Journal of Hazardous Materials 170 (2009) 495–505

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Bacterial biosurfactant in enhancing solubility and metabolism of petroleum hydrocarbons

N.K. Bordoloi, B.K. Konwar*

ONGC Centre for Petroleum Biotechnology, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur-784028, India

ARTICLE INFO

Article history:
Received 19 March 2008
Received in revised form 8 April 2009
Accepted 15 April 2009
Available online 22 May 2009

Keywords:
Petroleum hydrocarbon
Biosurfactant
Solubility
Metabolism

ABSTRACT

Biosurfactant can make hydrocarbon complexes more mobile with the potential use in oil recovery, pumping of crude oil and in bioremediation of crude oil contaminant. In the investigation, bacterial isolates capable of utilizing poly-cyclic aromatic hydrocarbons like phenanthrene, pyrene and fluorene were used. A gradual decrease of the supplemented hydrocarbons in the culture medium was observed with corresponding increase in bacterial biomass and protein. The medium having the combined application of fluorine and phenanthrene caused better biosurfactant production (0.45 g l^{-1}) and (0.38 g l^{-1}) by *Pseudomonas aeruginosa* strains MTCC7815 and MTCC7814. The biosurfactant from MTCC7815 (41.0 μg ml^{-1}) and MTCC7812 (26 μg ml^{-1}) exhibited higher solubilization of pyrene; whereas, MTCC8165 caused higher solubilization of phenanthrene; and that of MTCC7812 (24.45 μg ml^{-1}) and MTCC8163 (24.49 μg ml^{-1}) caused more solubilzation of fluorene. Higher solubilization of pyrene and fluorene by the biosurfactant of MTCC7815 and MTCC7812, respectively enhanced their metabolism causing sustained growth. Biosurfactants were found to be lipopeptide and protein–starch–lipid complex in nature and they could reduce the surface tension of pure water (72 mN m^{-1}) to 35 mN m^{-1}. The critical micelle concentration (CMC) was also lower than the chemical surfactant sodium dodecyl sulphate (SDS). They differed in quantity and structure. The predominant rhamnolipids present in biosurfactants were Rha–C_8–C_{10} and Rha–C_{10}–C_8.

1. Introduction

Hydrocarbons are mostly insoluble in water and their bioavailability is limited by their adsorption in to the soil matrix. According to White [1] petroleum refining and transport activities are major contributors to localize the environmental contaminations. Polycyclic aromatic hydrocarbons (PAHs) are the major contaminants of crude oil and they require physical means to be solubilized in aqueous phase on addition of surfactants. According to Alexander [2], Laha et al. [3] and Kim et al. [4] surfactant amendment may enhance the oil mobility and increase its availability improving the biodegradation rates.

Microbial biosurfactants exert some influence on interfaces in both aqueous solutions and hydrocarbon mixtures. These properties cause micro-emulsions in which micelle formation occurs where hydrocarbons can solubilize in water, or water in hydrocarbons [5]. Generally, biosurfactants are classified in five major groups, viz. glycolipids, phospholipids and fatty acids, lipopeptides (lipoprotein), polymeric and particulate biosurfactant. Biosurfactant production is generally associated with the utilization of hydrocarbons by microbial community. Certain hydrocarbon degrading bacteria and yeast produce appreciable amounts of phospholipids and fatty acids when grown on *n*-alkanes [6]. The saturated fatty acids in the range of C_{12}–C_{14} and the complex fatty acids containing hydroxyl groups and alkyl branches [7]. The spontaneous release and function of biosurfactants are often related to hydrocarbon uptake; therefore, they are predominantly synthesized by hydrocarbon degrading microorganisms.

At low concentrations, surfactants are soluble in water, and with increasing concentrations, they form micelle in solution. The concentration at which micelle begins to form is called the critical micelle concentration (CMC); above the CMC, biosurfactants can solubilize petroleum hydrocarbons in soil–water systems, but some biosurfactants may increase the water solubility of hydrocarbon molecules below the CMC [2]. Therefore, biosurfactants may be useful in degradation of soil contaminating hydrocarbons. The aim of the present investigation was to evaluate biosurfactants and their effective concentration to enhance the biodegradation of petroleum hydrocarbons in the contaminated soil, make the crude oil complex in the under-ground reservoirs more mobile for enhanced oil recovery and better transportation through pipe lines. Generally, biosurfactants are microbial metabolites with the typical amphiphilic structure of a surfactant, where the hydrophobic moiety is either a long chain fatty acid, hydroxy fatty acid, or

* Corresponding author at: Department of Molecular Biology and Biotechnology, Tezpur University (Central), Napaam, Tezpur-784028, Dist. Sonitpur, Assam, India. Tel.: +91 03712 267172.
E-mail addresses: bkkon@tezu.ernet.in, naba@tezu.ernet.in (B.K. Konwar).

0304-3894/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2009.04.136

α-alkyl-β-hydroxy fatty acid and the hydrophilic moiety can be a carbohydrate, an amino acid, a cyclic peptide, a phosphate, a carboxylic acid alcohol, etc. [8]. Physical and chemical properties, surface tension reduction, and stability of the emulsion formed are very important in the search for a potential biosurfactant. Glycolipids, the most commonly isolated and studied biosurfactants, are carbohydrates in combination with long chain aliphatic acids or hydroxy aliphatic acids.

2. Materials and methods

2.1. Biosurfactant producing microorganisms

Biosurfactant producing bacterial strains belonging to *Pseudomonas aeruginosa*: MTCC7812, MTCC7814, MTCC7815, MTCC8163 and MTCC8165 were isolated from petroleum contaminated soil samples collected from different oil fields of Assam Asset. These bacterial isolates were sub-cultured on nutrient agar (1 lit agar 15 g, NaCl 5 g, peptone 5 g, yeast extract 2 g, beef extract 1 g and pH 6.8) or Luria–Bertani medium (LB 1 lit: casein enzymic hydrolysate 10 g, yeast extract 5 g, sodium chloride 5 g, agar 1.5% and pH 7.0) agar plates before use as inoculums.

2.1.1. Media and microbial growth in the presence of PAHs

The mineral salt medium [MSM 1 l: urea 2 g, $(NH_4)_2SO_4$ 2 g, Na_2HPO_4 3.61 g, KH_2PO_4 1.75 g, $Mg_2SO_4{\cdot}7H_2O$ 0.2 g, $CaCl_2{\cdot}2H_2O$ 50 mg, $FeSO_4{\cdot}7H_2O$ 1 mg, $CuSO_4{\cdot}7H_2O$ 50 μg, H_3BO_4 10 μg, $MnSO_4{\cdot}5H_2O$ 10 μg, $ZnSO_4{\cdot}7H_2O$ 70 μg, MoO_3 10 μg and pH 7.0] was used for culturing the bacteria. Analytical grade chemicals were purchased from Merck (Germany). The pH was measured using the Cyberscane 500 pH meter. The pH was adjusted by using 0.1N standard NaOH and 0.1N standard HCl (Merck, Germany). PAHs dissolved in N,N-dimethylformamide (5%, v/v) was taken in a sterile 250 ml Erlenmeyer flask, shaken in an orbital shaking incubator at 200 rpm and 37 °C before addition to the MSM to give a final concentration of 200 mg l^{-1}, as described by Boonchan et al. [9]. Phenanthrene was used as the second or co-carbon source at a final concentration of 100 mg l^{-1}.

Replicated batch cultures were grown in 250 ml Erlenmeyer flasks containing 100 ml of MSM supplemented with pyrene/fluoerene/fluoerene and phenanthrene as the carbon source. All culture flasks were maintained in a rotary shaker incubator at 32 °C, pH 7.0 and 200 rpm. Non-inoculated flasks and those without PAHs were used as controls.

PAHs were purchased from Merck–Schuchardt, Germany; acetone and HPLC grade hexane from Merck Ltd., Mumbai, India; and crude oil was collected from Oil and Natural Gas Commission, Assam, India.

2.1.2. Determination of bacterial growth

The use of pyrene, fluorene, and phenanthrene as the carbon source for the growth of bacteria was assessed by their protein concentration and residual PAHs in the culture medium after 48 and 96 h of culture following the method of Vila et al. [10]. Bacterial dry biomass was determined by pelleting the bacterial cells through centrifugation following the method of Makkar and Cameotra [11]. Prior to pelleting the bacteria, the residual PAH from the culture broth was separated by a mixture of chloroform and methanol as described by Zhang et al. [12]. The protein concentration was measured using the flask contents of duplicate cultures by a modification of the Lowry method [13].

