Biotechnology Reports 7 (2015) 55–63

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Stimulation of rhamnolipid biosurfactants production in *Pseudomonas aeruginosa* AK6U by organosulfur compounds provided as sulfur sources

Wael Ismail [a,*], Sultanah AL. Shammary [a], Wael S. El-Sayed [b,1], Christian Obuekwe [c], Ashraf M. El Nayal [a], Abdul Salam Abdul Raheem [a], Abdulmohsen Al-Humam [d]

[a] Life Sciences Department, Biotechnology Program, College of Graduate Studies, Arabian Gulf University, Manama, Bahrain
[b] Taibah University, Faculty of Science, Biology Department, Al-Madinah Al-Munawarah, 344, Saudi Arabia
[c] Department of Biological Sciences, College of Science, Kuwait University, Kuwait
[d] Research & Development Center, P.O. Box 62, Dhahran, Saudi Aramco, Saudi Arabia

ARTICLE INFO

Article history:
Received 1 December 2014
Received in revised form 21 February 2015
Accepted 2 March 2015
Available online 21 May 2015

Keywords:
Surface tension
Biodesulfurization
Dibenzothiophene

ABSTRACT

A *Pseudomonas aeruginosa* AK6U strain produced rhamnolipid biosurfactants to variable extents when grown on $MgSO_4$ or organosulfur compounds as sulfur sources and glucose as a carbon source. Organosulfur cultures produced much higher biosurfactants amounts compared to the $MgSO_4$ cultures. The surface tension of the growth medium was reduced from 72 mN/m to 54 and 30 mN/m in cultures containing $MgSO_4$ and 4,6-dimethyldibenzothiophene (4,6-DM-DBT), respectively. AK6U cultures produced different rhamnolipid congener profiles depending on the provided sulfur source. The dibenzothiophene (DBT) culture produced more diverse and a higher number of rhamnolipid congeners as compared to the DBT-sulfone and $MgSO_4$ cultures. The number of mono-rhamnolipid congeners in the DBT culture was also higher than that detected in the DBT-sulfone and $MgSO_4$ cultures. Di-rhamnolipids dominated the congener profiles in all the analyzed cultures. The sulfur source can have a profound impact on the quality and quantity of the produced biosurfactants.

1. Introduction

Biosurfactants are amphiphilic tensioactive natural products produced by a diversity of microorganisms [16,39]. They are capable of reducing the surface and interfacial tension [39]. Moreover, effective biosurfactants are characterized by their ability to enhance the aqueous solubility of hydrophobic compounds and to emulsify hydrocarbons in water [16]. As compared to petroleum-based (synthetic) surfactants, biosurfactants are environmentally compatible, less or non-toxic, more efficient, stable under extreme conditions and can be produced from inexpensive renewable substrates [38]. These advantages render biosurfactants, interesting biotechnological products that have various applications, and are likely to replace synthetic surfactants in the future [16,36].

Environmental applications of biosurfactants include bioremediation and soil washing. They can also be applied in various processes in the oil industry such as microbial enhanced oil recovery, upgrading of crude oil, clean-up of oil containers and storage tanks, and formulation of petrochemicals [34]. Biosurfactants also offer many interesting biomedical and agricultural applications [9,30]. The commercialization of biosurfactants has been impeded by the high production costs and low yield of the producing strains. There is ongoing research and development work aiming to improve the economics of the biosurfactant production process. This can be achieved by optimizing various aspects like the yield, the growth conditions, the downstream processing, etc. [24].

Many studies have investigated the effect of the carbon source in the growth medium on biosurfactants production by some microorganisms. Both the type and concentration of the carbon source were shown to be essential determinants of biosurfactants yield and physicochemical properties. Hydrophobic carbon sources were found to be superior to hydrophilic ones in promoting biosurfactants production [1,33,37]. Other growth medium components that were found to have an impact on biosurfactants

* Corresponding author. Tel.: +973 36146948; fax: +973 17239664.
E-mail address: waelame@agu.edu.bh (W. Ismail).
[1] Permanent address: Ain Shams University, Faculty of Science, Microbiology Department, 11566 Cairo, Egypt.

http://dx.doi.org/10.1016/j.btre.2015.03.001

production include the nitrogen source and some ions like Mg, Ca, K, Na, Fe and trace metals [1,39].

In general, there is a lack of knowledge regarding biosurfactants production in relation to biodegradation/biotransformation of heterocyclic compounds [32]. Although the sulfur source is an essential component of the growth medium, its influence on the microbial production of biosurfactants has not been studied in detail. Recently, Ismail et al. [21] showed that the sulfur source plays a role in rhamnolipid biosurfactants yield via changing the expression of the *rhlABC* genes. The authors studied the influence of dibenzothiophene and dibenzothiophene-sulfone as compared to inorganic sulfate. Accordingly, in the present study we wanted to answer the question: does the sulfur source affect the diversity and numbers of the produced rhamnolipid congeners? Moreover, we investigated the effect of more organosulfur compounds on biosurfactants production.

2. Materials and methods

2.1. Materials

Sterile filters (Stericups, 0.22 μm) were from Millipore (USA). Arab light crude oil and kerosene were obtained from Saudi Aramco (Kingdom of Saudi Arabia).

2.2. Culture media and growth conditions

Commercially available Luria–Bertani (LB) agar and broth media were prepared according to the instructions of the supplier. Sulfur-free mineral salts medium (MSM) was prepared from stock solutions according to Gilbert et al. [19]. The MSM was supplemented with vitamin solution and trace elements [35,41]. The carbon source was glucose (10 mM) and the sulfur source was either $MgSO_4 \cdot 7H_2O$ (1 mM and 0.1 mM) or an organosulfur compound (0.1 mM). Sulfur-free cultures (no exogenous sulfur source) containing glucose as a carbon source were also included. The tested organosulfur substrates were dibenzothiophene (DBT), benzothiophene (BT), 4-methyldibenzothiophene (4-MDBT), 4,6-dimethyldibenzothiophene (4,6-DM-DBT), and dibenzyl sulfide (DBS). All organosulfur compounds were added to the MSM from 100 mM ethanol stocks except 4,6-DM-DBT, which was prepared in acetone. $MgCl_2 \cdot 6H_2O$ (1 mM) was added instead of $MgSO_4$ when organosulfur compounds were added as a sole sulfur source. If not mentioned otherwise, all liquid cultures were incubated in an orbital shaker (180 rpm) at 30 °C. All cultures on solid media were incubated at 3 0°C for 48 h. Liquid cultures were routinely grown in 250-mL Erlenmeyer flasks containing 100 mL of the growth medium. Uninoculated medium was routinely included as a negative control. Data are means of duplicate or triplicate measurements from two or three independent experiments, respectively. Error bars in the graphs represent standard error.

2.3. Enrichment and isolation of the AK6U strain

The enrichment of bacteria was performed in MSM containing glucose as a carbon source and DBT as a sulfur source. The inoculum was soil samples contaminated with used lubricating oil, benzene, and diesel collected from the neighborhood of mechanic workshops in Fahaheel area, State of Kuwait. The soil samples were collected from the top surface layer (10 cm in depth) in clean plastic containers and kept at room temperature in the laboratory. Soil samples (2 g) were inoculated into 100 mL of sterilized MSM. The enrichment flasks were incubated on an orbital shaker for 7 days until turbidity and/or change in color appeared. Subsequently, aliquots (1 mL) from those original enrichments was transferred to fresh medium with the same composition and further incubated under the same conditions for the same period of time. This sub-culturing was repeated 4 times.

