Production of Biosurfactant Using Different Hydrocarbons by *Pseudomonas aeruginosa* EBN-8 Mutant

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The present investigation dealt with the use of previously isolated and studied gamma-ray mutant strain *Pseudomonas aeruginosa* EBN-8 for the production of biosurfactant by using different hydrocarbon substrates viz. *n*-hexadecane, paraffin oil and kerosene oil, provided in minimal medium, as the sole carbon and energy sources. The batch experiments were conducted in 250 mL Erlenmeyer flasks, containing 50 mL minimal salt media supplemented with 1% (w/v) hydrocarbon substrate, inoculated by EBN-8 and incubated at 37 °C and 100 rpm in an orbital shaker. The sampling was done on 24 h basis for 10 d. The surface tension of cell-free culture broth decreased from 53 to 29 mN/m after 3 and 4 d of incubation when the carbon sources were paraffin oil and *n*-hexadecane, respectively. The largest reduction in interfacial tension from 26 to 0.4 mN/m was observed with *n*-hexadecane, while critical micelle dilution was obtained as 50 × CMC for paraffin oil as carbon source. When grown on *n*-hexadecane and paraffin oil, the EBN-8 mutant strain gave 4.1 and 6.3 g of the rhamnolipids/L, respectively. These surface-active substances subsequently allowed the hydrocarbon substrates to disperse readily as emulsion in aqueous phase.

Key words: Biosurfactant, Hydrocarbons, *Pseudomonas aeruginosa*

Introduction

Biosurfactants are a structurally diverse class of compounds consisting of a hydrophobic portion, which usually consists of saturated or unsaturated (hydroxy) fatty acids or fatty alcohols, and a hydrophilic portion, which is composed of mono-, oligo- or polysaccharides, amino acids or peptides or carboxylate or phosphate groups (Lang and Wullbrandt, 1999). The major classes of biosurfactants are: glycolipids, phospholipids, neutral lipids, fatty acids, lipopolysaccharides, lipoproteins-lipopeptides, flavolipids, complete cell surface itself and those not fully characterized (Bodour *et al.*, 2004; Desai and Banat, 1997). Biosurfactants are extracellular macromolecules produced by bacteria, yeast and fungi, and in particular by natural and recombinant bacteria when grown on different carbon sources. The microbial surfactants have gained attention in recent years due to their commercial importance, diverse desirable characteristics such as biodegradability, selectively effectiveness, low toxicity, ecological acceptability and their ability to be produced from cheaper substrates (Ishigami, 1997; Makkar and Cameotra, 1997). They are finding vast potential applications in environmental protection, petroleum, food, mining, agriculture, pharmaceutical, textile, leather and other industries (Banat *et al.*, 2000; Desai and Banat, 1997). Biosurfactants create micro-emulsions in which micelle formation occurs where hydrocarbons can be solubilized in water and *vice versa* (Ashtaputre and Shah, 1995; Haditirto *et al.*, 1989). They mainly reduce surface and interfacial tensions both in aqueous solutions and hydrocarbon mixtures, therefore increasing the aqueous concentrations of poorly soluble compounds which lead to improving the accessibility of these substrates to the microorganisms (Banat, 1995). These emulsification properties have been demonstrated to enhance the hydrocarbon degradation in the environment, hence making them potential tool for the oil spill pollution control (Banat *et al.*, 2000).

The conditions that promote biosurfactant production and control its chemical nature were determined for several microorganisms and observed

to be very dependent on the substrate and microbe used (Georgiou *et al.*, 1992; Kokub *et al.*, 1992). *Pseudomonas aeruginosa* produced rhamnolipids on C_{12} *n*-alkanes (Robert *et al.*, 1989), and increased production was observed under the phosphate-limiting conditions (Mulligan *et al.*, 1989) or with the exhaustion of nitrogen in the media (Ramana and Karanth, 1989). Rhamnolipids produced by *P. aeruginosa* are among the most effective surfactants, containing one or two 3-hydroxy fatty acids of various lengths, linked to a mono- or dirhamnose moiety (Deziel *et al.*, 2000). They are biodegradable and have potential ability to be used as replacements for synthetic surfactants (Kosaric, 1993). They lower the surface tension of distilled water from 72 to 25–30 mN/m and the interfacial tension to 1 mN/m against *n*-hexadecane (Lang and Wagner, 1987), and also emulsify alkanes and stimulate the growth of their producing organisms (Hisatsuka *et al.*, 1971).

