Indian Journal of Marine Sciences
Vol. 32(3), September 2003, pp. 202-207

Biosurfactants production by the quinoline degrading marine bacterium *Pseudomonas* sp. strain GU 104, and its effect on the metabolism of green mussel *Perna viridis* L.

Joanita Coelho, C. U. Rivonkar*, N. S. Bhavesh., M. Jothi & U. M. X. Sangodkar

Department of Marine Sciences and Biotechnology, Goa University, Taleigao Plateau, 403 206, Goa, India

*[E-mail: cur63@yahoo.com; cur63@rediffmail.com]

Received 18 February 2002, revised 2 July 2003

Biosurfactants produced by bacteria in marine ecosystems are involved in the degradation of hydrocarbons. In the present study, large-scale production of biosurfactants was demonstrated in a quinoline degrading marine bacterium *Pseudomonas* sp. strain GU 104. Studies were also carried out in experimental set ups to understand the effect of biosurfactants, along with the metabolites of quinoline, on the physiology of the green mussel *Perna viridis*. Acetylcholinesterase (AchE), lactate dehydrogenase (LDH), phenoloxidase and α-amylase activities from specific organs were analysed. The findings of the present study indicate that biosurfactant, as well as quinoline intermediates, produced by *Pseudomonas* sp. strain GU 104 do not have a significant effect on the physiology of *Perna viridis*.

[**Key words**: Biosurfactants, enzymes, green mussel]

The large scale production and transportation of petroleum and its products has resulted in dispersion of oil in the marine environment through various activities. A diversified group of petroleum products are amenable to microbial degradation. The activities of microbes involving various complex biodegradation pathways produces a series of primary metabolites[1,2] which act as synergistic intermediates that accelerate the process of biodegradation. One class of these compounds is susceptible to microbial action, thereby solubilizing the substrates, and displays distinct features of surfactants. To understand the effect of the biosurfactant produced by *Pseudomonas* sp. strain GU 104 on a sentinel organism of the marine environment, the effect of biosurfactant on the green mussel *Perna viridis* was tested in a marine experimental set up. These edible bivalves are sedentary in nature and are widely used as indicator species for environmental pollution[3]. *Perna viridis* is a filter feeder (0 – 62 μm), mainly on microscopic organisms from the aquatic medium. The activities of key enzymes of the mussel such as acetylcholine esterase, lacate dehydrogenase, phenol-oxidase and α-amylase were used as criteria for the physiological impact on the bivalves. The present investigations attempt to evaluate the impact of synthetic surfactant and biosurfactant produced by *Pseudomonas* sp. strain GU 104 on the physiology of the green mussel using the activity of enzymes.

*For correspondence

Materials and Methods

The production of biosurfactant, along with quinoline intermediates such as 2,4,6-hydroxyquino-line, 2,4-dimethyl quinoline and 8-hydroxycoumarin, was obtained[4] using a quinoline degrading marine bacterium *Pseudomonas* sp. strain GU 104 isolated from coastal waters of Goa[4]. The culture was grown on artificial seawater medium (~ 35 ‰) enriched with Bombay High crude oil and other hydrocarbons like n-hexadecane, petrol and kerosene at a concentration of 0.2 % v/v. Cells were cultivated in 250 ml conical flasks containing 100 ml sterile medium supplement-ed with 0.02 % quinoline on a rotary shaker at 32-34°C. Bacterial growth was monitored regularly by measuring the optical density at 600 nm with a spectrophotometer (Spectronic 1201). Large-scale production of biosurfactant, along with quinoline intermediates, was achieved in a 1 litre fermentor (BIOFLO model 7013, New Brunswick Scientific Co., USA) using mannitol (2 %) as a carbon source. Reiling *et al.*[5] have reported that *Pseudomonas* sp. has been used to yield biosurfactant from glucose and mannitol sources. Hence, mannitol was used in the subsequent work along with crude oil. Production of

the biosurfactant by the bacterial strain was confirmed by the reduction of surface tension in the medium. During the process of production of biosurfactant, a series of intermediate compounds were observed.

Isolation of crude biosurfactant

A crude preparation of biosurfactant was obtained following the method described by Reddy *et al*[6]. The culture broth obtained after a period of six days was filtered and centrifuged at 12,000 g for 30 minutes to separate the cells from other debris. The supernatant was treated with three volumes of chilled acetone and allowed to stand for 12 hrs at 4°C. The precipitate thus obtained was washed with n-hexadecane, collected by centrifugation (6000 g for 15 minutes) and dried at room temperature.

