Production of biosurfactant by *Pseudomonas* spp. isolated from industrial waste in Turkey

Tayfun KAYA[1], Belma ASLIM[2], Ergin KARİPTAŞ[1],*
[1]Department of Biology, Faculty of Arts and Science, Ahi Evran University, Kırşehir, Turkey
[2]Department of Biology, Faculty of Science, Gazi University, Ankara, Turkey

Abstract: In this study, 26 *Pseudomonas* spp. were isolated from a stream polluted by factory waste and from petroleum-contaminated soil. The surface tension (ST) of the cultures was used as a criterion for the primary isolation of biosurfactant-producing bacteria. Biosurfactant production was quantified by ST reduction, critical micelle concentration (CMC), emulsification capacity (EC), and cell surface hydrophobicity (CSH). Two of the isolates, *P. aeruginosa* 78 and 99, produced rhamnolipid biosurfactant. The strains started rhamnolipid production in the logarithmic phase. They decreased the ST of the culture from 73 dyne/cm^2 to 29 and 33 dyne/cm^2, and the CMC of produced rhamnolipids were 115 and 130 mg/L, respectively. *P. aeruginosa* 78 and 99 strains emulsified benzene and n-hexane at the highest rates, and the surfaces of these strains were 73% and 65% and 62% and 72% more hydrophobic for benzene and toluene, respectively.

Key words: Rhamnolipid, *Pseudomonas* spp., surface tension, critical micelle concentration, emulsification activity, cell surface hydrophobicity

1. Introduction

Surface active agents are substances that can reduce the surface and interface tension in liquids and liquid systems (i.e. oil/water, air/water, or liquid/solid) and can be produced through synthetic (surfactant) or microbial (biosurfactant) methods (Christofi and Ivshina, 2002; Khalladi et al., 2009). These substances have the same amphiphilic properties in that they both have the character of an emulsifier (i.e. contain both hydrophobic and hydrophilic parts), which allows the blending and spreading of phases in immiscible liquid systems like oil/water (Beal and Betts, 2000; Cassidy et al., 2002; Moraes et al., 2002; Cohen et al., 2003). However, because they have a low toxic effect; are biologically degradable and environmentally friendly; and display highly specific activity under extreme temperature, pH, and salinity conditions, biosurfactants have advantages over chemical surfactants as biotechnological products (Makkar and Cameotra, 1999; Beal and Betts, 2000; Cassidy et al., 2002; Moraes et al., 2002; Maneerat, 2005; Khalladi et al., 2009; Pornsunthorntawee et al., 2009; Long et al., 2013a). However, biosurfactants cannot compete with synthetic surfactants, due to the cost associated with their production via microorganisms (Makkar and Cameotra, 1999; Maneerat, 2005).

A biosurfactant is a biological material that has crucial intracellular (regulatory molecules, food stores, etc.), extracellular (cell hydrophobicity and mycelium formation), and intercellular (quorum sensing, biofilm formation, antagonism, and microbial community structure) roles in the physiology and ecology of microorganisms (Hamme et al., 2006). Many microorganisms produce biosurfactants of different characteristics and structures. These structures include glycolipid (rhamnolipid and trehalose lipid), lipid-protein complex (such as peptide-lipid, viscosin, and surfactin), fatty acids, and phospholipid or polymeric structures (Kosaric, 1992; Banat et al., 2000; Maneerat, 2005; Antunes et al., 2006). The best-known biosurfactant is the rhamnolipid produced by some *Pseudomonas* species. In particular, rhamnolipids produced by *P. aeruginosa* species have high emulsification activity and affinity due to their low surface tension and critical micelle concentration (Desai and Banat, 1997; Raza et al., 2007). Rhamnose sugar found in the structure of rhamnolipid bestows a hydrophilic quality to the biosurfactant structure, while the β-hydroxydecanoic fatty acid chain gives a hydrophobic quality to the structure (Lang and Wullbrandt, 1999; Youssef et al., 2005), enabling the rhamnolipid to function as an emulsifier for immiscible substances. Growth of microorganisms on hydrocarbons is often associated

with the production of compounds that can aid in the emulsification of hydrophobic substances in the growth medium (Calvo et al., 2002). This property is important for the containment of environmental pollution; due to the affinity of biosurfactants they are used for the degradation of oil and oil-derived environmental polluters, for example petroleum and heavy metals (Maier and Soberon-Chavez, 2000; Celik et al., 2008; Ozturk et al., 2012). Bacterial cell surface hydrophobicity is one of the most serious factors influencing bacterial adhesion to various surfaces (Zita and Hermansson, 1997). Adherence of a microorganism to a surface may occur through a hydrophobic effect provided the associating sites have sufficiently high densities of nonpolar areas (Gogra et al., 2010). Another property of rhamnolipid biosurfactants is their low critical micelle concentration, which is considered a hallmark of quality for surface-active agents and is expressed as the minimum surface active agent concentration for the lowest surface tension (Mata-Sandoval et al., 1999). Studies have been carried out to obtain new biosurfactant-producing isolates with a view to procuring new biosurfactants. Such studies generally make use of areas polluted with oil and oil-derivatives or factory waste for the isolation of biosurfactant-producing microorganisms (Beal and Betts, 2000; Cassidy et al., 2002; Tabatabaee et al., 2005; Whang et al., 2009).

The present study was conducted with the following aims: to screen strains of *Pseudomonas* spp. for biosurfactant production from a stream polluted by factory waste (Çorlu, Tekirdağ, Turkey) and from petroleum-contaminated soil (Batman Province, Turkey), select the strains producing the highest percentage yield of rhamnolipid, and examine rhamnolipids in terms of emulsification activity and cell surface hydrophobicity.

2. Materials and methods

2.1. Isolation and identification of microorganisms
In order to obtain microorganism isolates, samples collected from the Çorlu (Tekirdağ) stream contaminated with factory waste and from the soil around a Batman oil refinery were inoculated into Pseudomonas agar base (PAB) medium selective for *Pseudomonas* spp., as described by Atlas and Parks (1997). After the Gram properties of isolates grown in PAB culture were examined, Gram-negative isolates were identified using an analytical profile index (API) biochemical test kit (bioMérieux, France). In line with the data obtained, identification of the isolates was confirmed by creating protein profiles (Laemmli, 1970) with the help of biochemical and physiological tests such as oxidase, denitrification, gelatine hydrolisation, starch hydrolisation, pigmentation, and development at 5–24 °C. For conducting tests and creating protein profiles, *P. aeruginosa* ATCC 27853 and *P. stutzeri* DSM 6082 strains were used as test bacteria.

