0273-2289/1559-0291 (Online)/06/134/0129/$30.00

Production and Properties of a Biosurfactant Applied to Polycyclic Aromatic Hydrocarbon Solubilization

B. Mahanty, K. Pakshirajan,* and V. V. Dasu

Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, 781039, India, E-mail: pakshi@iitg.ernet.in

Received November 10, 2005; Revised January 2, 2006; Accepted January 8, 2006

Abstract

Microorganisms isolated from a soil sample collected from a gasoline filling station (located in Guwahati) were tested for their pyrene- and anthracene-degrading potential. Preliminary studies showed the ability of the organism to grow on carbon-free mineral medium (CFMM) supplemented with pyrene as the sole source of carbon. The organisms were found to produce a bioemulsifier when grown on CFMM with glucose or glycerol and/or pyrene as the carbon source. The organisms could also utilize anthracene when grown on mineral salt medium along with 2% glycerol. Within 2 d, anthracene concentration dropped less than 30% of the original concentration. Approximately 100 mg of the emulsifier was isolated from 25 mL of the 5-d-grown culture. The emulsifier was tested to produce emulsion with both an aliphatic and an aromatic group of hydrocarbons and resulting emulsions were found to be stable for a long period of time when kept at 10–15°C. The emulsifier was also quite stable in a pH range of 3.0–11.0. In a concentration range of 0.5–10 mg/mL, it resulted in a linear increment of apparent pyrene and anthracene solubility in water.

Index Entries: Soil microbes; biosurfactant; bioemulsifier; emulsifying activity; polycyclic aromatic hydrocarbons.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) belong to a class of environmental pollutants, which are also the byproduct of incomplete combustion of organic matters. In developed and developing countries, the major sources of PAHs are petroleum products such as coal tar, creosote, and

*Author to whom all correspondence and reprint requests should be addressed.

vehicle emission. Because of the genotoxicity and other toxic effects of PAHs on plant and animal systems and owing to their high molecular weight, remediation of PAHs is of great concern. Although biodegradation of low molecular weight hydrocarbons is reported, reports of biodegradation of compounds with more than a four-ring system are rare. Studies with more than a four-ring system come with inherent difficulty, because their detection methodology is poorly characterized and high-purity commercial standards are unavailable. Environmental bioremediation of PAH-contaminated soil is often limited by its low aqueous solubility. Because PAHs remain tightly bound to the soil matrix, bioavailability is often a limiting factor for biodegradation *(1)*. Enhancing bioavailability of PAHs may be better served by the production of biosurfactant *(2)* in *cis-* from PAHs degrading microbes itself or in *trans-* from other members of the consortium. Biosurfactant enhances the aqueous solubility of the hydrocarbons and, thus, bioavailability via micelle/microemulsion formation or immobilization. Potential applications of biosurfactant or bioemulsifier have gained considerable attention compared to their chemical counterpart.

Biosurfactant can be produced by a diverse group of bacteria, fungi, and yeast and can be of various chemical natures. By evolution bacteria have adapted themselves to feeding on hydrophobic substrates by manufacturing and using a surface-active product that helps the bacteria to adsorb, emulsify, wet, or disperse or solubilize the water-immiscible material. Biosurfactant can also be produced as an antimicrobial agent, in response to other bacteria. Most microbial surfactants are complex molecules, comprising different structures that include lipopeptides, glycolipids, polysaccharide protein complex, fatty acids, and phospholipids.

For potential application of bioemulsifier produced by hydrocarbon-degrading microbes for bioremediation of a contaminated site, it is essential to understand the environmental conditions at which it gives maximum efficiency. In this article, we discuss different aspects of the bioemulsifier produced by PAHs utilizing microbial culture isolated from soil contaminated with hydrocarbon. The soil was obtained from a gasoline filling station. We also report characterization of the bioemulsifier produced based on its solvent specificity and stability.

Materials and Methods

Chemicals

Pyrene (>95% pure) was purchased from Sigma Aldrich, anthracene was purchased from Merck, and all other solvents were from SRL. Media constituents used for mineral media were from HiMedia.

Media and Supply of Carbon Source

The carbon-free mineral medium (CFMM) contained 3.0 g/L of NH_4NO_3, 2.2 g/L of Na_2HPO_4, 0.8 g/L of KH_2PO_4, 0.01 g/L of $MgSO_4 \cdot 7H_2O$,

0.005 g/L of $FeCl_3 \cdot 6H_2O$, and 0.005 g/L of $CaCl_2 \cdot 2H_2O$. The final pH of the medium was adjusted to 7.2 with 0.1 *N* NaOH, and the medium was sterilized by autoclaving at 121°C for 20 min.

