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Characterization of Rhamnolipid Produced by *Pseudomonas aeruginosa* Isolate Bs20

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Abstract Rhamnolipid produced by *Pseudomonas aeruginosa* isolate Bs20 is viscous sticky oily yellowish brown liquid with a fruity odor. It showed solubility at aqueous pH>4 with optimum solubility at pH 7–7.5 and freely soluble in ethyl acetate. This biosurfactant has a very high surface activity as it could lower the surface tension of water to 30 mN/m at about 13.4 mg/L, and it exhibited excellent stabilities at high temperatures (heating at 100°C for 1 h and autoclaving at 121°C for 10 min), salinities (up to 6% NaCl), and pH values (up to pH 13). The produced biosurfactant can be used in the crude form either as cell-free or cell-containing culture broth of the grown bacteria, since both preparations showed high emulsification indices ranged between 59% and 66% against kerosene, diesel, and motor oil. These characters make the test rhamnolipid a potential candidate for use in bioremediation of hydrocarbon-contaminated sites or in the petroleum industry. High-performance thin-layer chromatography densitometry revealed that the extracted rhamnolipid contained the two most active rhamnolipid homologues dirhamno dilipidic rhamnolipid and monorhamno dilipidic rhamnolipid at 44% and 56%, respectively, as compared to 51% and 29.5%, respectively, in a standard rhamnolipid preparation. The nature and ratio of these two rhamnolipid homologues showed to be strain dependent rather than medium-component dependent.

Keywords Rhamnolipid · *Pseudomonas aeruginosa* · Characterization

Introduction

Microbial surfactants have diverse chemical structures. They include glycolipids, phospholipids, fatty acids, neutral lipids, lipopeptides, and lipid-containing polymers, such as lipoproteins, lipopolysaccharide–protein complexes, and polysaccharide–protein–fatty

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acid complexes [1]. The molecular composition, structure, and the physicochemical and biological properties of a large number of biosurfactants from various microorganisms including bacteria, yeasts, and fungi have been elucidated [2–4].

Rhamnolipids produced by *Pseudomonas aeruginosa* strains are among the most effective surfactants [5]. They posses low average minimum surface tension (30–32 mN/m), low critical micelle concentration (CMC) reaching 5 mg/l, high average emulsifying activity, and high affinity for hydrophobic organic molecules [6]. Those properties confer optimal characteristics to rhamnolipids and make them potential carriers of pollutants in soil systems [5]. Being microbially produced, rhamnolipids' high biodegradability provides them additional advantage over synthetic surfactants when performing soil washing and bioremediation processes [5]. Other advantages of rhamnolipids over other biosurfactants are their ease of isolation from the culture (they are exo-biosurfactants) and the fact that they can be produced using cheap hydrophobic or hydrophilic substrates such as hydrocarbons, vegetable oils, carbohydrates, or even wastes from the food industry [5].

Rhamnolipids produced by *Pseudomonas* grown with different carbon sources have been traditionally reported as mixtures of the homologues L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate ($RhC_{10}C_{10}$), L-rhamnopyranosyl-β-hydroxydecanoate (RhC_{10}), 2-*O*-L-rhamnopyranosyl-β-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate ($Rh_2C_{10}C_{10}$), and 2-*O*-L-rhamnopyranosyl-β-L-rhamnopyranosyl-β-hydroxydecanoate (Rh_2C_{10}) as classified by Syldatk and Wagner [4]. Several authors reported that rhamnolipid is produced by *P. aeruginosa* as mixtures of different homologues where the monorhamnolipid $RhC_{10}C_{10}$ was the predominant component [7–9]. Champion et al. [10] and Zhang and Miller [8] reported the presence of other monorhamnolipids with different fatty acid chains (C_{18}, C_{22}, and C_{24}) as minor components of the monorhamnolipid mixture, but their percentages were not quantified.

While an extra rhamnose ring confers more hydrophilicity to rhamnolipids (monorhamnolipids vs. dirhamnolipids), additional carbons in the fatty acid chains can increase their hydrophobicity. These properties can affect the stability of rhamnolipids in the aqueous phase (as monomers or micellar conglomerates), their capability to solubilize hydrophobic organic compounds, and the bioavailability of such compounds [5]. More hydrophilic rhamnolipids like RhC_{10} or Rh_2C_{10} yielded CMC values as high as 200 mg/l [4], whereas lower values of 5–60 mg/l have been reported for mixtures containing mainly $RhC_{10}C_{10}$ monorhamnolipid [6]. The dirhamnolipid $Rh_2C_{10}C_{10}$ shows intermediate CMC values of 40–65 mg/l [11]. The $RhC_{10}C_{10}$ monorhamnolipid showed higher micellar solubilization capacity than $Rh_2C_{10}C_{10}$ with phenanthrene. It has been documented that the composition of a rhamnolipid mixture will greatly influence its performance as a carrier of contaminants and its capability to enhance contaminants bioavailability [5]. The complexity of the *Ps. aeruginosa* genetic regulation involved in rhamnolipid production is an important problem for the construction of strains with the enhanced production of these biosurfactants [12].

As supported by the previously stated facts, there is a need to further characterize this important biomolecules using various physicochemical and chromatographic methods. Therefore, this study aimed at: (1) characterizing rhamnolipid biosurfactant produced by *P. aeruginosa* isolate BS20 to determine its potential fields of application as well as (2) studying the possibility of use of the produced biosurfactant in the crude forms to reduce costs associated with their use.

Materials and Methods

Microorganisms

The microorganism used was *P. aeruginosa* isolate BS20, a promising rhamnolipid producer obtained through an extensive screening program [13].

