Appl Biochem Biotechnol (2011) 164:1444–1460
DOI 10.1007/s12010-011-9225-z

Production and Physico-chemical Characterization of a Biosurfactant Produced by *Pseudomonas aeruginosa* OBP1 Isolated from Petroleum Sludge

Pranjal Bharali · Bolin Kumar Konwar

Received: 18 September 2010 / Accepted: 1 March 2011 /
Published online: 6 April 2011

Abstract *Pseudomonas aeruginosa* strain OBP1, isolated from petroleum sludge, was used to produce biosurfactant from a modified mineral salt medium with 2% *n*-hexadecane as sole source of carbon. The crude biosurfactant was fractionated using TLC and HPLC. Using FTIR spectroscopy, ^{1}H NMR, and LC-MS analyses, the chemical structure of the purified fraction of crude biosurfactant was identified as rhamnolipid species. The LC-MS spectra show that monorhamnolipid (L-rhamnopyranosyl-β-hydroxydecanoyl-β- hydroxydecanoate, Rha-C_{10}-C_{10}) was produced in abundance with the predominant congener $[M–H]^-$ ions for L-rhamnopyranosyl-L-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rha-Rha-C_{10}-C_{10}). Seven different carbon substrates and five nitrogen sources were examined for their effect on rhamnolipid production. Using *n*-hexadecane (20 g/l) as carbon substrate and urea along with $(NH_4)_2SO_4$ (2 g/l each) as nitrogen source was found to be the best, with a maximum yield of 4.8 g/l. The biosurfactant reduced the surface tension of water to 31.1 mNm^{-1} with a critical micelle concentration of 45 mg/l. The biosurfactant showed a better emulsifying activity against a variety of hydrocarbon and achieved a maximum emulsion index of 82% for diesel. The purified biosurfactant showed a significant antibacterial activity against *Staphylococcus aureus* at a minimum inhibitory concentration of 8 μg/ml.

Keywords Biosurfactant · Rhamnolipid · *n*-Hexadecane · *Pseudomonas aeruginosa*

Introduction

Biosurfactants are produced by a wide variety of organisms including bacteria, fungi, and yeast. These are amphiphilic molecules and have the ability to accumulate at the interface of two media which ultimately reduce the surface tension and interfacial tension between the phases. These properties create micro-emulsion, leading to micelle formation in which hydrocarbons can solubilize in water or water in hydrocarbons. The biosurfactants exhibit

P. Bharali (✉) · B. K. Konwar
Department of Molecular Biology & Biotechnology, Tezpur University,
Napaam, Tezpur, Assam 784028, India

several of the superior attributes in contrast to chemical surfactants such as biodegradability—thus problems of toxicity and bioaccumulation in the ecosystem are avoided [1, 2]—higher foaming , high selectivity [3, 4], low critical micelle concentration (CMC) [5]—specific at extreme temperature, pH, and salinity [6]—and the ability to be synthesized from renewable feedstock [7]. Therefore, they have been widely used in the bioremediation of pollutants, enhanced oil recovery process, health care, and food industries [7].

Structurally, biosurfactants are classified into glycolipid, phospholipids, fatty acids, neutral lipids, lipopeptide, and lipid-containing polymer such as lipoproteins, lipopolysaccharide–protein complexes, and polysaccharide–protein–fatty acid complexes [1]. Among them, rhamnolipid belongs to the class glycolipid, produced primarily by the genus *Pseudomonas*. Rhamnolipids are promising surfactants owing to their several characteristics such as low average minimum surface tension (30–32 mN/m), low CMC reaching up to 5 mg/l, high average emulsifying activity, and higher affinity for hydrophobic organic molecules [8]. *Pseudomonas aeruginosa* is known to produce a mixture of rhamnolipid homologues which may differ in the chain length of fatty acids (C_8–C_{14}) or in the number of rhamnose units [9]. Until now, 28 different homologues of rhamnolipid have been reported, out of which two major types of rhamnolipid species are L-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxydecanoate ($RhC_{10}C_{10}$) and L-rhamnopyranosyl-L-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxydecanoate ($Rh_2C_{10}C_{10}$) [10–12] . Genetic regulation for the synthesis of rhamnolipid by sequential glycosyl transfer has been proposed by Burger et al. [13]. Similarly, Ochsner et al. [14] isolated and characterized the gene involved in the regulation of rhamnolipid biosynthesis. The genetic regulation involved in rhamnolipid production is very much complex in *P. aeruginosa*, which is an important problem for the construction of efficient bacterial strains for the enhanced production of rhamnolipid [15].

Although rhamnolipid is an effective surfactant and well suitable for the application of bioremediation of oil pollution [16], the main lacunae however for the commercial application of rhamnolipid are its low yield and high production cost [17]. Therefore, there is a great need to develop an efficient rhamnolipid-producing strain and a low cost-effective processing technique. In addition to these, there is also a need to search for cheap renewable feedstock for its production.

In the light of this, the present investigation was started with an attempt on the isolation of a *P. aeruginosa* strain OBP1 from petroleum sludge capable of producing rhamnolipid, optimization of its production, laboratory-scale production using different carbon sources mainly by the components of crude oil, physico-chemical characterization, and characterization of its antibacterial activity.

Materials and Methods

Microorganism

The bacterial strain was isolated from petroleum sludge deposited outside the oil drilling sites in Assam, India. The production of glycolipid-type biosurfactant by the bacterial strains was detected by using Siegmund and Wagner technique [18]. Bacterial strain was grown on MSM with 2% *n*-hexadecane and containing 0.2 g/l CTAB and 0.005 g/l of methylene blue. After 48 h of incubation at 37 °C, the plates were inspected for the presence of dark blue halos around the bacterial colony. This indicates the specific interaction between the cationic surfactant CTAB and anionic glycolipid biosurfactant in

the presence of basic dye methylene blue. The highest biosurfactant-producing strain was selected and identified morphologically and biochemically. The isolated strain was maintained on nutrient agar slant and stored at 4 °C. Subculturing was done at an interval of 2 weeks. For further investigation, the strain was stored in cryovials at −80 °C.

Media and Growth Condition

For the production of rhamnolipid, the inoculum was prepared by transferring the single bacterial colony from the nutrient agar plate into the nutrient broth. The culture was incubated at 37 °C at 180 rpm in an orbital shaker overnight. The medium used to grow *P. aeruginosa* OBP1 strain was modified mineral salt medium consisting of 2.0 g urea, 2.0 g $(NH_4)_2SO_4$, 3.61 g Na_2HPO_4, 1.75 g KH_2PO_4, 0.2 $MgSO_4{\cdot}7H_2O$, 50 mg $CaCl_2{\cdot}2H_2O$, 1.0 mg $FeSO_4{\cdot}7H_2O$, 50 μg $CuSO_4{\cdot}7H_2O$, 10 μg $MnSO_4{\cdot}5H_2O$, 10 μg H_3BO_3, 70 μg $ZnSO_4{\cdot}7H_2O$, 10 μg MnO_3, and 20 ml of carbon source such as *n*-hexadecane (sterilized by passing through a 0.2-μm membrane filter) and the volume was increased up to 1.0 l with distilled water. The pH of the medium was initially adjusted to 6.8 by using 6 N HCl. After overnight culturing, an aliquot of 5 ml of culture was inoculated into 100 ml MSM and incubated at 37 °C and 180 rpm in an orbital shaker for 15 days. During the course, samples were taken from the culture to monitor cell growth, rhamnolipid concentration, and surface tension.

