In Vitro Analysis of Enhanced Phenanthrene Emulsification and Biodegradation Using Rhamnolipid Biosurfactants and *Acinetobacter calcoaceticus*

N.D. Henry and M. Abazinge

Abstract The ability of biosurfactants and *Acinetobacter calcoaceticus* to enhance the emulsification and biodegradation of phenanthrene was investigated. Phenanthrene is a polycyclic aromatic hydrocarbon (PAH) that may be derived from various sources, for example incomplete combustion of petroleum fuel, thus it occurs ubiquitously throughout the environment. Phenanthrene biodegradation has been reported to be greatly enhanced in the presence of surfactants (Cuny et al. 1999; Chen et al. 2001). It is weakly soluble in water (1.2 mg L^{-1}, 1 atm, 25 °C); therefore, its' biodegradation is strictly limited by its bioavailability (Chen et al. 2001). Emulsification assays were carried out to assess the stability of emulsions formed between phenanthrene and water in the presence of rhamnolipid biosurfactants. An increase in emulsion stability has been shown to equal an increase in bioavailability of a hydrophobic PAH (Dean et al. 2001). Emulsion stability was determined by height of emulsion layer and optical density measurements. Results show phenanthrene and water emulsifications were stabilized for a period up to 10 days at levels ranging from 70–80% with the use of un-encapsulated biosurfactants. Microencapsulated biosurfactants stabilized the emulsion up to 89% for 15 days. Experimental microcosm studies to assess biodegradation rates were carried out over 15 days in 40-mL bioreactors. The reactors were sampled at t = 0, 3, 6, 9, 12, 15 days. Biodegradation rates were determined from measurements of carbon dioxide respiration and phenanthrene concentrations. Results show that on average, more phenanthrene was mineralized (96.4% over 15 days) by bacteria amended with non-encapsulated rhamnolipid biosurfactant (NERhBS).

Keywords Bioremediation · biosurfactants · phenanthrene · microencapsulation

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1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are prevalent contaminants in the environment as a result of fossil fuel combustion and by-product waste from industrial activities (Banat 1995; Dean et al. 2001). Biodegradation by microorganisms is one of the primary methods of removal of PAHs from contaminated sites (Wong et al. 2004). One of the main reasons for prolonged persistence of PAHs in contaminated environments is their low water solubility, which restricts their bioavailability to biodegrading microorganisms (Barkay et al. 1999). Therefore, approaches to enhance PAH biodegradation often attempt to increase their apparent solubility by treatments such as addition of biosurfactants (Piehler and Paerl 1996; Dean et al. 2001).

Biosurfactants are biologically synthesized surface-active agents produced by many various microorganisms and represent a wide range of chemicals and molecular structures (Desai and Banat 1997; Barkay et al. 1999). They posses properties comparable to chemically produced surfactants and can be used for various applications many of them in environmental management (Maslin and Maier 2001; Ron and Rosenberg, 2001). In general, biosurfactants could enhance the apparent solubility of PAHs by micellar formation, which commences at the critical micelle concentration (CMC) and then solubility is proportional to surfactant concentration (Wong et al. 2004). However, biodegradation of PAHs is not always correspondingly enhanced by surfactants (Wong et al. 2004). Some researchers have found that addition of surfactants enhanced PAH biodegradation (Piehler and Paerl 1989; Barkay et al. 1999; Dean et al. 2001), whereas others reported no effect or inhibition by surfactants (Laha and Luthy 1991; Tiehm 1994; Volkering et al. 1995; Boonchan et al. 1998; Wong et al. 2004). The conflicting results may be due to varied interactions amongst microbial communities, types of PAHs targeted for biodegradation, or the type of surfactant used in the approach.

The goal of the work reported here was to test the efficiency of a microparticle system of biosurfactants, previously described in Henry et al. (2005), using emulsification assays and traditional biodegradation protocols. The use described here is only one potential application of the microparticle system. Rhamnolipid biosurfactants have been used as effective biopesticides on agricultural, horticultural and turf sites to eradicate certain pathogenic fungi (EPA 2004). This application of the microparticle system would be potentially effective in developing countries because it is inexpensive to formulate and it does not require clean-up of secondary waste, as none is produced. The objectives of the work are to assess the ability of encapsulated and non-encapsulated rhamnolipid biosurfactants to emulsify phenanthrene (PHE), a PAH; and to compare the capabilities of *Acinetobacter calcoaceticus* to degrade PHE in the presence of encapsulated and non-encapsulated rhamnolipid biosurfactants.

2 Materials and Methods

Chemicals: Phenanthrene (purity >98%; Aldrich Chemical Company, St. Louis, MO) was used in this study as the target PAH for biodegradation.