2.2. Media and growth conditions
In order to determine surfactant-producing strains among the isolated and identified ones, all strains were grown in nutrient broth culture in an agitating incubator (96 h, 37 °C, 120 rpm), and surface tensions of all cultures were measured. For this purpose, basal mineral salt medium (BMSM) culture, as specified by Zhang et al. (2005), was used. The composition of the culture was as follows (g/L): $NaNO_3$ 4.0, NaCl 1.0, KCl 1.0, $CaCl_2.2H_2O$ 0.1, KH_2PO_4 3.0, $Na_2HPO_4.12H_2O$ 3.0, $MgSO_4$ 0.2, $FeSO_4.7H_2O$ 0.001; 2 mL of trace element stock solution was composed of (g/L): $FeCl_3.6H_2O$ 0.08, $ZnSO_4.7H_2O$ 0.75, $CoCl_2.6H_2O$ 0.08, $CuSO_4.5H_2O$ 0.075, $MnSO_4.H_2O$ 0.75, H_3BO_3 0.15, $Na_2MoO_4.2H_2O$ 0.05. The initial pH was adjusted to 6.8. The cultures were inoculated in BMSM with 3% glycerol as a carbon and energy source (Silva et al., 2009). The strains were inoculated into the BMSM culture at a rate of 2% after being activated twice, and active culture cell concentrations in the nutrient culture were adjusted to McFarland 2. The cultivations were conducted in 1 L shaking (130 rpm/min) flasks containing 250 mL medium for 96 h. The temperature was controlled at 37 °C.

2.3. Measurement of surface tension
The surface tension of liquids was measured at 25 °C using a stalagmometer (Stalagmometer Rohr B Abgew.), as described by Caykara and Birlik (2005). The surface tension of the surfactant solutions was calculated using the following equation (Langmuir, 1917): $\gamma 0 = \gamma (n/n0)$, where $\gamma 0$ and γ are the surface tensions of the reference solvent (for water, $\gamma 0 = 73.49$ dyne/cm^2) and surfactant solution, and $n0$ and n are the drop numbers of the reference solvent and surfactant solution, respectively. Samples of the culture media strain were centrifuged at 10 rpm for 10 min.

2.4. Determination of time production of rhamnolipids and curve growth
The time production of rhamnolipids was determined with the drop-collapse test method, as modified by Bodour et al. (2003). Samples were taken from culture media every hour from the beginning of inoculation. The optical density was periodically measured at 600 nm with a spectrophotometer (Hitachi UV/VIS 1800).

2.5. Purification and isolation of rhamnolipids
After a 96-h incubation period, the pH of the culture was adjusted to 8.0 (using 10 M NaOH), and biomass was removed by centrifugation for 20 min at 10,000 × g. The supernatant pH was adjusted to 2 (using 3 M H_2SO_4), and an equal volume of chloroform–methanol (2:1) was added. The mixture was shaken for 10 min. Centrifugation was performed for 10 min at 10,000 × g, and the organic phase was removed. The extraction operation was repeated once more. The rhamnolipid product was concentrated from the pooled organic phases using a rotary evaporator (Heidolph, Laborota 4000). The thick yellowish product

was dissolved in methanol, filtered (Sterivex-GV, 0.22 mm; Millipore, Bedford, MA, USA), and concentrated again using the rotary evaporator (Mata-Sandoval et al., 1999).

2.6. Determination of rhamnolipid concentration
Concentration of the rhamnolipids, as stated above, was determined according to the phenol sulphuric method described by Dubois et al. (1956). The measurements were performed at 480 nm wavelength in a spectrophotometer.

2.7. Determination of critical micelle concentration value of rhamnolipid biosurfactants
Determination of the critical micelle concentration (CMC) of rhamnolipids was performed by measuring the surface tension of aqueous solutions. Purified rhamnolipids (obtained from 4-day culture) were dissolved in distilled water at several concentrations ranging from 50 mg/L to 160 mg/L. The measurements were carried out with a stalagmometer. CMC values were measured in triplicate (Moraes et al., 2002; Caykara and Birlik, 2005).

2.8. The effect of pH and temperature on surface tension
The effects of different pH values (2–12) and temperatures (5–60 °C) on the surface tension of rhamnolipids produced by *P. aeruginosa* 78 and 99 strains were tested in BMSM. The surface tensions of the culture were measured with a stalagmometer at different pH values and temperatures (Patel and Desai, 1997).

2.9. Emulsification assay
Emulsification activity of purified rhamnolipids was detected as described by Patel and Desai (1997). The assays of emulsification and the emulsification index were measured by adding 3 mL of hydrocarbons (benzene, n-hexane, toluene, and xylene) to 2 mL of aqueous phase containing 0.4 g/L of purified rhamnolipids and vortexing at 10,000 rpm for 2 min. While emulsification activity was measured after 24 h, the emulsification index was measured after 1 month [(height of emulsion layer/total height) × 100] (Patel and Desai, 1997).

2.10. Cell surface hydrophobicity assay
Cell surface hydrophobicity was determined as described by Rosenberg et al. (1980). For this purpose, the active bacteria culture growth on the nutrient broth media was harvested (10,000 × g, 10 min) at the exponential growth phase, washed twice in PBS (NaCl, 8; K_2HPO_4, 21; KH_2PO_4, 0.34; pH 6.8; respectively, g/L), and resuspended in PBS to an OD 600 of 0.6. Thereafter, 1 mL of test hydrocarbon (benzene, n-hexane, toluene, and xylene) was added to test tubes containing 3 mL of washed cells. After blending in a vortex mixer for 90 s, the tubes were left to stand for 30 min to allow the 2 phases to separate; then, the OD of the aqueous phase was measured. Hydrophobicity was calculated from 3 replicates as the percent decrease in optical density of the original bacterial suspension due to cells partitioning into the hydrocarbon layer. Percent

hydrophobicity was calculated by the following equation: hydrophobicity % = [(OD 600 before mixing – OD 600 after mixing)/(OD 600 before mixing) × 100] (Rosenberg et al., 1980).