PAH stock (100 g/mL) was prepared in dimethyl sulfoxide (DMSO). For production of biosurfactant by the culture, the medium *(3)* used consisted of 4 g/L of Na_2HPO_4, 1.5 g/L of KH_2PO_4, 1 g/L of NH_4Cl, 0.2 g/L of $MgSO_4 \cdot 7H_2O$, 0.005 g/L of iron ammonium citrate, 0.01 g/L of $CaCl_2$, and 2% (w/v) glycerol; the pH was set at 7.2.

Bacterial Growth and Culture Conditions

Hydrocarbon-contaminated soil was collected from a gasoline filling station (located in Guwahati). Bacterial inoculate was obtained by shaking 20 g of the contaminated soil with 100 mL of CFMM supplemented with pyrene and glucose at a concentration of 50 mg/L and 10 m*M*, respectively, at 27°C with agitation (120 rpm) in a rotating orbital shaker for 12 h. Approximately 5 mL of the culture supernatant was mixed with 45 mL of fresh CFMM supplemented with pyrene and glucose and allowed to grow under the same culture conditions. Culture enrichment was obtained by extensive subculturing with pyrene and decreasing the amount of glucose. Culture purity was verified by plating in a nutrient agar plate at every subculturing step.

Degradation of Anthracene

To determine degradation of anthracene by the mixed culture, cells were grown on mineral medium supplemented with 25 mg/L of anthracene, along with 2% glycerol as cosubstrate, at 27°C with shaking (180 rpm). Samples taken at a specific time interval were extracted with an equal volume of DMSO and centrifuged at 18,894*g* for 10 min at 4°C to separate the cells. The supernatant liquid was filtered through a 0.45-µm syringe filter to remove any insoluble anthracene particles. Anthracene was analyzed using an ultraviolet (UV) spectrophotometer (Carry50; Varian) taking a 1:1 mixture of CFMM and DMSO as a blank. Characteristics of the absorbance peak of anthracene were analyzed to correlate the result of standard anthracene absorbance spectra.

Isolation of Biosurfactant

Biosurfactant production was observed by the formation of a stable emulsion when 3-d-old culture supernatant was mixed with any organic solvent. The effectiveness of forming an emulsion was tested with a wide range of organic solvents such as benzene, toluene, xylene, and hexane. Bacterial cells were removed from the culture broth by centrifugation (14,926*g*, 4°C, 15 min). Cell-free broth was again filtered through a 0.45-µm Millipore filter to remove extraneous matter present in the culture supernatant. The filtrate was then saturated with 50% (w/v) ammonium sulfate. After standing overnight at 4°C, the turbid suspension was centri-

fuged (16,000 rpm, 4°C, 20 min). The precipitate so obtained was dissolved in water, dialyzed against deionized water, and lyophilized, yielding partially purified biosurfactant.

Kinetics of Bioemulsifier Production

Biosurfactant production kinetics were analyzed in CFMM supplemented with glycerol. Data were collected at 12-h intervals for five consecutive days. The culture was centrifuged at 14,926*g* at 4°C for 10 min; the cell palette was dried at 50°C until reaching constant weight. The pH of the supernatant was measured. Surfactant concentration was measured indirectly by emulsifying activity and again checked with the amount of ammonium sulfate precipitate (data not shown).

PAH Solubilization Assay

All solubilizations were performed in ddH_2O. Stock solutions (10 mg/mL) of pyrene and anthracene prepared in acetone or hexane were distributed into Eppendorf tubes to yield 500 µg of PAH/tube. The tubes were left open inside an operating chemical fume hood to remove solvents, and 1 mL of biosurfactant solution containing a known amount of biosurfactant was added. All experiments were performed in duplicate. The tubes were capped and incubated overnight at 27°C with shaking. Samples were filtered through a 0.45-µm filter to remove any undissolved particulate PAH. Samples were analyzed for pyrene concentration using a UV spectrophotometer at 337 nm and for anthracene at 360 nm taking surfactant solution of proper concentration as a blank.

PAH Solubilization Kinetics

To determine the kinetics of solubilization, 500 µg of anthracene or pyrene was crystallized in the bottom of a 1-mL quartz cuvet. The cuvet was placed in a holder of a SPEKOL 1200 (Analytik Jena AG) spectrophotometer, and 1 mL of prefiltered (0.45 µm; Millipore) assay solution containing desired concentrations of surfactant was added to the cuvet. Solubilization was carried out without shaking. Samples analyzed for anthracene and pyrene, respectively, were taken at room temperature every 5 min for about 3 h with the parallel nonsynchronized mode of the kinetics software package provided by the manufacturer. Surfactant solution was used as a control. Absorbency readings were converted to concentrations by extrapolation from a standard calibration curve that was constructed by saturated PAH solution in *n*-hexane.