The aim of this study was to microbially produce biosurfactant by using separate hydrocarbon substrates: *n*-hexadecane, paraffin oil or kerosene oil, as carbon and energy source present in minimal media in shake flasks at (37 ± 1) °C and 100 rpm in an orbital shaking incubator. The fermentation process was monitored by measuring parameters such as surface tension (ST), interfacial tension (IFT), pH value, critical micelle dilution (CMD), emulsification activity (E-24), substrate utilization, and biomass and rhamnolipids accumulation in the culture media. Also the kinetic parameters such as yield factors relating the biosurfactant production to the hydrocarbon substrate consumption and the biosurfactant production to the dry biomass were calculated.

Materials and Methods

Carbon sources

Well-known hydrocarbons such as *n*-hexadecane (Merck), paraffin and kerosene oils (from a local market) were used as carbon sources, in the minimal salt medium, for the production of biosurfactants by their microbial transformation.

Microorganisms

The hydrocarbon utilizing microorganism *Pseudomonas aeruginosa* S8, isolated from coastal waters (Shafeeq *et al.*, 1989a), and its gamma-ray induced mutant, designated as *Pseudomonas aeruginosa* EBN-8 (Iqbal *et al.*, 1995), maintained at NIBGE, Faisalabad, were used for biosurfactants production. The strains were streaked on (2.8%; w/v) nutrient agar (Oxoid) plates at (37 ± 1) °C for 24 h for fresh growth. A single colony was transferred to (0.8%; w/v) nutrient broth (Oxoid) for 48 h. The cells were collected by centrifugation (at 7,740 × *g* and 4 °C for 15 min), washed with normal saline (0.9%) and resuspended to an optical density of 0.7 at 660 nm. This cell suspension was used as an inoculum.

Shake flask experiment

The composition (g/L) of mineral salt medium prepared in distilled water was as follows: NH_4NO_3 (1.0); KH_2PO_4 (1.0); K_2HPO_4 (1.0); $MgSO_4 \cdot 7H_2O$ (0.2); $CaCl_2 \cdot 2H_2O$ (0.2) and $FeCl_3 \cdot 6H_2O$ (0.05) (Bushnell and Hass, 1941). The pH value of the medium was adjusted at 7.0 by K_2HPO_4. The minimal medium was sterilized in an autoclave at 121–124 °C for 15 min. The chemicals were of analytical grade and used as received from the supplier without further purification.

The study was carried out in 250 mL Erlenmeyer flasks containing 50 mL liquid medium. 1% (v/v) inoculum was added with the help of a sterilized syringe to the minimal medium supplemented with (1%; w/v) pre-sterilized hydrocarbon substrates (*n*-hexadecane, paraffin oil or kerosene oil), as sole carbon sources, separately. The flasks were incubated at (37 ± 1) °C and 100 rpm in an orbital shaker (OGAWA SEIKI Co. Ltd., Japan; Model: OSI-503L). There was also a parallel set of control flaks having the same composition of nutrients and incubating condition, as specified for treated flasks, but without inoculum.

The sampling was carried out each day for 10 d. Whole flasks were removed from the shaker for the analysis of different attributes. All experiments were conducted in triplicate and the results are averages of the three readings.

Biomass estimation

An aliquot (50 mL) of the culture sample was centrifuged (Beckman; T2-HS Centrifuge with Rotor JA-20) (at 7,740 × *g* and 4 °C for 15 min) to collect the cells (Iqbal *et al.*, 1995). The cell-free culture broth (CFCB) was stored for determining surface-active properties and rhamnolipid contents, while the cell pellet was washed, resuspended in pre-sterilized distilled water and centrifuged again (7,740 × *g*, 4 °C, 15 min). The cell

pellet was then desiccated in an electric oven (D 06060, Model 400; Memmert) at 105 °C until a constant weight was achieved.