To isolate bacteria, samples from enrichment cultures were serially diluted in sterile saline solution (0.9% NaCl) and aliquots from those culture dilutions (100 μL) were spread over LB-agar plates and incubated for 72 h. Morphologically distinct single colonies from the spread plates were streaked across LB-agar plates three times consecutively for purification.

2.4. Growth of the AK6U strain on different sulfur sources

The AK6U strain was grown in sulfur-free MSM containing glucose as a carbon source and either $MgSO_4$ or an organosulfur compound as a sole sulfur source. Triplicate shake flasks were prepared for each sulfur source. Growth was monitored by measuring culture turbidity (optical density at 600 nm, OD_{600}) at various time intervals until the culture entered the stationary phase. The biomass yield was measured as dry cell weight by drying cell pellets at 105 °C for 15 h. Culture samples were retrieved at time intervals to monitor biosurfactants production as described later.

2.5. Kinetics of biosurfactants production

A preculture of AK6U was grown on glucose and 4,6-DM-DBT in basal sulfur-free MSM as described earlier until the culture turbidity (OD_{600}) reached 0.3. Aliquots (0.8 mL) from the pre-culture were inoculated into 2 L Erlenmeyer flasks (in triplicates) containing 800 mL of the same medium. The cultures were incubated at 37 °C in an orbital shaker (200 rpm). Growth was monitored by measuring the OD_{600} and biosurfactant production was monitored by measuring the surface tension at various time points as described later.

2.6. Oil displacement and emulsification index (E_{24}) assays

The oil-displacement and emulsification index (E_{24}) *assays* were conducted to test the AK6U cultures for biosurfactants production in cell-free culture supernatants as described [42]. Cell-free supernatants were prepared by centrifuging culture samples (1 mL) at 14,000 rpm for 5 minutes (4 °C). The hydrocarbon used for the emulsification assay was kerosene. The emulsification index assay was performed in duplicates using 48-h old batch cultures.

2.7. Measurement of surface tension

Surface tension was measured in cell-free supernatants retrieved from 48-h old batch cultures (25 mL) as described [21].

2.8. Preparation of crude rhamnolipids for congener composition analysis

The AK6U strain was grown in MSM containing glucose as a carbon source and either $MgSO_4$, DBT, or DBT-sulfone as a sulfur source according to published procedure [21]. Crude biosurfactants were extracted twice from the AK6U cell-free culture broth with chloroform-methanol mixture after acidification to pH 2. The crude rhamnolipid extract was partially purified as described by Kock et al. [22]. About 0.1 g of the solid crude rhamnolipid was resuspended in 1 mL sterile distilled water and re-extracted in equal volume of diethylether. The upper solvent phase containing the surfactant was carefully transferred to a diethyl ether-washed tube. The extraction was done three times and the recovered solvent phase was pooled and evaporated to dryness.

2.9. Analysis of hydroxyalkanoyloxyalkanoic acids (HAAs)

The hydroxyalkanoyloxyalkanoates present in different cultures were detected according to the method of Lepine et al. [25]. Cultures were first centrifuged before filtering using membrane filters of 0.2 μm pore size. Samples (50 μL) from the mixture of the filtrate (450 μL), acetonitrile and 40 mM ammonium acetate (100 μL) were injected into Waters Acquity UPLC-I-Class LC linked to WaterXEVO-G2QSTOF MS.

2.10. HPLC/MS analysis of rhamnolipid congeners

The rhamnolipid extract was dissolved in 10% acetonitrile prepared in 2 mM ammonium acetate, and 20 μL of this mixture was analyzed using Waters Xevo-G2SQTOF connected to Waters ultra performance LC model Acuity UPLC I-Class fitted with Acuity UPLC BEH C18 1.7 μm column (2.1 × 50 mm i.d.). Analysis was by means of electrospray ionization operating in the negative mode. Elution was done isocratically in 50% acetonitrile in water pumped at 0.6 mL /min; (elution time, 6 min). Conditions for MS were as follows: capillary, 3.0 kV; source temp, 117 °C sampling cone, 45 V; desolvation gas, N_2; desolvation gas flow rate, 500 L/h; desolvation temperature, 400 °C.

2.11. Amplification of the 16S rRNA gene and phylogenetic analysis

Isolation of genomic DNA, amplification of the 16S rRNA gene of AK6U, cloning and sequencing were performed as described [20]. Sequence was analyzed using Blast search facility on NCBI database (http//:www.ncbi.nhl.nih.gov). Closest relatives in GenBank database were determined and retrieved sequences were used to construct neighbor-joining phylogenetic trees using ClustalX program and MEGA5 software.

2.12. Sequence accession number

The 16S rRNA gene sequence obtained for strain AK6U has been deposited in GenBank under accession number AB922602.

2.13. Statistical analysis

One way analysis of variance (Tukey test with $p < 0.05$) was performed on the oil-displacement, emulsification index, and surface tension data with the JMP statistical software (version 10.0.2, SAS Corporation, Chicago, Illinois, USA).

3. Results

3.1. Isolation and identification of bacteria

Applying enrichment culture technique with DBT as a sole sulfur source, it was possible to isolate several bacterial strains. The different isolates grew in MSM containing glucose as a carbon source and DBT as a sole sulfur source. A culture of one isolate appeared greenish and revealed excessive foaming. This strain was designated as AK6U and was chosen for studying the influence of the sulfur source on biosurfactants production. Partial 16S rRNA gene sequence of AK6U revealed 99% identity to that of several *Pseudomonas aeruginosa* strains including *P. aeruginosa* L1, G2, RB-48, N002, BS8, ODE5_EKITI, PGS1, and M2. Therefore AK6U was assigned to this species. Phylogenetic analysis based on 16S rRNA gene sequence confirmed the affiliation of the strain AK6U to *P. aeruginosa* group (Fig. 1). Comparison with a reference database containing the sequences of different pseudomonads showed that strain AK6U constitutes a single branch close to *P. aeruginosa* group.

3.2. Growth of the AK6U strain on different sulfur sources

The AK6U strain grew in MSM containing glucose as a carbon source, while sulfur was supplied from different substrates. The sulfur sources utilized by AK6U include $MgSO_4$, DBT, BT, 4-MDBT, 4,6-DM-DBT, and DBS (Fig. 2). The growth pattern varied among the different AK6U cultures in terms of rate and biomass yield (Table 1). The highest biomass yield was measured in the glucose-DBS and glucose-4,6-DM-DBT cultures. The glucose-4,6-DM-DBT culture exhibited the highest growth rate. AK6U gave similar maximum biomass yields in cultures containing different