Determination of Biomass Content

Cell density in the culture medium was determined by counting the colony-forming units (cfu) following serial dilution. Using appropriate calibration, the cell number was converted to dry cell weight. Cell biomass content was measured by determining the protein concentration in the culture medium. After alkaline hydrolysis, the protein content was measured by Lowry method [19] using bovine serum albumin as the standard. Each observation was repeated thrice.

Rhamnolipid Quantification

Total rhamnolipid concentration in the sample was determined thrice by the method described by Chandrasekran and Bemiller [20]. The rhamnolipid value was calculated from the standard curve prepared with L-rhamnose and expressed as a rhamnolipid value by multiplying the rhamnose value by a coefficient of 3.4, which is obtained from the correlation of pure rhamnolipid/rhamnose.

Extraction and Purification of Rhamnolipid

Rhamnolipid Recovery

The collected supernatant was first centrifuged at 9,000×*g* for 20 min at 4 °C to remove the bacterial cells. The culture supernatant was acidified to pH 2 with 6 N HCl and allowed to stand overnight at 4 °C to precipitate the rhamnolipid. The precipitate was harvested by centrifugation at 9,000×*g* for 15 min at 4 °C. The recovered precipitate was extracted thrice with ethyl acetate at room temperature. The organic phase was collected in a round-bottom flask and connected to a rotary evaporator (Eyela, CCAS-1110, Rikakikai Co. Ltd., Tokyo) to remove the solvent. The process yielded a viscous honey-colored biosurfactant [21]. The

thick residue was washed twice with *n*-hexane to remove any residual *n*-hexadecane. Finally, the yellowish product was dissolved in ethyl acetate, filtered, and concentrated using a rotary evaporator.

TLC Analysis

The isolated biosurfactant was further analyzed by thin-layer chromatography (TLC) on silica gel 60 G (Merck) in chloroform/methanol/water (65:25:4, *v/v/v*). The plates were sprayed with orcinol–H_2SO_4 solution [12] and developed at 100 °C for 5 min. Positive spots were scraped and extracted with 3 ml of chloroform/methanol (2:1, *v/v*) in an elution column.

HPLC Analysis

The components of partially purified biosurfactant were fractionated using a gradient elution high-performance liquid chromatography (HPLC) (Waters 2487) with a dual λ absorbance UV detector. A Novapak C18 column with a dimension of 3.9×150 mm (Nova Pak, C18, Waters W92661M139, Ireland) was used. The mobile phase was kept at a flow rate of 0.4 ml/min and the sample injection volume was 20 μl. The mobile phase solutions were acetone/acetonitrile (30:70, *v/v*). All fractions eluted from the HPLC column were collected at different retention times [22]. The fractions were then evaporated at room temperature to remove all of the solvent part to obtain a purified biosurfactant. The fraction having the height reduction in surface tension of water was selected and further characterized.

Chemical Characterization of Purified Rhamnolipid

FT-IR Spectroscopy

For elucidating the chemical bonds or the functional groups present, the isolated rhamnolipid was subjected to FTIR analysis. For the study, a Nicolos Impact I 410, FTIR System, USA, was used. The pure lyophilized rhamnolipid of 1.0 g was ground with 100 mg of KBr and pressed with 7,500 kg for 30 s to result in a translucent KBr pellet. The spectra of the sample were collected at a resolution wave number accuracy of 4 and 0.01 cm^{-1}, respectively, and 32 scans with correlation for atmospheric CO_2.

LC-MS of the Purified Rhamnolipid

Rhamnolipid mixtures were separated and identified by liquid chromatography coupled to mass spectroscopy using a LC–MS–MS, Water-Q-TOF Premier. Samples were prepared by diluting with methanol (HPLC grade) with a concentration of 10 mg/l and 100 μl of the same was injected into a C8 WP-300 (5 μm) 150×4.6-mm column. The LC flow rate was 1.0 ml/min. For mobile phase, an acetonitrile–water gradient was used starting with 30% of acetonitrile for 4 min, followed by 30–100% acetonitrile for 40 min then standby for 5 min and return to the initial condition. MS was performed with a single quadrupole mass spectrometer equipped with a pneumatically assisted electrospray source, and negative ion mode was used. The capillary was held at a potential of −3.5 kV and the extraction voltage at −75 V. Full-scan data were obtained by scanning from *m/z* 100 to 750 in the centroid mode using a scan duration of 2.0 s and an inter-scan time of 0.2 s [23].

NMR Analysis of Purified Rhamnolipid

The purified rhamnolipid was dissolved in denaturated methanol and directly analyzed with ^{1}H nuclear magnetic resonance (NMR). The ^{1}H NMR spectra were obtained from a NMR 400 MHz spectrometer (JNM-ECST NMR, 4,000 MHz, JEOL).

Physiochemical Characterization

Surface Tension Assay

The surface tension of the supernatant was measured with a Krüss K12 tensiometer (Tensiometer K9, Krüss K9 ETS-S) accordingly to the De Noüy ring method [24]. At the end of each measurement, the platinum ring was rinsed thrice with water followed by washing three times with acetone and was allowed to dry in air. Culture supernatant of 25 ml was taken into a glass beaker of 50-ml volume and placed onto the platform of the tensiometer. The surface tension was measured at 25±1 °C. After introducing the platinum ring in the culture supernatant, it was slowly allowed to touch the liquid–air interface to measure the surface tension. The platinum ring was kept in the supernatant for a while in order to attain equilibrium conditions. The measurement was repeated thrice and an average value was obtained.

Determination of CMC

For the determination of CMC value, the culture supernatant was serially diluted and surface tension for each dilution was measured. The CMC value, expressed in mg/l, was estimated from the breakpoint of the surface tension versus the concentration. For calibrating the instrument, it was subjected to the determination of surface tension of the pure water at 25±1 °C.

Measurement of Emulsification Activity

The emulsification activity of the biosurfactant was measured in terms of emulsion stability [25] against the different hydrophobic sources to study the emulsion stability. A mixture of 2 ml supernatant and 3 ml kerosene was taken in a test tube and vortexed for 2 min and the height of emulsion layer was measured after 24 h. The emulsification index (E_{24}) was calculated by dividing the height of the emulsion layer by the total height of the mixture and multiplying by 100.

Antimicrobial Activity

The microbroth dilution method was performed to determine the minimum inhibitory concentration (MIC). The purified rhamnolipid was tested for antibacterial properties against four Gram-positive bacteria: *Bacillus circulans* (ATCC 61), *Bacillus subtilis* (ATCC 11774), *Staphylococcus aureus* (ATCC 11632), and *Staphylococcus epidermis* (ATCC 49461), and six Gram-negative bacteria: *Alcaligenes faecalis* (ATCC 8750), *Escherichia coli* (ATCC 9637), *Klebsilla pneumoniae* (ATCC 10031), *P. aeruginosa* (MTCC 7815), *Proteous vulgaris* (ATCC 49132), and *Enterobacter aerogenes* (ATCC 49132) respectively collected from the Department of Molecular Biology and Biotechnology, Tezpur University,

Assam. Rhamnolipid was first dissolved in sterilized DMSO and then sonicated. The dissolved rhamnolipid solution was diluted to a series of tenfold in culture broth, seeded in a 96-well culture plate, and then inoculated with a fresh bacterial inoculum. Inoculated microplates were incubated at 37 °C for 24 h. The viability of the treated cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium assay) [26]. The MIC was determined as the lowest concentration of biosurfactant required to inhibit the growth of each organism.