Rapid drop-collapsing test

The changes in surface activity of culture media with time were measured using the rapid drop-collapsing test (Jain *et al.*, 1991). This test is a quick way to see whether a biosurfactant is produced in the media or not. For this purpose a 10 μL drop of a sample of supernatant was placed on a kerosene oil-coated glass surface and the surface area of the drop was measured under a microscope after 5 min of equilibration. The procedure was repeated with distilled water of equal volume.

Measurement of surface and interfacial tensions

The equilibrated surface tension (ST) and interfacial tension (IFT) of CFCB were measured by the ring method (Margaritis *et al.*, 1979) using a digital tensiometer (K10T; Krüss, Germany). The IFT was measured by submerging the tensiometer ring in 10 mL CFCB and adding an equal volume of kerosene oil in the tensiometer vessel, so that the ring did not break through the upper oil layer before the interfacial film was ruptured (Akit *et al.*, 1981). The changes in pH value of the culture broth were also monitored by a digital pH-meter.

Critical micelle dilution

The biosurfactant concentration was expressed in terms of critical micelle dilution (CMD), estimated by measuring the surface tension at varying dilutions of the CFCB. The dilution at which the surface tension abruptly began to increase was termed as the critical micelle dilution. This was the factor by which the biosurfactant concentration exceeded the effective critical micelle concentration (CMC). The plots of ST vs. dilution times gave the value of CMD of the CFCB (Fig. 2). The reciprocal critical micelle dilution (CMD^{-1}) was determined by measuring the surface tension of serially diluted CFCB (Tahzibi *et al.*, 2004).

Emulsification activity

Emulsification activity (E-24) was determined at zero time and at the end of 10 d of incubation. This was performed by adding equal volumes of kerosene oil and CFCB of EBN-8 in a screw capped test tube (10 mL capacity) and vortexing at high frequency for 2 min. The emulsion was left to equilibrate at room temperature for 24 h. E-24 is the percentage of the height of the emulsified layer divided by the total height of the liquid column (Cooper and Goldenberg, 1987).

Hydrocarbon utilization

The residual hydrocarbons in the CFCB were determined according to Kokub *et al.* (1990). The culture was washed three times with *n*-hexane in 1:2 (v/v) ratio. The organic layer was separated by a separating funnel, evaporated to dryness under vacuum using a rotary evaporator at 30 °C and then oven-dried at 60 °C to a constant mass.

Extraction of biosurfactant

Biosurfactant was extracted from the CFCB using the modified method of Hisatsuka *et al.* (1971). The culture sample was centrifuged (at 7,740 × *g* and 4 °C for 15 min) to remove cells. The supernatant was separated, carefully, into a 10 mL test tube with the help of a syringe. Its pH value was set at 2 with 1 M HCl and left for overnight at 4 °C. Biosurfactant was extracted from supernatant (aqueous phase) with two equal volumes of chloroform/methanol (2:1, v/v) mixture (Tahzibi *et al.*, 2004). The separated organic layer was removed using a separating funnel after equilibrating for 10 min. The combined extracts were concentrated under vacuum using a rotary evaporator to get the crude extract at 40 °C.

Quantification of rhamnolipids

The extracts obtained were dissolved in distilled water to determine rhamnolipids in terms of rhamnose equivalents by the orcinol method (Chandrasekaran and Bemiller, 1980). A 333 μL sample of the supernatant, obtained after centrifugation as mentioned earlier, was extracted twice with 1 mL diethyl ether. Ether fractions were evaporated to dryness and 0.5 mL of distilled H_2O was added. To 100 μL of each sample, 900 μL of a solution containing 0.19% orcinol (in 53% H_2SO_4) were added. After heating (at 80 °C for 30 min), the samples were cooled to room temperature and the absorbance was measured at 421 nm. The rhamnolipid concentrations were calculated from a standard curve prepared with L-rhamnose and expressed as rhamnose equivalents (RE) (mg/mL). The rhamnolipid contents were calculated as

3.4 times the rhamnose contents (Benincasa *et al.*, 2004).