2.11. Statistical analysis
SPSS 11.0 software was used for the statistical analyses. The correlation between rhamnolipid production and surface tension was examined using Pearson's correlation. In addition, the correlation was used for determining any significant difference between emulsification activity and cell surface hydrophobicity of the strains. All analyses were run in triplicate for each replication.

3. Results and discussion

3.1. Identification of isolates
Samples taken from contaminated water and soil were cultivated in PAB culture, a selective culture medium for *Pseudomonas* spp., and among the isolates that grew in this culture, 26 gram-negative isolates were identified using API. According to API results, isolates 78 and 99 were identified as *P. aeruginosa* (99.9%), and the other 24 were identified as *P. stutzeri* (Table 1). Additionally, biochemical and physiological tests were conducted on isolated strains and reference strains, and protein profiles (Figure 1) of all were created and compared to confirm API results. When Gram reaction, cell morphology, biochemical, and physiological tests were compared with the protein profiles of the strains, it was determined that strains 78 and 99 were similar to *P. aeruginosa* ATCC 27853 and the others resembled *P. stutzeri* DSM 6082 (Table 1). In addition, the isolates presented in Table 1 were observed to grow in PAB culture, to appear in the form of gram-negative rods, and to grow at 42 °C but not at 5 °C. On the basis of these data, *P. stutzeri* has a wider distribution than *P. aeruginosa* in soil/water samples contaminated with industrial waste and oil.

3.2. Determination of biosurfactant-producer strains
In this study, 26 *Pseudomonas* spp. isolates obtained from stream water contaminated with factory waste (Maneerat, 2005) and soil contaminated with oil (Banat et al., 2000) were used. All strains were developed in nutrient broth culture, the surface tension of which was measured using a stalagmometer. Measurement results showed (Table 2) that only 2 strains, *P. aeruginosa* 78 and 99, reduced the surface tension from 73 dyne/cm² to 29 and 33 dyne/cm², respectively. Therefore, these 2 strains were chosen for use in other studies. Other researchers isolated biosurfactant-producer microorganisms from areas contaminated with industrial and oil waste and used them in their studies. For instance, Tuleva et al. (2002) reported that they isolated *P. putida* strain, a biosurfactant producer that can create surface-active agents that reduce surface tension of the

Table 1. Results of API, biochemical, and physiological tests used in the identification of *Pseudomonas* spp. isolates.

Code	Strains	API%	D	GH	SH	O	P	LO
P. aeruginosa ATCC 27853			+	+	–	+	B–G	G.U..
78	*P. aeruginosa*	99.9	–	+	–	+	B–G	S
99	*P. aeruginosa*	99.9	+	+	–	+	B–G	S
P. stutzeri DSM 6082			+	–	+	+	–[b]	G.U.
A5	*P. stutzeri*	99.9	+	–	+	+	–[b]	S
A6	*P. stutzeri*	99.9	+	–	+	+	–[b]	S
A8	*P. stutzeri*	99.9	+	–	+	+	–[b]	S
C3	*P. stutzeri*	99.9	+	–	+	+	–[b]	S
C6	*P. stutzeri*	99.9	+	–	+	+	–[b]	S
C7	*P. stutzeri*	99.9	+	–	+	+	–[b]	S
N6	*P. stutzeri*	99.9	–	–	+	+	–[b]	S
N7	*P. stutzeri*	99.9	+	–	+	+	–[b]	S
S21	*P. stutzeri*	99.9	+/–[a]	–	+	+	–[b]	S
28	*P. stutzeri*	99.9	–	–	–	+	–[b]	S
30	*P. stutzeri*	99.9	+	–	+	+	–[b]	S
125	*P. stutzeri*	98.7	+	–	+	+	–[b]	S
T1	*P. stutzeri*	94.7	+	–	+	+	–[b]	W
T2	*P. stutzeri*	98.2	+/–[a]	–	–	+	–[b]	W
T3	*P. stutzeri*	99.9	+	–	+	+	–[b]	W
T4	*P. stutzeri*	97.2	+	–	+	+	–[b]	W
T5	*P. stutzeri*	99.9	+	–	+	+	–[b]	W
T6	*P. stutzeri*	99.9	+	–	+	+	–[b]	W
T7	*P. stutzeri*	94.7	+	–	+	+	–[b]	W
T8	*P. stutzeri*	94.7	+	–	+	+	–[b]	W
T9	*P. stutzeri*	99.9	+	–	–	+	–[b]	W
T10	*P. stutzeri*	94.7	+	–	+	+	–[b]	W
T11	*P. stutzeri*	94.7	+	–	+	+	–[b]	W
T12	*P. stutzeri*	94.7	+	–	+	+	–[b]	W