Assay of Emulsification Activity

Emulsification activity was assayed by the method of Cirigliano and Carman *(4,5)*. A diluted 1-mL sample was mixed with 0.5 mL of test organic solvent and vortexed for 2 min. The resulting emulsion was allowed to stand for 2 min, after which its turbidity was measured at 600 nm using a

SPEKOL 1200 (Analytik Jena AG) photoemission diode spectrometer. One unit of emulsification activity was defined as the amount of emulsifier that effected an emulsion with an absorbance of 1.0 at 600 nm.

Solvent Specificity of Bioemulsifier

Different organic solvents, both aliphatic and aromatic, were tested for their emulsifying activity and emulsion stability. Emulsion stability was analyzed with emulsification activity *(4,5)*. Emulsified solutions were allowed to stand for about 30 min at room temperature, and then absorbance readings at 600 nm were taken every 5 or 10 min for about 1 h. The decay constant K_d *(6)*, a measure of stability of the emulsion, is calculated by the following expression:

$$K_d = \frac{d(\log A)}{dt} \tag{1}$$

in which A is the absorbance at 600 nm and t is time.

Effects of Environmental Factors on Bioemulsification

Effect of pH

Culture supernatants (1 mL) of 5-d culture grown in CFMM were adjusted to various pH values in the range of 2.0-12.0 and incubated for 1 h at 4°C. Xylene (0.5 mL) was added into each of them and vortexed for 2 min. The resulting emulsions were tested for their activity and stability as described in the previous section.

Effect of Temperature

Culture supernatants incubated at various temperatures for different time intervals were tested for their emulsification properties. Both emulsification activity and stability of the treated culture supernatants were measured.

Other Analytical Methods

Cell biomass was measured by absorbance at 600 nm**.** Extracellular protein was measured by using the modified Lowry method *(7)* and extracellular glycosides by the phenol–sulfuric acid method *(8)*. Cell hydrophobicity was measured by bacterial adherence to hydrocarbons according to the method described by Zhang and Miller *(9)*. Cell hydrophobicity was determined using kerosene as the organic system, and the following expression was used to quantify the same:

$$\%\ \text{hydrophobicity} = \frac{(A_0 - A_f)}{A_0} \times 100 \tag{2}$$

in which A_0 is the initial absorbance of the cells (optical density [OD] = 600 nm) in aqueous phase; and A_f is the final absorbance of the cells (OD = 600 nm) in aqueous phase after mixing with kerosene and standing for 10 min, to separate the phases.

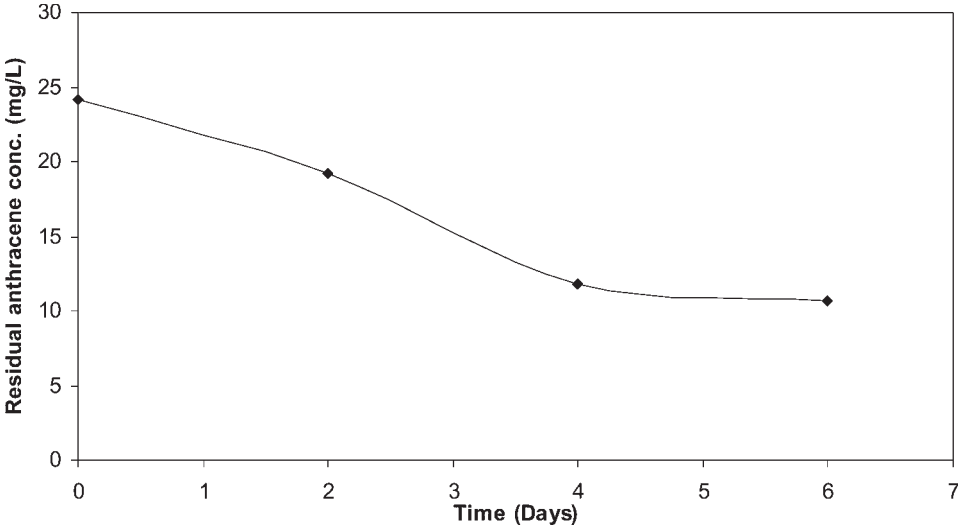

Fig. 1. Anthracene uptake by PAHs utilizing culture.