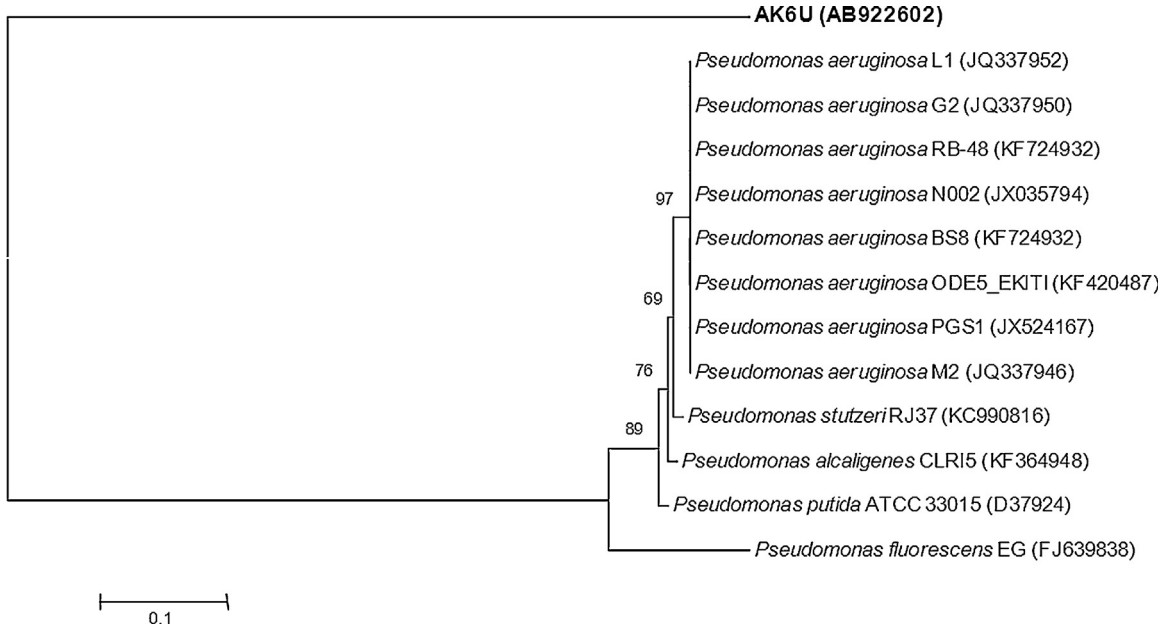

Fig. 1. Neighbour-joining consensus tree based on 16S rRNA gene sequence analysis showing the relationship between the AK6U strain and closest relatives along with other related members of the genus *Pseudomonas* in GenBank database. The bar represents 0.01 substitutions per site and bootstrap values (indicated at the nodes) were calculated from 1000 trees. GenBank accession numbers are given between parentheses.

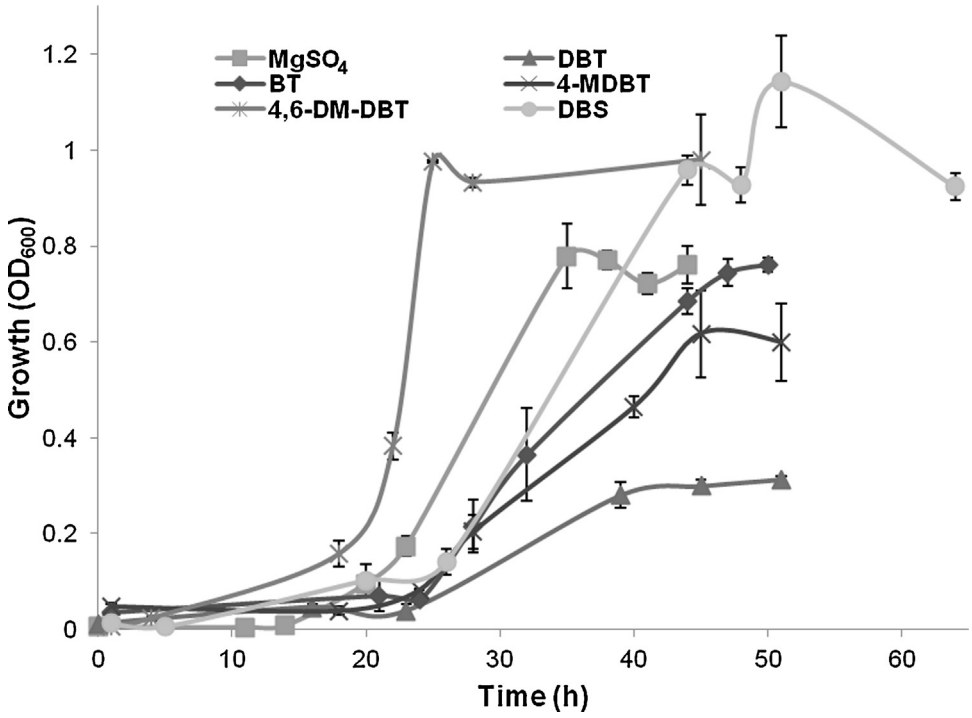

Fig. 2. Growth curves of AK6U cultures on glucose as a sole carbon source and different sulfur sources.

Table 1
Growth parameters of AK6U cultures growing on glucose as a carbon source and different sulfur sources.

Sulfur source	Generation time (h)	Specific growth rate (h^{-1})	Biomass yield (g dcw L^{-1})
$MgSO_4$	6.5	0.05	0.33
DBT	25	0.012	0.2
BT	15	0.02	0.27
4-MDBT	20	0.015	0.18
4,6-DM-DBT	5	0.06	0.5
DBS	15	0.02	0.5

concentrations of $MgSO_4$ (1 mM and 0.1 mM). Moreover, AK6U could not grow in sulfur-free MSM and lysis of the inoculum was obvious. The maximum OD_{600} attained in the sulfur-free cultures after 48-h incubation was 0.09 ± 0.01.

3.3. Influence of the sulfur source on biosurfactants production by AK6U

The AK6U cultures exhibited various degrees of foaming which increased with time. In general, the foam in cultures containing organosulfur substrates was very much higher than that observed in $MgSO_4$ cultures, which appeared almost foam-free (Fig. 3). Cell-free supernatants retrieved from the AK6U cultures caused

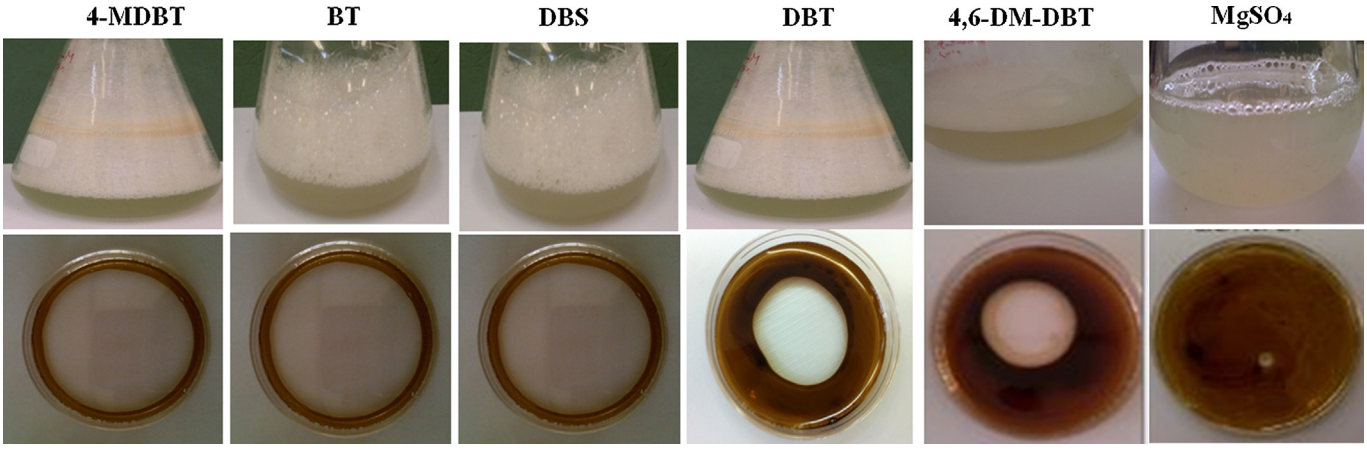

Fig. 3. Images showing the foaming and oil displacement activity of AK6U cultures grown on glucose as a carbon source and different sulfur sources.

clearing or displacement of crude oil in the oil displacement assay (Fig. 3). Statistically significant differences ($p < 0.0001$) in oil displacement activity were recorded between cultures containing $MgSO_4$ on one hand and those containing organosulfur compounds on the other hand. The oil displacement activity in cultures containing organosulfur substrates was much higher than that detected in cultures containing $MgSO_4$ as a sole sulfur source. No significant differences ($p > 0.05$) in the oil displacement activity were observed among the cultures containing 4-MDBT, BT, or DBS. On the contrary, cultures containing the later sulfur sources exhibited significantly higher oil displacement activity as compared to the DBT and 4,6-DM-DBT cultures. AK6U cultures containing either 1 mM or 0.1 mM of $MgSO_4$ exhibited similar foaming and oil displacement pattern. Similar to the foaming pattern, the oil displacement activity of the cultures increased with the incubation time (Fig. 4). In general, cultures showing higher foaming also gave larger oil displacement zones.