Biosurfactant: Rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa* (Jeneil Biosurfactant Co., Saukville, WI) were used because of their known ability to solubilize and emulsify hydrophobic organic compounds (Al-Tahhan et al. 2000). This study involved the use of microencapsulation as a means to deliver biosurfactants to target PAHs. Microencapsulation involves enclosing a desired substance within a biodegradable polymer, forming a microparticle. The desired substance is then released over time as the polymer degrades. The development of the microparticle system used here has been described by Henry et al. (2005).

Acinetobacter calcoaceticus (ATCC 31012): *Acinetobacter calcoaceticus* was obtained from the American Type Culture Collection (ATCC) as a pure culture. Strain ATCC 31012 (*A. calcoaceticus*-RAG-1) was used in this study. Freeze-dried bacteria were rehydrated with 0.5 mL of nutrient broth and mixed well. Aseptically, the total mixture of the vial was transferred to a test tube of nutrient broth medium (5–6 mL). The culture was incubated at 30 °C and allowed to grow for about 4 days, depending on the bacteria response.

Determination of emulsifying activity: A micro-modification of the emulsification assay described by Navon-Venezia et al. (1995) and Cooper and Goldberg (1997) was used to measure emulsifying activity. Samples to be tested were prepared by introducing 2.5 mL of each rhamnolipid biosurfactant (RhBS) solution and PHE into 10 mL glass test tubes. The samples were incubated at room temperature for 1 h. The height of the emulsion layer between the two aqueous phases was determined after the 1 h settling period. Changes in the emulsion layer were noted until the emulsion stabilized.

In order to assess the effectiveness of the microparticle system, the emulsification assay was further modified to account for release of the biosurfactant from the microparticles. Ten milligrams of microparticles were added to a 10 mL test tube containing 4 mL of TM buffer (20 mM Tris-HCl buffer [pH 7.0], 10 mM $MgSO_4$), and then 2.5 mL of PHE was added. The samples were incubated at room temperature for 24 h before any observations were made to allow time for biosurfactant to be released from the microparticles. The changes in height of the emulsion layer between the two aqueous phases were determined after the 1 h settling period and the initial 24 h incubation period, as described above.

Biodegradation microcosm study: Aqueous degradation tests were performed to estimate PHE degradation rates by *A. calcoaceticus*, grown in nutrient broth, supplemented with minimal salts at 30 °C, in the presence of non-encapsulated rhamnolipid biosurfactants (NERhBS) and encapsulated rhamnolipid biosurfactants (ERhBS). The bacterial culture medium, which consisted of 0.5 g K_2HPO_4, 1.0 g of NH_4Cl, 2.0 g of Na_2SO_4, 2.0 g of KNO_3, 0.2 g of $MgSO_4{}^*7\ H_2O$, and 0.002 g $FeSO_4{}^*7\ H_2O$ per liter of distilled water, was prepared in accordance with the procedure described by Wong et al. (2004).

Aqueous PHE concentrations were analyzed by high performance liquid chromatography (HPLC), equipped with a UV detector (254 nm). Samples (50 µL) were injected into a C_{18} reverse phase column (4.6 × 250 mm) and eluted with an isocratic mobile phase consisting of 80% acetonitrile and 20% water (v/v) delivered at a flow rate of 1.0 mL min^{-1}. Eluted peaks were monitored by UV absorption at 254 nm.

3 Results

Phenanthrene emulsification by rhamnolipid biosurfactant: Figure 1 summarizes the time required for RhBS to form a stable emulsion with PHE. NERhBS formed a stable emulsion more quickly than ERhBS; however the ERhBS held a more stable emulsion for a longer period of time. The NERhBS held a stable emulsion for up to 10 days, however after that time period, the sample vials began to mold so the results were hindered. A stable emulsion was observed for up to 360 h with the ERhBS, which was significantly higher ($p < 0.05$) than that observed with the NERhBS.

In order to quantify the volume of PHE emulsified by RhBS, the Emulsification Index (EI), determined based on the equation described by Bodour et al. (2004),