2.2. PAHs biodegradation

PAHs degradation by bacteria was determined by quantitating the amount remained in the culture broth at different time intervals. The residual PAHs in the flasks were extracted with a mixture of chloroform and methanol (v/v, 20:10) as described by Zhang et al. [12]. In the present investigation, the analysis for PAHs was done by using Waters HPLC system, USA (HPLC: Waters 515 Pump). The extracted sample 1 ml was filtered through a 0.4 μm pore sized-filter and an aliquot of 20 μl of the filtrate was analyzed for PAHs content by high performance liquid chromatography (HPLC: Waters 2487 dual λ absorbance detector) on a reverse-phase C_{18} Nova pak column (3.9 mm × 150 mm) by using isocratic elution with acetonitrile–water. Flow rate was adjusted to 1.0 ml min^{-1} and elution of PAHs was monitored at 273, 250 and 253 nm [14]. The decrease in the amount of pyrene, flourene and phenanthrene was estimated by measuring the peak area of UV absorbance, respectively and by comparing with the peak area of the control flasks. The concentrations of pyrene, fluorene and phenanthrene were expressed as mean and standard deviation based on results obtained with triplicate flasks.

2.3. Measurement of pyrene, flourene, phenanthrene and crude oil uptake by bacterial strains

Pyrene, fluorene, phenanthrene and crude oil uptake by bacterial strains was measured by spectrophotometric rate assay as described by Stringfellows and Aitken [14].

Bacterial cells 1.0×10^7 (final volume 3 ml in 20 mM phosphate buffer containing 150 mM NaCl, pH 7) were placed in 3.5 ml quartz cuvettes in Backman DU-530 life science UV/vis Spectrophotometer (USA) and separately taking 60 μg of pyrene, fluorene, phenanthrene and crude oil (in 10 μl of acetone) were injected in to each of the cuvettes. A decreased concentration at A_{273}, A_{250}, A_{253} and A_{254} was measured from 0 s to 60 min following the addition of PAHs and crude oil. PAHs and crude oil uptake was also measured in the presence of suspension of heat-killed cells. From a standard curve of representative PAHs and crude oil, the decreased concentration of PAHs and crude oil was calculated and results thus obtained were expressed as μg of PAHs and crude oil uptake by 1.0×10^7 bacterial cells.

To study the bacterial uptake of biosurfactant-solubilized PAHs and crude oil, stock solutions of PAHs and crude oil (in acetone) were incubated overnight with biosurfactants (0.5 mg/ml) of the respective bacterial isolates at 32 °C and then 60 μg each of biosurfactant-solubilized PAHs and crude oil was injected in to the cuvette containing 1.0×10^7 bacterial cells (final volume 3 ml).

2.4. PAHs and crude oil solubilization assay

PAHs and crude oil solubilization assay was carried out by the 'test tube solubilization assay' as described by Barkay et al. [15]. Aliquots of 60 μg PAHs and crude oil (from 6 mg/ml stock in hexane) were distributed in to test tubes and kept open inside an operating chemical fume hood to remove the solvent. Assay buffer (20 mM Tris–HCl, pH 7) 3 and 1 ml of biosurfactant (0.5 mg/ml) were added. Tubes were capped and incubated in vertical position overnight at 32 °C with shaking (200 rpm) in dark. Samples were filtered through 1.2 μm pore-sized filter (Whatman); 2.0 ml was removed in a clean test tube to which 2 ml hexane was added prior to extraction by vortexing for 2 min. The emulsion was centrifuged at 10,000 rpm for 10 min to separate aqueous and hexane phases. PAHs and crude oil in the hexane extracts were measured spectrophotometrically at A_{273}, A_{250}, A_{253} and A_{254} nm and also by using the calibration curve of PAHs and crude oil in hexane, the concentrations of PAHs and crude oil were determined. Control experiments were also run in parallel where no biosurfactant was added to PAHs and crude oil before extraction with hexane.

Table 1
Bacterial growth and yield of biosurfactant in pyrene and pyrene + phenanthrene containing media in 96 h of culture (mean ± SD).

P. aeruginosa Strains	Carbon source		After 48 h of growth		After 96 h of growth	
	Pyrene	Phenanthrene	Dry biomass ($g\,l^{-1}$)	Yield of biosur-factant ($g\,l^{-1}$)	Dry biomass ($g\,l^{-1}$)	Yd of biosur-factant ($g\,l^{-1}$)
MTCC7815	+	–	0.80 ± 0.01	0.20 ± 0.01	1.00 ± 0.10	0.30 ± 0.01
	+	+	1.00 ± 0.10	0.25 ± 0.01	1.20 ± 0.20	0.50 ± 0.01
MTCC7812	+	–	0.60 ± 0.02	0.15 ± 0.01	0.80 ± 0.01	0.20 ± 0.01
	+	+	0.70 ± 0.01	0.17 ± 0.01	1.00 ± 0.01	0.25 ± 0.01
MTCC7814	+	–	0.90 ± 0.02	0.20 ± 0.01	1.00 ± 0.10	0.26 ± 0.01
	+	+	1.10 ± 0.10	0.18 ± 0.01	1.20 ± 0.2	0.35 ± 0.01
MTCC8163	+	–	0.50 ± 0.01	0.10 ± 0.01	0.80 ± 0.02	0.18 ± 0.01
	+	+	0.72 ± 0.02	0.20 ± 0.01	0.75 ± 0.01	0.23 ± 0.01
MTCC8165	+	–	0.60 ± 0.01	0.18 ± 0.01	0.90 ± 0.03	0.28 ± 0.01
	+	+	1.20 ± 0.10	0.24 ± 0.01	1.40 ± 0.10	0.45 ± 0.02

2.5. Isolation of biosurfactant and determination of surface-active properties

The crude biosurfactant from each bacterial strain was isolated following the method of Makkar and Cameotra [11].

Surface tension and critical micelle dilution (CMD^{-1} and CMD^{-2}) of the biosurfactant were determined using a Du-Nouy Tensiometer (Kruss 9KT Tensiometer, Kruss, Germany) at room temperature (25 °C) with the ring correction mode of the instrument. All surface activities were tested on cell-free culture broths and aqueous solutions of purified biosurfactants. Cells from the fermentative broths were pelleted by centrifugation at 12,000 rpm for 15 min in Sorval 5B centrifuge at 4 °C.

2.6. Determination of chemical composition of biosurfactant

The chemical composition like protein, carbohydrate and lipid in biosurfactans of *P. aeruginosa* strains MTCC7815, MTCC7812, MTCC8163, MTCC8165 and MTCC7814 was determined following the standard methods of Lowry et al. [13], Dubois et al. [16] and Folch et al. [17].

2.7. Properties of biosurfactant

2.7.1. Light absorption by biosurfactants

While separating biomass from the culture broth, the surface activity of different fractions of the biosurfactant was analyzed. For assaying water-immiscible substrates, the recovered residual hydrocarbons were tested for surface activity, but negative results were confirmed by the tensiometer (Kruss 9KT Tensiometer, Kruss, Germany). The clumping biomass recovered by centrifugation or from the interface of aqueous and oil layers was washed with PBS buffer and dried. The same was resuspended in distilled water. The biomass did not show significant surface tension reduction. The biomass after centrifugation and then again mixing with medium did not show surface activity. The bacterial cells and oil-free-aqueous phase showed surface activity, and its acidification resulted in the precipitation of biosurfactant.

The crude biosurfactant obtained after acid precipitation was dissolved in 0.05 M Na_2HCO_3 and subjected to the wavelength scan to determine the light absorption at 190–510 nm to find the maximum absorption (λ_{max}).

2.7.2. Activity of fractionated biosurfactant by chromatography

Crude biosurfactant was purified by using column chromatography and TLC. Biosurfactants were eluted with a linear gradient of methanol (80%) and 10 mM potassium phosphate buffer, pH 6.0 (20%) at a flow rate of 1 ml min^{-1}. Detection was monitored at 210 nm and the peak showing the maximum biosurfactant activity was lyophilized and stored. Specific activity of the purified fraction and that of the crude was defined as the amount of biosurfactant required to reduce the surface tension by 1 mN m^{-1} at 25 °C.

2.7.3. FT-IR spectra of biosurfactant

The IR (Nicolett Impact 410 spectrometer) spectra of the biosurfactant samples were recorded using KBr pellet. Spectra showing the functional group were used to study the composition of the biosurfactant. The absorption spectra were plotted using a built-in plotter. The spectra were obtained from 500 to 4000 wave numbers (cm^{-1}). Samples were prepared by dispersing the solid uniformly in a matrix of dry nujol (KBr) mull, compressed to form an almost transparent disc.