API %: percent identified of isolates, D: denitrification test, GH: gelatine hydrolisation test, SH: starch hydrolisation test, O: oxidase test, P: pigmentation, LO: location of isolates and strains obtained, +: positive, –: negative; a: suspicious, b: not pigmentation but yellow colony, B-G: blue-green pigmentation, G.U.: Gazi University Biotechnology Laboratory, S: soil, W: water.
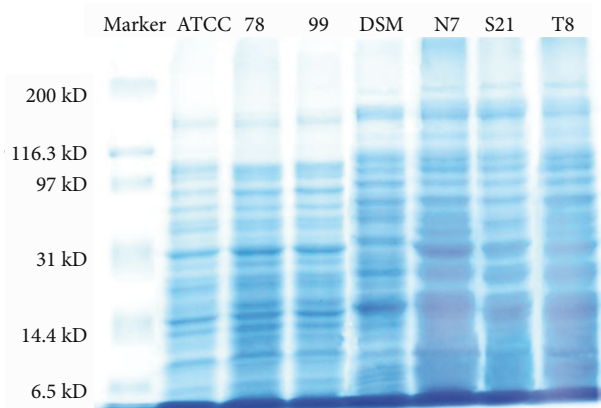
Figure 1. SDS-PAGE of whole-cell proteins of *Pseudomonas aeruginosa* (ATCC 27853, 78, 99) and *Pseudomonas stutzeri* (DSM 6082, N7, S21, T8).
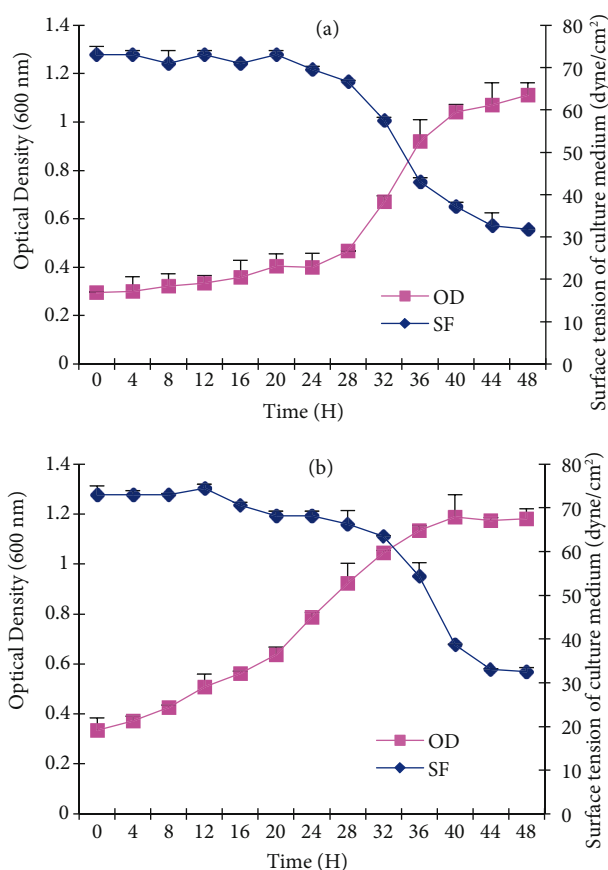

Figure 2. Surface tension (depending on time) in culture medium and growth curve of a) *P. aeruginosa* 78 (n = 3) and b) *P. aeruginosa* 99. Figure also indicates mean ± standard error, n = 3.

culture, from industrial waste. In a similar study, it was stated that biosurfactant-producer bacteria could be found more commonly in areas contaminated with hydrocarbons such as oil (Margesin and Schinner, 2001; Arutchelvi et

al., 2011). Considering the data obtained in the study, the possibility of isolating biosurfactant-producer strains from soil contaminated with oil is high.

The genus *Pseudomonas* is capable of using different substrates, such as glycerol, mannitol, fructose, glucose, n-paraffins, molasses, and vegetable oils, which are all known as good carbon and energy sources for producing rhamnolipid-type biosurfactants (Boulton et al., 1987; Santa Anna et al., 2002; Onbasli and Aslim, 2009). *P. aeruginosa* 78 and 99 were able to use 3% glycerol and produced 287 and 286 mg/L of rhamnolipids, decreasing the surface tension of the culture from 73 dyne/cm² to 29 and 33 dyne/cm² at the end of 4 days of incubation, respectively. These strains showed an ability to use glycerol for rhamnolipid production. These findings are consistent with the observations reported by Santa Anna et al. (2002), Itoh et al. (1971), and Rashedi et al. (2005), who stated that the best results were obtained using glycerol as substrate (Guerra-Santos et al., 1986; Rashedi et al., 2005). Furthermore, in a study conducted by Onbasli and Aslim (2009) the strains of *P. aeruginosa* examined produced rhamnolipids at 250–300 mg/L. Moreover, Khoshdast et al. (2011) suggested that rhamnolipids produced from the strain *P. aeruginosa* Ma01 decreased the surface tension of the culture from 70 dyne/cm² to 30 dyne/cm² at the end of incubation.

The correlation between rhamnolipid production and surface tension was significant, according to statistical analysis ($P < 0.01$).

3.3. Determination of rhamnolipid production time and curve growth

The correlation between rhamnolipid production time and curve growth on culture media was examined, and the results are presented in Table 3. To clarify, rhamnolipid production time in culture media was determined by drop-collapse method, and the results were verified by measuring surface tensions of samples taken from the same culture media. Accordingly, depending on rhamnolipid production, the diameter of drop samples taken from BMSM culture medium grew after 28 h in the culture medium of *P. aeruginosa* 78 strain and after 32 h in the culture medium of *P. aeruginosa* 99 culture medium (Table 3). Similarly, surface tension measured on the culture media was observed to drop after 28 h in *P. aeruginosa* 78 strain and after 32 h in *P. aeruginosa* 99 (Figure 2). Based on those results, *P. aeruginosa* 78 strain starts producing rhamnolipids at 28 h, and *P. aeruginosa* 99 strain begins to produce rhamnolipids at 32 h. *P. aeruginosa* 78 strain entered the log phase at 28 h and the stationary phase at 48 h, while *P. aeruginosa* 99 strain entered the log phase at 20 h and the stationary phase at 40 h. A comparison of reproduction curves and rhamnolipid production of the strains revealed that *P. aeruginosa* 78 strain

Table 2. The surface tensions measured on the culture media and amount of rhamnolipid produced on nutrient broth medium by isolated and identified *Pseudomonas* spp.

Strain	Code	N	Surface tension (dyne/cm²)[a]		Rhamnolipid (mg/L)	
			Mean	SD	Mean	SD
P. aeruginosa	78	3	29	1.7	287	5.2
P. aeruginosa	99	3	33	1.7	286	1.7
P. stutzeri	A5	3	73	3.5	9	1.69
P. stutzeri	A6	3	71	1.7	13	1.68
P. stutzeri	A8	3	70	5.2	6	1.70
P. stutzeri	C3	3	72	1.7	22	3.4
P. stutzeri	C6	3	72	3.5	10	1.69
P. stutzeri	C7	3	71	1.7	7	5.1
P. stutzeri	N6	3	70	1.7	3	1.7
P. stutzeri	N7	3	69	1.7	10	3.4
P. stutzeri	S21	3	72	1.7	21	3.5
P. stutzeri	28	3	70	6.9	30	3.5
P. stutzeri	30	3	70	3.5	5	3.6
P. stutzeri	125	3	67	1.7	20	3.5
P. stutzeri	T1	3	69	5.21	3	5.21
P. stutzeri	T2	3	70	1.7	5	1.7
P. stutzeri	T3	3	68	3.5	6	1.69
P. stutzeri	T4	3	71	3.6	16	1.7
P. stutzeri	T5	3	72	1.6	20	0
P. stutzeri	T6	3	70	1.6	4	1.7
P. stutzeri	T7	3	70	3.3	11	1.7
P. stutzeri	T8	3	69	3.5	8	1.7
P. stutzeri	T9	3	73	5.2	21	3.5
P. stutzeri	T10	3	69	5.2	10	1.7
P. stutzeri	T11	3	70	1.7	7	3.4
P. stutzeri	T12	3	71	1.69	17	1.69

[a]: preliminary surface tension on culture medium 73 dyne/cm², the measurements were carried out at the end of a 96-h cultivation period at room temperature and pH 7.