Results and Discussion

Bacterial Strain and Culture Conditions

Bacterial strain OBP1 produces a significant amount of 4.75 g/l of rhamnolipid when grown on hydrocarbon. Microscopic studies confirmed its Gram negativity, motility, and rod-shaped characters. The electron microscopic images of the bacterial strains are shown in Fig. 1. The surface tension of the culture medium was reduced to 31 mN/m after 15 days of culture and became constant. The increase in the biomass and quantification of rhamnolipid produced in the culture was determined and recorded for 15 days and the data are shown in Fig. 2. In addition, the biochemical and physiological studies suggested that the strain OBP1 was closest to *P. aeruginosa*.

Fractionation of Crude Biosurfactant

The partially purified biosurfactant obtained after solvent extraction from the culture supernatant of *P. aeruginosa* OBP1 was further purified by different chromatographic techniques. The rhamnolipid isolated by TLC technique was compared with those already described by various authors and identified according to Monteiro et al. [12]; lower spot with R_f 0.36 and upper spot with R_f 0.55, representing the monorhamnolipid and dirhamnolipids, respectively, are shown in Fig. 3. The major TLC fraction with highest surface tension activity was further successfully separated into five main sub-fractions eluted from the HPLC column at different elution times and are presented in Fig. 4. The

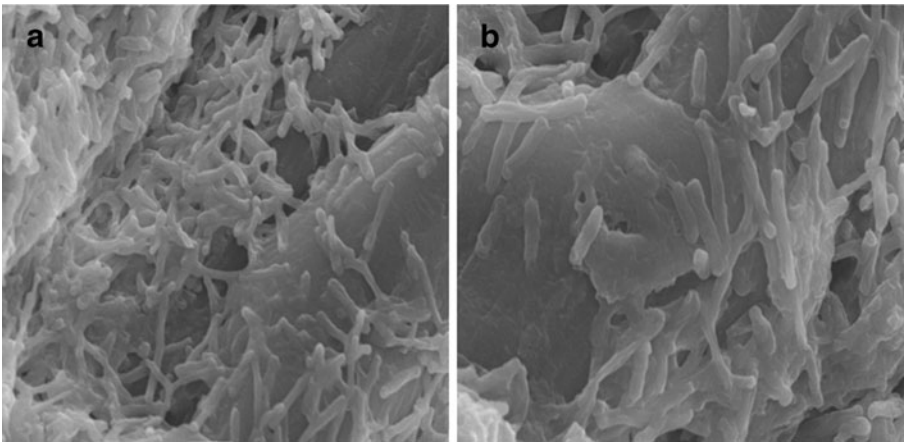

Fig. 1 **a**, **b** SEM micrographs of a 96-h culture of *P. aeruginosa* OBP1 on *n*-hexadecane as carbon source

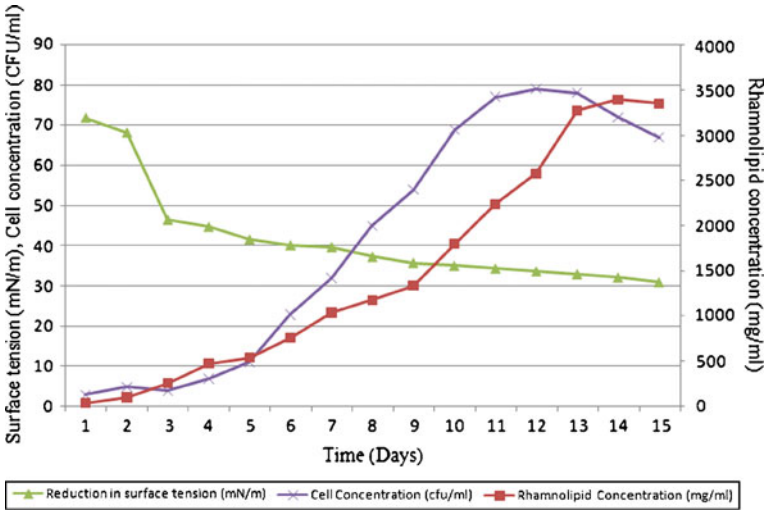

Fig. 2 Profile of cell growth, reduction in surface tension of the culture medium, and rhamnolipid production by *P. aeruginosa* OBP1 in MSM medium with 2% *n*-hexadecane. Rhamnolipid levels were measured by orcinol method. Surface tension was measured by using digital tensiometer and cell growth was monitored by plate count method. Values are averages of three independent experiments. Rhamnolipid (mg/ml), *filled square*; surface tension (mN/m), *filled triangle*; bacterial growth (CFU/ml), *multiplication symbol*

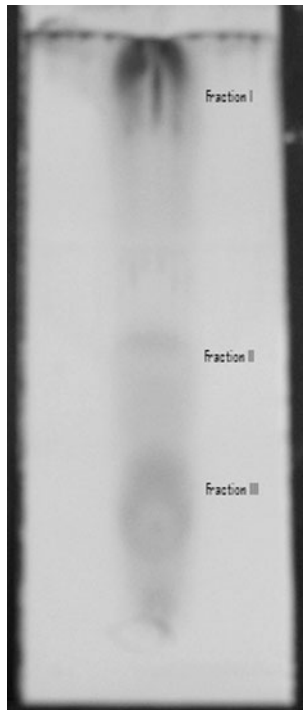

Fig. 3 Thin-layer chromatogram of isolated biosurfactant by *P. aeruginosa* OBP1 showing three main fractions having R_f values of 0.74, 0.56, and 0.36, respectively

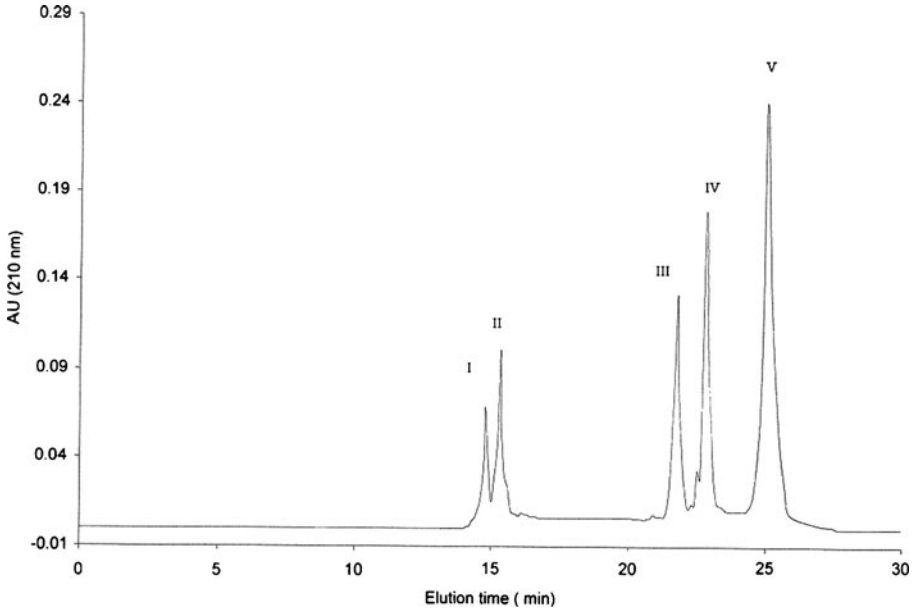

Fig. 4 HPLC chromatogram of the TLC fraction (spot with R_f value of 0.36) using a Waters C18 Nova pack RP-HPLC column

fourth fraction showed the highest surface activity that reduces the surface tension of water from 72 to 31.1 mN/m and was further characterized.