Biosurfactants production by AK6U was confirmed by the decrease in surface tension and enhanced emulsification power of culture samples. Biosurfactants produced in the different AK6U cultures reduced the surface tension of the growth medium to various extents (Fig. 5). This was revealed by measuring the surface tension in cell-free culture supernatants. Surface tension reduction was much greater in cultures containing organosulfur substrates + glucose (from 71 to 30 mN/m, for some cultures) than in the corresponding cultures having $MgSO_4$ as a sole sulfur source (from 71 to 54 mN/m). The lowest surface tension was observed in cell-free supernatants from the DBT and 4,6-DM-DBT cultures (30 mN/m). The differences in surface tension between the $MgSO_4$ cultures and those of the organosulfur cultures were statistically significant ($p < 0.0001$). Moreover, significant differences in surface tension were observed among the cultures containing the different organosulfur substrates ($p < 0.0001$) with the exception of the DBT and the 4,6-DM-DBT cultures. The two latter cultures had similar surface tension ($p > 0.05$). There was no difference in the minimal surface tension between AK6U cultures containing different concentrations of $MgSO_4$. Interestingly, despite lack of growth, surface tension of the sulfur-free cultures was reduced to 40.2 ± 0.2 mN/m within 24 h.

The biosurfactants produced in the different AK6U cultures with organosulfur substrates emulsified kerosene (Fig. 5). No emulsions were produced with samples from the $MgSO_4$ cultures or from the uninoculated medium. There was no significant difference between the emulsification indices of the different organosulfur cultures ($p > 0.05$).

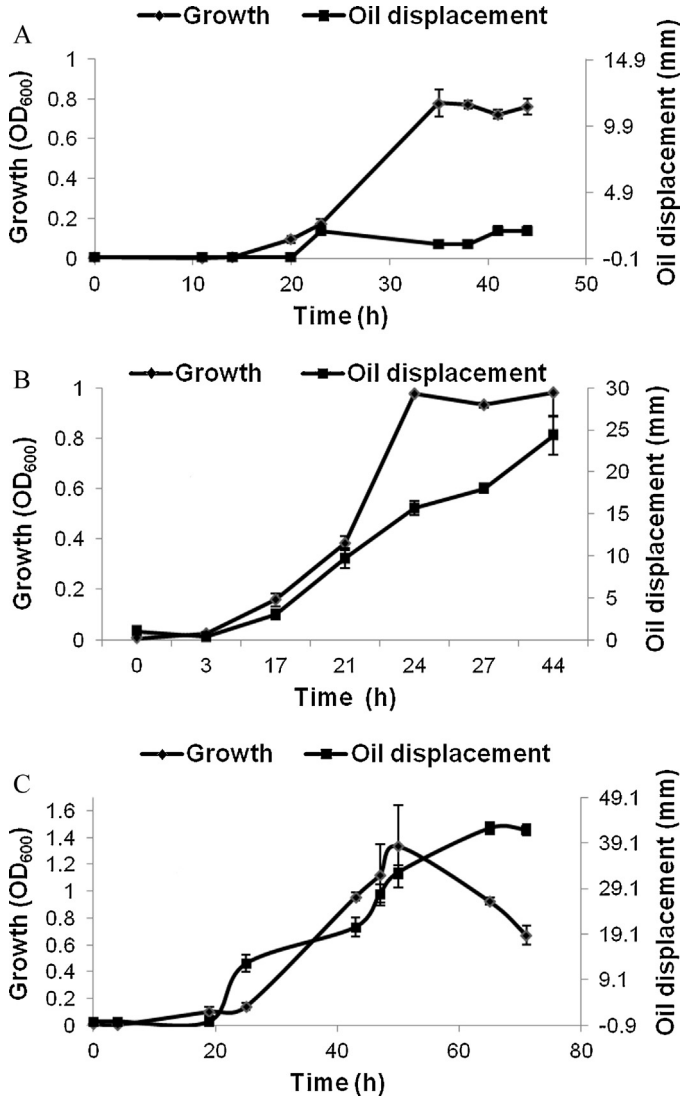

Fig. 4. Temporal changes in the oil displacement activity (biosurfactants production) of AK6U cultures grown on glucose as a carbon source and $MgSO_4$ (A), 4,6-DM-DBT (B) or DBS (C) as a sole sulfur source.

3.4. Rhamnolipid congeners profile

The profiles of rhamnolipid congeners produced by AK6U differed markedly depending on the provided sulfur source. A typical mass spectrum of rhamnolipids produced in cultures of AK6U is shown in Fig. 6. In the DBT/glucose medium (Table 2), 10 different congeners were detected, while 5 and 7 congeners were detected in both DBT-sulfone/glucose and $MgSO_4$/glucose cultures, respectively. Of the ten congeners produced in DBT/glucose cultures, six were di-rhamnolipids. The most abundant congeners produced in DBT/glucose medium were the di-rhamnolipid L-Rha–L-Rha–C_{10}–C_{10} (41.5%), while the mono-rhamnolipid, L-Rha–C_{10}–C_{10} constituted 32% of the total rhamnolipids. In DBT-sulfone/glucose cultures, the dominant rhamnolipids produced were di-rhamnolipids (4 of 5) in which L-Rha–L-Rha-C_{10}–C_{10} was the most abundant (65%). Both L-Rha–L-Rha–C_{12}–C_{10} and L-Rha–C_{10}–C_{10} were produced at comparable levels (9.4 and 10.4%, respectively).

As observed in both DBT/glucose and DBT-sulfone/glucose cultures, di-rhamnolipids were most prevalent in the $MgSO_4$/glucose culture in which they constituted six of the seven detected congeners. The most prevalent congeners were L-Rha–L-Rha–C_{10}–C_{10} (65.3%) and L-Rha–C_{10}–C_{10} (10.5%). Irrespective of the growth medium, the unsaturated congener varieties produced were di-rhamnolipid derivatives of terminal dodecanoic acids (L-Rha–L-Rha–C_{10}–$C_{12:1}$ and L-Rha–L-Rha–$C_{12:1}$) (Table 3). The unsaturated mono-alkanoate was found only in DBT/glucose cultures. Although both L-Rha–L-Rha–C_{10}–C_{10} and the L-Rha–C_{10}–C_{10} were produced in the three media, their ratios varied markedly. In the presence of DBT/glucose, the ratio of L-Rha–L-Rha–C_{10}–C_{10}: L-Rha–C_{10}–C_{10} was 1.3:1, while in DBT-sulfone/glucose and $MgSO_4$/glucose cultures the ratios were 6.9:1 and 6.2:1, respectively. On the other hand, the ratios of L-Rha–L-Rha–C_{10}–C_{10} and L-Rha–L-Rha–C_{10} produced in DBT/glucose, DBT-sulfone/glucose and $MgSO_4$/glucose cultures were 13:1, 8:1, and 37:1, respectively.