Culture Media

Soybean oil–mineral salts medium (SMSM) was used consisting of (per liter): soybean oil (20 ml), $NaNO_3$ (2.5 g), $MgSO_4 \cdot 7H_2O$ (2.85 g), $ZnSO_4$ (0.16 g), $FeCl_3 \cdot 6H_2O$ (0.013 g), $MnCl_4 \cdot H_2O$ (0.2 g), $Na_2MoO_4 \cdot 2H_2O$ (0.012 g), H_3BO_3 (0.062 g), $BaCl_2$ (0.208 g), $CoSO_4 \cdot 7H_2O$ (0.028 g), NaCl (1 g), KCl (1 g), H_3PO_4 85% (10 ml), and distilled H_2O to 1,000 ml, and the pH was adjusted to 7.2 with KOH pellets. Mineral salts medium (MSM), a basal medium for rhamnolipid production developed by Bodour et al. [14], was used for certain rhamnolipid production experiments.

Chemicals

All chemicals were of high quality from available grades supplied by (unless otherwise indicated) El-Nasr Chemicals (Adwic), Egypt.

Production of Rhamnolipid

Seed culture was prepared by transferring a loopful from a fresh culture grown onto nutrient agar slant into 25 ml of MSM contained in 250-ml Erlenmeyer flask. The flask was incubated at 250 rpm and 30 °C for 36 h.

Rhamnolipid production was carried out in 1-l Erlenmeyer flasks containing 200-ml aliquots of the fermentation medium under test (MSM or SMSM). The flasks were inoculated with the seed culture at 2% *v*/*v* and incubated under shaking conditions (250 rpm) and at 30°C for 2 days.

Extraction of Rhamnolipid

The cell suspension was centrifuged (Hietech® Biofuge) 10,000×*g* for 5 min to prepare the cell-free supernatant (CFS). The CFS was acidified with 1 N HCl to pH 2 and left overnight at 4°C. The cloudy CFS was twice extracted with an equal volume of ethyl acetate in a separating funnel. The pooled organic phase was evaporated under vacuum (Buchi, Germany) at 40°C. The obtained brownish oily residue was characterized as such or after being dissolved in 0.1 M sodium phosphate buffer, pH 7.2 (the crude rhamnolipid solution).

Analytical Methods

Measuring Surface Tension Using Tensiometers

The measurement of surface tension was conducted using a *du* Nouy ring tensiometer (Krüss) according to Ozdemir et al. [15]. The tested solution was placed into a clean glass

beaker (50 ml) specific for the surface tensiometer (Krüss). Before conducting the experiment and between each pair of measurements, the sample cup was washed three times with distilled water and acetone in series and then allowed to dry; the platinum ring was also similarly treated, and then it was flamed till redness and left to cool.

Measurement of Rhamnolipid Emulsifying Activity

Measurement of the emulsification activity of rhamnolipid-containing crude preparation (CFS) was conducted by measuring the emulsification index (E_{24}) using different oil phases according to Cooper and Goldenberg [16] with a slight modification in calculating the emulsification index (E_{24}). The emulsification index (E_{24}) was calculated by determining the percentage volume occupied by the emulsion after 24 h to the total volume of the liquid phase [17, 18] by measuring the emulsion height and knowing the tube internal diameter.

Characterization of the Produced Rhamnolipid

Physical Characterization

The physical characterization of rhamnolipid was mainly performed using rhamnolipid solutions prepared in 0.1 M sodium phosphate buffer (pH 7.2). In some cases, however, crude preparations (CFS or crude culture broth without cell removal) containing rhamnolipid at 3.5 g/l were used (prepared from cultures in SMSM as mentioned in extraction of rhamnolipid).

The crystalline appearance of the extracted rhamnolipid was determined according to Dubey and Juwarkar [19] by examining the precipitated crystalline rhamnolipid, recovered from an overnight incubated (at 4°C) acidified (to pH 2 using 1 N HCl) rhamnolipid solution, under a light microscope at a magnification of ×40, and the films were photographed. After acidification, the final concentration of rhamnolipid was 50 mg/l.

Critical Micelle Concentration and Surface Tension Lowering Values A crude rhamnolipid solution was prepared at a concentration of 200 mg/l. Then, it was appropriately serially diluted (200–0 mg/l), and the surface tension values of the prepared dilutions were measured using a *du* Nouy ring-type tensiometer from Krüss and as mentioned previously. The lowest surface tension value reached by rhamnolipid was recorded. The CMC of the test rhamnolipid was estimated from the intercept of two straight lines extrapolated from the concentration-dependent and concentration-independent sections of a curve plotted between rhamnolipid concentration and surface tension values [20]. In parallel, the CMC of standard rhamnolipid was also determined. CMC values were used for the tentative estimation of the percentage purity of rhamnolipid (RL) [21, 22]:

$$\text{Purity } (\%) = (\text{CMC}_{\text{standard RL}}/\text{CMC}_{\text{test RL}}) \times 100$$

The emulsification index (E_{24}) was used to measure the emulsification activity of crude rhamnolipid preparations (CFS and culture broth containing 3.5 g/l rhamnolipid) against different oil phases: hexadecane, linseed oil, kerosene, diesel (solar), and motor oil (Pennzoil oil 10W-40) and carried out as mentioned previously.

Stability at High Temperatures Thermal stability was determined mainly according to Turkovskaya et al. [23] using the crude biosurfactants solutions. Test tubes, containing

10 ml of rhamnolipid solution (50 mg/l), were prepared and incubated in an oven at 100°C for different time intervals (0, 5, 10, 20, 40, and 60 min). After incubation, the compensation of the volume was made if necessary; then, the percentage of surface tension reduction was determined from the following equation:

$$\%\ \mathrm{ST\ reduction} = [(\mathrm{ST_{initial}} - \mathrm{ST_{final}})/\mathrm{ST_{initial}}] \times 100$$

Moreover, another set of rhamnolipid solution was autoclaved at 121°C for 10 min, and the percentage surface tension reduction values were measured for treated and untreated samples.

In addition, the stability to autoclaving (121°C for 10 min) was also determined using rhamnolipid-containing CFS by determining emulsification index (E_{24}) before and after autoclaving [24].

Stability at Different Salinities Rhamnolipid solutions were prepared at 50 mg/l containing different NaCl concentrations (0, 0.3, 0.9, 3, and 6 g%, *w*/*v*), and the surface tension of these solutions was measured at 30°C [25].