Effect of Carbon and Nitrogen Sources on Rhamnolipid Production

Both carbon and nitrogen sources play a crucial role in the production of rhamnolipid [15, 27]. In this work, different carbon sources on the basis of their increasing complexity and hydrophobicity, including glucose, glycerol, vegetable oils—sunflower and soybean oil—and three different hydrocarbons (*n*-hexadecane, octadecane, and crude oil) were used to examine their effectiveness on rhamnolipid production and the results obtained are presented in Table 1. The bacterial strain *P. aeruginosa* 0BP1 was able to produce a maximum of 4.57 g/l and a minimum of 0.12 g/l rhamnolipid in *n*-hexadecane- and

Table 1 Effect of carbon substrate on rhamnolipid production by *P. aeruginosa* OBP1 in MS medium with 2 g $(NH_4)_2SO_4$+2 g urea. Values are the mean of three separate experiments ± S.D. within 10–15%

Carbon source	Biomass (g/l)	Rhamnolipid yield (g/l)	Surface tension (mN/m)	Interfacial tension (mN/m)	F_{CMC} dilution
Glucose	5.04	0.12	50.7	2.7	1.0
Glycerol	4.96	0.39	47.2	2.4	1.0
n-Hexadecane	4.87	4.57	31.1	1.5	4.7
Octadecane	4.83	4.21	31.9	1.5	4.1
Crude oil	3.71	2.53	32.7	1.8	3.7
Sunflower oil	2.63	1.89	37.9	1.9	1.7
Soybean oil	2.51	1.71	38.3	1.9	1.4

glucose-supplemented medium, respectively. Meanwhile, 0.12 and 0.39 g/l of rhamnolipid were produced with 5% glucose and glycerol, which are much lower than that obtained from *n*-hexadecane, octadecane, and crude oil. On the basis of the details in Table 1, *n*-hexadecane was found to be an excellent carbon source for the production of rhamnolipid, followed by octadecane and crude oil. According to Perfumo et al. [28], hydrocarbons are excellent carbon sources for the production of rhamnolipid from *P. aeruginosa* strains. This ineffectiveness of glucose and glycerol in the production of rhamnolipid is due to their high solubility and being more readily available to the bacterial cells. Thus, there is no need for the cells to produce biosurfactant to enhance their solubility or availability. From the results, it is clear that hydrophobic substrates such as *n*-hexadecane, octadecane, and crude oil could induce the biosurfactant production [17]. In contrast, vegetable oils from sunflower and soya bean were found to be comparatively less efficient in rhamnolipid production but much better than glucose and glycerol and are represented graphically in Fig. 5. According to various authors, vegetable oils are a more efficient carbon source for rhamnolipid production by *P. aeruginosa* strains as compared to glucose, glycerol, and hydrocarbons [15, 29]. The present study showed that the complex hydrocarbons were better than easily available carbon sources such as glucose, glycerol, and vegetable oils in the production and yield of rhamnolipid. This clearly suggested that carbon sources preference for rhamnolipid productivity entirely depends on the bacterial strain.

The type of nitrogen source markedly affects the production of rhamnolipid [17, 30]. The production of biosurfactant by *P. aer*uginosa strain OBP1 was examined in the presence of various organic (urea and yeast extract) and inorganic nitrogen (NH_4NO_3, $(NH_4)_2SO_4$, and $NaNO_3$) sources. The inorganic sources appeared to be efficient for *P. ae*ruginosa strain OBP1 for the production of rhamnolipid, having a maximum yield of 3.14 g/l as shown in Table 2. The inorganic nitrogen sources such as NH_4NO_3 and $NaNO_2$ caused a similar growth of bacterial strain and biosurfactant production. Organic nitrogen source such as urea was found to be effective against yeast extract, leading to a high rhamnolipid yield as shown graphically in Fig. 6. Using urea and $(NH_4)_2SO_4$ together at the concentration of 2 g/l, the yield was 4.57 g/l, which was much higher compared with the individual nitrogen sources.

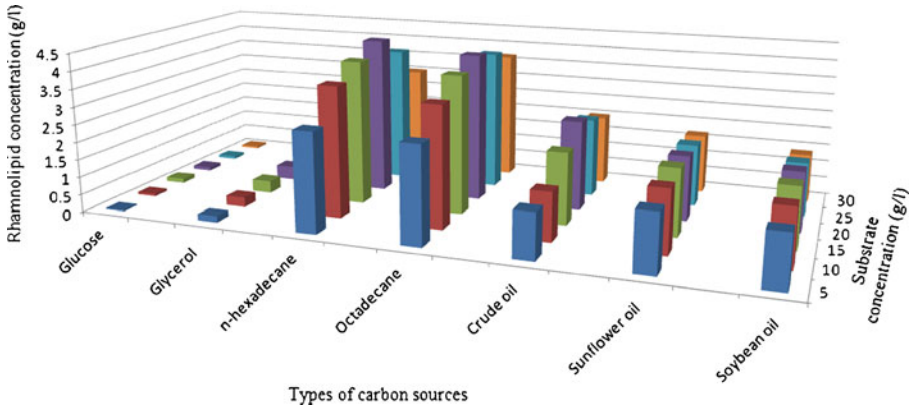

Fig. 5 Effect of the type and the concentration of carbon substrate on rhamnolipid production by *P. aeruginosa* OBP1 in MSM medium with 2 g $(NH_4)_2SO_4$+2 g urea. Values are the mean of three separate experiments ± 1S.D. within 10–15%

Table 2 Effect of nitrogen source on rhamnolipid production by *P. aeruginosa* OBP1 in MS medium with 2 g $(NH_4)_2SO_4$+2 g urea. Values are the mean of three separate experiments ± S.D. within 10–15%

Nitrogen source	Biomass (g/l)	Rhamnolipid yield (g/l)	Surface tension (mN/m)	Interfacial tension (mN/m)	F_{CMC} dilution
No nitrogen	0.51	0.10	41.4	2.2	1.0
NH_4NO_3	3.19	3.06	38.4	2.0	2.6
$(NH_4)_2SO_4$	3.78	3.14	36.7	1.9	3.0
$NaNO_3$	2.76	2.03	39.2	2.1	1.4
Urea	2.34	2.32	37.3	1.9	2.2
Yeast extract	2.63	2.16	37.8	1.9	1.7
$(NH_4)_2SO_4$ + urea	4.87	4.57	31.1	1.5	4.7
$(NH_4)_2SO_4$ + yeast extract	4.81	4.11	33.7	1.8	3.5