3.5. Kinetics of biosurfactants production using 4,6-DM-DBT as a sole sulfur source

The AK6U strain grew in MSM at 37 °C with glucose as a sole carbon source and 4,6-DM-DBT as a sole sulfur source. The surface tension of the growth medium decreased to a minimum within

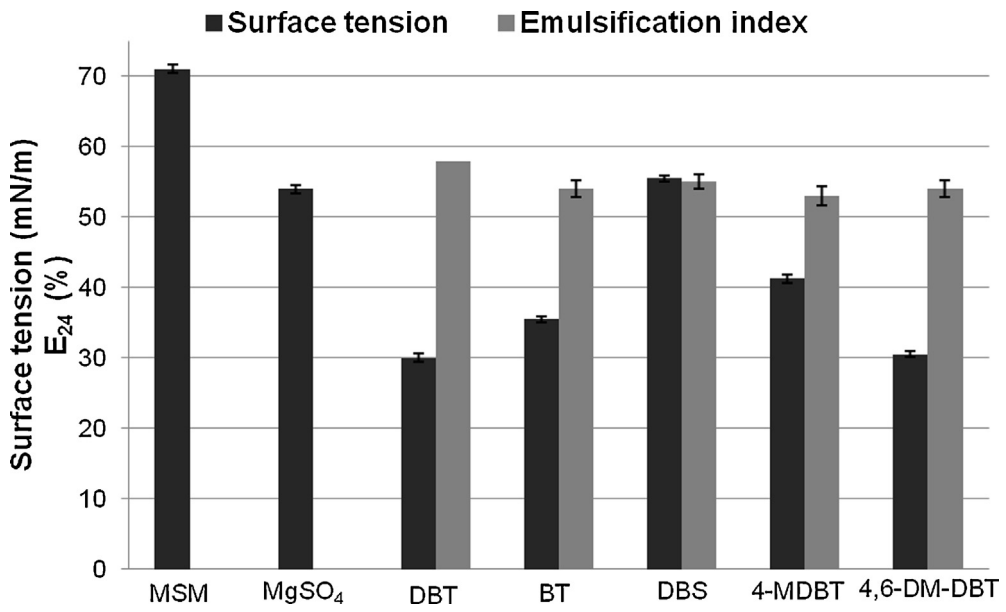

Fig. 5. Surface tension and emulsification power of AK6U cultures grown on glucose as a carbon source and different sulfur sources. MSM: mineral salts medium.

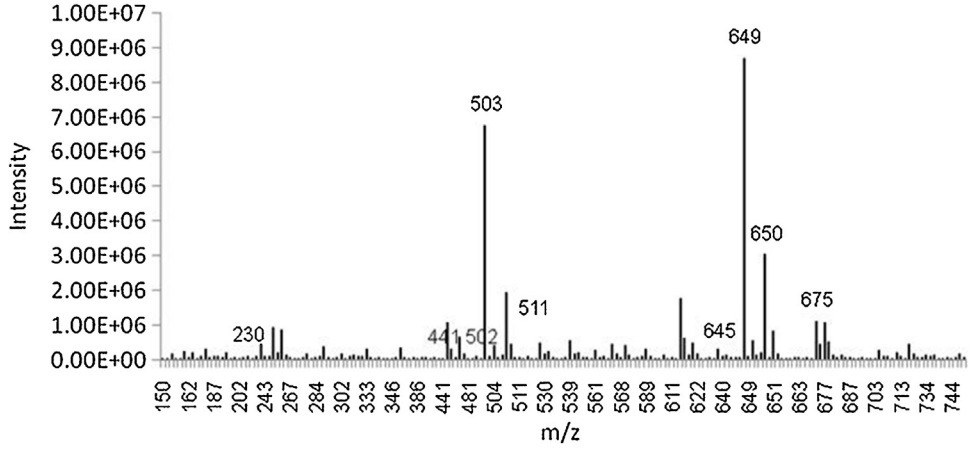

Fig. 6. The mass spectrum of precipitated rhamnolipid produced by AK6U in MSM containing glucose as a carbon source and DBT as a sole sulfur source.

24 h of incubation (Fig. 7). This reduction in surface tension was coincident with the exponential growth phase. After 24 h, no further decrease in surface tension occurred.

4. Discussion

To conduct this study, it was important to isolate a bacterial strain having the phenotypes of biosurfactants production and utilization of organosulfur compounds as sulfur sources (biodesulfurization). This was achieved by isolating the AK6U strain from enrichment cultures using DBT as a sole sulfur source. Some authors reported the isolation of bacteria capable of utilizing DBT as a sole sulfur source from hydrocarbons- and crude oil-contaminated soil [10,15]. The AK6U strain was identified as a *P. aeruginosa* strain due to high 16S rRNA gene sequence identity. Nonetheless, AK6U seems to have a unique evolutionary trend revealed by clustering in a separate phylogenetic branch.

The significance of sulfur for bacterial growth was demonstrated by the disability of the AK6U strain to grow in sulfur-free MSM. Chayabutra et al. [11] reported similar results for *P. aeruginosa* resting cells inoculated in medium lacking sulfur or nitrogen. Production of similar maximum biomass yields in AK6U cultures containing different $MgSO_4$ concentrations indicates that sulfur was not growth-limiting at the low concentration of 0.1 mM. Utilization of several organosulfur substrates as sole sulfur sources by AK6U reveals its broad biodesulfurization spectrum. Some bacteria are known to be biodesulfurization-competent [28]. The observed variation in terms of growth profile and biomass yield could be attributed to different biodesulfurization pathways and/or different metabolic enzymes activity. Also, it can not be excluded that the chemical structure of the organosulfur substrate affects its bioavailability and, hence, its utilization. It is known that 4,6-DM-DBT, for instance, is more recalcitrant than DBT toward microbial desulfurization via the 4S pathway [23]. As compared to the other organosulfur substrates, the higher specific growth rate and biomass yield observed in the 4,6-DM-DBT cultures could be due to several reasons. The less foaming in the 4,6-DM-DBT cultures might have led to relatively better aeration. It is also tempting to propose that the type of rhamnolipids (congener profile) produced in the 4,6-DM-DBT cultures facilitated better access to the substrate and consequently, enhanced the growth rate in the 4,6-DM-DBT cultures. The congeners fingerprint has been reported to influence the physicochemical properties of rhamnolipid biosurfactants [12,14]. Biosurfactants can also be

Table 2
Relative abundance (%) of rhamnolipid congeners produced in DBT, DBT-sulfone and $MgSO_4$ cultures in the presence of glucose as a carbon source.

	Rhamnolipid congener	Pseudo-molecular ion	Sulfur source		
			DBT	DBT-sulfone	$MgSO_4$
1	Rha–C_8–C_8	447			
2	Rha–rha –C_8–C_8	593			
3	Rha–rha–C_8–C_{10}	621	2.19		3.5
4	Rha–rha–C_{10}–C_8	621			
5	Rha–C_8–C_{10}	475	5.16		
6	Rha–C_{10}–C_8	475			
7	Rha–rha–C_8–$C_{12:1}$	647			
8	Rha–rha–$C_{12:1}$–C_8	647			
9	Rha–rha–C_{10}–C_{10}	649	41.48	64.95	65.27
10	Rha–C_{10}–C_{10}	503	32.17	9.43	10.46
11	Rha–rha–C_{10}–$C_{12:1}$	675	5.25	7.32	5.63
12	Rha–C_{10}–$C_{12:1}$	529	2.28		
13	Rha–$C_{12:1}$–C_{10}	529			
14	Rha–rha–$C_{12:1}$–C_{10}	675			5.63
15	Rha–rha–C_{12}–C_{10}	677	5.11	10.39	5.74
16	Rha–rha–C_{10}–C_{12}	677			
17	Rha–C_{12}–C_{10}	531	1.13		
18	Rha–C_{10}–C_{12}	531			
19	Rha–rha–$C_{12:1}$–C_{12}	703			
20	Rha–rha–C_{10}–$C_{14:1}$	703			
21	Rha–rha–C_{12}–C_{12}	705			
22	Rha–rha–C_8	451			
23	Rha–rha–C_{10}	479	3.18	7.92	3.81
24	Rha - C_8	305			
25	Rha–rha–$C_{12:1}$	505	2.1		
26	Rha–rha–C_{12}	507			
27	Rha–C_{10}	333			
28	Rha–C_{12}	361			
	Total	28	10	5	7

Table 3
Relative abundance (%) of HAAs in samples DBT, DBT-sulfone and $MgSO_4$ cultures.