Determination of emulsification activity of the crude biosurfactant

The isolated crude biosurfactant was dissolved in double distilled water at a concentration of 1 mg ml^{-1} to which 30 µl of n-hexadecane was added and the mixture was shaken vigorously for two minutes. The mixture was allowed to stand for 10 minutes and the optical density was measured[6] at 610 nm. The increase in optical density with respect to the control was measured. One unit of the emulsification index is defined as one unit change in the optical density at 610 nm. The emulsification index with different carbon sources for cell free supernatant and crude samples were calculated (Table 1).

Measurement of surface tension

The surface tension was measured at 25°C using Traube's stalganometer and the equation

$$\gamma \text{ (surface tension)} = \gamma_o \text{ x } (m\ m_o^{-1})$$

where γ_o is the surface tension of double distilled water, m_o is the mass of double distilled water per one drop and m is the mass of sample for one drop.

Table 1 — Emulsification index (D_{610}±SD) in different carbon sources

Substrate	Emulsification index (D_{610})	
	Cell free culture supernatant	Crude sample
Crude oil	0.415±0.010	0.061±0.007
n-Hexadecane	0.385±0.027	0.042±0.009
Kerosene	0.301±0.026	0.024±0.006
Mannitol	0.308±0.031	0.012±0.004
Glucose	0.286±0.037	0.016±0.007
Sucrose	0.278±0.017	0.009±0.005

Measurement of cell hydrophobicity

A modified assay of microbial adhesion to hydrocarbon[8] was used to determine changes in cell surface hydrophobicity during growth using crude oil and mannitol as substrates. The sedimented cells were washed twice with n-hexadecane for making them completely free from any adhering hydrocarbons. The amount of optical density absorbed at 550 nm of the aqueous phase with respect to that of the initial cell suspension was measured and hydrophobicity was estimated by calculating the percentage of cells adhering to n-hexadecane[9].

Gas chromatographic analysis of hydrocarbon

The hydrocarbons present in the ethyl acetate extracts of fermentation broths of *Pseudomonas* sp. strain GU104 grown on quinoline were determined by gas chromatographic analysis (Schimadzu GC 14B model, Japan) through a capillary column programmed at 6°C $minute^{-1}$ using nitrogen as carrier gas.

Development of marine experimental set-up

The experimental set-up consisted of glass culture tanks ($10 \times 10 \times 10$ inch) with a tapering bottom connected to an outlet for elimination of faeces in order to maintain water quality (Fig. 1). Seawater in the experimental set-up was continuously aerated through an online compressor pump fitted with an air filter. Twelve mussels were maintained in each experimental tank with a water capacity of 15 litres.

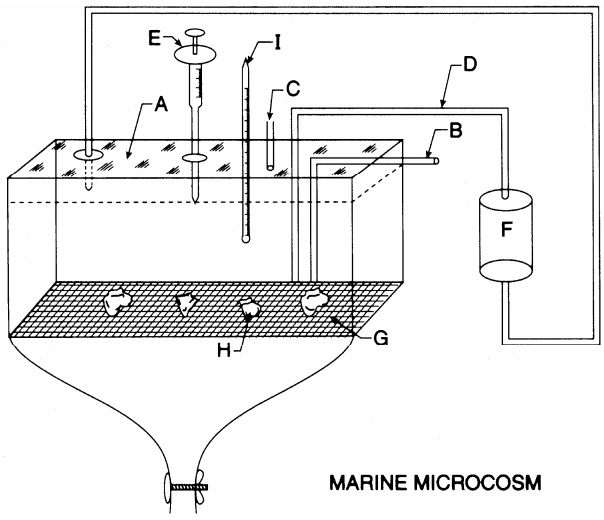

Fig. 1 — A model of the experimental set-up used in the present study (A = Thermocol cover to seal aquarium tank, B = Air inlet, C = Air outlet, D = Water circulation tube, E = Biosurfactant and Synthetic surfactant addition point, F = External sand filter, G = False bottom, H = Mussels, I = Thermometer)