Optimization of Rhamnolipid Production

P. aeruginosa strain 0BP1 was cultured in modified mineral salt medium supplemented with urea and ammonium sulphate as nitrogen source. Glucose, glycerol, *n*-hexadecane, octadecane, crude oil, and sunflower and soya bean oil were used as carbon sources in the media. Experimentally, it has been found that *P. aeruginosa* strain 0BP1 was able to produce biosurfactant in a medium supplemented with 2% *n*-hexadecane with a maximum yield of 4.57 g/l, followed by octadecane and crude oil. The culture medium attained a lower surface tension of 31.1 mN/m and a higher emulsification index of 82% in *n*-hexadecane-containing medium. Strains belonging to the above species were reported to produce biosurfactant on water-immiscible hydrocarbon sources [31, 32] and readily soluble and available sources like glucose, mannitol, or glycerol [33–35]. It was found that a gradual increase in the *n*-hexadecane concentration causes a decrease in the cell growth and emulsification activity.

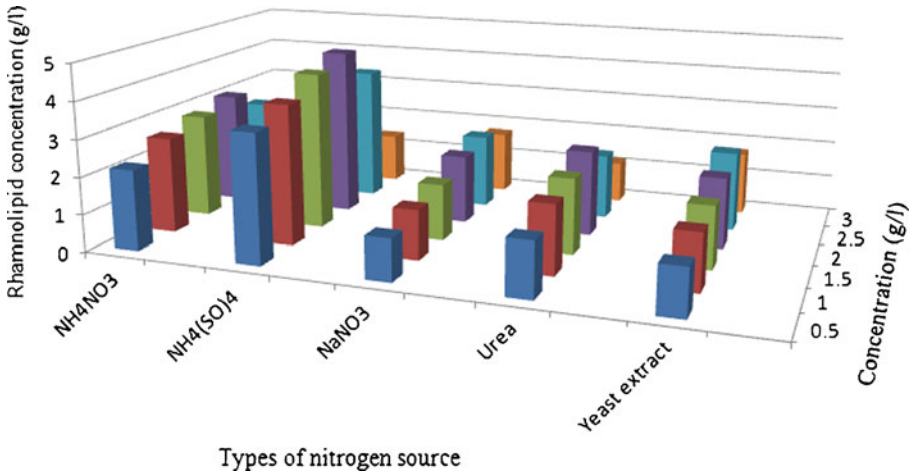

Fig. 6 Effect of the type and the concentration nitrogen sources on rhamnolipid production by *P. aeruginosa* OBP1 in MSM medium with 20 g/l *n*-hexadecane as sole source of carbon. Values are the mean of three separate experiments ± S.D. within 10–15%

The production of biosurfactant was examined in the presence of various organic and inorganic nitrogen sources. Urea and $(NH_4)_2SO_4$, each at 2 mg/l, were found to be optimum for the production of biosurfactant, with a maximum yield of 4.57 g/l. The phosphate source KH_2PO_4 and essential metal ions Mg^{2+}, Fe^{2+}, and Ca^{2+} supplied in the form of $MgSO_4{\cdot}7H_2O$ (g/l), $FeSO_4{\cdot}7H_2O$ (g/l), and $CaCl_2{\cdot}2H_2O$ (g/l), respectively, attributed to higher biosurfactant production. Adding of trace elements like Zn^{2+}, Mn^{3+}, $BO_3{}^{3+}$, and Mn^{2+} in the medium significantly enhanced cell growth and biosurfactant production. Culture condition also plays a crucial role in the production of biosurfactant [36]. The culture medium was kept at a pH of 6.8, 180 rpm, and a temperature of 37 °C for 15 days. Define media like Luria broth, M9, and basal salt medium were used for the production of biosurfactant. Out of these, modified mineral salt medium was found to be the best from the point of biosurfactant production. The behavior of biosurfactant production was studied by determining the rhamnolipid concentration in the culture medium directly at a specific interval for 15 days by orcinol method showing that the production was low during the exponential phase of growth. However, the concentration of the biosurfactant increases in the culture medium at the stationary phase of growth, referring to the accumulation of biosurfactant in the culture as a secondary metabolite. A similar observation was reported by Patel and Desai [37].

Chemical Characterization of Rhamnolipid

The rhamnolipid secreted extracellularly into the culture medium was recovered by means of acid precipitation method directly from the culture supernatant [38]. *P. aeruginosa*, which produces a rhamnolipid, has homologues that differ in the number of rhamnose molecules as well as in the length and composition of alkyl chain [34]. The molecular composition of the isolated biosurfactant was evaluated by FTIR. The important bands which were located at 2,927.03, 2,860.39, 1,725, and 1,300–1,100 cm^{-1} confirmed the presence of glycolipid-type biosurfactant [39, 40]. The bands appearing at 3,430.19 cm^{-1} denoted the presence of –OH stretching of the hydroxyl group. The strong adsorption peaks at 3,000–2,700 cm^{-1} showed the presence of –CH aliphatic stretching bands. The characteristic peaks for the ester compounds were observed at 1,725.32 cm^{-1}, conforming –C=O stretching vibrations of carbonyl groups. Another strong peak at 1,638.1 cm^{-1} showed the presence of –COO– stretching vibrations of carbonyl groups. Other absorption peaks between 1,300 and 1,000 cm^{-1} confirmed the presence of ester carbonyl groups which corresponded to the presence of –C–O– deformation vibrations as shown in Fig. 7. The important adsorption bands obtained suggested the presence of rhamnolipid in the isolated freeze-dried culture precipitate.

In ^{1}H NMR, characteristic chemical shifts at 0.88 ppm showed the presence of $–CH_3$ and similarly 1.58 ppm for $–CH_2–CH_2–CH_2–$, 2.67 ppm for $–CH_2–COO–$, 4.27 for –O–C–H, and 5.298 ppm for $–COO–CH_2–$ [12, 15, 30,]. The characteristic chemical shifts present in the ^{1}H NMR spectra confirmed the presence of two species of rhamnolipid. All three parameters such as chemical shifts of the adsorption frequency, coupling, and integral height indicated the molecular structure as that of L-rhamnosyl-ß-hydroxydecanoate (monorhamnolipid) and L-rhamnosyl-L-rhamnosy-β-hydroxydecanoate (dirhamnolipid).