Stability at Different pH A set of rhamnolipid solutions was adjusted to pH values of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13 using 1 N HCI or 1 N NaOH at a final biosurfactant concentration of 50 mg/l. The surface tension of the resultant solutions was measured using a *du* Nouy ring tensiometer [11, 16].

HPTLC of the Crude Rhamnolipid Extract

Rhamnolipid produced by *P. aeruginosa* isolate BS20 was extracted as mentioned previously from cultures in basal MSM and in SMSM media. The reference glucose was prepared at 0.125 g% in the sodium phosphate buffer (pH 7.2). The standard rhamnolipid (25 g%; obtained from AgSciTech, Logan, UT, USA) and the two crude rhamnolipid extracts (prepared from cultures in MSM and SMSM medium) were diluted in the 0.1 M sodium phosphate buffer (pH 7.2) to reach a final concentration of 1.25 g%. Glucose was used as a standard together with the standard rhamnolipid as described previously by Vogt Singer and Finnerty [26] and Matsufuji et al. [27].

High-performance thin-layer chromatography (HPTLC) was carried out basically as described by Matsufuji et al. [27] using a CAMAG thin-layer chromatography (TLC) system composed of an automatic TLC sampler (CAMAG Linomat 5), automatic development chamber (CAMAG ADC2), detector (CAMAG TLC Scanner 3), and an electronic integrator (winCATS software). Two aliquots (5 and 7.5 μl) of the standard rhamnolipid solution and an aliquot (15 μl) from each of the two crude rhamnolipid extracts were band applied (6 mm) onto an HPTLC precoated silica gel $60F_{254}$ plate (10×10 cm). The samples were loaded at a dosage speed of 50 nl/s under nitrogen stream. The samples were developed (ascending) using 10 ml of the mobile phase of $CHCl_3/CH_3OH/H_2O$ (65:25:4, *v*/*v*/*v*), in plates preconditioned for 3 min, to a migration distance of 85 mm. The plate was air dried, sprayed with orcinol reagent (0.19% orcinol in 53% H_2SO_4), and then put in a hot-air oven at 120°C for 15 min. The developed chromatogram was scanned in remission type, absorbance mode at 550 nm (λ_{max} of rhamnolipid as determined experimentally). The signals recovered from the scanner were integrated into absorbance chromatograms from which peak areas were automatically calculated using the winCATS software.

Results

Physical Properties

Organoleptic Characters Physical state, color, and odor were determined for crude rhamnolipid residue obtained after extraction. Crude rhamnolipid extract of the tested strain appeared as viscous sticky oily residue with amber to yellowish brown color and fruity pineapple like odor.

Solubility The solubility of the crude rhamnolipid residue left after extraction was tested in some solvents. The test rhamnolipid was soluble in aqueous solution at pH>4 with optimum solubility at pH 7–7.5. It is also soluble in organic solvents like methanol, chloroform, and ethylether and freely soluble in ethylacetate.

Crystalline Appearance

From Fig. 1, it appears that the test rhamnolipid is characterized by its parallel acicular feathery-like crystals with rhomboid heads appearing at low frequency.

Critical Micelle Concentration and Surface Tension Lowering Values

From Fig. 2, it appears that rhamnolipid lowered the surface tension of water from about 70 to 30 mN/m for test rhamnolipid and to 29.5 mN/m for standard rhamnolipid. The CMC values for test and standard rhamnolipids were found to be 13.4 and 9.85 mg/l, respectively. Although purity can be more accurately calculated using chromatographic methods as will be shown later, it was calculated by comparing the CMC values between the test and the standard [22] using the following proposed relation:

$$\text{Percent of impurities} = \text{Purity}_{\text{Std}} \times [(\text{CMC}_{\text{Test}}/\text{CMC}_{\text{Std}}) - 1] \times 100$$

$$\text{Percent purity} = (100 - \text{percent of impurities})$$

Using the above equation, it was found that the purity of test rhamnolipid was about 65% as compared to 98% for the standard rhamnolipid.

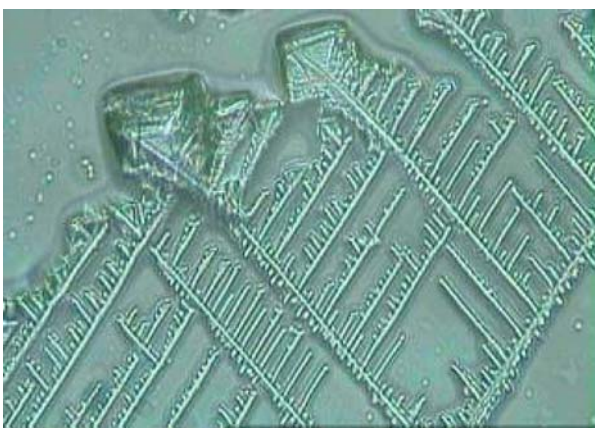

Fig. 1 Crystalline appearance of the recovered crystals of test rhamnolipid examined under a light microscope at a magnification power of ×40

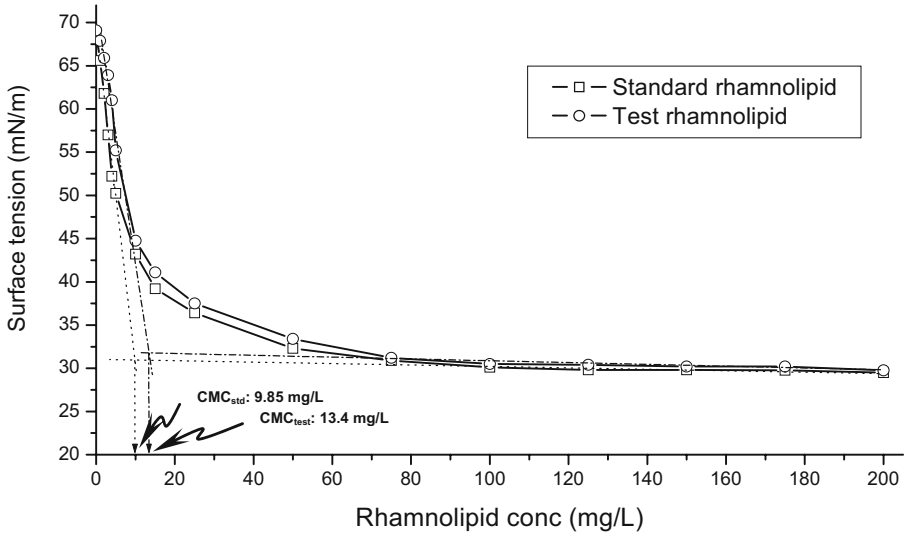

Fig. 2 A plot of surface tension as a function of concentration of standard and test rhamnolipid at pH 7.2 in 0.1 M Na_2PO_4 buffer