Results and Discussion

Surface-active properties

The surface tension of distilled water (72 mN/m) dropped to 52–53 mN/m in the minimal media containing 1% (w/v) hydrocarbon, without inoculum (*i.e.* control). The initial drop in surface tension might be due to interaction of media components. There was a substantial decrease in ST and IFT, after growth of the organisms on different hydrocarbons (Table I). This decrease in ST and IFT was used as an indication of the biosurfactant production in the batch culture media of *Pseudomonas aeruginosa* EBN-8 mutant grown on different hydrocarbons. The mutant reduced the ST of CFCB from about 53 to below 30 mN/m on 3rd, 4th and 7th day of incubation on paraffin oil, *n*-hexadecane and kerosene oil, respectively. The strain reduced the IFT in the CFCB from about 26 mN/m to 0.4, 0.6 and 4 mN/m on the same respective days (as mentioned for ST reduction of media below 30 mN/m) for the carbon sources *n*-hexadecane, paraffin oil and kerosene oil, respectively. The results of ST and IFT are displayed in Fig. 1 and Table I. The collapsing of a drop from culture broth on oil-coated surface indicated the presence of some biosurfactant and the non-biosurfactant-containing drop remained stable.

Critical micelle dilution and E-24

Critical micelle dilution is also an indication of biosurfactant concentration in the CFCB (Makkar

Table I. Surface-active properties of control (abiotic) and cell-free culture broth (CFCB) of mutant strain EBN-8 on different hydrocarbons (1%; w/v) in minimal media (Bushnel and Hass, 1941) grown in shake flasks [at (37 ± 1) °C and 100 rpm in an orbital shaker], after 10 d of incubation. The values are the average of three replicates.

Carbon source	Surface tension			Interfacial tension*			$CMD^{-1,a}$	E-24[b]
	[mN/m]		(% reduction)	[mN/m]		(% reduction)	[mN/m]	(%E)
	Control	CFCB	CFCB	Control	CFCB	CFCB	CFCB	CFCB
n-Hexadecane	52 ± 1	28.9 ± 0.5	44.40 ± 0.08	26 ± 1	0.4 ± 0.1	98.46 ± 0.32	30 ± 0.1	71 ± 0.5
Paraffin oil	53 ± 1	29.0 ± 0.6	45.28 ± 0.10	26 ± 1	0.6 ± 0.1	97.69 ± 0.29	30 ± 0.2	73 ± 0.7
Kerosene oil	54 ± 1	29.5 ± 0.5	45.37 ± 0.08	26 ± 1	4.0 ± 0.1	84.61 ± 0.17	60 ± 0.5	61 ± 0.5

[a] CMD^{-1}, reciprocal critical micelle dilution.
[b] E-24, emulsification activity.
* Interfacial tension of CFCB was measured against kerosene oil.