The mussels (25-30 mm) were collected from a rocky shore from a distance of about 5 km from the laboratory. No supplementary feeding was adopted for the mussels during the experimental period. The *in vitro* studies on risk assessment of the biosurfactant was performed in ten experimental tanks. Only one control was maintained whereas other experimental tanks were maintained in triplicate (SLS 0.001 %, BS 5 %; BS 10 %). The quantity of bisurfactant added to each 15 liter experimental set-up was 750 ml for 5 % concentration, and 1500 ml for 10 % concentration. In the case of a tank with SLS (Sodium Lauryl Sulphate; 0.001 %), 0.15 ml was added to a water tank capacity of 15 litres using a micropipette. The water in the culture tank was replenished every alternate day, wherein 25 % of water was exchanged and, accordingly, biosurfactants and SLS were added so as to maintain the same concentration of the surfactants. The control tank with artificial seawater (~ 35 ‰) was also replenished every alternate day. The experimental tanks were maintained at 26°C; *p*H 7.6 and a natural photoperiod of 12 hours light and 12 hours darkness. In the present study, specific activity of the below mentioned enzymes was measured among the mussels exposed to biosurfactants at concentrations of 5 % and 10% (v/v). Sodium lauryl sulphate was used as control without biosurfactant at a concentration of 0.001 %. The experiments were conducted over a period of 40 days.

Acetylcholinesterase (AchE) assay

Live mussels were removed from the experimental culture tanks at an interval of four days and sacrificed for AchE activity. Mussels were deshelled and the tissue was homogenised in phosphate buffer (*p*H = 7.0) at low temperature (4°C). The homogenate was centrifuged (4000 g at 4°C). Supernatant obtained was used as crude sample for the enzyme and protein assay. Protein analysis was carried out according to Lowry *et al.*[10] using bovine serum albumin as standard. The specific activity of all the enzymes studied in the present investigations was done in triplicate for each sampling day. The assay for AchE activity was done using the method described by Ellman *et al.*[11]. The reaction mixture was prepared with mussel tissue and the same was monitored at 405 nm on a spectrophotometer (Spectronic 1201). One AchE enzyme unit was expressed[12] as change of 0.001 optical densities at 405 nm using a 1 cm cuvette at 25°C. Specific activity was expressed as μ mol $min^{-1}mg^{-1}$protein.

Lactate dehydrogenase (LDH) assay

The mussels were dissected and the gills were washed with 0.25 *M* sucrose solution. Thereafter the gills were homogenized using a teflon glass homogeniser to obtain the gill homogenate. The homogenate was centrifuged at 10,000 g for 10 minutes in a Sorval RC5C centrifuge**.** The resulting supernatant was assayed using lactate as substrate[13]. The specific activity of LDH was expressed as μmol $min^{-1}mg^{-1}$ protein.

Phenoloxidase assay

The byssal gland of mussel was removed and homogenate was prepared using phosphate buffer (*p*H 6.8). The homogenate was centrifuged at 5000 g for 15 minutes. The resulting supernatant was assayed[14] for the phenoloxidase activity and the specific activity of phenoloxidase was expressed as μmol $min^{-1}mg^{-1}$ protein.

α-Amylase assay

The digestive gland of the mussel was removed and homogenised with 0.2 *M* citrate-phosphate buffer (*p*H = 7.0). The homogenate was centrifuged at 7,000 g for 5 minutes. The supernatant was then assayed for α-amylase activity[15]. The specific activity of α-amylase was expressed as μmol min^{-1} mg^{-1} protein.

Results and Discussion

An increasing use of petroleum and its products has resulted in the discharge of oil in the aquatic environment, which due to its toxicity, disrupts the normal metabolism of an organism[12]. These products undergo various physico-chemical changes and are susceptible to biodegradation. During the present study, the production of biosurfactant by marine *Pseudomonas* sp. strain GU 104, which are able to mineralise quinoline and other selected components of crude oil, was monitored by checking the emulsification index in the cell free culture supernatant. The production of biosurfactant using the microbial culture was found to be highest on the eighth day, as indicated by the emulsification index (Fig. 2). Earlier studies[16,17] have demonstrated that microbial populations isolated from coastal water bodies produced biosurfactants under both aerobic and anaerobic conditions. Surface associated bacteria are important in the biodegradation of hydrophobic organic pollutants and surfactants[18]. Among the few sugars such as glucose, sucrose and mannitol used in the present study, mannitol was the most effective for

surfactant production (in both the cell free supernatant and crude sample) as determined by the measurement of the emulsification index (Fig. 2). Cooper *et al.*[19] have also reported the production of biosurfactant using kerosene as a carbon source, showing an increased emulsification activity, and others[20,21] reported that maximum emulsification activity was observed after six days for mannitol and crude oil supplemented media.