2.7.4. GC analysis of biosurfactant

Analysis of the biosurfactant was performed by using Varian 3800 gas chromatography with FID detector. Helium served as the carrier and make up gas. Liquid biosurfactant samples were analyzed by using CPSil 8 low bleed (30 m × 0.25 mm × 0.25 μm) coupled with a CP-Sil 5 CB low bleed/MS (30 m × 0.25 mm × 0.25 μm) column. The column temperature was 80–240 °C for 30 min with 5 °C/min increment and hold at 240 °C for 30 min. The injector temperature was 240 °C and the injector was in split-less mode. The detector temperature was programmed at 300 °C.

2.8. Statistical analysis

The necessary experiments were carried out thrice and each observation was repeated five times. The mean data, standard deviations and Student's '*t*'-tests were calculated as per standard procedures [18].

3. Results

3.1. Growth of bacteria in PAH containing medium

The bacterial strains were separately cultured in MSM supplemented with pyrene, phenanthrene and fluorene for 12 days. The increase in protein concentration as the index for bacterial growth and utilization of the hydrocarbon component was estimated at an interval of 2 days. Data thus obtained are presented in Tables 1–3.

The concentration of phenanthrene decreased dramatically in the culture medium over the next 12 days. The strains MTCC7815, MTCC7812 and MTCC7814 exhibited the maximum utilization of phenanthrene with the reduction of its concentration to 70, 85 and 87 μg, respectively from the initial application of 180 μg. In the case of pyrene-supplemented medium, no significant growth was observed in the initial 24 h period of culture. MTCC7814 and MTCC8165 exhibited better utilization of pyrene with the reduction

Table 2
Bacterial biomass and yield of biosurfactant in fluorene and fluorene + phenanthrene-supplemented media in 96 h of culture (mean ± SD).

P. aeruginosa strains	Carbon source		After 48 h of growth		After 96 h of growth	
	Fluorene	Phenanthrene	Dry biomass ($g\,l^{-1}$)	Yd of biosur-factant ($g\,l^{-1}$)	Dry biomass ($g\,l^{-1}$)	Yd of biosur-factant ($g\,l^{-1}$)
MTCC7815	+	–	0.60 ± 0.01	0.20 ± 0.01	0.80 ± 0.01	0.30 ± 0.01
	+	+	1.00 ± 0.04	0.28 ± 0.01	1.30 ± 0.06	0.45 ± 0.03
MTCC7812	+	–	0.50 ± 0.01	0.18 ± 0.01	0.80 ± 0.01	0.25 ± 0.01
	+	+	0.60 ± 0.01	0.18 ± 0.01	1.00 ± 0.05	0.29 ± 0.01
MTCC7814	+	–	0.80 ± 0.02	0.23 ± 0.01	0.90 ± 0.01	0.28 ± 0.01
	+	+	1.20 ± 0.05	0.21 ± 0.01	1.50 ± 0.04	0.38 ± 0.02
MTCC8163	+	–	0.70 ± 0.01	0.15 ± 0.02	0.90 ± 0.03	0.20 ± 0.01
	+	+	0.80 ± 0.03	0.23 ± 0.01	1.10 ± 0.1	0.25 ± 0.01
MTCC8165	+	–	0.50 ± 0.01	0.21 ± 0.01	1.10 ± 0.2	0.30 ± 0.01
	+	+	0.80 ± 0.02	0.27 ± 0.02	1.50 ± 0.06	0.35 ± 0.03

Table 3
Reduction of crude oil, phenanthrene, pyrene, and fluorene from the culture medium as acted upon by the bacterial isolates following the addition of biosurfactant (mean ± SD).

Carbon sources	Bacterial isolates	Biosurfactant	Reduction of crude oil and components in the media (μg) in				
			0	15	30	45	60 min
Crude oil	MTCC7815	–	0	6.82 ± 0.15	10.00 ± 0.30	10.24 ± 0.02	10.65 ± 0.14
		+	0	36.88 ± 0.08	40.21 ± 0.06	40.31 ± 0.02	**40.31** ± 0.02
	MTCC7812	–	0	1.62 ± 0.02	2.54 ± 0.21	4.40 ± 0.18	4.49 ± 0.07
		+	0	8.81 ± 0.02	11.37 ± 0.31	11.45 ± 0.06	**11.53** ± 0.77
	MTCC7814	–	0	4.52 ± 0.07	7.87 ± 0.47	9.05 ± 0.10	10.2 ± 0.08
		+	0	42.82 ± 0.14	44.79 ± 0.84	45.92 ± 0.10	**45.92** ± 0.71
	MTCC8163	–	0	3.91 ± 0.09	5.21 ± 0.07	8.38 ± 0.58	10.24 ± 0.08
		+	0	34.14 ± 1.04	35.28 ± 0.22	35.28 ± 0.02	**35.28** ± 0.26
	MTCC8165	–	0	4.43 ± 0.40	8.73 ± 0.05	9.00 ± 0.14	9.14 ± 0.11
		+	0	10.75 ± 0.73	18.64 ± 0.28	19.60 ± 0.37	**20.31** ± 0.52
Phenanthrene	MTCC7815	–	0	7.90 ± 0.20	10.58 ± 0.16	12.30 ± 0.10	12.57 ± 0.16
		+	0	17.08 ± 0.02	18.32 ± 0.07	19.47 ± 0.52	**20.55** ± 0.13
	MTCC7812	–	0	9.12 ± 0.10	9.69 ± 0.15	11.23 ± 0.05	11.72 ± 0.10
		+	0	21.37 ± 0.31	42.00 ± 0.65	43.12 ± 0.10	**43.87** ± 0.79
	MTCC7814	–	0	16.50 ± 0.43	23.50 ± 0.49	23.71 ± 0.08	23.71 ± 0.06
		+	0	14.26 ± 0.38	25.14 ± 0.05	**25.22** ± 0.06	**25.05** ± 0.17
	MTCC8163	–	0	21.12 ± 0.13	22.34 ± 0.09	22.39 ± 0.11	22.44 ± 0.13
		+	0	15.75 ± 0.36	28.12 ± 0.21	28.12 ± 0.18	**28.37** ± 0.54
	MTCC8165	–	0	7.31 ± 0.08	11.30 ± 0.23	10.95 ± 0.23	10.95 ± 0.14
		+	0	42.61 ± 0.55	33.01 ± 0.11	33.26 ± 0.18	**33.84** ± 0.20
Pyrene	MTCC7815	–	0	16.01 ± 0.15	15.02 ± 0.13	15.84 ± 0.30	15.68 ± 0.17
		+	0	18.29 ± 0.48	27.80 ± 0.13	35.12 ± 0.09	**41.70** ± 0.32
	MTCC7812	–	0	5.77 ± 0.28	8.07 ± 0.03	8.26 ± 0.08	8.37 ± 0.04
		+	0	5.50 ± 0.43	20.91 ± 0.22	26.42 ± 0.35	**26.97** ± 0.09
	MTCC7814	–	0	9.19 ± 0.17	20.32 ± 0.09	20.80 ± 0.13	21.77 ± 0.14
		+	0	14.13 ± 0.36	21.45 ± 0.21	21.45 ± 0.13	**21.54** ± 0.05
	MTCC8163	–	0	1.04 ± 0.01	3.40 ± 0.14	3.60 ± 0.16	4.15 ± 0.04
		+	0	8.79 ± 0.08	11.83 ± 0.27	15.33 ± 0.08	**15.67** ± 0.17
	MTCC8165	–	0	7.94 ± 0.75	14.35 ± 0.14	14.87 ± 0.09	15.64 ± 0.17
		+	0	20.43 ± 0.17	20.98 ± 0.19	21.35 ± 0.12	**21.71** ± 0.09
Fluorene	MTCC7815	–	0	2.45 ± 0.19	4.91 ± 0.46	5.94 ± 0.10	8.27 ± 0.16
		+	0	5.15 ± 0.76	9.12 ± 0.22	10.17 ± 0.52	**11.23** ± 0.13
	MTCC7812	–	0	0.48 ± 0.05	6.91 ± 0.17	15.87 ± 0.05	16.17 ± 0.10
		+	0	11.37 ± 0.81	24.27 ± 0.32	24.36 ± 0.10	**24.45** ± 0.79
	MTCC7814	–	0	13.00 ± 0.23	14.14 ± 0.07	14.52 ± 0.08	14.75 ± 0.06
		+	0	21.91 ± 0.28	48.26 ± 0.26	49.04 ± 0.06	**49.56** ± 0.17
	MTCC8163	–	0	4.34 ± 0.25	10.60 ± 0.21	11.30 ± 0.11	11.65 ± 0.33
		+	0	18.71 ± 0.11	20.36 ± 0.0.19	13.80 ± 0.23	**24.49** ± 0.54
	MTCC8165	–	0	4.12 ± 0.02	6.19 ± 0.26	6.70 ± 0.18	6.70 ± 0.14
		+	0	16.30 ± 0.09	16.06 ± 0.07	16.69 ± 0.30	**17.01** ± 0.20
Crude oil	Heat-killed bacterial cells	–	0	0	0	0	0
		+	0	0	0	0	0
Phenanthrene		–	0	0	0	0	0
		+	0	0	0	0	0
Pyrene		–	0	0	0	0	0
		+	0	0	0	0	0
Fluorene		–	0	0	0	0	0
		+	0	0	0	0	0