Negative ion mode with an extraction voltage up to −75 V was used to produce pseudomolecular ions $[M–H]^-$ of rhamnolipid which is attributed to the loss of H^+ proton from the carboxylic group [34]. The purified rhamnolipid sample was diluted in methanol with a concentration of 10 mg/ml. The mass spectrum of the purified biosurfactant showed a mixture of rhamnolipid with a molecular weight between 333 and 649 with intense molecular ions at *m/z* 333.09, 503.18, 504.18, 531.20, and 649.20. The *m/z* values obtained

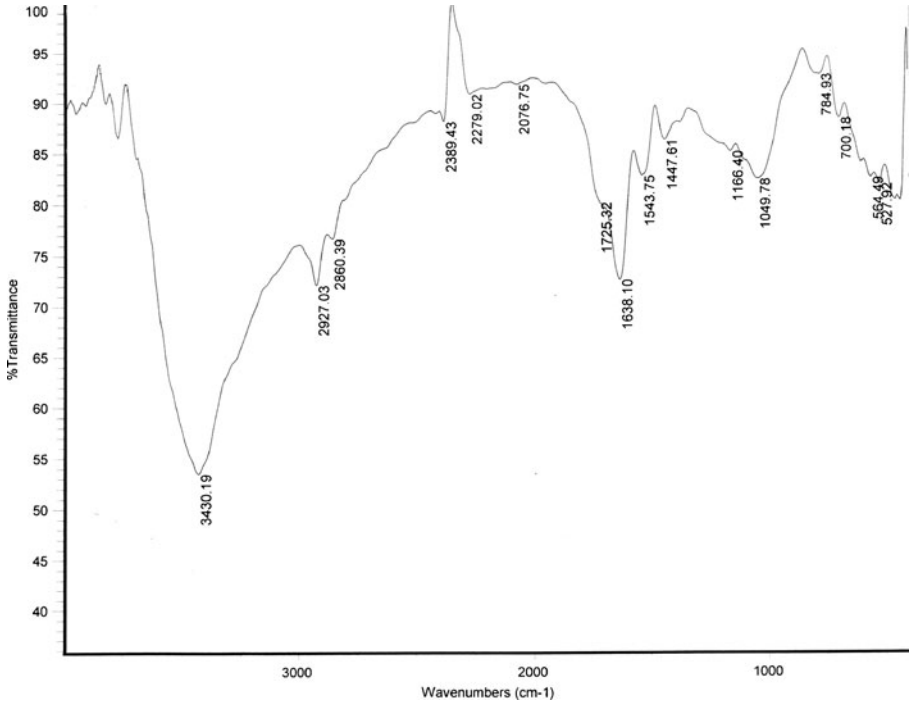

Fig. 7 FTIR spectrum of the purified biosurfactant produced by *P. aeruginosa* OBP1 in MSM medium with 20 g/l *n*-hexadecane as sole source of carbon

were consistent with the molecular structure of Rha-C_{10}, Rha-C_{10}-C_{10}, Rha-C_{10}-C_{12}, and Rha-Rha-C_{10}-C_{10}, respectively. The spectra obtained when the carbon source was *n*-hexadecane showed the predominant congener $[M–H]^-$ ions for Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10} and constituting 60% of the entire mixture as shown in Fig. 8. In general, the results showed the presence of a relatively higher abundance of monorhamnolipid than dirhamnolipid. Generally, most of the reported *P. aeruginosa* strains know how to produce dirhamnolipid [29, 34]. However, few literatures reported the predominance of monorhamnolipid [30, 35, 36]. The rhamnolipid composition and predominance of a particular type of congener depends on various factors like type of carbon substrate [31, 35, 41], culture conditions [36], age of the culture [42], and the most important by the strain *P. aeruginosa* [34].

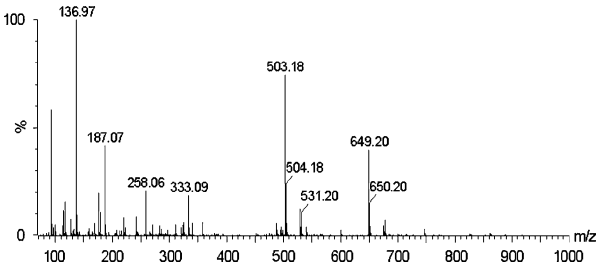

Fig. 8 The mass spectra of the two co-eluting congeners, Rha-C_{10}-C_{10} (*m*/*z* 503) and Rha-Rha-C_{10}-C_{10} (*m*/*z* 649) in the liquid chromatography–electrospray mass spectroscopy

Physiochemical Characterization of Rhamnolipid

The efficiency of a surfactant is measured by the concentration required for it to cause a significant reduction of the surface tension of water. The biosurfactant produced in the liquid culture of *P. aeruginosa* species could reduce the surface tension of water from 72 to 29 mN/m with a critical micelle concentration in the range of 5–60 mg/l, depending on the components of the mixture of rhamnolipid [5].The partially purified biosurfactant was dissolved in water and serially diluted to prepare different concentrations. The surface tension of each dilution was measured and presented in Fig. 9. The biosurfactant lowers the surface tension of water from 72 to 31.1 mN/m but could not decrease it beyond this value even after increasing the concentration of biosurfactant in water. The isolated purified biosurfactant is a mixture mainly of six rhamnolipid species. The dominant species was Rha-C_{10}-C_{10} followed by Rha-Rha-C_{10}-C_{10}. According to Zhang and Milller [43] and Zhang et al. [44], monorhamnolipid is less soluble and is adsorbed to surfaces more strongly, requiring higher CMC for the solubilization of hydrocarbons and binding with the metal ions much stronger than the homologues dirhamnolipid. Due to the variation in the rhamnolipid production, accumulation, complexity in its composition, number and proportion of homologues, and surface active behavior could cause a difference in the reduction of surface tension [41]. Due to these reasons, the reduction in surface tension and critical micelle concentration would vary as per the *P. aeruginosa* strains.

In the case of interfacial tension, biosurfactant reduces the interfacial tension value from 24 to 1.5 mN/m against *n*-hexadecane at a concentration of 100 mg/l. The interfacial tension values obtained were consistent with the previously reported values [41, 45, 46]. Abalos et al. [45] reported the surface tension value of 28.8 mN/m for Rha-Rha-C10 and 26 mN/m for a mixture of Rha-C_{10}-C_{10} and Rha-Rha-C_{10} by Syldatk et al. [47].

As the concentration of the surfactant increased, the surface tension of the culture medium decreased up to a certain value and became almost constant due to the saturation of surfactant molecules at the interface; further addition of surfactant would lead to the formation of micelle. The concentration at which the saturation occurs is known as critical micelle concentration and can be calculated from the surface tension of the concerned solution against the surfactant concentration [48]. As shown in Fig. 9, the CMC was approximately 45 mg/l in the culture medium. Surfactant beyond this concentration did not show any significant change in surface tension of the solution. The value of CMC obtained was consistent with the previously reported values by Van Dyke et al. [5].