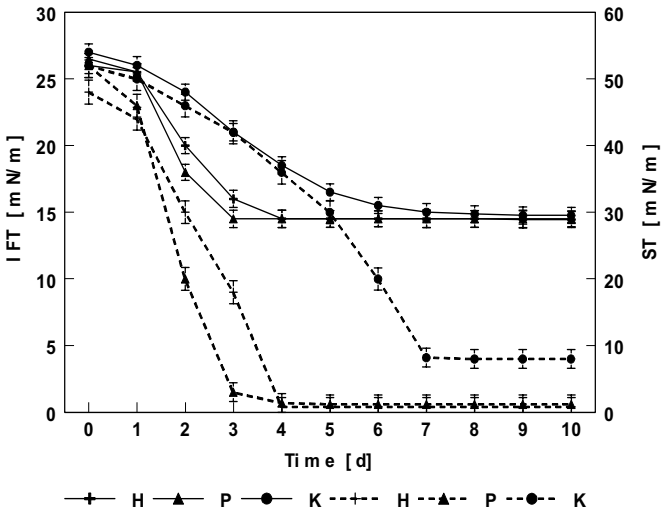

Fig. 1. Changes in surface tension (ST) and interfacial tension (IFT) of cell-free culture broth of mutant strain EBN-8 grown on 1% (w/v) hydrocarbon in minimal media at (37 ± 1) °C and 100 rpm vs. time of fermentation. Surface tension (——), interfacial tension (- - -); H, *n*-hexadecane; P, paraffin oil; K, kerosene oil. The values are averages from three cultures.

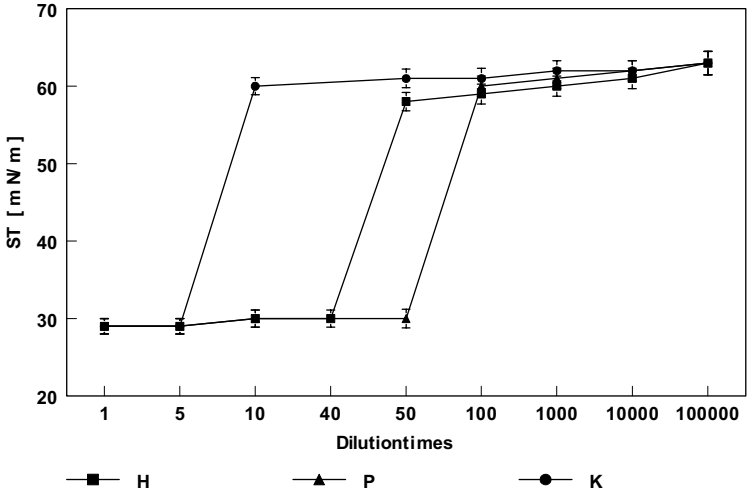

Fig. 2. Changes in surface tension (ST) serial dilution of cell-free culture broth (times) of mutant strain EBN-8 grown on 1% (w/v) hydrocarbon in minimal media at (37 ± 1) °C and 100 rpm. H, *n*-hexadecane; P, paraffin oil; K, kerosene oil. The values are averages from three cultures.

and Cameotra, 1997). The lower the CMD value the higher the dilution was required to cause a prominent increase in surface tension, thus higher was the biosurfactant concentration in the medium. The results of superfacial tension related to serial dilution of CFCB are displayed in Fig. 2. When the biosurfactant concentration became lower than CMC, the superfacial tension abruptly raised. The dilution limits (*i.e.* CMD) and CMD^{-1} of CFCB of EBN-8 on different carbon sources are given in Fig. 2 and Table I, respectively. The results showed that the biosurfactant produced in culture media with kerosene oil was not so significant because the ST of the medium abruptly rose to 60 mN/m on 10-times dilution. The CFCB of EBN-8 exhibited an emulsification index of more that 70% after 10 d of incubation on the carbon sources selected, except kerosene oil (Table I).

The pH values of the CFCB of EBN-8 with time, grown on different carbon sources, were lowered from 7.0 to about 6.0 and this was an indication of synthesis of some fatty acids along with biosurfactant in the culture media. The pH value of the control flasks remained 7.0 ± 0.1, which showed that there was no fermentative metabolism in the sterilized control.

Biomass and residual hydrocarbons

The assays of biomass formation, product accummulation and substrate utilization with time in the culture medium are displayed in Fig. 3. EBN-8 reached the stationary growth with 2.1, 2.0 and 1.7 g dry biomass/L on 2nd, 3rd and 4th day of fermentation on *n*-hexadecane, paraffin oil and kerosene oil, respectively. The lower growth on kerosene and paraffin oils might be due to the complexity of these substrates as compared to *n*-hexadecane. The biosurfactant production remained growth associated till the bacterial cell reached the stationary phase (Fig. 3). This might