Table 3. Drop collapse depending on time of samples obtained from culture media of *P. aeruginosa* 78 and 99.

Hour / Strains		4.	8.	12.	16.	20.	24.	28.	32.	36.	40.
		Drop diameter (mm) ± 0.00									
P. aeruginosa 78	BMSM	5.7	5.6	5.6	5.8	5.7	5.7	**6.2**	6.9	7.5	7.6
P. aeruginosa 99	BMSM	5.8	5.8	5.7	5.8	5.7	5.8	5.9	**6.1**	6.4	7.1

started rhamnolipid production in the early logarithmic phase, while *P. aeruginosa* 99 strain started producing rhamnolipids in the middle of the logarithmic phase and continued thereafter. Surface tensions measured in culture media of *P. aeruginosa* 78 and 99 strains in BMSM culture media after 96 h were 27 and 28 dyne/cm^2 respectively, while amounts of rhamnolipid produced were 396 and 282 mg/L, respectively. Previous studies reported that production of rhamnolipid biosurfactant started during the logarithmic and stationary phases of bacterial growth, and that the amount of production increased afterwards (Guerra-Santos et al., 1986; Zhang and Miller, 1995). In another study, it was noted that *P. fluorescens* 5064 strain started biosurfactant production in culture media after 30 h of incubation (Cui, 2004). Onbasli and Aslim (2009) stated that *P. luteola* and *P. putida* produced rhamnolipids of 0.23 and 0.24 g/L after 48 h and 0.38 and 0.36 g/L after 72 h, respectively. The results of our study are similar to those of the above-mentioned studies.

3.4. Determination of CMC value of rhamnolipid biosurfactants
CMC is defined as the minimum surface-active agent concentration required to minimise surface tension. This is a very important property in that it is a criterion used in comparing surface active agents with one another (Georgiou et al., 1992). CMC can be used as an indicator of the quality of any surfactant/biosurfactant. CMC values of rhamnolipids produced by *P. aeruginosa* 78 and 99 strains were 115 mg/L and 130 mg/L, respectively (Figure 3).

In a study by Moraes et al. (2002) the CMC value of the rhamnolipid biosurfactant produced by *P. aeruginosa* was 120 mg/L. It was reported that the CMC value of
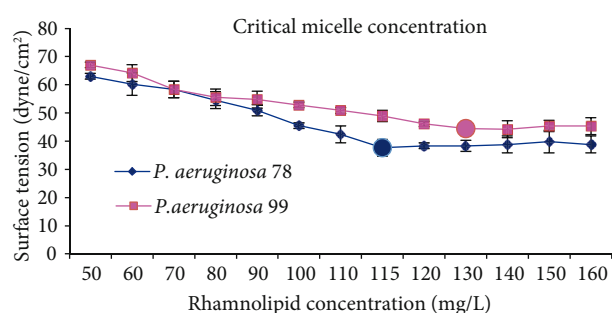

Figure 3. CMC value of rhamnolipids obtained from supernatant cultures of *P. aeruginosa* 78 and 99 strains. Figure also indicates mean ± standard error, n = 3.

rhamnolipid biosurfactants produced by *Pseudomonas* spp. species generally ranged between 50 and 200 mg/L. Therefore, our results are congruous. On the other hand, in another study (Khoshdast et al., 2011) the CMC value of a rhamnolipid with 97.5% purity was 10 mg/L. It is thought that the difference in CMC values observed in this study may be due to the purity degree of the rhamnolipid.

In addition, surface tension measurements in the culture media showed that surface tension of both strains reached the lowest level at 52 h and tended to level off thereafter. Thus, the strains reached the CMC value in rhamnolipid production in culture media at 52 h. In other words, *P. aeruginosa* strains started rhamnolipid production at the beginning of the logarithmic phase, the production gradually grew, and rhamnolipid concentration in the culture medium reached the CMC value in the stationary phase (Figure 2).

3.5. Effect of pH and temperature on surface tension

The effect of rhamnolipids, produced by *P. aeruginosa* 78 and 99 strains, on surface tension at different temperatures and pH values was examined, and the results are presented in Figures 4 and 5. As seen in Figure 4, pH markedly affected the surface activity of rhamnolipids. Acidic values exercised a more unfavourable effect on surface tension of rhamnolipids, relative to basic values. Similarly, in Figure 5 it is clear that low temperatures influenced surface tension, causing it to increase. Rhamnolipids showed an almost stable surface activity profile at pH values less than neutral (from 7 to 2). A more pronounced reduction in surface activity was observed at alkaline pH values, which could be ascribed to the formation of vesicles at these pH values (Khoshdast et al., 2011).

The most appropriate temperature values for the surface tension of rhamnolipids are 25 °C and above. Several researchers reported that activities of biosurfactants were not affected by extremes in temperature and pH (Kosaric, 1992; Kosaric, 2001; Christofi and Ivshina, 2002; Wong et al., 2003). However, rhamnolipids are known to go through stages of precipitation with acid and at low temperatures (+4 °C) during extraction. Therefore, acidity and low temperature play a negative role in the surface tension activity of rhamnolipids.

3.6. Emulsification activity and cell surface hydrophobicity

Biosurfactants enable the interaction of molecules by acting between contact surfaces of phases in immiscible systems like hydrocarbon/water (Delden and Iglew, 1998; Kosaric, 2001). Hydrophobic tail contacts hydrocarbons, while hydrophilic head interacts with water molecules; and this amphiphilic property bestows an emulsifier characteristic to surface-active substances. The same is true for hydrophobicity. When surface-active agents are on bacterial cell surfaces, as the hydrophilic head binds to the cell surface and the hydrophobic tail to hydrocarbons, they give a hydrophobic character to cell surfaces (Dubois et al., 1956; Desai and Banat, 1997; Christofi and Ivshina,

2002; Nayak et al., 2009). Thus, emulsification activity and hydrophobicity are correlated.