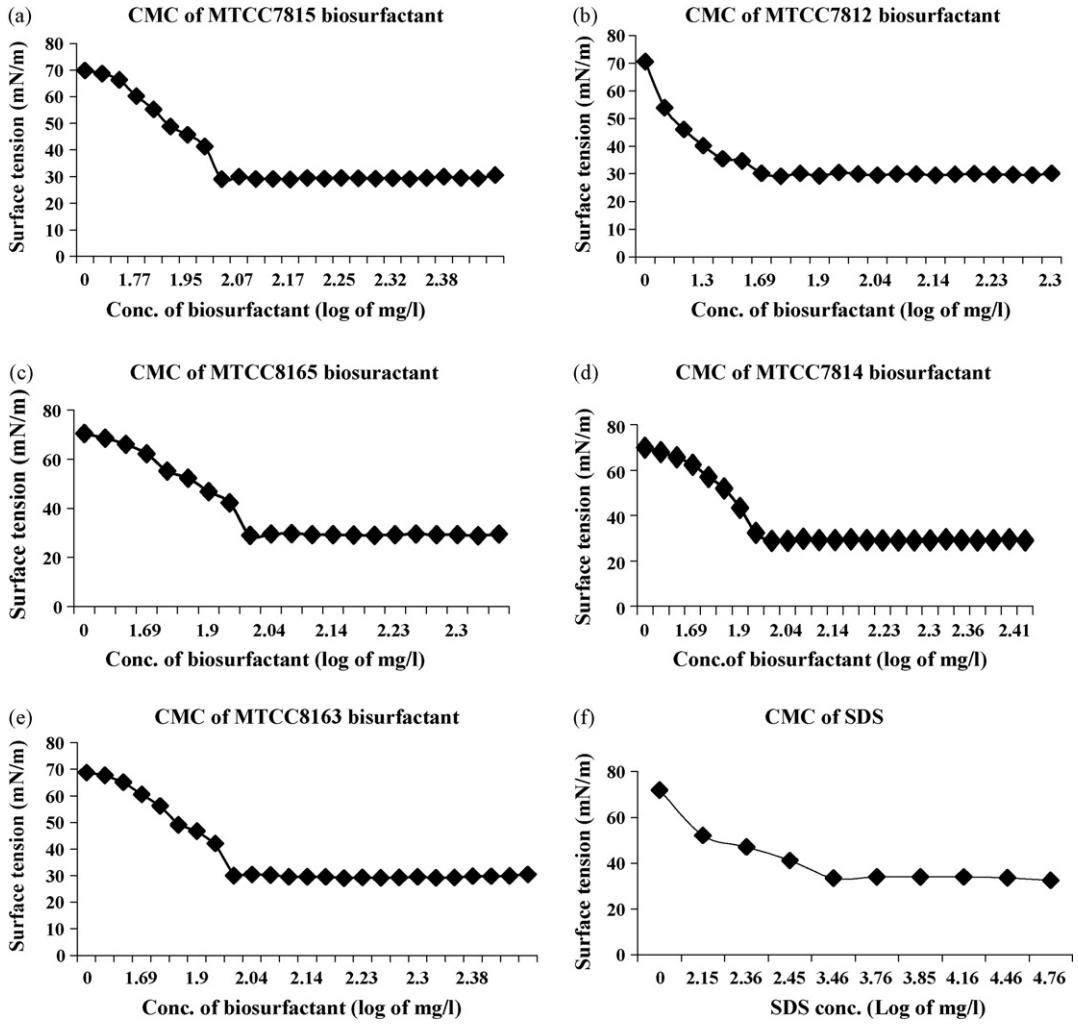

Fig. 1. CMC of biosurfactant from (a) MTCC7815, (b) MTCC7812, (c) MTCC8165, (d) MTCC7814, (e) MTCC8163 and (f) SDS (mean ± S.D.).

of its concentration to 93 and 89 μg, respectively. In fluorene-supplemented medium, MTCC7814, MTCC7815 and MTCC8163 exhibited higher utilization with the reduction of concentration to 89, 90 and 92 μg.

3.2. Biosurfactant production during microbial growth on PAHs

The bacterial isolates produced biosurfactant in pyrene or fluorene-supplemented medium with or without the addition of phenanthrene. Results obtained are presented in Tables 1 and 2. The yield of biosurfactant in the culture supernatant increased dramatically after 96 h of culture. The bacterial isolates exhibited better biosurfactant yield of 0.23–0.5 g l^{-1} at 96 h of culture supplemented with pyrene and phenanthrene as compared to the medium without phenanthrene (0.18–0.30 μg l^{-1}). Concomitantly, the bacterial biomass of 0.7–1.20 g l^{-1} increased to 0.75–1.4 g l^{-1} after 96 h of culture.

The medium having the combined addition of fluorene and phenanthrene caused better biosurfactant yield of 0.45 and 0.38 g l^{-1} in the case of bacterial strains MTCC7815 and MTCC7814, respectively for the entire growth period of 96 h. In the same medium, the bacterial biomass increased from 0.6 to 1.2 g l^{-1} at 48 h of inoculation to a maximum of 1.0–1.5 g l^{-1} at 96 h of culture. The uptake of crude oil and its components increased significantly in all bacterial cultures on addition of biosurfactant.

Uptake of crude oil and PAHs like pyrene, fluorene and phenanthrene was also measured in the presence of a suspension of heat-killed bacterial cells. Heat-killed bacterial strains were cultured separately in the medium supplemented with the addition of biosurfactant; but no uptake was observed.

3.3. Chemical composition of biosurfactant

The chemical composition of biosurfactans isolated from *P. aeruginosa* strains was assayed and the data are presented in Table 4.

The protein and lipid content of MTCC7815-biosurfactant was 26.5 and 32.77%, respectively with a meager 1.2% content of carbohydrate. A similar pattern (protein 25.6 and lipid 31.8%) was recorded in the case of MTCC7812-biosurfactant with the carbohydrate content 1.5% only. But, in the case of MTCC8163-biosurfactant

Table 4
Chemical composition of biosurfactant from the bacterial isolates.

Biosurfactant from *P. aeruginosa strains*	Protein content (%)	Carbohydrate content (%)	Lipid content (%)
MTCC7815	26.50	1.2	32.77
MTCC7812	25.6	1.5	31.8
MTCC8163	20.60	35.75	6.41
MTCC8165	41.25	25.6	15.33
MTCC7814	16.50	40.25	20.28

carbohydrate (35.75%) and protein (20.6%) content was higher as against low lipid content of 6.41%. In the case of MTCC8165 and MTCC7814 protein content was 41.25, 16.5%; carbohydrate 25.6, 40.25%; and lipid 15.33, 20.28%, respectively).

3.4. Critical micelle concentration (CMC) of bio- and chemical surfactants

The CMC values of the biosurfactants were determined by separately measuring the surface tension of different concentrations (log of mg l^{-1}) of bacterial biosurfactants and data thus obtained are presented in Fig. 1(a–f).

3.5. Solubilization of PAHs and crude oil by biosurfactants

The effect of biosurfactants on the solubility of PAHs (phenanthrene, pyrene and fluorene) and crude oil was determined in the presence of biosurfactant at the rate of 500 μg ml^{-1}. Data obtained are presented in Fig. 4. The solubility of PAHs and crude oil in the mineral salt medium was higher due to the addition of biosurfactant isolated from five bacterial strains MTCC7812, MTCC7814, MTCC7815, MTCC8183 and MTCC8165. Pyrene was solubilized more by the biosurfactant isolated from MTCC7815 (41 μg ml^{-1}) and MTCC7812 (26 μg ml^{-1}).