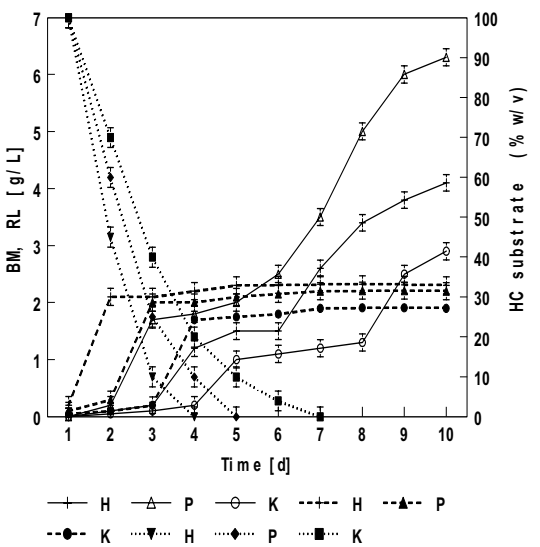

Fig. 3. Kinetics of biomass (BM), rhamnolipids (RL) and substrate with time of the culture broth of mutant strain EBN-8 grown on 1% (w/v) hydrocarbon (HC substrate) in minimal media at (37 ± 1) °C and 100 rpm, for different times. Rhamnolipid (——), dry biomass (- - -), and hydrocarbon substrate (·····). H, *n*-hexadecane; P, paraffin oil; K, kerosene oil. The values are averages from three cultures.

help the cells in the adherence to the substrate molecules and degrade them (Bharathi and Vasudevan, 2001). The substrates: *n*-hexadecane, paraffin and kerosene oils were entirely consumed by microbes after 4th, 5th and 7th day of incubation, respectively. Though a weak parallel relationship was observed between growth, substrate utilization and rhamnolipid production, but as soon as the carbon source was consumed, the bacterial growth reached the stationary phase, and a considerable increase in rhamnolipid production was observed under growth limiting condition. This showed that rhamnolipids were the secondary metabolites of this strain, as also observed by other hydrocarbons (Desai and Banat, 1997).

Biosurfactant's extraction and quantification

The profile of rhamnolipids production, when EBN-8 was cultivated on minimal media with 1% (w/v) hydrocarbon substrate, is shown in Fig. 3. The strain was able to produce 2.9, 4.1 and 6.3 g rhamnolipids/L with 45.4, 44.4 and 45.3% reduction in surface tension, and 84.6, 98.5 and 97.7% drop in IFT at the end of 10 d of incubation in shake flasks, following growth on kerosene oil, *n*-hexadecane and paraffin oil, respectively. Although, the surface tension of medium with kerosene oil dropped significantly, yet its IFT reached 4 mN/m in contrast to below 1 mN/m in the case of other carbon sources tested. This might be due to the emulsion formation between CFCB and kerosene oil, which was problematic during IFT measurement.

The yields of the crude extracts and the rhamnolipids are shown in Table II. These results indicate that 65% of the crude extract were rhamnolipids, when paraffin oil was used as a carbon source by EBN-8. Santa Anna *et al.* (2002) obtained 0.26, 0.13 g rhamnose/L (or 0.88, 0.44 g rhamnolipids/L, respectively) with paraffin oil and *n*-hexadecane by *Pseudomonas aeruginosa* PA1, respectively. Sim *et al.* (1997) obtained 0.4 g rhamnolipids/L with paraffin by *Pseudomonas aeruginosa* UW-1 after 13 d of incubation. Our results were more significant than those recorded by Robert *et al.* (1989) and Matsufugi *et al.* (1997), who were unable to produce rhamnolipids by *Pseudomonas aeruginosa* 44T1 and *Pseudomonas aeruginosa* IFO 3929 strains, respectively, by growing on *n*-paraffin as carbon source.