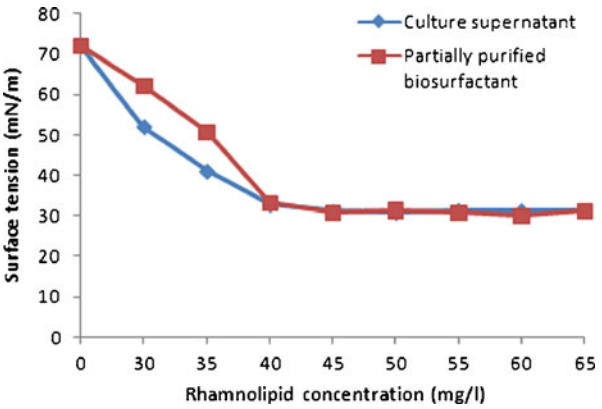

Fig. 9 Determination of the CMC from the surface tension of serially diluted solutions of isolated biosurfactant and the MSM medium having 2% *n*-hexadecane as carbon source produced by *P. aeruginosa* OBP1. The data shown here are mean values of triplicates

Table 3 Emulsification indices and decay constant, K_d, of various emulsifying substrates by the culture supernatant of *P. aeruginosa* OBP1. The data shown here are mean values of triplicates

Hydrocarbon substrate	Emulsification index	Decay constant (K_d)
Toluene	1.78	−7.61
Benzene	1.83	−3.08
n-Hexadecane	2.79	−0.81
Nonadecane	1.89	−3.43
Lubricating oil	2.87	−0.43
Kerosene	2.84	−0.87
Diesel	2.92	−0.15
Petrol	2.67	−0.36
Crude oil	2.72	−0.49
Sunflower oil	1.13	−11.83
Peanut oil	1.07	−34.00
Castor oil	1.23	−9.41

The emulsification ability is an important factor of any surfactant. Therefore, it was assessed for the rhamnolipid produced in the culture broth by determining the emulsification indices (E_{24}) against different water-immiscible substrates, mainly different oil phases. The culture broth showed appreciable emulsification indices, as shown in Table 3, with crude oil, waste lubricating oil, kerosene, diesel, petrol, *n*-hexadecane, naphthalene, anthracene, and soya bean and sunflower oils. The cell-free supernatant was found to form stable water-in-oil emulsion efficiently with all the tested hydrophobic substrates but failed to form a stable emulsion with vegetable oils. Various authors reported the dependence of emulsification activity of rhamnolipid on different carbon sources used for its production of rhamnolipid [36, 37]. The maximum emulsification activity was against diesel followed by *n*-hexadecane. All of the test hydrocarbons were found to form a stable emulsion and was expressed as decay constant ($-K_d$). From the literature, it has been found that rhamnolipid can efficiently emulsify and stabilize emulsions with various types of water-immiscible substrates such as linseed oil, almond oil, mineral oil [32], diesel and kerosene [28], diesel, hexadecane, linseed oil, kerosene, and diesel and motor oil [49]. The results showed that the biosurfactant produced from the hydrocarbon substrates such as

Table 4 Antibacterial effect of rhamnolipid based on minimal inhibitory concentration (μg/ml) on the bacterial growth

Microorganism	Minimal inhibitory concentration (μg/ml)
Bacillus subtilis (ATCC 11774)	19
Bacillus circulans (ATCC 61)	11
Staphylococcus aureus (ATCC 11632)	08
Staphylococcus epidermidis (ATCC 49461)	12
Alcaligenes faecalis (ATCC 8750)	57
Escherichia coli (ATCC 9637)	64
Klebsilla pneumoniae (ATCC 10031)	>78
Pseudomonas aeruginosa (MTCC 7815)	37
Enterobacter aerogenes (ATCC 13048)	31
Proteus vulgaris (ATCC 49132)	>105

n-hexadecane could emulsify different hydrocarbons significantly, which conformed to their applicability against different hydrocarbon pollution such that to enhance the availability of the recalcitrant hydrocarbons [15, 50].

Biosurfactants are known to cause the inhibition of microbial growth by affecting the release of intracellular material or by inhibiting respiration [51]. A surfactant was reported to be more toxic towards Gram-positive bacteria compared to Gram-negative bacteria due to differences in the cell wall structure composition and osmolarities. The MIC values of rhamnolipid for the inhibition of microbial growth are presented in Table 4. The MIC values were much less for Gram-positive bacteria, indicating its effectiveness at a low concentration; however, the MIC values were much higher for Gram-negative bacteria because of the surface proteins and lipopolysaccharide which are the two main constituents of the cell wall. These structures are known to protect the cell membrane from surfactant attack [52]. Rhamnolipid mixture was found to be more effective against Gram-positive bacteria, such as *B. subtilis* and *S. aureus*, but less effective against *Alcaligenes faecalis*. Very limited information is available for the antibacterial activities of rhamnolipid. Lang et al. [52] reported the antibacterial property of RL1/RL3 against the growth of *B. subtilis* at a MIC value of 35 μg/ml and of *Staphylococcus epidermidis* at 350 μg/ml. Hommel et al. [53] reported another type of glycolipid, produced by *Torulopsis apicola*, showing antimicrobial activity against a number of Gram-positive bacteria. Kitamoto et al. [54] reported MIC values ranging from 3.1 to 25 μg/ml as being effective against bacteria and less effective against fungus (MIC>400 μg/ml). Haba et al. [42] reported the antimicrobial activities of rhamnolipid mixtures against a number of both Gram-positive and Gram-negative bacteria and fungal strains.

Conclusion

In the present study, a potential biosurfactant-producing *P. aeruginosa* strain OBP1 isolated from the petroleum sludge was found to be capable of producing rhamnolipid effectively from a wide range of hydrocarbons. *n*-Hexadecane and two different nitrogen sources, urea and $(NH_4)_2SO_4$, together were found to significantly increase the production of rhamnolipid. The isolated rhamnolipid was analyzed by FTIR, LC-MS, and ^1H NMR which confirmed the presence of different species of rhamnolipid. Monorhamnolipid (Rha-C_{10}-C_{10}) was produced in abundance with the predominant congener $[M–H]^-$ ions for Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10} which constituted 60% of the entire mixture. The purified rhamnolipid was able to reduce the surface tension of pure water from 72 to 31.1 mN/m and interfacial tension up to 1.5 mN/m with a CMC value of 45 mg/l. The biosurfactant was also found to be antimicrobial against bacteria. The culture supernatant exhibited a high emulsifying activity and the effectiveness was much higher against the hydrocarbons, indicating the possibility of use in bioremediation.

Acknowledgement This work was financed by the Oil and Natural Gas Corporation, India. The authors would like to thank Dr. A. J. Thakur and Mr. S. Das for their technical support.

References

1. Hardegger, M., Koch, T. A. K., Ochsner, U. A., Fiechter, A., & Reiser, J. (1994). *Applied and Environmental Microbiology, 60*, 3679–3687.
2. Shoham, Y., Rosenberg, M., & Rosenberg, E. (1983). *Applied Environmental Microbiology, 46*, 573–577.