Results and Discussion

Anthracene Mineralization

Weighted average data from the anthracene absorption spectrum of the sample indicated an approx 60% reduction in the initial concentration in 5 d (Fig. 1). Maximum mineralization was observed within the first 4 d. No appreciable degradation was shown after d 6. All studies were performed along with glycerol as an alternate source of carbon. The rate of anthracene uptake was found to be 3.7 mg/(L·d), comparable with that reported for a *Pseudomonas aeruginosa* strain (3.90 mg/[L·d]) *(10)*.

Bacterial Growth and Emulsifier Production

During the bacterial growth in anthracene (Fig. 2), it was observed that for up to 12 h there was no appreciable change in the emulsifying activity of the culture supernatant. However, after that period and up to 21 h the emulsifying activity increased exponentially, after which it decreased slowly. Because the increase in the amount of bioemulsifier is synchronous to the growth of the microbial culture, it is reasonable to say that bioemulsifier production is growth associated *(11)*. In contradiction to our results, various other investigators *(12,13)* have reported that bioemulsifier production is maximum during the stationary phase of growth.

With respect to the effect of carbon source on bioemulsifier produced by the culture, it was observed that irrespective of the presence or absence of any PAH in the media, the culture supernatant showed maximum emulsification activity when grown in glycerol as cosubstrate. However, when glucose was used as cosubstrate, the emulsification activity of the culture supernatant was less (data not shown). This difference in emulsification

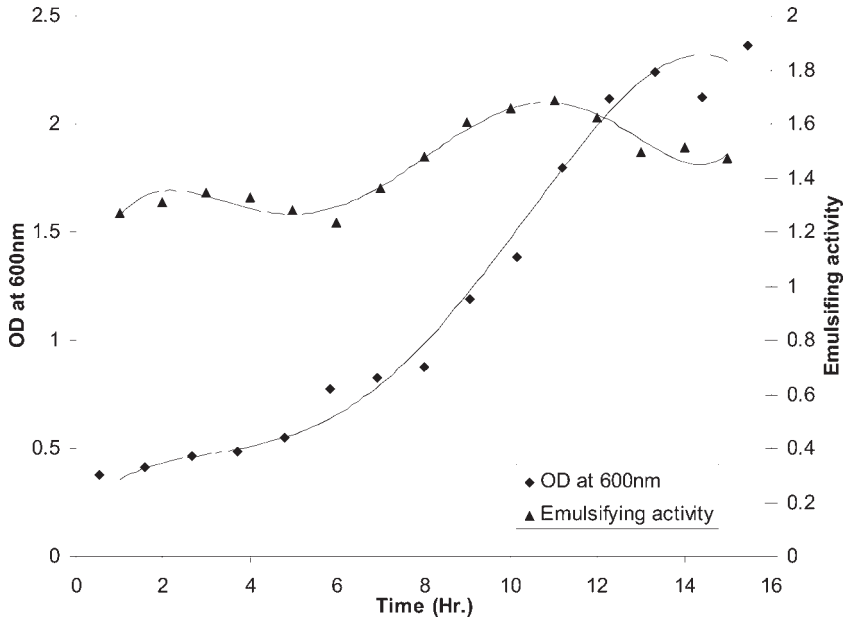

Fig. 2. Relationship between bioemulsifier production (emulsifying activity) and culture growth (OD at 600 nm).

activity of the culture supernatant obtained from glucose- or glycerol-containing medium may be owing to the difference in chemical composition of the two substrates. This also indicates that the microbial culture is able to produce a bioemulsifier without any inducer such as PAHs.

Isolation and Purification of Bioemulsifier

It is known that bioemulsifiers produced by a microorganism may be of various types based on their chemical composition *(11)* such as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, and polymeric and particulate compounds. Preliminary investigations aimed at isolating the bioemulsifier produced were based on chloroform-methanol (for lipid nature) and ammonium sulfate precipitation (for protein). A concentrated bioemulsifier was obtained when culture supernatant was subjected to ammonium sulfate precipitation. The precipitate so obtained was dialyzed overnight against distilled water to obtain partially purified product. The supernatant remaining after ammonium sulfate precipitation showed no emulsifying activity with either xylene or *n*-butanol, indicating that the bioemulsifier was completely extracted.

Protein assay based on a modified Lowry method *(7)* showed that the partially purified bioemulsifier contained 10% protein (bovine serum albumin [BSA] equivalent). The phenol–sulfuric acid method *(8)* for determining total carbohydrate (as described in Materials and Methods) showed 7% of carbohydrate content in the bioemulsifier.