Bacterial cultures grown on mannitol and crude oil supplemented media were monitored every alternate day for cell surface hydrophobicity. An increase in the adhering property of n-hexadecane was found in the bacterial culture with the advancement of time reaching a saturation level after six and ten days for mannitol and crude oil, respectively (Fig. 3). Further, it was noticed that the correlation coefficient (γ) between cell surface hydrophobicity and emulsification index showed a positive correlation for the cultures supplemented with mannitol ($\gamma = 0.786$; $p < 0.001$) and crude oil ($\gamma = 0.794$; $p < 0.001$). Deziel *et al.*[9] have computed cell surface hydrophobicity with reference to n-hexadecane which suggests that percentage adherance increases with time. Surface tension in the mannitol supplemented medium decreased from 58 mN m^{-1} to 38 mN m^{-1}, indicating the production of biosurfactant. In agreement, a substantial decrease in the surface tension due to the production of biosurfactant have been reported by earlier workers[21,22].

In order to ascertain the hydrocarbon solubilizing activity of the biosurfactant produced by *Pseudomonas* sp. strain GU 104, the samples extracted with chloroform and the control, were subjected to gas chromatographic analysis. The control showed negligible traces of n-hexadecane (Fig. 4A), whereas the extracts of cell free supernatants demonstrated a peak indicating the production of intermediate compound namely 2- hydroxyquinoline[23] during the production of biosurfactant by

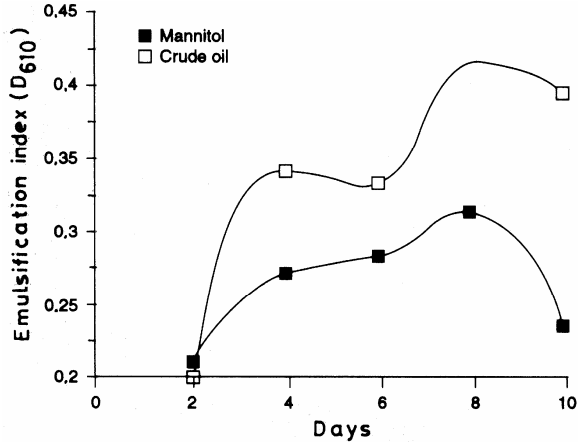

Fig. 2 — Emulsification index (D_{610}) of biosurfactant produced by bacteria grown on mannitol and crude oil.

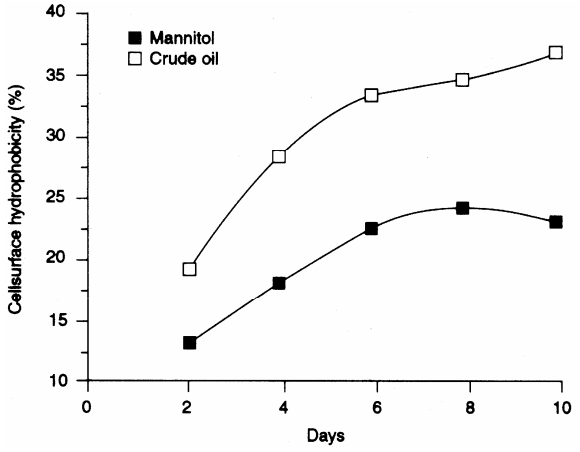

Fig. 3 — Cell hydrophobicity (%) of mannitol and crude oil.