	HAAs	Pseudo-molecular ion	Sulfur source		
			DBT	DBT-sulfone	$MgSO_4$
1.	C_8–C_8	301		29.11	21.41
2.	C_8–C_{10}	329	32.15		
3.	C_{10}–C_8	329			
4.	C_8–C_{12}	357		14.51	
5.	C_{12}–C_8	357			
6.	C_{10}–C_{10}	357			
7.	C_{10}–C_{12}	385		8.43	
8.	C_{12}–C_{10}	385			
9.	C_{12}–C_{12}	413	67.86	20.33	62.44
10.	C_8–$C_{12:1}$	355		7.24	
11.	$C_{12:1}$–C_8	355			
12.	C_{10}–$C_{12:1}$	383			
13.	$C_{12:1}$–C_{10}	383			
14.	C_{12}–$C_{12:1}$	411		20.4	16.17
15.	$C_{12:1}$–C_{12}	411			
	Total	15	2	6	3

detrimental to growth depending on the mode of action. They can enhance the solubility of toxic metabolic intermediates, reduce bioavailability of micellar hydrocarbons, and interfere with the membrane uptake processes through alteration of cell surface properties [26,44]. In this regard, Wang et al. [43] reported that biosurfactants interact variably with different hydrocarbons. Some metabolic intermediates can be toxic and might inhibit growth and/or desulfurization activity depending on the pathway. 2-Hydroxybiphenyl, the dead-end product of the 4S biodesulfurization pathway, is known to inhibit growth and biodesulfurization activity [2,10]. Moreover, it can be postulated that utilization of the sulfur retrieved from 4,6-DM-DBT for growth occurs via mechanisms that differ from those involved in the utilization of the other organosulfur substrates. All these suggestions or speculations are based on the assumption that the enhanced growth of the 4,6-DM-DBT cultures is due to better sulfur supply. However, this needs in-depth investigations of the pathways underlying the removal of sulfur from the organosulfur substrates and the subsequent utilization and incorporation into biomass.

The culture foaming and the oil displacement activity clearly indicate biosurfactants production by the AK6U strain [18,30]. Biosurfactants production by bacteria inhabiting oil- and hydrocarbons-contaminated soil has been reported [5,6]. The much higher foaming and oil displacement power of cultures containing organosulfur substrates, compared to the inorganic sulfate cultures, suggest production of higher amounts of biosurfactants. This can be attributed to enhancement or stimulation of biosurfactants production in the presence of organosulfur substrates [21]. Many bacteria produce biosurfactants when they are challenged with hydrophobic compounds [37]. It is tempting to propose that biosurfactants produced by AK6U might play a role by enhancing the bioavailability of the organosulfur substrates via solubilization, thus, making them more accessible for the bacterial cells [3,18].

The greater reduction of surface tension in the growth medium and the emulsification power results further confirm the proposed effect of the organosulfur substrates as stimulators of biosurfactants production. Nayak et al. [31] reported that an efficient biosurfactant decreases the surface tension of the growth medium more than 20 mN/m. This agrees with the results reported here. Moreover, Borole et al. [8] reported reduction of the surface tension of the growth medium (from 70 to 35 dyn/cm) during biodesulfurization of DBT. The coincidence of surface tension reduction with increase in the biomass in the 4,6-DM-DBT cultures confirms the growth-associated mode of biosurfactants production. It provides further evidence for the importance of biosurfactants in the utilization of 4,6-DM-DBT [4,17]. The observed variations in surface tension among the AK6U cultures might reflect the different physicochemical properties of the various sulfur sources. It was reported that inherent aqueous solubility and biodegradability of the substrates can affect the biosurfactants yield [7,17]. Accordingly, the similar biosurfactants productivity and biomass yield of the 1 mM and 0.1 mM $MgSO_4$ cultures can be reconciled. In both cases, the cells have no sulfur limitations due to the good solubility of $MgSO_4$. In contrast, although the concentration of the organosulfur substrates and $MgSO_4$ (0.1 mM) was equimolar, biosurfactants production was enhanced in the organosulfur cultures. Therefore, it is the accessibility, not the quantity, of the sulfur source that dictates biosurfactants productivity. The hydrophobic nature of the organosulfur compounds makes them less bioavailable than $MgSO_4$. In a previous study on *P. aeruginosa* [11], rhamnolipid biosurfactants production increased by reducing the iron or nitrogen content of the growth medium. This is in contrast to our results which showed that reduction of the inorganic sulfate content had no effect on rhamnolipid production. Based on this, we assume that different nutrients may impact biosurfactants production via different mechanisms.

The low surface tension of the sulfur-free cultures is intriguing. Chayabutra et al. [11] reported production of rhamnolipids by resting cells of *P. aeruginosa* in sulfur-free medium. Production of biosurfactants by AK6U in the sulfur-free cultures can be excluded because actually there was no growth and AK6U produces biosurfactants in a growth-associated mode. The AK6U inoculum originated from LB-grown cells and we know that AK6U produces biosurfactants in LB medium (unpublished). Nonetheless, a carry-over from the pre-cultures is excluded because the cells were washed twice before inoculation. The only plausible explanation is that due to cell disintegration, some surface-active compounds like

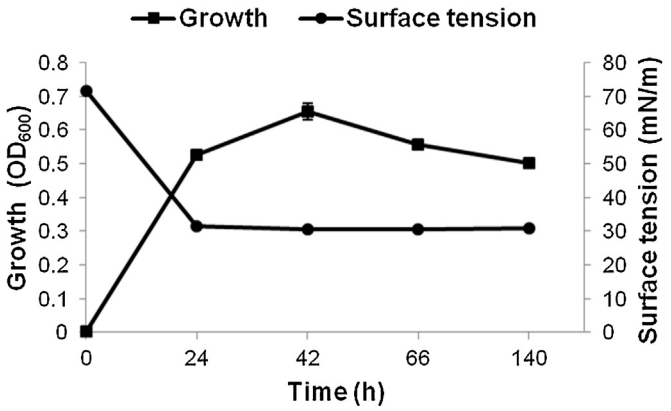

Fig. 7. Temporal changes in surface tension during growth of AK6U on glucose as a carbon source and 4,6-DM-DBT as a sulfur source.

rhamnolipids and free HAAs were released into the medium and reduced the surface tension. According to Daniels et al. [13],*N*-acylhomoserine lactones (quorum sensing signal molecules) can also cause surface tension changes.

The differences in surface tension among the AK6U cultures could also be due to qualitative and/or quantitative differences in the produced biosurfactants. Such qualitative and quantitative differences were exhibited in the rhamnolipid congeners produced in cultures containing organosulfur (DBT, DBT-sulfone) and inorganic sulfur substrates. The variations in rhamnolipid congener profiles reflect the recently reported differences in the *rhlABC* gene expression among the DBT, DBT-sulfone, and inorganic sulfate cultures [21]. Moreover, in line with our results, it has been reported that rhamnolipid composition depends on many factors such as the producing strain, culture conditions, and medium composition [27,29]. The higher proportion of di-rhamnolipids as compared to mono-rhamnolipids (in the DBT and DBT-sulfone cultures) is in a good agreement with the previously reported higher expression level of the *rhlC* gene compared to the *rhlB* gene [21]. Furthermore, the predominance of the di-rhamnolipid L-Rha–L-Rha–C_{10}–C_{10} congener in all the analyzed cultures agrees with data reported in the literature [40].