In the case of crude oil, the biosurfactant from the strains MTCC8165 showed 20 μg ml^{-1} more solubilization as compared to the control. The biosurfactant of the strain MTCC8165 displayed three times more solubilization of phenanthrene as compared to the biosurfactant of MTCC7812. The biosurfactants from MTCC7812 and MTCC8163 with 24.45–24.49 μg ml^{-1} concentration exhibited more solubilization of fluorene.

3.6. Activity of fractionated biosurfactant by chromatography

Fractionation by TLC of the crude biosurfactants isolated from all five bacterial strains MTCC 7815, MTCC8165, MTCC7814, MTCC7812 revealed three spots (S1, S2 and S3) and MTCC8163 revealed two spots (S1 and S2). Each fractionated spot was assessed for surface tension and R_f value calculated. Data obtained are presented in Table 5. The spot S3 of MTCC7815 and S2 of MTCC7812 with the R_f values 0.52 and 0.72, respectively exhibited the maximum biosurfactant activity. Similarly, the crude biosurfactants obtained by acid precipitation of the cell-free supernatant of MTCC8163 and MTCC8165 while subjecting to TLC resolved two (S1 and S2 of MTCC 8163) and three (S1, S2 and S3 of MTCC 8165) spots, respectively. When individual spots were tested for the surface-active properties, the spot S2 of MTCC8163 and MTCC8165 with the R_f values of 0.18 and 0.72, respectively exhibited higher biosurfactant activity in terms of surface tension. These spots were subjected to further purification on the same TLC plate with the solvent system of chloroform:methanol:water (60:30:3, v/v). Fractionation of crude biosurfactant from MTCC7814 by the TLC resolved three spots. When spots were tested for surface-active properties, the spot S3 with the R_f value of 0.53 exhibited higher biosurfactant activity.

3.7. Emulsification stability of biosurfactant

The emulsification index of the biosurfactants in kerosene was determined and data are presented in Table 6.

The emulsification index of the crude biosurfactant from MTCC7815, MTCC 7812, MTCC8163, MTCC8165 and MTCC7814 was found to be 65.33, 60.37, 50.51, 70.34 and 63.33%, respectively. The biosurfactant from MTCC8165 was the most stable with the decay constant of −0.0095 followed by MTCC7814 with the value of −0.0115. The biosurfactant from MTCC8163 with K_d value of −0.0216 was comparatively less stable as compared to the biosurfactant of other strains. The biosurfactants from MTCC8165 and MTCC7814 isolates were 8.2 and 6.8 times more stable than that of SDS, which had the K_d value −0.0788.

Table 5
Isolated biosurfactant fractions from MTCC7815, MTCC7812, MTCC8163, MTCC8165 and MTCC7814.

Fractions	Surface tension reduction (mN/m)	R_f value of spots
P. aeruginosa MTCC7815 cell-free extract	30.2	
Crude biosurfactant of MTCC7815	29.1	
TLC fractions (spots) S1	55.3	0.65
S2	35.8	0.25
S3	29.0	0.52
P. aeruginosa MTCC7812 cell-free extract	32.1	
Crude biosurfactant of MTCC7812	31.7	
TLC fractions (spots) S1	51.2	0.58
S2	31.5	0.72
S3	42.5	0.18
P. aeruginosa MTCC8163 cell-free extract	31.1	
Crude biosurfactant	30.4	
TLC fractions (spots) S1	59.0	0.62
S2	30.4	0.18
P. aeruginosa MTCC8165 cell-free extract	29.5	
Crude biosurfactant of MTCC8165	30.4	
TLC fractions (spots) S1	38.5	0.45
S2	30.3	0.72
S3	52.3	0.24
P. aeruginosa MTCC7814 cell-free extract	32.2	
Crude biosurfactant of MTCC7814	31.1	
TLC fractions (spots) S1	54.5	0.32
S2	38.5	0.25
S3	31.1	0.53

3.8. FT-IR spectra of the biosurfactant

The IR spectra of the biosurfactant from each of MTCC7815, MTCC7812, MTCC 8163, MTCC8165 and MTCC7814 in nujol are presented in Fig. 2(a–e).

3.9. GC–MS of biosurfactant

The biosurfactant purified from the culture media of each bacterial strain was assessed using a Varian GC–MS system and data thus obtained are presented in Fig. 3(a–e) and Table 7. In the assay, the biosurfactants of MTCC7815 and MTCC8163 exhibited peaks at almost the same retention time of 19 min. The pseudo-molecular ion at m/z 355 was the base peak of the spectrum, but some fragment ions were seen at m/z 147, 221,281 and 401. However, the purified biosurfactants from MTCC7812 and MTCC7814 showed peaks at the same retention time of 23 min. The pseudo-molecular ion at m/z 429 was the base peak of the spectrum, but some fragments of the ions were seen at m/z 147, 221, 281, 355 and 475. While the purified biosurfactant from MTCC8163 showed the base peak

Table 6
Emulsification index and emulsion stability of biosurfactants and chemical surfactant SDS (mean ± SD).

Source of crude biosurfactant/chemical surfactant	Kerosene	
	Emulsification index (%)	Decay constant (K_d)
SDS	40.67 ± 1.3	−0.0788
MTCC7815	65.33 ± 1.2	−0.0158
MTCC7812	60.37 ± 1.1	−0.0117
MTCC8163	50.51 ± 1.3	−0.0216
MTCC8165	70.34 ± 1.4	−0.0095
MTCC7814	63.33 ± 1.2	−0.0115

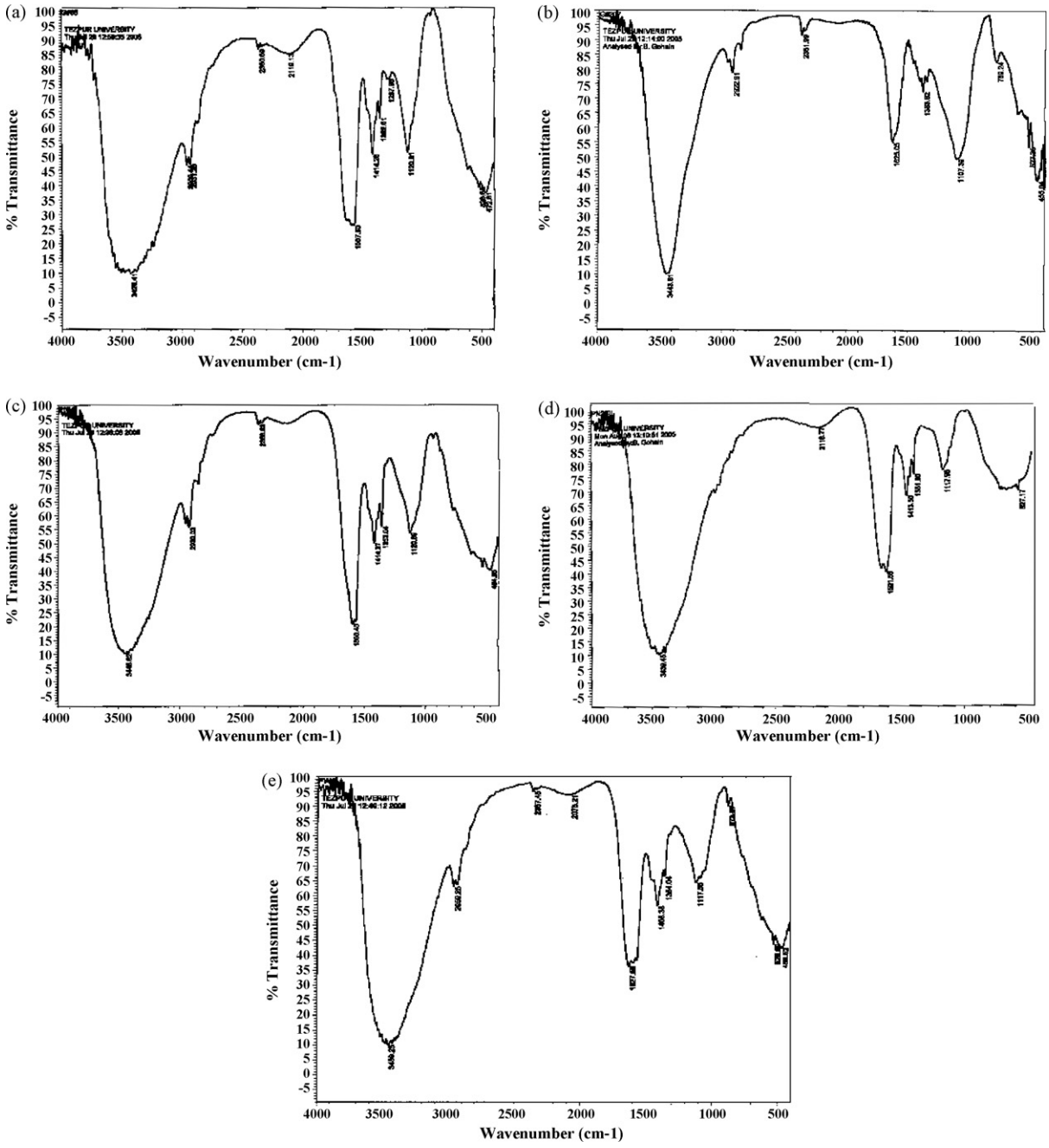

Fig. 2. FT-IR spectra of purified biosurfactant isolated from (a) MTCC7815, (b) MTCC7812, (c) MTCC8163, (d) MTCC8165 and (e) MTCC7814.