Emulsification Index (E_{24})

As shown in Fig. 3, rhamnolipid-containing CFS and culture broth showed appreciable emulsification indices with hexadecane, kerosene, diesel, and motor oil. The highest E_{24} values were with kerosene, diesel, and motor oil. The E_{24} of the rhamnolipid-containing CFS and crude culture broth were 62.5% and 59%, respectively, against kerosene, 54% and 63%, respectively, against diesel, and 62% and 66%, respectively, against motor oil. It is interesting to note that rhamnolipid-containing culture broth could emulsify linseed oil to an appropriate level (E_{24}=32%), although it was not emulsified to any detectable level using CFS.

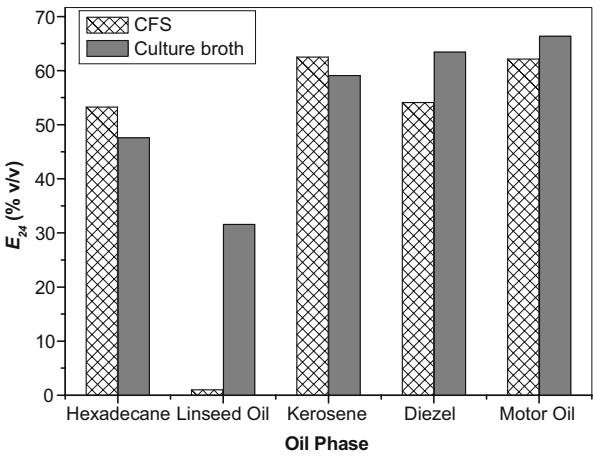

Fig. 3 E_{24} values of crude rhamnolipid-containing CFS and crude culture broth (3.5 g/l each) against different oils

Stability at High Temperatures

Figure 4 shows that, approximately, there was no reduction in surface activity upon exposure to 100°C for up to 1 h. It is interesting to note that autoclaving at 121°C for 10 min did not reduce surface activity to any degree.

As seen in Fig. 5, test rhamnolipid, in its crude CFS form, showed a minor reduction in their emulsifying activity upon autoclaving.

Stability at Different Salinities

From Fig. 6, it appears that the surface activity of test rhamnolipid was not affected by high sodium chloride concentrations up to 6%. In contrary, there was a slight increase in surface activity (as demonstrated by the decrease in surface tension) of rhamnolipid upon increasing the NaCl concentration up to 3%. Sodium chloride alone has nearly no effect on surface tension of the used buffer.

Stability at different pHs As seen in Fig. 7, the test rhamnolipid showed an almost stable surface activity profile over a wide range of pH (from 2 to 13). However, the maximum surface activity was reached at pH 7 to 8, and a slight reduction in surface activity was observed at pH values less than 6. A more pronounced reduction in surface activity was observed at higher pH values (pH≥9).

Chromatographic Characterization

HPTLC Densitometry

The different spots appearing on the chromatogram shown in Fig. 8 were scanned as mentioned in "Materials and Methods," and the recorded absorbance chromatograms for the different tracks are shown in Fig. 9.

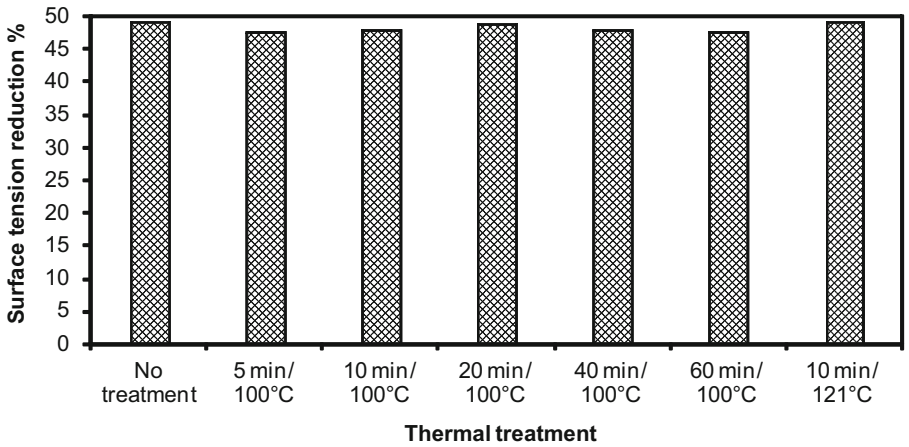

Fig. 4 Effect of temperature on surface activity of test rhamnolipid (50 mg/l in 0.1 M sodium phosphate buffer, pH 7.2). Surface activity was expressed in terms of percentage reduction in surface tension

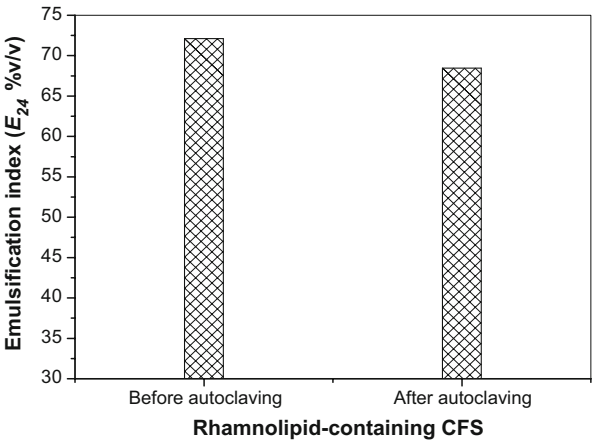

Fig. 5 Effect of autoclaving on emulsifying activity of rhamnolipid-containing CFS (3.5 g/l)

The produced absorbance chromatograms were processed using winCATS Planar Chromatography Manager software for measuring the peak area, retardation factor, and the area percentage of glucose and separated rhamnolipid homologues (Table 1).