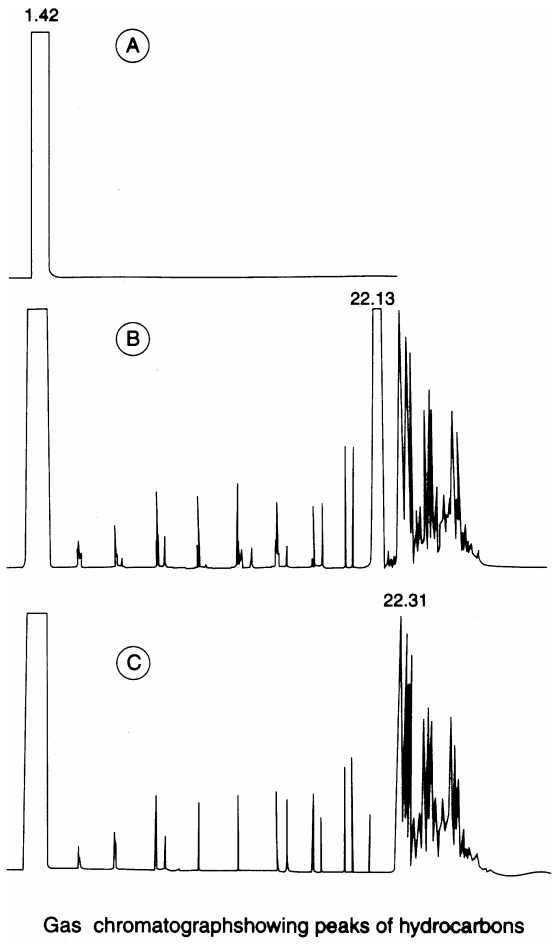

Fig. 4 — Gas chromatograph (A) control, (B) peak corresponding to 2-hydroxyquinoline, (C) peak due to 8-hydroxy coumarin.

Pseudomonas sp. (Fig. 4B). Reddy *et al.*[6] stated that the hydrocarbon-emulsifying factor could cause an appreciable amount of non-specific solubilisation of hydrocarbons in a fermentor. A number of other peaks were also observed in the extracted cell free culture supernatant (Fig. 4C). It can be noticed from the Fig. 4B and C, that there are a series of intermediate products that are produced. The retention time of 22.13 min (Fig. 4B) corresponds to intermediate product namely, 2-hydroxyquinoline, whereas the retention time of 22.31 min (Fig. 4C) corresponds to another intermediate product namely 8-hydroxy-coumarin[23]. Therefore the chromatographic analysis emphasize that a large number of intermediate products are formed during the breakdown of quinoline.

Biosurfactants produced in the marine environment may have an undesirable effect on biological communities[24]. In order to assess and study the influence of biosurfactants on the metabolism and physiology of organisms, *in vitro* experiments with *Perna viridis* were spiked with biosurfactant from the cell free supernatant produced from *Pseudomonas* sp. strain GU 104. Mussels were sacrificed at an interval of 4 days and the AchE, LDH, phenoloxidase and α-amylase activities were assayed. Livingstone[25] reported that activities of various enzymes, including oxidoreductases from the mussel *Mytilus edulis* from polluted and unaffected sites are good as indicators of pollution. Whilst the specific activity of AchE showed a sharp decrease among the mussels grown in sodium lauryl sulphate, there was no effect on specific activities of AchE from the mussels grown in the presence of biosurfactant from *Pseudomonas* sp. strain GU 104. Assays of the specific activity of LDH, AchE, phenoloxidase and ∝-amylase from specific organs for the duration of 40 days revealed a similar trend (Fig. 5). Initially for duration of about 8 days, the specific activity of the enzymes studied was quite stable, then progressively decreased with time in 5 % biosurfactant ($F = 4.215$; $p < 0.005$) and 10 % biosurfactant ($F = 4.60$; $p < 0.001$) (Table 2). Such observations reveal that the biosurfactant produced by the marine bacterial species in the tested concentrations does not affect the enzymatic activity in the mussels, whereas the specific activity of all enzymes of mussels exposed to sodium lauryl

Table 2 — Students' 't' test analysis showing significant levels

Control	Control	BS 5 %	BS 10 %	SLS 0.001 %
Control	—	0.36*	1.72*	3.88**
BS 5 %	—	—	1.26*-	4.215**
BS 10 %	—	—	—	4.60**