The DBT culture which produced greater number and types of congeners brought about greater decline in surface tension. Thus, the degree of congener diversity appears to correlate with the observed decrease in the surface tension determined in the different culture media. Moreover, the greater surface tension reduction in the DBT culture may be attributed to the larger proportion of mono-rhamnolipid congeners (4 out of 10) as compared to the DBT-sulfone (1 out of 5) and the $MgSO_4$ cultures (1 out of 7). In line with this assumption, it is known that rhamnolipid mixtures at the air–water interface exhibit Langmuir-like adsorption isotherms where mono-rhamnolipids are more surface-active than di-rhamnolipids [12]. Interestingly, the greater number and types of HAAs found in the DBT-sulfone culture appear to have contributed markedly to the surface tension reduction recorded at comparable levels in both the DBT-sulfone and $MgSO_4$ cultures. HAAs are known to possess surface activity. These observations highlight the possible impact of qualitative differences in rhamnolipid/HAAs produced in the different cultures on the observed surface tension changes.

The results of the HAAs analysis contradict the previously reported expression levels of *rhlA* in the DBT, DBT-sulfone and $MgSO_4$ cultures. It is not clear why the DBT culture exhibited much lower HAAs number and types, although it possessed the highest level for RhlA, which catalyzes the synthesis of the HAA dimers needed for rhamnolipids production [21]. Similarly, we can't see the reason why the DBT-sulfone culture had the largest number and diversity of HAAs while having the lowest *rhlA* expression level. It can be speculated that HAAs in the DBT culture are incorporated into rhamnolipids faster than the DBT-sulfone and the $MgSO_4$ cultures. Alternatively, one can envisage that other biosynthetic pathways are competing with rhamnolipids biosynthesis for HAAs [1].

5. Conclusions

The sulfur source induces qualitative as well as quantitative variations in rhamnolipid biosurfactants production. The influence of the sulfur source on biosurfactants production by AK6U appears to be dictated by the inherent polarity (aqueous solubility) of the substrate. The simultaneous phenotypes of biosurfactants production and utilization of organosulfur compounds as sulfur sources make AK6U a good candidate for bioremediation, biodesulfurization, as well as biotechnological production of biosurfactants.

Acknowledgements

The authors wish to acknowledge the financial support (Grant BIO01) provided by the Arabian Gulf University to conduct this study. We also thank Harine Gopal and the Science Analytical Facility (Project GS02/10, University of Kuwait) for analysis of rhamnolipids.

References

[1] A.M. Abdel-Mawgoud, R. Hausmann, F. Lepine, M.M. Muller, E. Deziel, Rhamnolipids: detection, analysis, biosynthesis, genetic regulation, and bioengineering of production, in: G. Soberón-Chávez (Ed.), Biosurfactants: from Genes to Applications, Microbiology Monograph, vol. 20, Springer-Verlag, Berlin, Heidelberg, 2011, pp. 13–55.
[2] A. Abin-Fuentes, M. El-S. Mohamed, D.I.C. Wang, K.L.J. Prather, Exploring the mechanism of biocatalyst inhibition in microbial desulfurization, Appl. Environ. Microbiol. 79 (2013) 7807–7817.
[3] G.A. Amin, S.A. Bazaid, M. Abd El-Halim, A two-stage immobilized cell bioreactor with *Bacillus subtilis* and *Rhodococcus erythropolis* for the simultaneous production of biosurfactant and biodesulfurization of model oil, Pet. Sci. Technol. 31 (2013) 2250–2257.
[4] S. Bandyopadhyay, R. Chowdhury, C. Bhattacharjee, Steady state performance of a bioreactor for production of near zero sulfur diesel (NZSD) and biosurfactant, J. Clean Energy Technol. 1 (2013) 89–193.
[5] S. Batista, A. Mounteer, F. Amorim, Isolation and characterization of biosurfactant/bioemulsifier producing bacteria from petroleum contaminated sites, Bioresour. Technol. 97 (2006) 868–875.
[6] A.A. Bodour, K. Drees, R.M. Miller-Maier, Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils, Appl. Environ. Microbiol. 69 (2003) 3280–3287.
[7] N.K. Bordoloi, B.K. Konwar, Bacterial biosurfactant in enhancing solubility and metabolism of petroleum hydrocarbons, J. Hazard. Mater. 170 (2009) 495–505.
[8] A.P. Borole, E.N. Kaufman, M.J. Grossman, V. Minak-Bernero, R. Bare, M.K. Lee, Comparison of the emulsion characteristics of *Rhodococcus erythropolis* and *Ecsherichia coli* SOXC-5 cells expressing biodesulfurization genes, Biotechnol. Prog. 18 (2002) 88–93.
[9] S.S. Cameotra, R. Makkar, Recent applications of biosurfactants as biological and immunological molecules, Curr. Opin. Microbiol. 7 (2004) 262–266.
[10] H. Chen, W. Zhang, Y.B. Cai, Y. Zhang, W. Li, Elucidation of 2-hydroxybiphenyl effect on dibenzothiphene desulfurization by *Microbacterium* sp. strain ZD-M2, Bioresour. Technol. 99 (2008) 6928–6933.
[11] C. Chayabutra, J. Wu, L.-K. Ju, Rhamnolipid production by *Pseudomonas aeruginosa* under denitrification: effects of limiting nutrients and carbon substrates, Biotechnol. Bioeng. 72 (2001) 25–33.
[12] M.L. Chen, J. Penfold, R.K. Thomas, T.J.P. Smyth, A. Perfumo, R. Marchant, I.M. Banat, P. Stevenson, A. Parry, I. Tucker, I. Grillo, Mixing behaviour of the biosurfactant rhamnolipid, with a conventional anionic surfactant, sodium dodecyl benzene sulfonate, Langmuir 26 (2010) 17958–17968.
[13] R. Daniels, S. Reynaert, H. Hoekstra, C. Verreth, J. Janssens, K. Braeken, M. Fauvart, S. Beullens, C. Heusdens, I. Lambrichts, D.E. De Vos, J. Vanderleyden, J. Vermant, J. Michiels, Quorum signal molecules as biosurfactants affecting swarming in *Rhizobium etli*, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 14965–14970.
[14] P. Das, X.-P. Yang, Z.M. Luyan, Analysis of biosurfactants from industrially viable *Pseudomonas* strain isolated from crude oil suggests how rhamnolipids congeners affect emulsification property and antimicrobial activity, Front. Microbiol. 5 (2014) , doi:http://dx.doi.org/10.3389/fmicb.2014.00696.