This study explored emulsifier properties of rhamnolipids produced by *P. aeruginosa* 78 and 99 strains on benzene, n-hexane, toluene, and xylene hydrocarbons as well as rhamnolipid affinity to cell surfaces of these hydrocarbons. Rhamnolipids produced by *P. aeruginosa* 78 and 99 strains emulsified benzene at a rate of 68% and 69%, n-hexane at a rate of 64% and 71%, toluene at a rate of 60% and 64%, and xylene at a rate of 46% and 36%, respectively. In order to determine the stability of the emulsifier characteristic of rhamnolipids, measurements were repeated after 30 days, and the results changed (8 ± 1%) (Table 4). Previous studies reported that rhamnolipids emulsified benzene at a rate of 60%–72%, n-hexane at 73%, toluene at 33%–100%, and xylene at 59% (Moraes et al., 2002; Bodour et al., 2003; Costa et al., 2005; Lotfabad et al., 2009). The results of previous studies were congruent with ours; rhamnolipids produced from the strains had a higher emulsifier quality for benzene, n-hexane, and benzene, relative to xylene. In a study conducted by Long et al. (2013b), it was found that the presence of rhamnolipid at a medium concentration of 1000 mg/L made 40% of water precipitate from waste crude oil.

The study also examined the affinity of the cell surface of rhamnolipid-producer *P. aeruginosa* 78 and 99 strains to benzene, n-hexane, toluene, and xylene hydrocarbons. The cell surfaces of the strains attracted benzene at a rate of 73% and 62%, n-hexane at a rate of 40% and 29%, toluene at a rate of 65% and 72%, and xylene at a rate of 60% and 53%, respectively (Table 4). It was reported in another study that n-hexadecane was attracted to the cell surface of the 21 BN strain of *P. putida* at a rate of 72% (Tuleva et al., 2002). In a study by Noordman and Janssen (2002) hexadecane was attracted to the cell surfaces of the strains of biosurfactant producers *P. aeruginosa* UG2, *Acinetobacter calcoaceticus* RAG1, *Rhodococcus erythropolis* DSM 43066, and *R. erythropolis* ATCC 19558
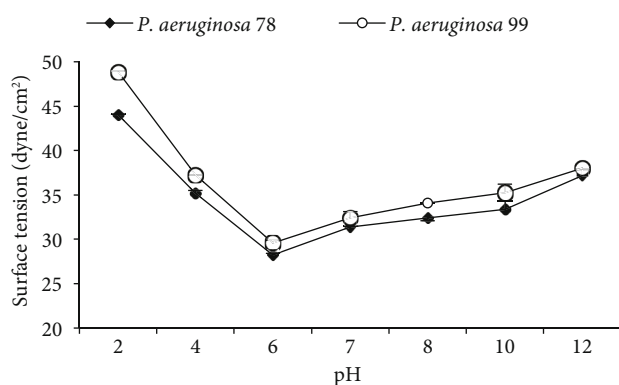

Figure 4. Surface tension of rhamnolipids with changing pH. Figure also indicates mean ± standard error, n = 3.
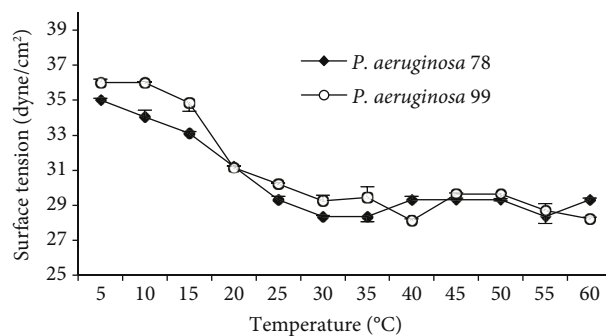

Figure 5. Surface tension of rhamnolipids with changing temperature. Figure also indicates mean ± standard error, n = 3.

Table 4. Emulsifying activity of rhamnolipids produced by *P. aeruginosa* 78 and 99 strains with different hydrocarbons and cell surface hydrophobicity (with hydrocarbons) of strains.

Strains	Hydrocarbon	N	E24%	SD	EI%	SD	CSH%	SD
P. aeruginosa 78	Benzene	3	68	1.7	65	3.5	73	5.2
	n-Hexane	3	64	1.69	63	1.7	40	3.4
	Toluene	3	60	1.7	59	1.7	65	3.5
	Xylene	3	46	3.5	44	5.2	60	5.1
P. aeruginosa 99	Benzene	3	69	1.69	61	1.7	62	3.4
	n-Hexane	3	71	1.7	71	3.5	29	1.7
	Toluene	3	64	1.7	62	1.7	72	5.2
	Xylene	3	36	1.69	35	3.4	53	3.5

E24: emulsification activity, measured after 24 h; EI: emulsification index, emulsification activity measured after 30 days; CSH: cell surface hydrophobicity.

at rates of 42%, 81%, 30%, and 12%, respectively. Similarly, it was observed that G1 strains of *P. aeruginosa* degraded 48% of 2.5% crude oil in 7 days (Celik et al., 2008). In addition, Arutchelvi et al. (2011) stated that rhamnolipids contributed to the attachment of microorganisms to plastic and high hydrophobic polymers and played a role in degrading them. It was established that cell surfaces had a more hydrophobic quality for benzene, toluene, and xylene than n-hexane. The correlation between emulsification activity and cell surface hydrophobicity was not significant according to statistical analysis ($P > 0.05$). An evaluation of emulsification activity and hydrophobicity results in terms of correlation between the 2 revealed that rhamnolipid produced by *P. aeruginosa* 78 and 99 strains had a poorer emulsifier quality for xylene, and that cell surfaces of the concerned strains had a lower affinity to n-hexane relative to other hydrocarbons. Accordingly, rhamnolipids with high emulsifier characteristics failed to bestow a high hydrophobic quality to cell surfaces.