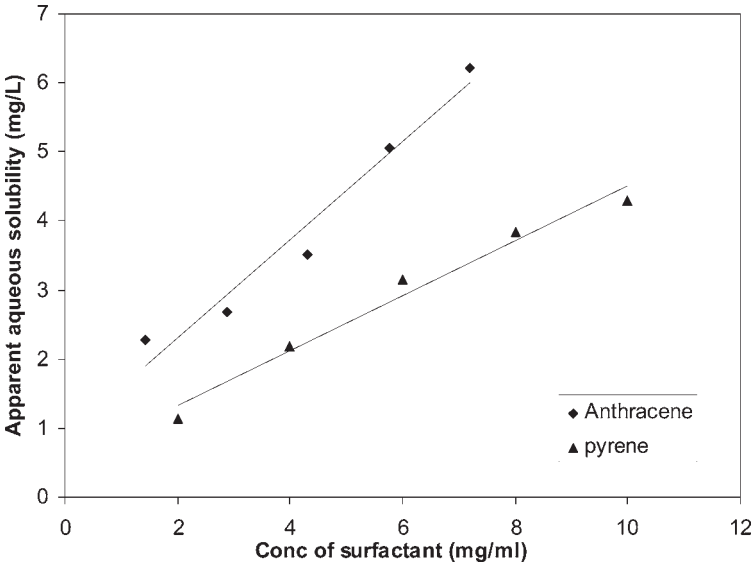

Fig. 3. Apparent aqueous solubility of pyrene and anthracene in presence of varying concentrations of bioemulsifier.

PAH Solubilization Studies

The intrinsic aqueous solubility of pyrene and anthracene is 0.175 and 0.08 mg/L, respectively *(14)*. This low solubility is one of the limiting factors affecting their bioavailability and subsequent mineralization. They can also emulsify hydrophobic compounds, form stable emulsions, and increase PAH solubility and consequently bioavailabity in the environment *(15)*. Figure 3 shows the apparent aqueous solubility of pyrene and anthracene in the presence of bioemulsifier. It is clear that the apparent solubility increased linearly with bioemulsifier concentration. The correlation coefficient (R^2) was found to be 0.96 and 0.97 for anthracene and pyrene solubility, respectively, owing to the bioemulsifier. The solubility of anthracene and pyrene was found to improve by about 78 and 25 times in the presence of 7.2 and 10 g/L of bioemulsifier, respectively.

PAH Solubilization Kinetics

The increase in anthracene and pyrene concentration in solution was monitored to evaluate how the surfactant affected the solubilization kinetics. Solubilization was rapid, reaching half of its final magnitude within the first 120 and 60 min for anthracene and pyrene, respectively, for a surfactant concentration of 7.2 g/L (Fig. 4). From the slope of the initial anthracene and pyrene solubilization kinetic curves, we observed that the rate of solubilization of pyrene (0.0333 mg/[L·min]) was faster compared to anthracene (0.0137 mg/[L·min]). The final equilibrium concentrations of the PAHs obtained owing to the bioemulsifier were 4.75 and 3.39 mg/L for pyrene and anthracene, respectively.

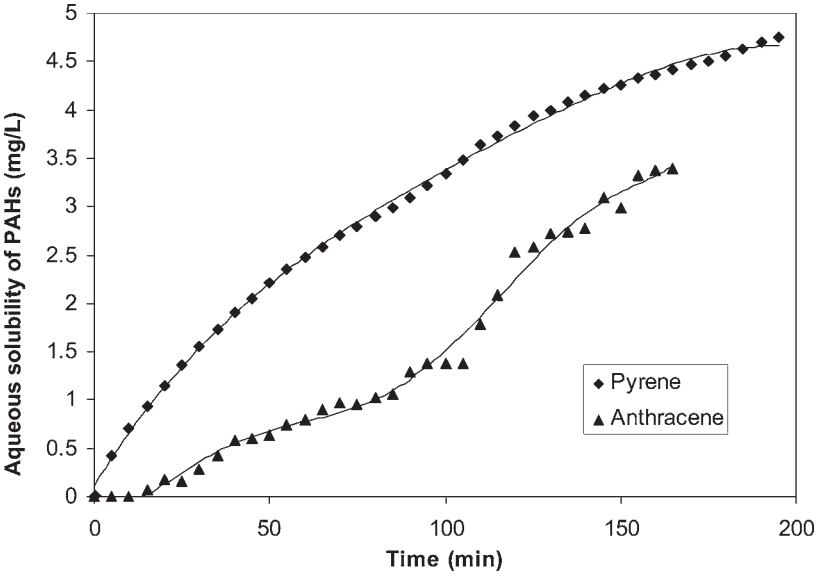

Fig. 4. Solubilization kinetic profiles of anthracene and pyrene with surfactant (7.2 g/L).