[15] F. Davoodi-Dehaghani, M. Vosoughi, A.A. Ziaee, Biodesulfurization of dibenzothiophene by a newly isolated *Rhodococcus erythropolis* strain, Bioresour. Technol. 101 (2010) 1102–1105.
[16] J.D. Desai, I.M. Banat, Microbial production of surfactants and their commercial potential applications, Microbiol. Mol. Biol. Rev. 61 (1997) 47–64.
[17] E. Deziel, G. Paquette, R. Villemur, F. Lepine, J. Bisaillon, Biosurfactant production by a soil *Pseudomonas* strain growing on polycyclic aromatic hydrocarbons, Appl. Environ. Microbiol. 62 (1996) 1908–1912.
[18] M.A. Dinamarca, A. Rojas, P. Baeza, G. Espinoza, C. Ibacache-Quiroga, J. Ojeda, Optimizing the biodesulfurization of gas oil by adding surfactants to immobilized cell systems, Fuel 116 (2014) 237–241.
[19] S.C. Gilbert, J. Morton, S. Buchanan, C. Oldfield, A. McRoberts, Isolation of a unique benzothiphene-desulfurization bacterium, *Gordona* sp. strain 213E (NCIMB 40816), and characterization of the desulphurization pathway, Microbiology 144 (1998) 2545–2553.
[20] W. Ismail, I.S. Al-Rowaihi, A.A. Al-Humam, R.Y. Hamza, A.M. El-Nayal, M. Bououdina, Characterization of a lipopeptide biosurfactant produced by a crude oil-emulsifying *Bacillus* sp. I-15, Int. Biodeterior. Biodegrad. 84 (2013) 168–178.
[21] W. Ismail, A.M. El Nayal, A.R. Ramadan, N. Abotalib, Sulfur source-mediated transcriptional regulation of the *rhlABC* genes involved in biosurfactants production by *Pseudomonas* sp. strain AK6U, Front. Microbiol. 5 (2014) , doi: http://dx.doi.org/10.3389/fmicb.2014.00423.
[22] A.K. Kock, O. Kappeli, A. Fiechter, J. Reiser, Hydrocarbon assimilation and production in *Pseudomonas aeruginosa* mutants, J. Bacteriol. 173 (1991) 4212–4219.
[23] J. Konishi, T. Onaka, Y. Ishii, M. Suzuki, Demonstration of the carbon-sulfur bond targeted desulfurization of benzothiophene by thermophilic *Paenibacillus* strain A11-2 capable of desulfurizing dibenzothiophene, FEMS Microbiol. Lett. 187 (2000) 151–154.
[24] M. Krishnaswamy, G. Subbuchettiar, K. Thiengungal, S. Panchaksharam, Biosurfactants: properties, commercial production and application, Curr. Sci. 94 (2008) 736–747.
[25] F. Lepine, E. Deziel, S. Milot, R. Villemur, Liquid chromatographic/mass spectrometric detection of the 3-(3-hydroxyalkanoyloxy) alkanoic acid precursors of rhamnolipids in *Pseudomonas aeruginosa* cultures, J. Mass Spectrom. 37 (2002) 41–46.
[26] Y. Liu, X. Ma, G. Zeng, H. Zhong, Z. Liu, Y. Jiang, X. Yuan, X. He, M. Lai, Y. He, Role of low-concentration monorhamnolipid in cell surface hydrophobicity of *Pseudomonas aeruginosa:* adsorption or lipopolysaccharide content variation, Appl. Microbiol. Biotechnol. 98 (2014) 1023–1041.
[27] J.C. Mata-Sandoval, J. Kanrns, A. Torrents, High-performance liquid chromatography method for the characterization of rhamnolipid mixtures produced by *Pseudomonas aeruginosa* UG2 on corn oil, J. Chromatogr. A 864 (1999) 211–220.
[28] F. Monot, M. Warzywoda, Microbial desulfurization of petroleum products, in: J.-P. Vandecasteele (Ed.), Petroleum Microbiology: Concepts, Environmental Implications, Industrial Applications, Vol. 2, Editions Technip, Paris, 2008, pp. 757–775.
[29] S.A. Monteiro, G.L. Sassaki, L.M. de Souza, J.A. Meira, J.M. de Araujo, D.A. Mitchel, L.P. Ramos, N. Krieger, Molecular and Structural characterization of the biosurfactant produced by *Pseudomonas aeruginosa* DAUPE 614, Chem. Phys. Lipids 147 (2007) 1–13.
[30] T. Morita, M. Konishi, T. Fukuoka, T. Imura, S. Yamamoto, M. Kitagawa, A. Sogabe, D. Kitamoto, Identification of *Pseudozyma graminicola* CBS 10092 as a producer of glycolipid biosurfactants, mannosylerythritol lipids, J. Oleo Sci. 57 (2008) 123–131.
[31] A.S. Nayak, M.H. Vijaykumar, T.B. Karegouder, Characterization of biosurfactant produced by *Pseudomonas* sp. PNK-04 and its application in bioremediation, Int. Biodeterior. Biodegrad. 63 (2009) 73–79.
[32] G. Pasternak, B. Kotwzan, Surface tension and toxicity changes during biodegradation of carbazole by newly isolated methylotrophic strain *Methylobacterium* sp. GPE1, Int. Biodeterior. Biodegrad 84 (2013) 143–149.
[33] J.F. Pereira, E.J. Gudina, R. Costa, R. Vitorino, J.A. Teixeira, J.A. Coutinho, L.R. Rodrigues, Optimization and characterization of biosurfactant production by *Bacillus subtilis* isolates towards microbial enhanced oil recovery applications, Fuel 111 (2013) 259–268.
[34] A. Perfumo, T.J. Smyth, R. Marchant, I.M. Banat, Production and roles of biosurfactants and bioemulsifiers in accessing hydrophobic substrates, in: K.N. Timmis (Ed.), Handbook of Hydrocarbon and Lipid Microbiology, vol. 1, Springer-Verlag, Berlin, Heidelberg, 2010, pp. 501–1512.
[35] N. Pfenning, Rhodocyclus purpureus gen. nov. a ring-shaped, vitamin B12 requiring member of the family *Rhodospirillaceae*, Int. Syst. Bacteriol. 28 (1978) 283–288.
[36] K.K. Sekhon, S. Khanna, S.S. Cameotra, Biosurfactant production and potential correlation with esterase activity, J. Pet. Environ. Biotechnol. 3 (2012) 133–142.
[37] S.N. Silva, C.B. Farias, R.D. Runfino, J.M. Luna, L.A. Sarubbo, Glycerol as substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCP0992, Colloids Surf. B 97 (2010) 174–183.
[38] A. Singh, J.D. Van Hamme, O.P. Ward, Surfactants in microbiology and biotechnology, Biotechnol. Adv. 25 (2007) 99–122.
[39] G. Soberón-Chávez, R.M. Maier, Biosurfactants: a general overview, in: G. Soberón-Chávez (Ed.), Biosurfactants: from Genes to Applications, Springer-Verlag, Berlin, Germany, 2011, pp. 1–11.
[40] A. Thanomsub, W. Pumeechockchai, A. Limtrakul, P. Arunrattiyakorn, W. Petchleelaha, T. Nitoda, H. Kanzaki, Chemical structures and biological activities of rhamnolipids produced by *Pseudomonas aeruginosa* B189 isolated from milk factory waste, Bioresour. Technol. 97 (2006) 2457–2461.
[41] J.D. Van Hamme, J.A. Odumeru, O.P. Ward, Community dynamics of a mixed-bacterial culture grown on petrol hydrocarbons in batch culture, Can. J. Microbiol. 46 (2000) 411–450.
[42] V. Walter, C. Syldatk, R. Hausmann, Biosurfactants: screening concepts for the isolation of biosurfactant producing microorganisms, Adv. Exp. Med. Biol. 672 (2010) 1–13.
[43] X.-B. Wang, C.-Q. Chi, Y. Nie, Y.-Q. Tang, Y. Tan, G. Wu, X.-L. Wu, Degradation of petroleum hydrocarbons (C60-C40) and crude oil by a novel *Dietzia* strain, Bioresour. Technol. 102 (2011) 7755–7761.
[44] O.P. Ward, Microbial biosurfactants and biodegradation, in: R. Sen (Ed.), Biosurfactants, Springer Science and Landes Bioscience, 2010, pp. 65–74.