 17–24 g L^{-1} after 7 and 13 days, respectively. In contrast, rhamnolipid levels produced when ethanol, glycerol, glucose and paraffin were used as carbon sources ranged from 0 to 1.2 g L^{-1}. Rhamnolipid content of crude extracts from plant oil-containing cultures ranged from 40–55%. A comparison of crude extracts on TLC plates is presented in Figure 1. Since the amount of rhamnolipid applied was taken from a fixed volume of culture, spot intensities give a relative comparison of rhamnolipid titres. Large intense spots corresponding to the components in the purified rhamnolipid standard were observed in flasks containing plant oils (Figure 1).

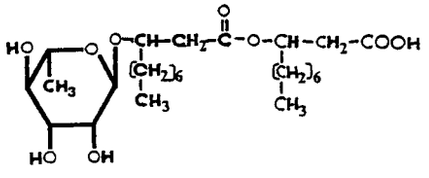
Canola oil was chosen as the carbon substrate for further studies as it gave highest rhamnolipid production (Table 1), closely followed by soya oil. The time course for growth and rhamnolipid accumulation by *Pseudomonas* UW-1 in Erlenmeyer flasks containing Canola oil as carbon source are illustrated in Figure 2. Rhamnolipid production began after the exponential phase of growth and reached a yield of 24.3 g L^{-1} after 9 days. Viable counts reached a maximum of 2.6×10^{10} CFU ml^{-1} after 2 days, during which time rapid utilisation of nitrogen was observed. The pH during growth remained stable at approximately 7.5.

Rhamnolipids produced by *Pseudomonas* UW-1, separated by TLC on silica gel-coated glass plates and recovered by extracting with chloroform : methanol (1 : 2 v/v), were characterised by nuclear magnetic resonance and mass spectrometry. ^{1}H NMR data for the mono- and dirhamnosyl lipids are provided in Table 2. ^{13}C NMR chemical shift data of the components are provided in Table 3. Fast atom bombardment ionization mass spectra of the monorhamnosyl lipid exhibited a peak at 550 corresponding to a molecular weight of 504 for the component and two sodium ions. A peak at 674 for the second component represents a molecular weight of 650 for the component plus one sodium ion. The structures of the two forms, the more mobile and less mobile components, when separated by TLC in chloroform : methanol : water (65 : 15 : 2 v/v/v) (Figure 3), were identified as l-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and l-rhamnosyl-l-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate, respectively (Figure 4).

The mono- and dirhamnosyl components were also separated preparatively by column chromatography. Twenty millilitre-fractions were collected and tested by TLC in order to monitor the separation and elution of the two components from the column. Neutral lipids were first eluted in fractions 18–38 in a yield determined by weight of 0.49 g. The more mobile monorhamnosyl component was then eluted (fractions 39–101), in a yield of 1.07 g. Then the less mobile component (fractions 102–111), was eluted, producing a yield of 0.89 g. Amounts of mono- and dirhamnosyl component recovered were 1.07 and 0.89 g, respectively. The purity of the combined mono- and dirhamnosyl fractions was qualitatively determined by TLC. The monorhamnosyl fraction contained no dirhamnosyl material whereas the dirhamnosyl fraction contained a trace amount of monorhamnosyl material (Figure 5). The purity of the dirhamnosyl fraction was established by HPLC to be 95% with monorhamnosyl contaminant amounting to 5% by weight (Figure 6). The ratio of biosurfactant produced by *P. aeruginosa* UW-1 as determined by HPLC, was 4 : 1 for mono- to dirhamnosyl components by weight.

The surfactant characteristics of mixed component rhamnolipid from strain *P. aeruginosa* UW-1 were determined. CMC value was 22 μg ml^{-1} and the biosurfactant reduced

L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate



L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate

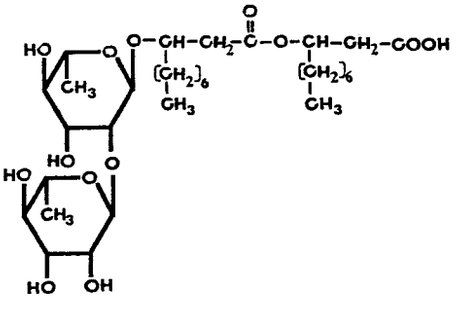


Figure 4 Structures of rhamnolipid components as determined by MS and NMR analyses.