of the spectrum at retention time 26 min with fragmentation of ion at *m*/*z* 503, but some fragment ions were also seen at *m*/*z* 147, 221, 281, 355, 429 and 475.

4. Discussion

4.1. Growth of bacterial strains and biosurfactant production

The bacterial strains used in the investigation were able to utilize PAH as the sole source of carbon and energy. This was evident as there was a decrease in the concentration of phenanthrene, pyrene and fluorene (Tables 1–3) in the medium supplemented with PAH with a concomitant increase in the bacterial dry biomass and protein. However, variation was observed in the utilization of phenanthrene, pyrene, fluorene and crude oil as the sole source of carbon and energy by the bacterial strains. There was a decrease in the concentration of these three PAHs in the media with a concomitant increase in the bacterial dry biomass and protein with respect to time. The utilization of phenanthrene (Table 3) by the bacterial strains as the sole source of carbon and energy was confirmed by its removal from the medium, with a corresponding increase in the bacterial protein. The concentration of phenanthrene decreased dramatically in the culture medium during the culture period of 12 days. The biosurfactant produced by the respective bacterial strains caused a significant increase in the metabolism of PAHs for their growth and energy production. Trzesicka-Mlynarz et al. [19] reported the utilization of pyrene, when supplemented with other forms of organic carbon by non-actinomycetes bacteria such as *P. aeruginosa*, *Pseudomonas putida* and *Flavobacterium* species.

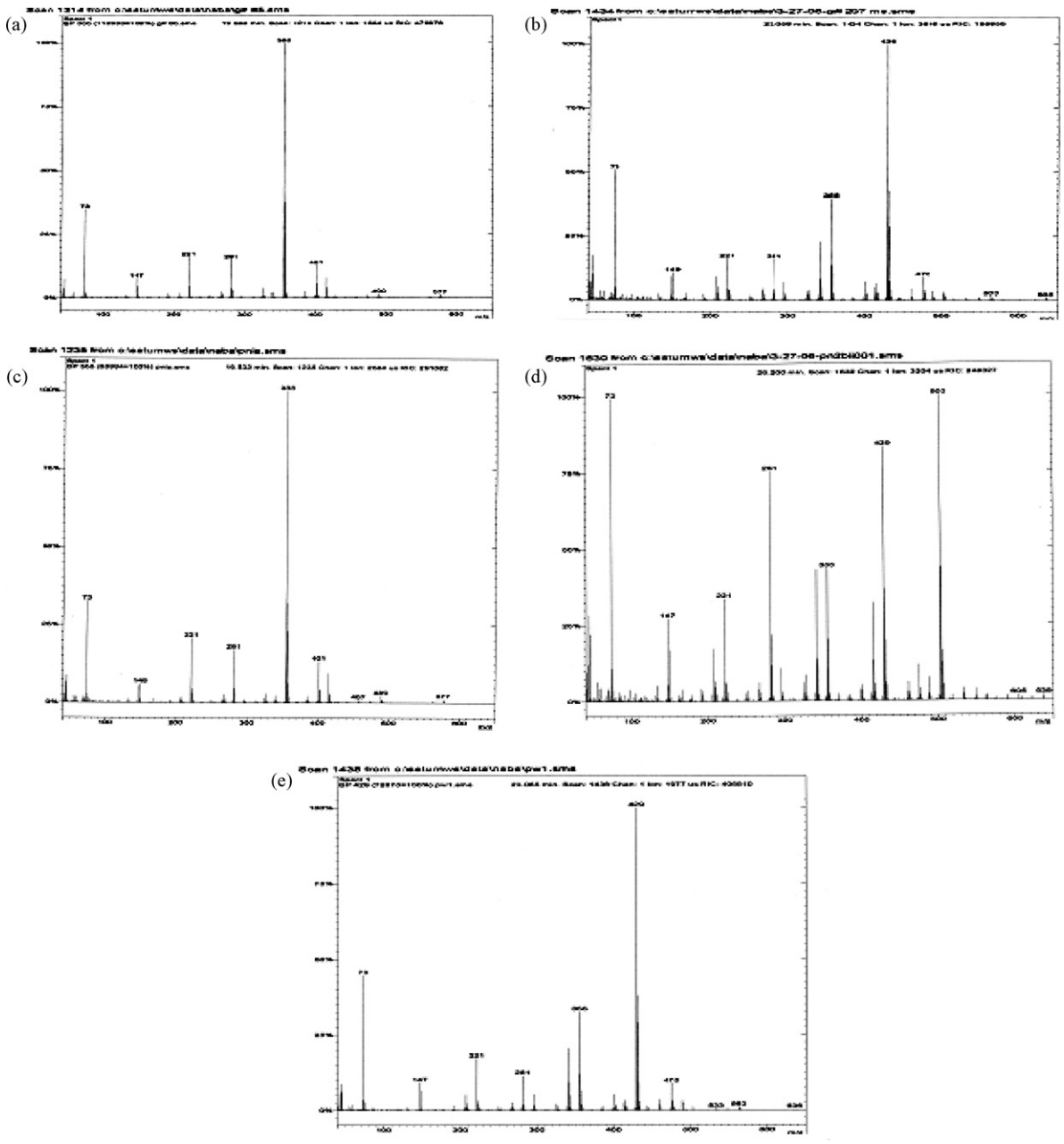

Fig. 3. GC–MS spectra of purified biosurfactants isolated from (a) MTCC7815, (b) MTCC7812, (c) MTCC8163, (d) MTCC8165 and (e) MTCC7814.

The bacterial strains MTCC7814 and MTCC8165 exhibited better utilization of pyrene (Tables 1 and 3) with increased biomass and protein production, and a concomitant reduction in pyrene content from the culture medium. The growth of the bacterial strains at the expense of fluorene (Tables 2 and 3) as the sole source of carbon suggested higher utilization of fluorene as well. The utilization of phenanthrene as the sole source of carbon and energy by the bacterial isolates was confirmed by its removal from the medium with a corresponding increase in the bacterial protein. Biosurfactant production by all five bacterial strains increased significantly when the medium was supplemented with phenanthrene along with pyrene and fluorene (Tables 1 and 2). The yield of biosurfactant in the culture supernatant with acid precipitation increased dramatically. The bacterial isolates exhibited better biosurfactant yield in medium supplemented with pyrene and phenanthrene as compared to the one without phenanthrene. Concomitantly, the bacterial biomass increased. This result might be viewed in the context of increased rate of co-metabolism of pyrene and phenanthrene [20,21]. Stringfellow and Aitken et al. [14] observed that *Pseudomonas saccharophila* P-15 could degrade pyrene on being induced by either phenanthrene or salicylate. Media having the combined addition of fluorene and phenanthrene caused better biosurfactant yield of 0.45 and 0.38 g l^{-1} in the case of bacterial strains MTCC7815 and MTCC7814, respectively for the entire growth period of 96 h. In the same medium, the bacterial biomass increased from 0.6 to 1.2 g l^{-1} at 48 h of inoculation to a maximum of 1–1.5 g l^{-1}. Bodour and Miller-Maier [22] reported the presence of wide diversity among the biosurfactant producing microbes suggesting biosurfactant production to be an important survival tool for the producing microbes and this has evolved to be an independent but parallel process. Biosurfactant producing bacteria are present in higher concentration in hydrocarbon-contaminated soils [23].