4. Conclusions

In this study, *P. aeruginosa* 78 and 99 produced rhamnolipid biosurfactants from petroleum-contaminated soil (Batman Province, Turkey). However, *P. aeruginosa*-producing rhamnolipids could not be isolated from a Çorlu stream contaminated with factory waste. Therefore, the possibility of isolating strains capable of producing rhamnolipids is much higher from the areas contaminated by petroleum. Under favourable conditions rhamnolipid synthesis occurred in the logarithmic phase of bacterial growth. An important result obtained in this research was that these strains decreased the surface tension of the culture from 73 dyne/cm^2 to 29 and 33 dyne/cm^2 with the CMC of produced

rhamnolipids 115 and 130 mg/L, respectively. The surface active qualities, surface tension, and CMC of the rhamnolipids produced by *P. aeruginosa* 78 and 99 made them eligible for use in place of synthetic surfactants. Nonetheless, regarding CMC value, purity of rhamnolipids is significant as well. The rhamnolipids were examined in terms of emulsification activity and cell surface hydrophobicity. At the end of this investigation, it was found that *P. aeruginosa* 78 and 99 strains emulsified benzene and n-hexane at the highest rates and that the surfaces of these strains were 73% and 65% and 62% and 72% more hydrophobic for benzene and toluene, respectively. Therefore, both *P. aeruginosa* 78 and 99 are likely to be used in place of synthetic surfactants owing to the characteristics of the produced rhamnolipids. Thanks to the hydrophobic surface properties of the rhamnolipid-producer bacteria, rhamnolipids can be used to reduce the contaminative burden of oil-derived hydrocarbons, which are among the foremost environmental polluters. Therefore, we need to arrive at a better understanding of the characteristics of biosurfactants, use them as alternatives to synthetic surfactants, and isolate more effective biosurfactant-producer microorganisms. As biosurfactants are amphiphilic substances, their emulsification activities and the hydrophobicity they conferred on cell surfaces were explored.

To support the conclusions of the current study detailed fundamental research should be performed on rhamnolipids produced by the aforementioned strains.

References

Antunes AA, Silva MLRB, Silva CAA, Campos-Takaki GM (2006). Characterization of *Chromobacterium violaceum* isolated from Paca River, Pernambuco, Brazil. Revista de Biologia E Ciências da Terra 1: 29–39.

Arutchelvi J, Joseph C, Doble M (2011). Process optimization for the production of rhamnolipid and formation of biofilm by *Pseudomonas aeruginosa* CPCL on polypropylene. Biochemical Engineering Journal 56: 37–45.

Atlas RM, Parks LC (1997). Handbook of Microbiological Media. 2nd ed. New York, NY, USA: CRC Press.

Banat IM, Makkar RS, Cameotra SS (2000). Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol 53: 495–508.

Beal R, Betts WB (2000). Role of rhamnolipid biosurfactants in the uptake and mineralization of hexadecane in *Pseudomonas aeruginosa*. J Appl Microbiol 89: 158–168.

Bodour AA, Drees KP, Maier RM (2003). Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid Southwestern soils. Appl Environ Microb 69: 3280–3287.

Calvo C, Martínez-Checa F, Toledo FL, Porcel J, Quesada E (2002). Characteristics of bioemulsifiers synthesised in crude oil media by *Halomonas eurihalina* and their effectiveness in the isolation of bacteria able to grow in the presence of hydrocarbons. Appl Microbiol Biotechnol 60: 347–351.

Cassidy DP, Hudak AJ Jr, Werkema DD, Atekwana EA, Rossbach S, Duris JD, Atekwana EA, Sauck WS (2002). In situ rhamnolipid production at an abandoned petroleum refinery. Soil Sediment Contam 11: 769–787.

Caykara T, Birlik G (2005). Swelling and adsorption properties of hydrophobic poly[(n- (3-(dimethylamino)propyl) methacrylamide)-co-(lauryl acrylate)] hydrogels in aqueous solutions of surfactants. Macromol Mater Eng 290: 869–874.

Celik GY, Aslim B, Beyatli Y (2008). Enhanced crude oil biodegradation and rhamnolipid production by *Pseudomonas stutzeri* strain G11 in the presence of Tween-80 and Triton X-100. J Environ Biol 29: 867–870.

Christofi N, Ivshina IB (2002). Microbial surfactants and their use in field studies of soil remediation. J Appl Microbiol 93: 915–929.

Cohen R, Ozdemir G, Exerowa D (2003). Free thin liquid films (foam films) from rhamnolipids: type of the film and stability. Colloids and Surfaces B: Biointerfaces 29: 197–204.

Costa SGVAO, Nitschke M, Haddad R, Eberlin MN, Contiero J (2005). Production of *Pseudomonas aeruginosa* LBI rhamnolipids following growth on Brazilian native oil. Process Biochem 41: 483–488.

Cui X (2004). Regulation of biosurfactant production by quorum sensing in *Pseudomonas fluorescens* 5064, the cause of broccoli head rot disease. PhD, The University of Edinburgh, Edinburgh, United Kingdom.

Delden CV, Iglew BH (1998). Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. Emerg Infect Dis 4: 551–560.

Desai JD, Banat IM (1997). Microbial production of surfactants and their commercial potential. Microbiol Mol Biol R 61: 47–64.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956). Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350–356.

Georgiou G, Lin S, Sharma MM (1992). Surface-active compounds from microorganisms. Bio/Technology 10: 60–65.

Gogra AB, Yao J, Sandy EH, Zheng SX, Zaray G, Koroma BM, Hui Z (2010). Cell surface hydrophobicity (CSH) of *Escherichia coli*, *Staphylococcus aureus* and *Aspergillus niger* and the biodegradation of diethyl phthalate (DEP) via microcalorimetry. Journal of American Science 6: 78–88.

Guerra-Santos LH, Käppeli O, Fiechter A (1986). Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant production on nutritional and environmental factors. Appl Microbiol Biot 24: 443–448.

Hamme JDV, Singh A, Ward OP (2006). Physiological aspects. Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. Biotechnol Adv 24: 604–620.

Itoh S, Honda H, Tomita F, Suzuki T (1971). Rhamnolipid produced by *Pseudomonas aeruginosa* grown on n-paraffin. J Antibiot 24: 855–859.

Khalladi R, Benhabiles O, Bentahar F, Moulai-Mostefa N (2009). Surfactant remediation of diesel fuel polluted soil. J Hazard Mater 164: 1179–1184.