3. Zajic, J. E., Guignard, H., & Gerson, D. F. (1977). *Biotechnology and Bioengineering, 19*, 1303–1320.
4. Razafindralambo, H., Poquot, M., Baniel, A., Popineau, Y., Hbid, C., Jacques, P., et al. (1996). *Journal of American Oil Chemical Society, 73*, 149–151.
5. Van Dyke, M. I., Couture, P., Brauer, M., Lee, H., & Trevors, J. T. (1993). *Canadian Journal of Microbiology, 39*, 1071–1078.
6. Velikonja, J., & Kosaric, N. (1993). *Biosurfactants: Production, properties, applications* (pp. 419–446). New York: Dekker.
7. Banat, I. M., Makkar, R. S., & Cameotra, S. S. (2000). *Applied Microbiology and Biotechnology, 53*, 495–508.
8. Mata-Sandoval, J. C., Karns, J., & Torrents, A. (1999). *Journal of Chromatography A, 864*, 211–220.
9. Syldatk, C., & Wagner, F. (1987). In N. Kosaric, W. L. Cairns, & N. C. C. Gray (Eds.), *Biosurfactants and biotechnology* (pp. 89–120). New York: Dekker.
10. Déziel, E., Lépine, F., Milot, S., & Villemur, R. (2000). *Biochimica et Biophysica Acta, 1440*, 244–253.
11. Soberón-Chavez, G., Lépine, F., & Déziel, E. (2005). *Applied Microbiology and Biotechnology, 68*, 718–725.
12. Monterio, S. A., Sassaki, G. L., de Souza, L. M., Meira, J. A., de Araújo, J. M., Mitchell, D. A., et al. (2007). *Chemistry and Physics of Lipids, 147*, 1–13.
13. Burger, M. M., Glaser, L., & Burton, R. M. (1966). *Methods in Enzymology, 8*, 441–445.
14. Ochsner, U. A., Koch, A. K., Fiechter, A., & Reiser, J. (1994). *Journal of Bacteriology, 176*, 2044–2054.
15. Maier, R. M., & Soberón-Chavez, G. (2000). *Applied Microbiology and Biotechnology, 54*, 625–633.
16. Mulligan, C. N. (2005). *Environmental Pollution, 133*, 183–198.
17. Wei, Y. H., Chou, C. L., & Chang, J. S. (2005). *Biochemical Engineering Journal, 27*, 146–154.
18. Siegmund, I., & Wagner, F. (1991). *Biotechnology Techniques, 5*, 256–268.
19. Lowry, O. H., Rosebough, N. J., Farr, A. L., & Randall, R. J. (1951). *Journal of Biological Chemistry, 193*, 265–275.
20. Chandrasekaran, E. V., & Bemiller, J. N. (1980). In L. Wrhiste & M. L. Wolfrom (Eds.), *Methods in carbohydrate chemistry* (Vol. III, pp. 89–97). New York: Academic.
21. Wu, J. Y., Yeh, K. L., Lu, W. B., Lin, C. L., & Chang, J. S. (2008). *Bioresource Technology, 99*, 1157–1164.
22. George, S., & Jayachandran, K. (2008). *Applied Biochemistry and Biotechnology, 58*, 428–434.
23. Abalos, A., Viñas, M., Sabaté, J., Manresa, M. A., & Solanas, A. M. (2004). *Biodegradation, 15*, 249–260.
24. Bodour, A. A., & Maier, R. M. (1998). *Journal of Microbiological Methods, 32*, 273–280.
25. Cooper, D. G., & Zajic, J. E. (1980). *Advances in Applied Microbiology, 26*, 299–253.
26. Price, P., & Mcmillan, T. J. (1990). *Cancer Research, 50*, 1392–6.
27. Sandoval, J. C. M., Karns, J., & Torrents, A. (1999). *Journal of Chromatography A, 864*, 211–220.
28. Perfumo, A., Banat, I. M., Canganella, F., & Marchant, R. (2006). *Applied Microbiology and Biotechnology, 72*, 132–138.
29. Sandoval, J. C. M., Karns, J., & Torrents, A. (2001). *Microbiological Research, 155*, 249–256.
30. Sim, L., Ward, O. P., & Li, Z.-Y. (1997). *Industrial Microbiology and Biotechnology, 19*, 232–238.
31. Rahman, K. S. M., Rahman, T. J., McClean, S., Marchant, R., & Banat, I. M. (2002). *Biotechnology Progress, 18*, 1277–1281.
32. Benincasa, M., Abalos, A., Oliveira, I., & Manresa, A. (2004). *Antonie van Leeuwenhoek, 85*, 1–8.
33. Arino, S., Marchal, R., & Vandecasteele, J.-P. (1996). *Applied Microbiology and Biotechnology, 45*, 162–168.
34. Déziel, E., Lépine, F., Dennie, D., Boismenu, D., Mamer, O. A., & Villemur, R. (1999). *Biochimica et Biophysica Acta, 1440*, 244–252.
35. Bodour, A. A., Drces, K. P., & Maier, R. M. (2003). *Applied Environmental Microbiology, 69*, 3280–3287.
36. Costa, S. G. V. A. O., Nitschke, M., Hadaad, R., Eberlin, M. N., & Contiero, J. (2006). *Process Biochemistry, 41*, 483–488.
37. Patel, R. M., & Desai, A. J. (1997). *Letters in Applied Microbiology, 25*, 91–94.
38. Benincasa, M., Contiero, J., Manresa, A., & Moraes, I. O. (2002). *Journal of Food Engineering, 54*, 283–288.
39. Tuleva, B. K., Ivanov, G. R., & Christova, N. E. (2002). *Z Naturforsch C, 57*, 356–360.
40. Pornsunthorntawee, P., Wongpanit, P., Chavadej, S., Abe, M., & Rujiravanit, R. (2008). *Bioresource Technology, 99*, 1589–1595.
41. Benincasa, M., & Accorsini, F. R. (2008). *Bioresource Technology, 99*, 3843–3849.
42. Haba, E., Abalos, A., Jauregui, O., Espuny, M. J., & Manresa, A. (2003). *Journal of Surfactants and Detergents, 6*, 155–161.
43. Zhang, Y., & Miller, R. M. (1995). *Applied Environmental Microbiology, 61*, 2247–2251.
44. Zhang, Y., Maier, W. J., & Miller, R. M. (1997). *Environmental Science and Technology, 31*, 2211–2217.

45. Abalos, A., Pinazo, A., Infante, M. R., Casals, M., Garcia, F., & Manresa, A. (2001). *Langmuir, 17*, 367–1371.
46. Mercadé, M. E., Manresa, M. A., Robert, M., Espuny, M. J., de Andŕes, C., & Guinea, J. (1993). *Bioresource Technology, 43*, 1–6.
47. Syldatk, C., Lang, S., & Wagner, F. (1985). *Z Naturfosch, 40*, 51–60.
48. Sheppard, J. D., & Mulligan, C. N. (1987). *Applied Microbiology and Biotechnology, 27*, 110–116.
49. Abdul-Mawgoud, A. M., Aboulwafa, M. M., & Hassouna, N. N. (2008). *Applied Biochemistry and Biotechnology, 2*, 329–345.
50. Banat, I. M. (1995). *Bioresource Technology, 51*, 1–12.
51. Kopecka-Leitmanova, A., Devinsky, F., Mlynarcik, D., & Lacko, I. (1989). *Drug Metabolism and Drug Interaction, 7*, 29–50.
52. Lang, S., & Wagner, F. (1993). Biosurfactants: Bioconversion of oils and sugars to glycolipids. In N. Kosaric (Ed.), *Biosurfactants: Production, properties, and applications* (pp. 251–268). New York: Dekker.
53. Hommel, R., Stüwer, O., Stuber, W., Haferburg, D., & Kleber, H. P. (1987). *Applied Microbiology and Biotechnology, 26*, 199–205.
54. Kitamoto, D., Yanagishita, H., Nakane, T., Kamisawa, C., & Nakahara, T. (1993). *Journal of Biotechnology, 29*, 91–96.
55. Finnerty, W. R. (1994). *Environmental Biotechnology, 5*, 291–295.
56. Nitschke, M., Costa, S. G. V. A. O., & Contiero, J. (2005). *Biotechnology Progress, 21*, 1593–1600.