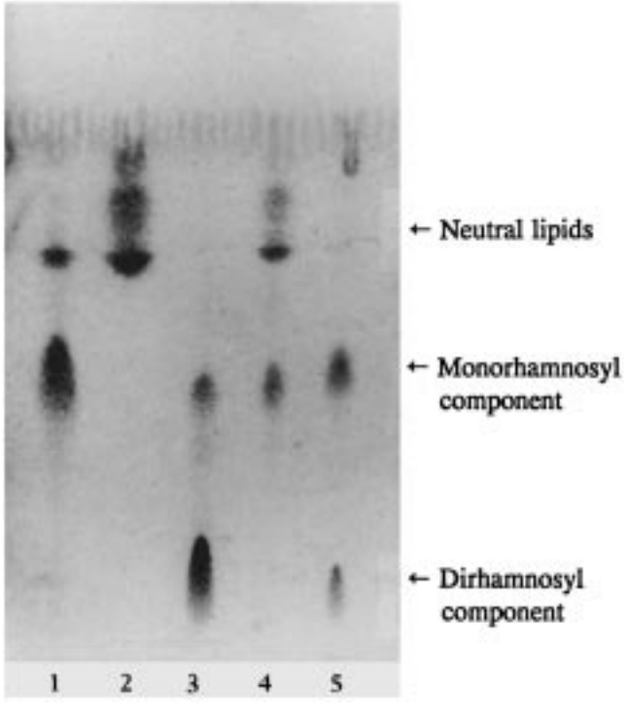


Figure 5 Thin layer chromatographs monitoring the separation of rhamnolipid components. Lane 1, more mobile rhamnolipid fraction; 2, neutral lipid fraction; 3, less mobile rhamnolipid fraction; 4, more mobile rhamnolipid fraction; 5, rhamnolipid standard (Shanghai Institute of Organic Chemistry, China). Rhamnolipid components were separated by liquid column chromatography on a 26 cm × 3.5 cm silica gel column. A 5-g sample of raw rhamnolipid was prepared in 10 ml of chloroform and eluted with various combinations of chloroform : methanol systems. The composition of each collected fraction was identified by TLC and combined. As shown here, standard and samples of the combined fractions were applied in 5.0-μl volumes onto the TLC plate and developed in a chloroform : methanol : water (65 : 15 : 2) solvent system.

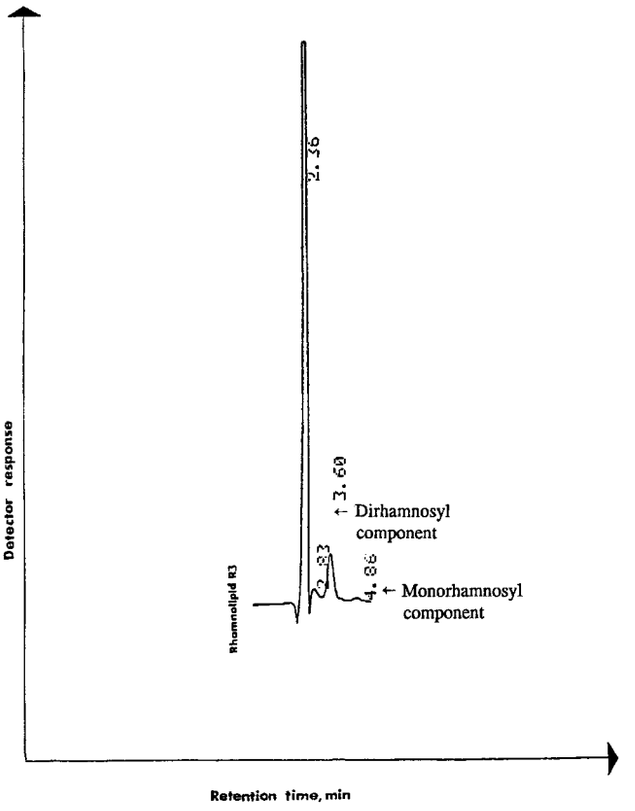


Figure 6 HPLC spectra indicating the purity levels of isolated dirhamnosyl component. It had a retention time of 3.60 min and the contaminant monorhamnosyl component had a retention time of 4.86 min. Separation was achieved on a Hi-Pore™ C_{18} reversed-phase column. Detection limit: 1 μg of rhamnolipid.

the surface tension of distilled water to 17.1 dynes cm^{-1}. The purified l-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and l-rhamnosyl-l-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate had the same CMC value of 40 μg ml^{-1} and decreased the surface tension of water to 27.7 and 30.4 dynes cm^{-1}, respectively.

Discussion

Conditions have been described for very high production of rhamnolipid biosurfactant by strain *P. aeruginosa* UW-1. A yield of 24.3 g L^{-1} was obtained in media containing 6% canola oil after a 9-day incubation. These findings are in contrast to the observations of Mulligan and Gibbs [16], who noted that maximum rhamnolipid synthesis was produced under conditions of nitrogen limitation using glucose, glycerol or N-paraffins as carbon sources. Syldatk *et al* [21] achieved a yield of 1.8 g L^{-1} in growing cultures of *Pseudomonas* DSM 2874. This yield was further increased two-fold using resting cells. Other approaches to developing biosurfactant production systems involved use of immobilised cells and continuous culture systems [13]. Mattei and coworkers [14] devised an efficient continuous biosurfactant recovery system.

The structures of *P. aeruginosa* UW-1 component rhamnolipids are the same as the predominant components found in other *Pseudomonas* strains [9,19,21], composed of 2-hydroxy-decanoyl-2-hydroxy-decanoate and one or two molecules of rhamnose. The accumulation of the rhamnolipid in the exponential and stationary phases when nitrogen concentration is low is consistent with the observations made by Manresa *et al* [12].

Development of a preparative silica gel chromatographic method for separation of the components resulted in complete purification of the l-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate fraction and recovery of l-rhamnosyl-l-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate which had a surfactant purity of 95%. An analytical separation procedure involving HPLC was also developed to effectively separate the component biosurfactants and indicated that the typical ratio of the latter components in cultures of *P. aeruginosa* UW-1 was 4 : 1.

In conclusion, the rhamnolipids produced by this strain are similar to those characterised from other *Pseudomonas* strains. This organism is a very efficient rhamnolipid producer and the culture conditions promote very high titres of rhamnolipid production.

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