Table 7
GC–MS data of biosurfactants along with other traits.

Fraction	Surface tension reduction to (mN/m)[a]	Amount recovered (mg)	Yield (%)	CMC (mg/l)	*m/z* (Da)[b]	Assignment
MTCC7815 CFE	30.2	130.0	100	130	–	
CB	29.1	92.33	71.0	110	–	
TLC fractions S1	55.3	0.115	0.08	140	–	
S2	35.8	0.210	0.16	130	–	
S3	29.0	2.70	2.07	110	147, 191, 221, 281, 327, 355, 401	
MTCC7812 CFE	32.1	125.00	100	140	–	
CB	31.7	75.00	60.0	110	–	
TLC fractions S1	51.2	0.120	0.09	130	–	
S2	31.5	1.85	1.48	110	–	
S3	42.5	0.145	0.11	140	147, 221, 281, 355, 401, 429, 475	Rha–C_8–C_{10} and C_{10}–C_8
MTCC8163 CFE	31.1	135	100	130	–	
CB	30.4	0.95	0.70	110	–	
TLC fractions S1	59.0	0.310	0.22	140	–	
S2	30.4	0.980	0.72	110	147, 221, 281, 355, 401	
MTCC8165 CFE	29.5	152.0	100	140	–	
CB	30.4	0.85	0.55	100	–	
TLC fractions S1	38.5	0.315	0.20	130	–	
S2	30.3	2.50	1.64	100	–	
S3	52.3	0.210	0.13	140	147, 221, 281, 355,429, 475, 503	Rha–C_8–C_{10} and C_{10}–C_8
MTCC7814 CFE	32.2	215.0	100	140	–	
CB	31.1	1.98	0.92	110	–	
TLC fractions S1	54.5	0.145	0.06	140	–	
S2	38.5	0.310	0.14	130	–	
S3	31.1	3.5	1.62	110	147, 221, 281, 355, 401, 429, 475	Rha–C_8–C_{10} and C_{10}–C_8

[a] CFE, cell-free extract.
[b] CB, crude biosurfactant (reduction in surface tension ($mN\,m^{-1}$) by 100 μg of biosurfactant, surface tension of the control was $72\,mN\,m^{-1}$).

4.2. Chemical nature of biosurfactants and their CMC

It was quite evident that biosurfactants from both MTCC7815 and MTCC7812 were lipopeptide in nature; MTCC8163 proteinostarchy; whereas, MTCC8165 and MTCC7814 produced biosurfactant being complex structure of proteins, carbohydrates as well as lipids (Table 4). The TLC (Table 5) fraction S3 of MTCC7815 and S2 of MTCC7812 exhibited the maximum biosurfactant activity. Similarly, the fraction S2 of MTCC8163 and MTCC8165 exhibited higher biosurfactant activity in terms of surface tension.

The Mili Q distilled water was found to have the surface tension of $72\,mN\,m^{-1}$ and the addition of biosurfactant reduced its surface tension to less than $35\,mN\,m^{-1}$. The CMC of biosurfactants from the bacterial strains MTCC7815, MTCC7812, MTCC 8163, MTCC 8165, and MTCC7814 were found to be 100, 110, 110, 100 and $110\,mg\,l^{-1}$, respectively. The CMC value of the chemical surfactant sodium dodecyl sulphate (SDS) was found to be $140\,mg\,l^{-1}$.

4.3. Emulsification stability of biosurfactant

The emulsion formed by the bacterial biosufactants was stable as was evident from the result of K_d (Table 5). The biosurfactant emulsion of each isolate was more stable than that of the tested chemical surfactant sodium dodecyl sulphate. MTCC8165-biosurfactant was the most stable with the decay constant of −0.0095 followed by MTCC7814 (K_d −0.0115). However, MTCC8163-biosurfactant with K_d value of −0.0216 was comparatively less stable. The biosurfactant of MTCC8165 and MTCC7814 was 8.2 and 6.8 times more stable than the SDS having a high K_d value of −0.0788.

4.4. Properties of biosurfactants

The spectra (Fig. 2) showed strong absorption bands at $3443\,cm^{-1}$ is found due to stretching vibration of –O–H group. The absorption at $1605–1625\,cm^{-1}$ is possible due to either stretching of –C=C or)C=O, stretching of carboxylate anion. This material behave as surface-active agent, therefore we presume the absorption at $1625\,cm^{-1}$ is due to presence of carboxylate anion. The absorption at $1120\,cm^{-1}$ is due to stretching vibration of –C–O–C of ether linkage. In the present investigation, the rhamnolipids with the longest fatty acid side chain tended to have slightly shorter retention time. The rhamnolipids produced by MTCC7812, MTCC8165, and MTCC7814 differed in both quantities and in structure (Table 7). Therefore, GC/MS spectrometry (Fig. 3) was used as a tool to elucidate the probable structure and determine to the molecular mass. On comparison of mass data obtained for active fractions and the mass numbers of rhamnolipid and lipopeptide complexes isolated from other bacterial strains by Bouchez et al. [24], Pajarron et al. [25] and Deziel et al. [26]; glycolipids of the above three bacterial strains could be identified as Rha–C_8–C_{10} and Rha–C_{10}–C_8. But, it could not be ascertained whether they were made up one or more fatty acid chains. Syldatk et al. [27] obtained hydrophilic rhamnolipids Rha–C_{10} and Rha–Rha–C_{10} from culture supernatants of resting *Pseudomonas* sp. DSM 2874. Their relative abundance was not reported, but they were apparently present in lower quantity than their two fatty acid-containing analogues, as was also reported by Arino et al. [28]. They reported the rhamnolipid profile of *P. aeruginosa* strain GL1 which was isolated from hydrocarbon-contaminated soils. The bacteria were grown with glycerol as carbon source. They observed a variety of mono- and dirhamnolipids containing one or two 3-hydroxy fatty acids and one or two rhamnoses represented 90% of all rhamnolipids. The fatty acids were predominantly C_{10} with some C_8, $C_{12:1}$ and C_{12}. Bouchez et al. [24] reported the rhamnolipids produced by a *Pseudomonas* species. They only observed Rha–C_{10}–C_{10} and Rha–Rha–C_{10}–C_{10}.

In most of the cases where some quantification results were presented, the predominant rhamnolipids were Rha–C_8–C_{10} and Rha–C_{10}–C_8 (Table 6). The most abundant 3-hydroxyl fatty acids were also C_8 and C_{10} observed in rhamnolipids. Deziel et al. [26] reported that the unsaturated fatty acid was always found at the terminal end of rhamnolipids.

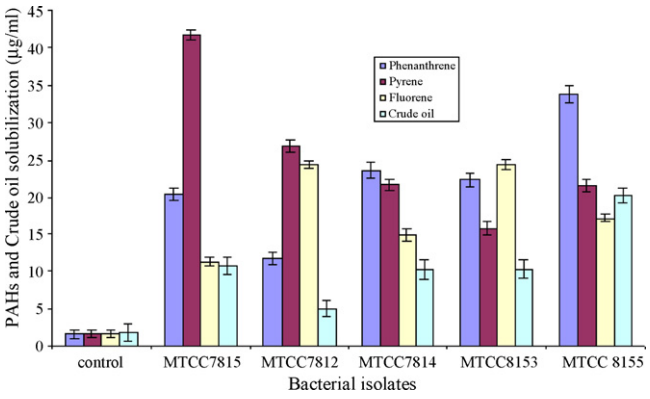

Fig. 4. Solubilization of PAHs and crude oil by the bacterial biosurfactants (mean ± S.D.).

In the present investigation, the biosurfactants produced by the bacterial strains MTCC7815 and MTCC8163 might be nearly identical demonstrating only minor differences in the isomers. The molecular mass of active biosurfactants from bacterial isolates were detected in the range from *m*/*z* 355 to 677 [29–31], but interestingly the *m*/*z* of the most active biosurfactant isoforms secreted by these two *P. aeruginosa* strains were detected in the range from *m*/*z* 147 to 401.

4.5. Solubilization of PAHs–crude oil and role of biosurfactant in culture media

The bacterial biosurfactant enhanced the solubilization of PAHs and crude oil (Fig. 4). Pyrene was solubilized more by the biosurfactant of MTCC7815 and MTCC7812; phenanthrene by MTCC8165; fluorene by MTCC7812 and MTCC8163; and crude oil by the biosurfactant of MTCC8165. The bacterial isolates MTCC7814, MTCC7815 and MTCC8163 showed the highest uptake of crude oil in a period of 60 min following the addition of biosurfactant. The crude oil components phenanthrene, pyrene and fluorene were utilized more in biosurfactant-supplemented media by the bacterial strains MTCC7812, MTCC7815 and MTCC7814, respectively.