From Fig. 9 (track B), it is clear that the number of homologues present in the standard rhamnolipid is four since four peaks were detected. These four homologues have the following R_f values: 0.26, 0.45, 0.52, and 0.67; the first and third peaks corresponded to the minor homologues, while the second and fourth peaks corresponded to the major homologues as known from their area percentages (Table 1, track B). The two major homologues were identified by the supplier as the rhamnolipid homologue dirhamno dilipidic rhamnolipid (RRLL) having an R_f value of 0.45 and the rhamnolipid homologue monorhamno dilipidic rhamnolipid (RLL) having an R_f value of 0.67.

The test rhamnolipid (Fig. 9; tracks D and E) showed the presence of two peaks; therefore, only two homologues are present in the test rhamnolipid. The two homologues have R_f values of 0.40 and 0.68 as shown in Table 1. The strong similarity between the R_f values of the two test rhamnolipid homologues and the RRLL and RLL homologues of the standard rhamnolipid confirm the identity of these two test homologues to be RRLL and

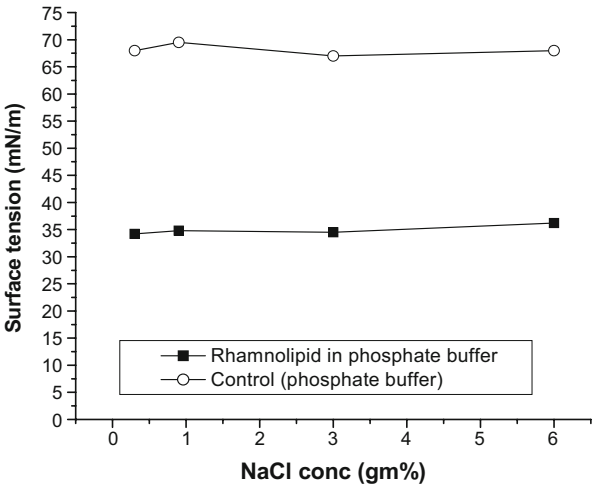

Fig. 6 Effect of salinity on surface activity (expressed in terms of surface tension values) of test rhamnolipid. Rhamnolipid was dissolved in 0.1 M sodium phosphate buffer, pH 7.2 at 50 mg/l. Surface tension was measured using a *du* Nouy ring tensiometer

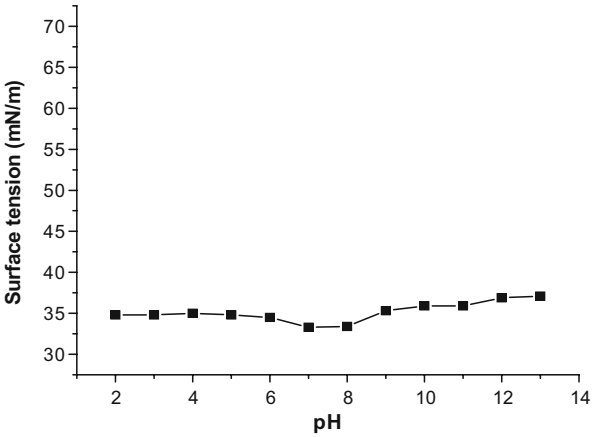

Fig. 7 Effect of pH on the surface activity of rhamnolipid solutions. Surface activity was monitored using surface tension measurement of rhamnolipid solutions in 0.1 M sodium phosphate buffer. The surface tension value of 0.1 M sodium phosphate buffer at pH 7.5 was 69.1 mN/m

RLL. The area percentages of the major rhamnolipid homologues found in the standard and test rhamnolipids produced in SMSM (an optimized medium) and MSM media were compared, and the results are shown in Table 2.

From Table 2, it is clear that the test rhamnolipid produced either in SMSM (an optimized medium) or in the basal medium (MSM) contained RRLL and RLL at nearly equal percentages of about 43% and 57%, respectively.

The purity of the test crude rhamnolipid extract could be calculated by comparing the total peak areas (TPA) per loaded weight of the test rhamnolipid to that of the standard (Table 3).

Purity of the test rhamnolipid (RL) was calculated using the following equation:

$$\text{Purity}_{\text{test RL}}(\%w/w) = [(\text{TPA}_{\text{test RL}}/\text{loaded wt}_{\text{test RL}})/(\text{TPA}_{\text{std RL}}/\text{loaded wt}_{\text{std RL}})] \times 98$$

By applying this equation, it was found that the purity of the test rhamnolipid produced in SMSM was 24.77% (~25%). However, this purity was 17.36% for rhamnolipid produced in MSM.

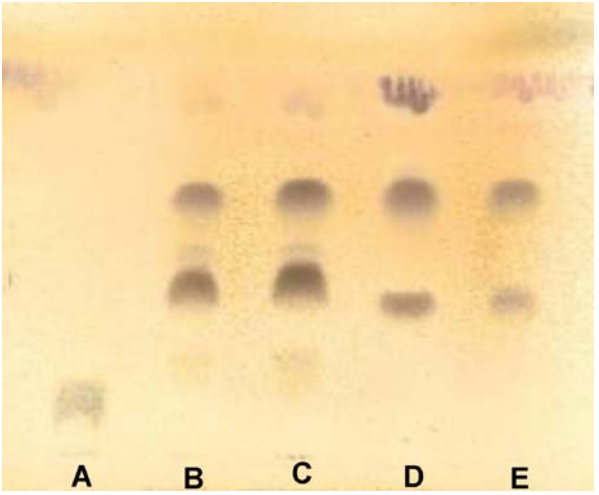

Fig. 8 HPTLC of the two crude rhamnolipid extracts produced by *P. aeruginosa* isolate BS20 developed against a standard rhamnolipid and a reference glucose. The plate was sprayed with orcinol reagent. *Track A*: 7.5 μl of 0.125 g% glucose solution; *tracks B* and *C*: 5.0 and 7.5 μl aliquots of 1.25 g% standard rhamnolipid solution, respectively; *tracks D* and *E*: 15 μl of test rhamnolipid solutions (1.25 g%) prepared from cultures of the tested isolate in SMSM for *track D* and in MSM for *track E*