Bioemulsifier Specificity, Activity, and Stability

The emulsification property of a compound depends on the organic solvent system used, i.e., aliphatic or aromatic. In the present study, the specificity of emulsion formation was highly variable, depending on the carbon source used in the growth medium of the culture. Although the culture grown on medium containing glycerol as the sole carbon source showed emulsification activity against aliphatic hydrocarbons such as *n*-butanol and *n*-hexane, it lost its activity toward aromatic hydrocarbons such as benzene, toluene, and xylene. However, when the culture was grown on CFMM supplemented with 50 mg/L of pyrene along with 2% glycerol as a secondary carbon source, activity against both aliphatic and aromatic hydrocarbons was observed. Hence, CFMM supplemented with both pyrene and glycerol was used for emulsion stability studies.

Table 1 shows the emulsification activity of the bioemulsifier toward different organic solvents and their stability, and Fig. 5 shows the linear form of the emulsion stability profile for different organic solvents.

From Table 1, it can be seen that emulsification activity toward the different solvents followed the order benzene > toluene > xylene > paraffin oil >kerosene > butane-1-ol > isoamyl alcohol > petroleum ether > *n*-hexane > sunflower oil. Singh et al. *(16)* reported a similar result, for which bioemulsifier showed activity against various hydrocarbons, with the maximum with aromatics and the least with normal paraffin. The stability of emulsion formed with different organic solvents is measured from the decay constant values *(9)*. Stability was found to follow the order benzene

Table 1
Emulsification Activity of Various Emulsifying Substrates by Culture Supernatant

Organic solvent	Emulsification activity (A_{600nm})[a]	Decay constant (K_d)[b]
n-Hexane	0.688	–12.52
Isoamyl alcohol	0.849	–3.312
Butane-1-ol	0.924	–3.60
Benzene	1.510	–1.008
Toluene	1.203	–12.38
Xylene	0.984	–1.296
Kerosene	0.949	–2.16
Sunflower oil	0.445	–3.45
Paraffin oil light	0.980	–50.54
Petroleum ether (30–40°C)	0.843	–16.27

[a]All absorbance values are the average of three consecutive readings after allowing a 10-min standing time.

[b]For determination of decay constant, after an initial 1-h standing time, readings were taken at 5-min intervals and expressed in day^{-1}.

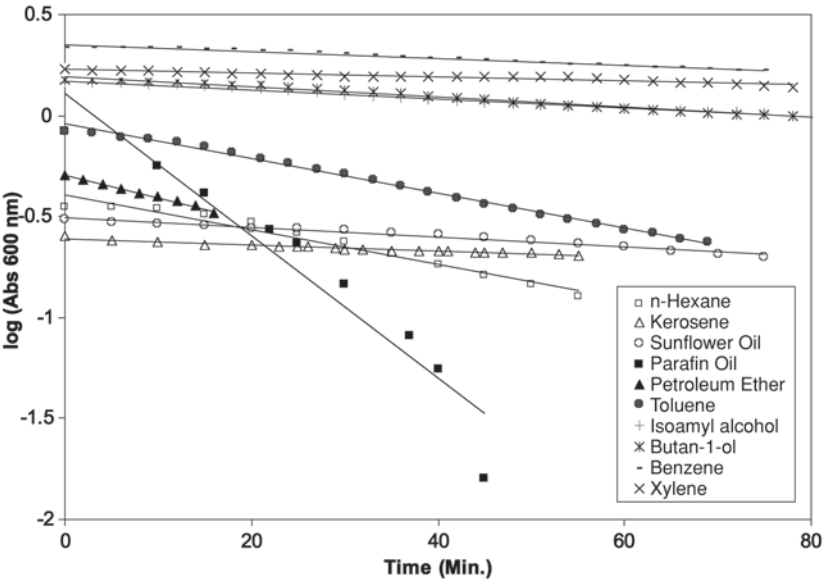

Fig. 5. Emulsion stability profile of bioemulsifier to different organic solvents.

> xylene > kerosene > isoamyl alcohol > sunflower oil > butane-1-ol > toluene > *n*-hexane > petroleum ether > paraffin oil.

Environmental Factors Affecting Bioemulsifier Activity

pH and temperature are known to be the important environmental factors that affect the performance of bioemulsifier. The present study was