The yield factors relating rhamnolipids production to substrate utilization ($Y_{P/S}$) and rhamnolipids production to dry cell biomass ($Y_{P/X}$) are given in Table II. The results were obtained as $Y_{P/S}$ = 0.716 g/g; $Y_{P/X}$ = 3.15 g/g, when paraffin oil (1%; w/v) was used as carbon source in minimal medium, after 10 d of incubation. Itoh *et al.* (1971) and Itoh and Suzuki (1972) obtained rhamnolipids using *n*-paraffin as carbon source by *Pseudomonas aeruginosa* KY 4025 with the yield factor ($Y_{P/S}$) of 0.094 g/g. Yamaguchi *et al.* (1976) determined $Y_{P/S}$ = 0.280 g/g for rhamnolipids produced on *n*-paraffin (50 g/L) by *Pseudomonas* sp. The yield factor ($Y_{P/X}$ = 1.78 g/g) with *n*-hexadecane by EBN-8 was comparable to 1.79 g/g, calculated from the data reported by Shafeeq *et al.* (1989b) with hexadecane as carbon source by *Pseudomonas aeruginosa* S8 strain (the parent of EBN-8).

The parent strain *Pseudomonas aeruginosa* S8, when grown on *n*-hexadecane (1%; w/v), decreased the surface tension of liquid medium from 52 (control) to 33 mN/m (36.54% reduction) and IFT from 26 (control) to 3.0 mN/m (88.5% reduction) against kerosene oil after 10 d of incubation. The emulsification index of CFCB of S8 grown on *n*-hexadecane was 64% and critical micelle dilution was as 6 × CMC. The S8 strain produced 1.9 g rhamnolipids/L, 1.07 g dry cell biomass/L with the yield factor $Y_{P/X}$ = 1.77 g/g at the end of 10 d of incubation. The EBN-8 mutant, in contrast, exhibited 6.77 and 10% more reduction in ST and IFT, respectively, and 9.8, 90.0, 53.66 and 43.68% more

Table II. Yields of crude extract, rhamnolipids, and yields of rhamnolipids related to dry cell biomass ($Y_{P/X}$) and to substrate utilization ($Y_{P/S}$) produced by mutant strain EBN-8 grown on 1% (w/v) hydrocarbon in minimal media at (37 ± 1) °C and 100 rpm in orbital shaker, after 10 d of fermentation. The values are averages from three cultures.

Carbon source	Crude extract	Rhamnolipids	$Y_{P/S}$	$Y_{P/X}$
	[g/L]	[g/L]	[g/g]	[g/g]
n-Hexadecane	9.1 ± 0.5	4.1 ± 0.2	0.53 ± 0.01	1.78 ± 0.02
Paraffin oil	9.6 ± 0.4	6.3 ± 0.3	0.72 ± 0.02	3.15 ± 0.04
Kerosene oil	6.8 ± 0.3	2.9 ± 0.1	0.35 ± 0.01	1.53 ± 0.02

E-24, CMD, rhamnolipids and dry biomass, respectively, when grown on *n*-hexadecane as compared to its parent strain.

Different oily carbon sources were used for biosurfactant production by the EBN-8 mutant in Erlenmeyer flasks in mineral salts media. All of them were found to support bacteria to grow and produce biosurfactant but to different extent, as shown in Fig. 3. The most preferred substrate was paraffin oil, producing 6.3 g rhamnolipids/L and 2.0 g dry biomass/L, and exhibiting 45.3% surface tension reduction of CFCB (with respect to control), CMD as 50 × CMC (Fig. 2), 73% emulsification activity and 0.6 mN/m interfacial tension against kerosene oil, at the end of 10 d of fermentation (Table I).

These results confirmed the formation of some biosurfactant (rhamnolipids), thereby allowing the hydrocarbon emulsification in water. It was found that at the earlier stage of growth the surface tension values remained approximately constant (Fig. 1). This might be attributed to the non-availability of the substrate to the bacteria. This noncompliance was compensated by microorganisms themselves, which produced biosurfactant to emulsify the hydrocarbon. This enhanced the surface area of the hydrocarbon, hence its transfer to the cells became feasible (Goma *et al.*, 1973), and resultantly, the biodegradation rate of the hydrocarbons by EBN-8 in the minimal media increased (Fig. 3).

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