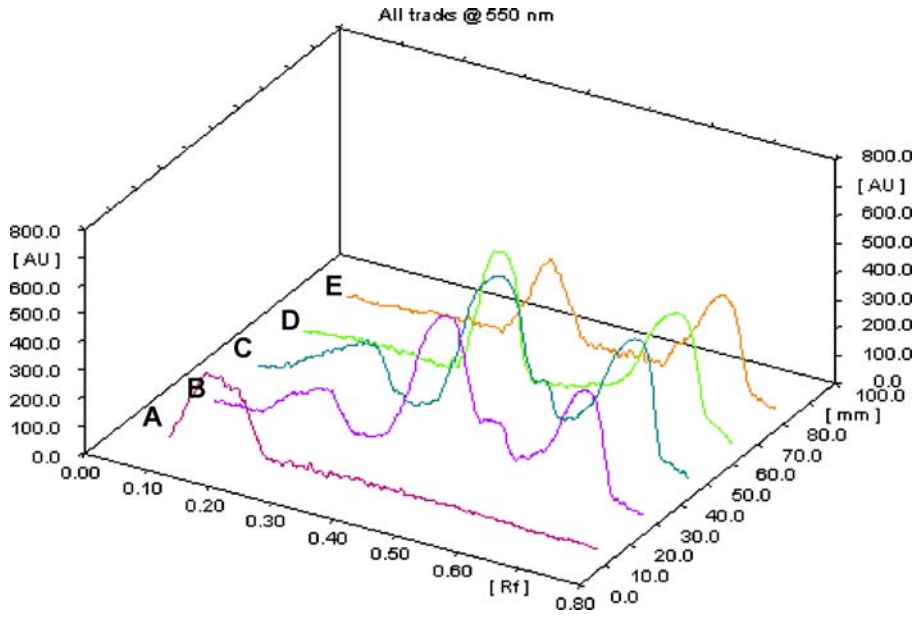

Fig. 9 3-D absorbance chromatograms measured at 550 nm for the different tracks obtained in Fig. 8. These chromatograms were developed using winCATS Planar Chromatography Manager software

The standard glucose preparation was incorporated in this experiment to study the mathematical relation between glucose (GL) and rhamnolipid (RL) for the possible use of standard glucose instead of standard rhamnolipid as an internal standard in TLC densitometry, with the former being more cheap and available. The following equation was used for the calculation of that relation:

$$\text{Relation RL/GL} = [(\text{TPA}_{\text{Std RL}}/\text{loaded wt}_{\text{Std RL}})/(\text{TPA}_{\text{std GL}}/\text{loaded wt}_{\text{std GL}})]$$

By applying this equation, it was found that 1 mg rhamnolipid corresponded to 0.594 mg glucose.

Table 1 HPTLC parameters for the different tracks (A, B, D, and E) obtained in Fig. 2.

Track	Peak number	Max R_f	Area	Area percent
A (7.5 μl reference glucose [0.125%])	1	0.14	20,020.7	100.00
B (5 μl standard rhamnolipid [1.25%] obtained from AgSciTech)	1	0.26	9,616.6	12.12
	2	0.45	40,748.1	51.37
	3	0.52	5,546.8	6.99
	4	0.67	23,412.1	29.51
D (15 μl test rhamnolipid [1.25%] produced in SMSM[a])	1	0.40	26,246.3	43.66
	2	0.68	33,867.6	56.34
E (15 μl test rhamnolipid [1.25%] produced in MSM[b])	1	0.40	18,058.0	43.03
	2	0.68	24,119.8	56.97

[a] SMSM medium

[b] MSM medium

Table 2 The relative percentages of the RLL and RRLL homologues in standard rhamnolipid and test rhamnolipid produced in SMSM and MSM media.

Rhamnolipid homologue	The relative percentages of RRLL and RLL rhamnolipid homologues in		
	Standard rhamnolipid (%)	Test rhamnolipid from optimized medium[a] (%)	Test rhamnolipid from basal medium[b] (%)
RRLL	51.37	43.66	43.03
RLL	29.51	56.34	56.97

[a] SMSM

[b] MSM

Discussion

The characterization of rhamnolipid biosurfactant produced by *Pseudomonas* isolate BS20 included organoleptic, solubility, crystalline characteristics, and chromatographic characterization using HPTLC. Rhamnolipid biosurfactants are promising environmental molecules for bioremediation purposes; therefore, the stability of test rhamnolipid at high temperature, salinities, and over a wide range of pH was studied.

Physical Properties

Physical State and Organoleptic Characters and Solubility

Crude rhamnolipid extract appeared as viscous sticky oily residue with amber to yellowish brown color and fruity pineapple like odor.

Test rhamnolipid is soluble in aqueous solution at pH>4 with optimum solubility at pH 7–7.5. It is also soluble in organic solvents like methanol, chloroform, ethylether, and freely soluble in ethylacetate. It was observed that rhamnolipids have their optimum aqueous solubility at neutral to alkaline pHs. This phenomenon is attributed to their acidic nature. Rhamnolipids contain a single free carboxylic acid group corresponding to the β-hydroxy fatty acid moiety, and this confers the anionic nature of rhamnolipids. It was reported that as the pH increases from 5 to 8, the negative charge of the polar head increases [10], and this is reflected by increased aqueous solubility.

Crystalline Appearance

Rhamnolipid crystals appeared as parallel acicular feathery like crystals (Fig. 1) with rhomboid heads appearing at low frequency. This crystalline shape differs from that reported by Dubey and Juwarkar [19]. Their produced rhamnolipid crystals appeared fine

Table 3 Total peak areas (TPA) in the HPTLC tracks of standard glucose, standard rhamnolipid, and test rhamnolipid (produced in SMSM and MSM media.