*-p < 0.005 SLS – Sodium Lauryl Sulfate
**-p < 0.001 BS – Biosurfactant

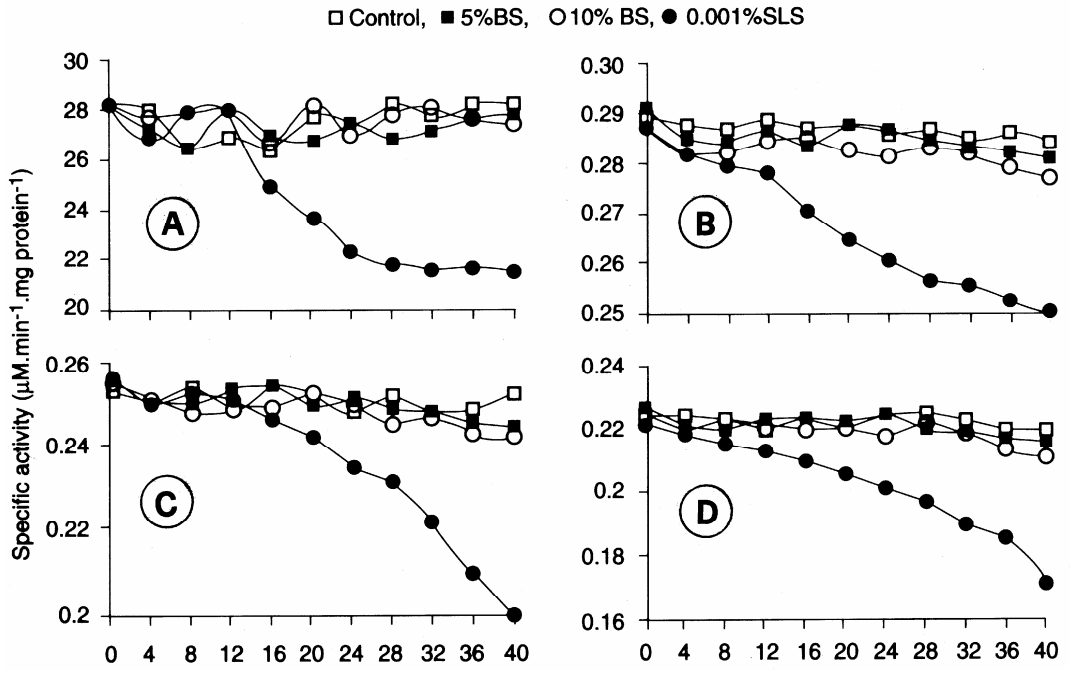

Fig. 5 — Specific activity of enzymes: (A) Acetylcholinesterase expressed as µmol min^{-1} mg $^{-1}$protein, (B) Lactatedehydrogenase expressed as µmol min^{-1} mg $^{-1}$protein, (C) Phenoloxidase expressed as µmol min^{-1} mg $^{-1}$ protein, (D) α-amylase expressed as µmol min^{-1} mg $^{-1}$ protein in control & biosurfactant (5 % and 10 % v/v) and sodium lauryl sulfate (0.001 %).

sulphate showed a marked decline after ten days. However, Koskova & Kozlovskaya[26] carried out similar investigations and showed that synthetic surfactants reduce the rate of fertilisation and development in *Mytilus edulis*. The effect of biosurfactants as compared to synthetic surfactants is found to be less detrimental to the biotic communities. Hence, it appears that propagation of biosurfactants through application of bacterial population as a comparatively viable option for bioremediation of marine oil spills could be explored.

Acknowledgement

The authors are thankful to the Department of Biotechnology, Govt. of India for the financial assistance and support [for grants BT/R&D/11/22/91-IV and BT/PRO 083/R&D/12/14/96]. Technical assistance of T. Walke and S. Sawant are gratefully acknowledged.

References

1 Hales S G, Watson G K, Dodgson K S, & White G F, A comparative study of the biodegradation of surfactant sodium dodecyle triethonisulfate by four detergent degrading bacteria, *J Gen Microbiol,* 132 (1986) 953-961.

2 Griffiths E T, Hales S G, Russel N J, & White G F, Identification of hydrophobic metabolites formed during biodegradation of alkyl ethoxysulfate surfactants by *Pseudomonas* sp. DESI, *Biotechnol Appl Biochem*, 9 (1987) 217-229.

3 Goldberg E D, The mussel watch – A first step in global marine monitoring, *Mar Pollut Bull*, 6 (1975) 111.

4 Coelho J, Fernandes N, Bharadwaj M, Rodrigues J & Sangodkar U M X , Efficacy of marine mixed bacterial cultures in the microcosm for clean up of tar balls, In: *Proceedings of National symposium of Frontiers in Applied Environmental Microbiology,* edited by Mohandas A & Bright Singh I S (Cochin Univ. Sci & Tech., Cochin, India), 1998; pp. 49-53.

5 Reiling H E, Thanneiwyss U, Guerrasantos L H, Hirt R, Kappeli O & Feichter A , Pilot plant production of rhamnolipid biosurfactant by *Pseudomonas aeruginosa*, *Appl Environ Microbiol*, 51 (1986) 985-989.