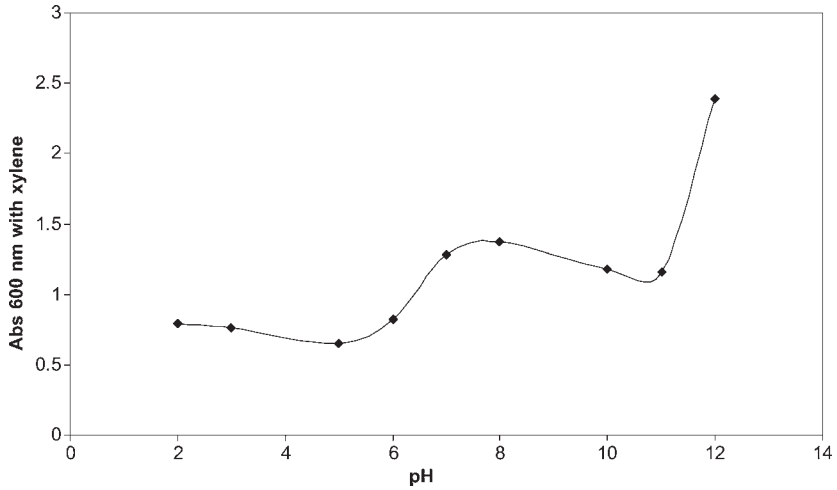

Fig. 6. Emulsifying activity at different pH values as determined with xylene as organic solvent.

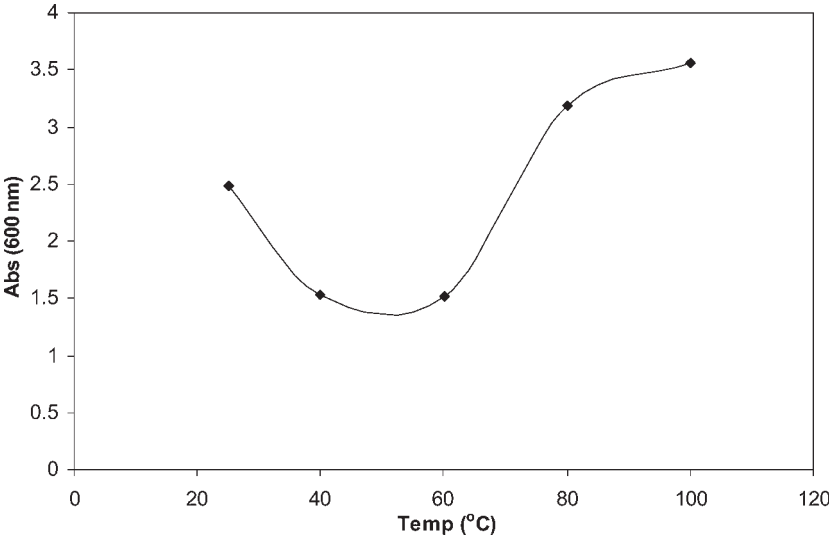

Fig. 7. Effect of temperature on emulsifying activity.

conducted with xylene, which shows moderate emulsifying activity and stability. Figures 6 and 7 show emulsifying activity toward xylene at different pHs and temperatures, respectively. It can be seen that the activity of the bioemulsifier tends to be more at an alkaline pH greater than 6.0, with the highest activity at pH 12.0. However, the stability of the emulsion at lower pH was found to be greater compared with the stability at higher pHs (Fig. 8). This emulsifier was found to be better than liposan (showing emulsifying activity between pH 2.0 and 5.0) *(17)* or bioemulsifier produced by

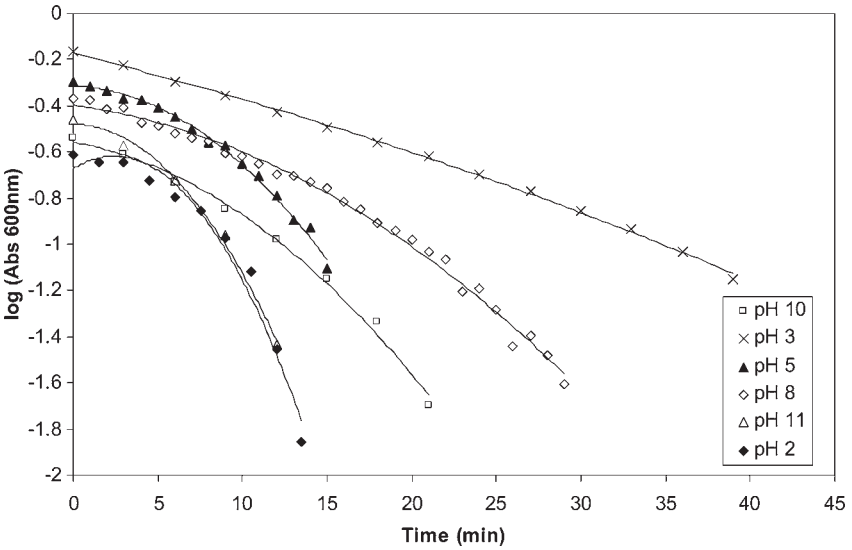

Fig. 8. Stability profile of emulsion formed at different pH values with xylene.