	Standard glucose	Standard rhamnolipids[a]	Test RL from SMSM	Test RL from MSM
TPA	20,020.7	79,323.6	60,113.9	42,177.8
Loaded wt (mg)	0.009375	0.0625	0.1875	0.1875
TPA/Loaded wt	2.14×10^{6}	1.27×10^{6}	3.21×10^{5}	2.25×10^{5}

[a] Standard rhamnolipid purity, 98%

rectangular in shape. However, these different crystalline shapes may be attributed to differences in the composition of rhamnolipids produced. The aforementioned authors did not characterize their produced rhamnolipids.

Critical Micelle Concentration and Surface Tension Lowering Value

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 the surfactant concentration increases, the surface tension of the surfactant solution decreases up to a certain value and then becomes almost constant due to the interface saturation with the surfactant molecules. The surfactant concentration at which this phenomenon occurs is known as the CMC and is determined from the break point of the surface tension versus concentration curve [28]. For practical purposes, it is important to distinguish between an effective biosurfactant and an efficient biosurfactant. Effectiveness is measured by the minimum value to which the surface tension can be reduced, whereas efficiency is measured by the biosurfactant concentration required to produce a significant reduction in the surface tension of water. The latter can be known from the CMC of the biosurfactant [29].

Test rhamnolipid showed a surface tension-lowering profile that was very similar to that of the standard rhamnolipid (Fig. 2). The data showed the high effectiveness of rhamnolipids as they could lower the surface tension of water from about 70 to 30 mN/m for the test rhamnolipid and to 29.5 mN/m for the standard rhamnolipid. Rhamnolipid showed to be highly efficient as well, since the CMCs for both of the test and standard preparations were found to be 13.4 and 9.85 mg/l, respectively. These results are very similar to that published by Zhang and Miller [11]. The different CMC values may have resulted from differences in purity and composition of rhamnolipid [20]. Moreover, a range of CMC values between 10 and 230 mg/l have been reported for rhamnolipids from different microbial sources [30]. In this study, the low purity (25% as calculated using TLC densitometry techniques) of the test rhamnolipid may be responsible for the difference in CMC values between the test and standard rhamnolipid [28].

In the present study, it was found that the purity of the test rhamnolipid produced in SMSM as calculated from its CMC value was 65%, while its purity as calculated chromatographically was 25%. The former method is dependent on biosurfactant activity, while the latter method is dependent on biosurfactant concentration. This discrepancy may explain the lack of coherency between the biosurfactants activity and biosurfactant concentration that was noticed during rhamnolipid production experiments (Abdel-Mawgoud et al., submitted).

Emulsification Index

The emulsifying power is another important character of any surfactant. Therefore, it was assessed for the test rhamnolipid in the crude CFS or crude culture broth forms using the emulsification index (E_{24}) against different oil phases.

Rhamnolipid-containing CFS and culture broth showed appreciable emulsification indices with hexadecane, kerosene, diesel, and motor oil. The highest E_{24} values were with kerosene, diesel, and motor oil. It is interesting to note that the rhamnolipid-containing culture broth could emulsify linseed oil to an appropriate level (E_{24}=32%), although it was not emulsified to any detectable level using CFS.

It should be realized that the emulsifying activity was measured using an oil/water ratio of 3:2, which means that the oil phase constitutes 60% of the total volume. This means that E_{24} values greater than or equal to 60 entails a complete emulsification of the oil phase.

This condition occurred with rhamnolipid-containing CFS against kerosene, rhamnolipid-containing culture broth against diesel, and with both crude forms against motor oil.

The ability of rhamnolipids to emulsify crude oil products (hexadecane, kerosene, diesel, and motor oil), which may facilitate their microbial assimilation, could be useful for pollution treatment of oil-contaminated environments [12, 18].

The stability of surfactin to drastic conditions such as (elevated temperature, high salinities, and over a wide range of pH values) deserved to be studied to assess their applicability in bioremediation where such conditions may prevail.

Stability at High Temperatures

Test rhamnolipid was prepared at an appropriate discriminative concentration (lowest concentration that can produce maximum reduction in surface tension value of water), which was determined from Fig. 2. This concentration was selected to be at the linear declining part of the curve just preceding the plateau where minor changes in rhamnolipid activity (that may result from the exposure to the tested conditions) will be accompanied with measurable changes in surface tension. This concentration was 50 mg/l of rhamnolipid.

Test rhamnolipid solutions showed very good thermal stability. There was no reduction in surface activity (surface tension activity) to any degree upon exposure to 100°C for up to 1 h (Fig. 4). It is interesting to note that autoclaving at 121°C for 10 min did not also reduce surface activity (surface tension or emulsifying activity; Figs. 4 and 5).

Purification accounts for up to 60% of the total production cost of biosurfactants [31]. Because of economic considerations in the oil industry, most biosurfactants would require either whole-cell culture broths or crude preparations [31]. Therefore, the applicability of test rhamnolipid at high temperatures in their crude form (CFS) without prior costly extraction steps was additionally investigated. Upon autoclaving of test rhamnolipid-containing CFS at 121°C for 10 min, it was found that it tolerated well autoclaving conditions as such treatment resulted in a minor reduction in its emulsifying activity.

The thermal stability exhibited by the test rhamnolipid either in the crude CFS or the extracted form widens the scope of its applicability at conditions where high temperatures prevail as in microbially enhanced oil recovery (MEOR).

Stability at Different Salinities

It was necessary to study the effect of salinities on the activity of the test rhamnolipid to investigate its applicability in the bioremediation of contaminated marines. It was found that the surface activity of test rhamnolipid was not affected to any degree by high salinities (Fig. 6), even at salinities reaching double the highest sea salinity in the world (3%; http://en.wikipedia.org/wiki/Seawater). It is interesting to note that high NaCl concentrations caused a slight increase in surface activity since there was a detectable decrease in surface tension up to 3% NaCl. This phenomenon was interpreted by the finding of another study conducted by Helvaci et al. [25]. They stated that electrolytes directly affect the carboxylate groups of the rhamnolipids. The solution/air interface has a net negative charge due to the ionized carboxylic acid groups at pH 6.8 with strong repulsive electrostatic forces between the rhamnolipid molecules. This negative charge is shielded by the Na^+ ions in the electrical double layer in the presence of NaCl, causing the formation of a close-packed monolayer and consequently a decrease in CMC and surface tension values [25].