6 Reddy P G, Singh H D, Pathak M G, Bhagat S D, & Baruah J N, Isolation and functional characterisation of hydrocarbon produced by a *Pseudomonas* sp. *Biotechnol Bioengg,* 25 (1983) 387-401.

7 Morikawa M, Daido H, Takao T, Murata S, Shimonishi Y & Imanaka T, A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain 1538, *J Bacteriol,* 175 (1993) 6459-6466.

8 Rosenberg M, Basic and applied aspects of microbial adhesion at the hydrocarbon:water interface, *Crit. Rev. Microbiol*., 18 (1991)159-173.

9 Dezeil E, Paquetta G, Willermur R, Lepine F & Bisaillon J, Biosurfactant production by soil *Pseudomonas* strain growing on polycyclic aromatic hydrocarbons, *Appl Environ Microbiol,* 62 (1996) 1908-1912.

10 Lowry O H, Rosebrough N J, & Randall R J, Protein measurement with the folin phenol reagent, *J Biochem Chem,*193 (1951) 265-275.

11 Ellman G L, Courtney K O, Andres V & Featherstone R M, A new and rapid calorimetric determination of acetylcholinesterase activity, *Biochem Pharmacol*, 7 (1961) 88-95.

12 Galgani F, Bocquene G, Truquet D, Burgeot T, Chiffolean J F & Claisse D, Monitoring of pollutant biochemical effects on marine organisms of the French coast, *Oceanol Acta*, 15 (1992) 353-364.

13 Vassault, A, Lactatedehydrogenate – U.V. method with pyrite and NADH, In: *Methods in enzymatic analysis,* edited by J Begmeyer & M Grable (Verlag Chemie, Florida) 13 (1985) 118-126.

14 Bharati M S & Ramalingam K, Electrophoretic study of the enzyme phenol oxidase from the enzyme gland in the foot, *Perna viridis* L, *J Exp Mar Biol Ecol,* 70 (1983) 128-132.

15 Sabhapati U & Teo I H, A kinetic study of the α-amylase from the digestive gland of *Perna viridis* L, *Comp Biochem Physiol*, 101B (1992)73-77.

16 Safeeq M, Kokub D, Khalid Z M, Khan A M & Malin K A, Degradation of different hydrocarbons and production of biosurfactant by *Pseudomonas aeruginosa* isolated from coastal waters, *MIRCEM Journal*, 5 (1989) 505-510.

17 Yakimov M M, Timmis K N, Wray V & Fredrickson H L, Characterisation of new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniforms* BAS 50, *Appn Environ Microbiol*, 61 (1995)1706-1713.

18 White G F, Russel N J & Day M J, A survey of sodium dodecyle sulfate (SDS) resistance and alkylsulfatase production from clean and polluted river sites, *Environ Pollut*, 37 (1985)1-11.

19 Cooper D G, Zajii J F & Gerson D F, Production of surface active lipids by *Corynebacterium lepus*, *Appl Environ Microbiol*, 37 (1979) 4-10.

20 Jariyami K L, Wati S R & Joshi SR, Surfactant production by *Pseudomonas stutzcri*, *J Microb Biotechnol,* 7 (1992) 18-21.

21 Javaheri M, Jenneman G E, McInerney M J & Knapp R M, Anaerobic production of a biosurfactant by *Bacillus licheniforms* JF2, *Appl Environ Microbiol*, 50 (1985) 698-700.

22 Mercade M E, Monleor L, de Andrew C, Rodon I, Martinez M J E & Mauresa A, Screening and selection of surfactant producing bacteria from waste lubricating oil, *J Appl Bact*, 81 (1996)161-166.

23 Shukla O P, Microbial transformation of quinoline by a *Pseudomonas* sp, *Appl. Environ. Microbiol*, 51 (1986) 1332-1342.

24 Owen S A, Russell N J, Home W A & White G F, Re-evaluation of the hypothesis that biodegradable surfactants stimulate surface attachment of competent bacteria., *Microbiology,* 143 (1997)3649-3659.

25 Livingstone D R, Responses of microsomal NADPH-cytochrome *C* reductase activity and cytochrome P450 in digestive glands of *Mytilus edulis* and *Littoriana littorea* to environmental and experimental exposure to pollutants, *Mar Ecol Prog Ser*, 46 (1988) 37-43.

26 Koskova L A & Kozlovskaya V I, Toxicity of synthetic surfactant and detergents to aquatic animals – A survey, *Hydrobiol J*, 15 (1979) 67-73.