5. Conclusion

The bacterial strains could utilize PAH as the sole source of carbon and energy which was evident from the decreased concentration of phenanthrene, pyrene and fluorene in the culture medium. The bacterial isolates exhibited better biosurfactant yield in the medium supplemented with pyrene and phenanthrene. Biosurfactants from the bacterial isolates MTCC7815 and MTCC7812 were lipopeptide in nature; MTCC8163 proteino-starchy; whereas, MTCC8165 and MTCC7814 were complex structure of proteins, carbohydrates and lipids. There was the presence of an aliphatic chain in the C–H stretching with two lactone carbonyl groups, where as, in the C=O stretching there was one. Glycolipids in the biosurfactants produced by the bacterial isolates were Rha–C_8–C_{10} and Rha–C_{10}–C_8. The most abundant 3-hydroxyl fatty acids were also C_8 and C_{10}. The molecular mass of the active biosurfactants from the bacterial isolates was *m*/*z* 355–677.

The addition of biosurfactant could reduce the surface tension of water from 72 to 35 mN m^{-1}. The CMC of biosurfactants was lower (100–110 mg l^{-1}) than the chemical surfactant SDS (140 mg l^{-1}). The emulsion formed by the bacterial biosufactant was more stable than that of SDS. The uptake of crude oil and its components increased significantly in all bacterial cultures on the addition of biosurfactant.

Acknowledgments

We gratefully acknowledge the receipt of research grant from the Oil and Natural Gas Corporation, India. We also thank Prof. D. Konwer, Department of Energy, Tezpur University, Napaam for his valuable suggestions during the course of the investigation.

References

[1] K.L. White, An overview of immunotoxicology and carcinogenic polycyclic aromatic hydrocarbons, Environ. Carcinogen Rev. C 4 (1986) 163–202.
[2] M. Alexander, Biodegradation and Bioremediation, Academic Press, San Deigo, CA, 1994, pp. 302.
[3] S. Laha, Z. Liu, D.A. Edwards, R.G. Luthy, Surfactant solubilization of phenanthrene in soil-aqueous systems and its effects on bioremediation, Ads. Chem. Ser. 244 (1995) 339–361 (Aquatic Chem.).
[4] H.S. Kim, E.J. Lim, S.O. Lee, J.D. Lee, T.H. Lee, Purification and characterization of biosurfactants from *Nocardia* sp. L-417, Biotechnol. Appl. Biochem. 31 (2000) 249–253.
[5] I.M. Banat, Biosurfactants production and possible uses in microbial enhanced oil enhanced oil recovery and oil pollution remediation: a review, Bioresour. Technol. 51 (1995) 1–12.
[6] M.C. Cirigliano, G.M. Carman, Purification and characterization of liposan, a bioemulsifier from *Candida lipolytica*, Appl. Environ. Microbiol. 50 (1985) 846–850.
[7] A. Kretschmer, H. Bock, F. Wagner, Chemical and physical characterization of interfacial-active lipids from *Rhodococcus erythropolis* grown on *n*-alkanes, Appl. Environ. Microbiol. 44 (1982) 864–870.
[8] G. Georgiou, S.C. Lin, M.M. Sharma, Surface active compounds from microorganisms, Bio/Technology 10 (1992) 60–65.
[9] S. Boonchan, M. Britz, G.A. Stanley, Degradation and mineralization of high molecular weight polycyclic aromatic hydrocarbons by defined fungal–bacterial cocultures, Appl. Environ. Microbiol. 66 (2000) 1007–1019.
[10] J. Vila, Z. Lopez, J. Sabate, C. Minguillon, A.M. Solanas, M. Grifoll, Identification of a novel metabolite in the degradation of pyrene by *Mycobacterium* sp. strain AP1: actions of the isolate on two- and three-ring polycyclic aromatic hydrocarbons, Appl. Environ. Microbiol. 67 (2001) 5497–5505.
[11] R.S. Makkar, S.S. Cameotra, Production of biosurfactant at mesophilic and thermophilic conditions by a strain of *Bacillus subtilis*, J. Ind. Microbiol. Biotechnol. 20 (1998) 48–52.
[12] H. Zhang, A. Kallimanis, A.I. Koukkou, C. Drainas, Isolation and characterization of novel bacteria degrading polycyclic aromatic hydrocarbons from polluted Greek soils, Appl. Microbiol. Biotechnol. 65 (2004) 124–131.
[13] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[14] W.T. Stringfellow, M.D. Aitken, Competitive metabolism of naphthalene, methyl-naphthalenes and fluorene by phenanthrene-degrading *Pseudomonads*, Appl. Environ. Microbiol. 61 (1995) 357–362.
[15] T. Barkay, S. Navon-Venezia, E.Z. Ron, E. Rosenberg, Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier Alasan, Appl. Environ. Microbiol. 65 (1999) 2697–2702.
[16] M. Dubois, K.A. Giles, J.K. Hamilton, P.A. Roberts, F. Smith, Colorimetric method of determination of sugar and related substances, Anal. Chem. 28 (1956) 350–356.
[17] J. Folch, M. Lees, G.H.S. Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol Chem. (1956) 497–509.
[18] W.W. Daniel, Biostatistics: A Foundation for Analysis in the Health Sciences, 7th edition, John Wiley and Sons, Inc., New York, 2000, pp. 166–167.
[19] D. Trzesicka-Mlynarz, O.P. Ward, Degradation of polycyclic aromatic hydrocarbons (PAHs) by a mixed culture and its component pure cultures obtained from PAH-contaminated soil, Can. J. Microbiol. 41 (1995) 470–476.
[20] A.A. Bodour, K.P. Drees, R.M. Maier, Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils, Appl. Environ. Microbiol. 69 (2003) 3280–3287.
[21] E.J. McKenna, Biodegradation of polynuclear aromatic hydrocarbon pollutants by soil and water microorganisms, in: 70th Annu. Mtg. Am. Inst. Chem. Eng., New York, 1997, p. 335.
[22] A.A. Bodour, R.M. Miller-Maier, Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorganisms, J. Microbiol. Methods 32 (1998) 273–280.
[23] C.E. Cerniglia, Biodegradation of polycyclic aromatic hydrocarbons, Biodegradation 3 (1992) 351–368.
[24] M. Bouchez, D. Blanchet, B. Besnainou, J. P. Vandecasteele, Substrate availability in phenanthrene biodegradation: transfer mechanism and influence on metabolism. Appl. Microbiol. Biotechnol. 43: 952–960.
[25] A.M. Pajarron, C.G. De Koster, W. Heerma, M. Schmidt, J. Haverkamp, Structure identification of natural rhamnolipid mixtures by fast atom bombardment tandem mass spectrometry, J. Glycoconjug. 10 (1993) 219–226.
[26] E. Deziel, F. Lepine, D. Dennie, D. Boismenu, O.A. Mamer, R. Villemur, Liquid chromatography/mass spectrometry analysis of mixture of rhamnolipids produced by *Pseudomonas aeruginosa* strain 57RP grown on mannitol or naphthalene, J. Biochem. Biophys. Acta 1440 (1999) 244–252.

[27] C. Syldatk, S. Lang, F.Z. Wagner, Chemical and physical characterization of four interfacial- active rhamnolipids from *Pseudomonas* sp. DSM 2874 grown on *n*-alkanes, Z. Naturforsch 40C (1985) 61–67.
[28] S. Arino, R. Marchal, J.P. Vandecasteele, Identification and production of a rhamnolipid biosurfactant by a *Pseudomonas* species, Appl. Microbiol. Biotechnol. 45 (1996) 162–168.
[29] C. Chayabutra, J. Wu, L.K. Ju, Rhamnolipid production by *Pseudomonas aeruginosa* under denitrification: effects of limiting nutrients and carbon substrate, Biotechnol. Bioeng. 72 (2001) 25–33.
[30] E. Haba, A. Abalos, O. Ja'uregui, M.J. Espuny, A. Manresa, Use of liquid-chromatography–mass spectroscopy for studying the composition and properties of rhamnolipids produced by different strains of *Pseudomonas aeruginosa*, J. Sur. Det. 6 (2003) 155–216.
[31] M. Benincasa, A. Abalos, I. Oliveria, A. Manresa, Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock, A. van Leeuw. 85 (2004) 1–8.