Khoshdast H, Sam A, Vali H, Noghabi KA (2011). Effect of rhamnolipid biosurfactants on performance of coal and mineral flotation. International Biodeterioration & Biodegradation 65: 1238–1243.

Kosaric N, Cairns WL, Gray NCC (1987). Biosurfactants and Biotechnology. New York, NY, USA: Marcel Dekker Inc.

Kosaric N (1992). Biosurfactants in industry. Pure Appl Chem 64: 1731–1737.

Kosaric N (2001). Biosurfactants and their application for soil bioremediation. Food Technol Biotechnol 39: 295–304.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

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

Long X, Zhang G, Han L, Meng Q (2013a). Dewatering floated oily sludge by treatment with rhamnolipid. Water Research 47: 4303–4311.

Long X, Zhang G, Shen C, Sun G, Wang R, Yin L, Meng Q (2013b). Application of rhamnolipid as a novel biodemulsifier for destabilizing waste crude oil. Bioresource Technology 131: 1–5.

Lotfabad TB, Shourian M, Roostaazad R, Najafabadi AR, Adelzadeh MR, Noghabi KA (2009). An efficient biosurfactant-producing bacterium *Pseudomonas aeruginosa* MR01, isolated from oil excavation areas in south of Iran. Colloids and Surfaces B: Biointerfaces 69: 183–193.

Maier RM, Soberon-Chavez G (2000). *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. Appl Microbiol Biot 54: 625–633.

Makkar RS, Cameotra SS (1999). Biosurfactant production by microorganisms on unconventional carbon sources. J Surf Det 2: 237–241.

Maneerat S (2005). Production of biosurfactants using substrates from renewable-resources. Songklanakarin J Sci Technol 27: 675–683.

Maneerat S (2005). Biosurfactants from marine microorganisms. Songklanakarin J Sci Technol 27: 1263–1272.

Margesin R, Schinner F (2001). Bioremediation (natural attenuation and biostimulation) of diesel-oil contaminated soil in an alpine glacier skiing area. Appl Environ Microb 67: 3127–3133.

Mata-Sandoval JC, Karns J, Torrents A (1999). High-performance liquid chromatography method for the characterization of rhamnolipid mixtures produced by *Pseudomonas aeruginosa* UG2 on corn oil. J Chromatogr A 864: 211–220.

Moraes IO, Benincasa M, Alegre RM (2002). Production and characterization of rhamnolipids produced by a newly isolated strain of *Pseudomonas aeruginosa*. Braz J Food Technol 5: 145–149.

Nayak AS, Vijaykumar MH, Karegoudar TB (2009). Characterization of biosurfactant produced by *Pseudoxanthomonas* sp. PNK-04 and its application in bioremediation. Int Biodeter Biodegr 63: 73–79.

Noordman WH, Janssen DB (2002). Rhamnolipid stimulates uptake of hydrophobic compounds by *Pseudomonas aeruginosa*. Appl Environ Microb 68: 4502–4508.

Patel RM, Desai AJ (1997). Biosurfactant production by *Pseudomonas aeruginosa* GS3 from molasses. Lett Appl Microbiol 25: 91–94.

Onbasli D, Aslim B (2009). Biosurfactant production in sugar beet molasses by some *Pseudomonas* spp. J Environ Biol 30: 161–163.

Ozturk S, Kaya T, Aslim B, Tan S (2012). Removal and reduction of chromium by *Pseudomonas* spp. and their correlation to rhamnolipid production. Journal of Hazardous Materials 231–232: 64–69.

Pornsunthorntawee O, Chavadej S, Rujiravanit R (2009). Solution properties and vesicle formation of rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa* SP4. Colloids and Surfaces B: Biointerfaces 7: 26–15.

Rasshedi H, Jamshidi E, Assadi M, Bonakdarpour B (2005). Isolation and production of biosurfactant from *Pseudomonas aeruginosa* isolated from Iranian southern wells oil. Int J Environ Sci Tech 2: 121–127.

Raza ZA, Rehman A, Khan MS, Khalid ZM (2007). Improved production of biosurfactant by a *Pseudomonas aeruginosa* mutant using vegetable oil refinery wastes. Biodegradation 18: 115–121.

Rosenberg M, Gutnick D, Rosenberg E (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol Lett 9: 29–33.

Santa Anna LMS, Sebastian GV, Menezes EP, Alves TLM, Santos AS, Pereira N Jr, Freire DMG (2002). Production of biosurfactants from *Pseudomonas aeruginosa* PA1 isolated in oil environments. Brazilian Journal of Chemical Engineering 19: 159–166.

Silva GP, Mack M, Contiero J (2009). Glycerol: a promising and abundant carbon source for industrial microbiology. Biotechnol Adv 27: 30–39.

Tabatabaee A, Assadi MM, Noohi AA, Sajadian VA (2005). Isolation of biosurfactant producing bacteria from oil reservoirs. Iranian J Env Health Sci Eng 2: 6–12.

Tuleva BK, Ivanov GR, Christova NE (2002). Biosurfactant production by a new *Pseudomonas putida* strain. Verlag der Zeitschrift für Naturforschung Tübingen 57: 356–360.

Whang LM, Liu PW, Ma CC, Cheng SS (2009). Application of rhamnolipid and surfactin for enhanced diesel biodegradation—effects of pH and ammonium addition. J Hazard Mater 164: 1045–1050.

Wong JWC, Fang M, Zhao Z, Xing B (2003). Effect of surfactants on solubilization and degradation of phenanthrene under thermophilic conditions. J Environ Qual 33: 2015–2025.

Youssef NH, Duncan KE, McInerney MJ (2005). Importance of 3-hydroxy fatty acid composition of lipopeptides for biosurfactant activity. Appl Environ Microb 71: 7690–7695.

Zhang G, Wu Y, Qian X, Meng Q (2005). Biodegradation of crude oil by *Pseudomonas aeruginosa* in the presence of rhamnolipids. J Zhejiang Univ SCI 6: 725–730.

Zhang Y, Miller RM (1995). Effect of rhamnolipid (biosurfactant) structure on solubilization and biodegradation of n-alkanes. Appl Environ Microb 61: 2247–2251.

Zita A, Hermansson M (1997). Determination of bacterial cell surface hydrophobicity of single cells in cultures and in wastewater in situ. FEMS Microbiol Letters 152: 299–306.