Bacillus stearothermophilus VR-8 isolate (emulsification activity stable over pH 2.0–8.0) *(18)*. By contrast, Alasan, bioemulsifier from *Acinetobacter radioresistens*, shows maximum activity in an acidic pH range (3.3–9.2) and maximum activity at pH 5.0 *(19)*.

Emulsifying activity of the bioemulsifier varied with the incubation temperature. It is very clear from Fig. 7 that the emulsifying activity of the emulsifier decreased up to 50°C, after which the emulsifying activity increased rapidly up to 100°C. A similar increase in emulsifying activity (activation) has also been reported previously *(19)* with Alasan.

Conclusion

Microorganisms capable of degrading anthracene and pyrene isolated from a hydrocarbon-contaminated soil were tested for bioemulsifier production. The bioemulsifier thus produced was dependent on the type of carbon source in the medium. Although both the media with and without pyrene induced bioemulsifier production, the specificity of the bioemulsifier toward different organic solvents was found to be higher when both carbon sources were present. Ammonium sulfate precipitation followed by dialysis of the culture supernatant resulted in partially purified bioemulsifier. Protein assay and carbohydrate assay indicated that it contained 10% protein (as BSA equivalent) and 7% carbohydrate (as glucose equivalent).

The stability and activity of the emulsion formed with various organic solvents were tested and a trend was observed. Whereas alkaline pH favored more emulsifying activity, acidic pH favored more stability of the emulsion produced. After incubation at 100°C for 30 min, the emulsifier not

only retained its emulsifying activity but also showed increased emulsifying activity and stability for the higher temperature of incubation.

The bioemulsifier produced in our study can be a valuable resource for enhancing bioavailability and subsequent mineralization of PAHs in a contaminated system.

References

1. Cerniglia, C. E. (1993), *Curr. Opin. Biotechnol*. **4,** 331–338.
2. Desai, J. D. and Banat, I. M. (1997), *Microbiol. Mol. Biol. Rev.* **61,** 47–64.
3. Bodouur, A. A. and Miller-Maier, R. M. (1998), *J. Microbiol. Methods* **32,** 273–280.
4. Cirigliano, M. C. and Carman, G. M. (1984), *Appl. Environ. Microbiol.* **46,** 747–750.
5. Cirigliano, M. C. and Carman, G. M. (1985), *Appl. Environ. Microbiol.* **50,** 847–850.
6. Kim, S. H., Lim, E. J., Lee, S. O., Lee, J. D., and Lee, T. H. (2000), *Biotechnol. Appl. Biochem*. **31,** 249–253.
7. Hartree, E. F. (1972), *Anal. Biochem.* **48,** 422–427.
8. Herbert, D., Phipps, P. J., and Strange, R. E. (1971), in *Methods in Microbiology, Vol. 5B,* Norris, J. R. and Ribbons, D. W., eds., Academic, London, pp. 266–291.
9. Zhang, Y. and Miller, R. M. (1992), *Appl. Environ. Microbiol.* **58,** 3276–3282.
10. Jacques, R. S. J., Santos, E. C., et al. (2005), *Int. Biodeterioration Biodeg.* **56,** 143–150.
11. Mulligan, C. N. (2005), *Environ. Pollut.* **133(2),** 183–198.
12. Sarubbo, L. A., Marcal, M. C., Neves, M. L., Silva, M. P., Porto, A. L., and Campos-Takaki, G. M. (2001), *Appl. Biochem. Biotechnol*. **95(1),** 59–67.
13. Patil, J. R. and Chopade, B. A. (2001), *J. Appl. Microbiol.* **91(2),** 290–298.
14. Barkay, T., Navon-Venezia, S., Ron, E. Z., and Rosenberg, E. (1999), *Appl. Environ. Microbiol.* **65,** 2697–2702.
15. Cameotra, S. S. and Bollag, J. M. (2003), *Crit. Rev. Environ. Sci. Technol.* **30,** 111–126.
16. Singh, M., Saini, V. S., Adhikari, D. K., Desai, J. D., and Sista, V. R. (1990), *Biotechnol. Lett.* **12(10),** 743–746.
17. Cirigliano, M. C. and Carman, G. M. (1984), *Appl. Environ. Microbiol*. **48(4),** 747–750.
18. Gurjar, M., Khire, J. M., and Khan, M. I. (1995), *Lett. Appl. Microbiol*. **21(2),** 83–86.
19. Navon-Venezia, S., Zosim, Z., Gottlieb, A., Legmann, R., Carmeli, S., Ron, E. Z., and Rosenberg, E. (1995), *Appl. Environ. Microbiol.* **61,** 3240–3244.