Chemical surfactants, however, are deactivated by 2–3% salt concentration [32]. Therefore, it can be concluded that the test rhamnolipid is an excellent candidate, if

compared to chemical surfactants, to be used in the bioremediation of contaminated marines where high salinities prevail.

Stability at Different pHs

The stability of test rhamnolipid at different pH values is also an important issue that can affect its application spectrum. The test rhamnolipid showed an almost stable surface activity profile over a wide range of pH values (from 2 to 13). However, the maximum surface activity was reached at pH 7 to 8 (Fig. 7).

In conclusion, the physical characterization revealed that test rhamnolipid has an excellent surface and emulsifying activities as well as it showed high stabilities at elevated temperatures, high salinities, and over a wide pH range. These properties make rhamnolipid biosurfactants potential candidates to be used in bioremediation of contaminated sites and in the petroleum industry (MEOR) where drastic conditions commonly prevail. In addition, crude rhamnolipid-containing preparations as CFS and culture broth (without cell removal) gave high emulsifying activities against different oils. This complies with the nowadays need for the use of biosurfactants in their crude form for the purpose of cost reduction. The crude culture broth form is particularly applicable in the field of the oil industry where lower purity specifications are required [33]. Therefore, crude culture broth-containing rhamnolipid is a candidate for direct use in the field of the oil industry.

Chromatographic Characterization

In a previous study conducted by Abdel-Mawgoud et al. [13], TLC experiments revealed that the test rhamnolipid is composed of different homologues. Therefore, in this study, the nature and population of these different homologues present in the crude test rhamnolipid extract were studied in comparison with a standard rhamnolipid (obtained from AgSciTech) using the HPTLC densitometry technique. In addition, the effect of medium components on the nature and relative percentages of the different homologues of the produced rhamnolipid was also investigated. This was performed by comparing the HPTLC densitometry profile of two crude rhamnolipid extracts: One was prepared from a culture of the tested isolate in MSM, while the other was prepared from a culture of the tested isolate in SMSM.

Results of HPTLC revealed that the number of homologues present in standard rhamnolipid was four (Figs. 8 and 9). The first and third peaks corresponded to the minor homologues while the second and fourth peaks corresponded to the major homologues (Table 1, track B). The two major homologues were identified by the supplier as RRLL for the spot having an R_f value of 0.45 and RLL for the spot having an R_f value of 0.67; the other minor homologues were identified as dirhamno monolipidic rhamnolipid (RRL) and monorhamno monolipidic rhamnolipid (RL). RLL was identified by the supplier as L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate, while RRLL was identified as 2-*o*-L-rhamnopyranosyl-β-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate. Therefore, chemically, rhamnolipids are glycosides of rhamnose (6-deoxymannose) and β-hydroxydecanoic acid, and the relative mobility of rhamnolipid homologues on the polar TLC is determined by the number of polar rhamnose moieties present. The less mobile homologues were the more polar ones (RRL and RRLL; the former minor and the latter major) that appeared as the lower spots. However, the more mobile were the less polar ones (RL and RLL; the former minor and the latter major) that appeared as the upper spots in Fig. 8.

Test rhamnolipid, however, contained only two homologues (Figs. 8 and 9). The strong similarity between the R_f values of the two test rhamnolipid homologues and the RRLL and RLL homologues of the standard rhamnolipid confirm the identity of these two test homologues to be RRLL and RLL (Table 1).

The relative distribution of the two major homologues (RRLL and RLL) in the test rhamnolipid extracts obtained from MSM and SMSM was nearly constant (approximately 43% and 57%) whatever the nature of production medium used is (MSM or SMSM; Table 2). This finding means that the population (relative percentages) of the different rhamnolipid homologues produced is mainly strain dependent rather than being culture condition dependent.

Upon comparing the test rhamnolipid homologues with those of the standard rhamnolipid, it was found that the percentage of the RRLL homologue is slightly higher in the standard rhamnolipid than its percentage in test rhamnolipid. However, the percentage of RLL in test rhamnolipids is much higher, by about two times, than its percentage in standard rhamnolipid. The rhamnolipid homologues (RRL and RL) were found in the standard rhamnolipid preparation but not in the test one. It was reported that, among the different rhamnolipid homologues, RRLL and RLL are the most active, the latter being more active than the former as known from their separate CMC values [5, 30]. Therefore, it can be concluded that the two most active homologues (RRLL and RLL) are present in the test rhamnolipid at 100%. However, these two homologues are present in the standard rhamnolipid at 80% only. In addition, the most active homologue (RLL) is present at a relatively high percentage if compared to the standard rhamnolipid. As a result, the test rhamnolipid has advantages over the standard one in two aspects: One is the increased activity per weight, and the second is the ease of purification of the most active homologues (RRLL and RLL).

The purity of the two crude rhamnolipid extracts was assessed using the data collected from the HPTLC densitometry experiment. It was found that the purity of the test rhamnolipids extract produced in SMSM was ~25%; however, it was only ~17% for the rhamnolipid extract produced in MSM. This finding adds a new advantage to SMSM (an optimized medium) over the basal medium (MSM), since the former not only enhanced higher levels of rhamnolipid production by the test isolate BS20 (Abdel-Mawgoud et al., submitted) but also resulted in a relatively more pure rhamnolipid.

The standard glucose preparation was eluted beside standard and test rhamnolipids in HPTLC densitometry for the aim of using glucose as a reference for rhamnolipid assay. It was found that 1 mg rhamnolipid corresponded to 0.594 mg glucose as calculated from the peak area of their spots per unit weight of their respective loaded amounts (Table 3). This finding is very useful, as it allows the application of the more cheap and available glucose as a reference standard instead of standard rhamnolipids in HPTLC densitometry experiments for assaying rhamnolipids.

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