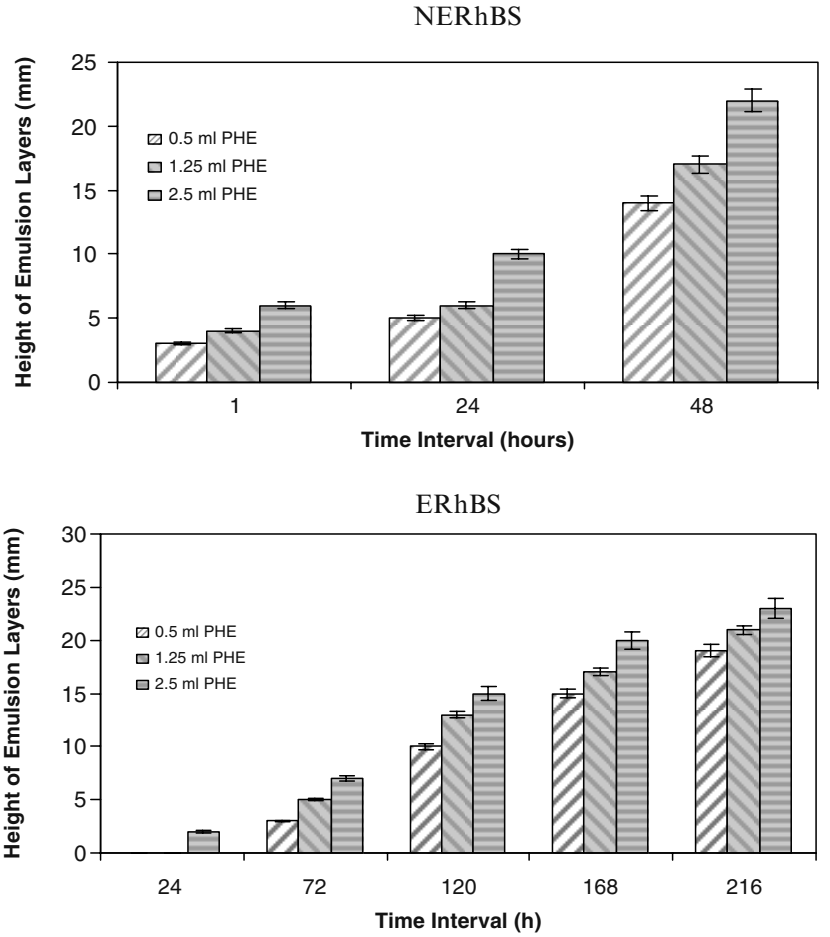

Fig. 1 Changes in height of emulsion layers of NERhBS and ERhBS up to stabilization. NERhBS achieved a stable emulsion after only 48 h, while ERhBS achieved the same at approximately 216 h

was used. The RhBS was a strong and stable emulsifier. PHE concentrations of 5 mg L^{-1} exhibited EI of up to 89% by ERhBS with biosurfactant concentration of 45 mg L^{-1} after 360 h (Table 1). There was no significant difference ($p > 0.05$) among the EI in the ERhBS treatments, showing that biosurfactant concentration had no influence on the results. Similar results were shown in the NERhBS treatments, wherein biosurfactants with concentrations of 30 and 45 mg L^{-1} had no significant difference ($p > 0.05$) in the emulsion stability. After 168 h, the NERhBS test vials became turbid and the presence of mold formation was obvious. A decrease in the height of the emulsion layer was also detected.

Phenanthrene biodegradation in the presence of biosurfactants and *A. calcoaceticus*: Biodegradation of PHE in the presence of NERhBS, ERhBS and A. calcoaceticus was evaluated by determining the change in PHE concentration in solution phase (Fig. 2) over a 15 day incubation period. A. calcoaceticus was able to utilize PHE as a carbon source and degrade it accordingly. The PHE biodegradation percentage values (mean ± SD%) after 6 days for NERhBS + AC, ERhBS + AC and AC treatments were 58 ± 3%, 40 ± 2% and 29 ± 2%, respectively. At the end of the incubation period, 96 ± 4%

Table 1 Emulsion activity

Emulsifying activity of biosurfactant with phenanthrene										
Biosurfactant concentration	Emulsification index NERhBS					Emulsification index ERhBS				
	E_{24}	E_{48}	E_{120}	E_{168}	E_{264}	E_{24}	E_{48}	E_{120}	E_{168}	E_{360}
30 mg L^{-1}	80	80	80	80	75	0	46	74	85	85
45 mg L^{-1}	80	80	80	80	78	0	35	75	89	89

The emulsion index (E = vol. of emulsion layer × total vol^{-1}) × 100. Values represent mean percentages of emulsion of the oil layer in the test tubes. EI values for ERhBS increase over time because biosurfactants are consistently released from microparticles during the emulsification assay.

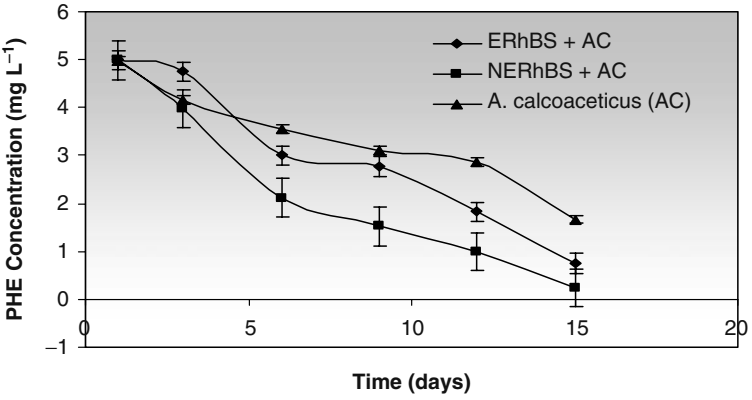

Fig. 2 Biodegradation of phenanthrene (mg L^{-1}) in the microcosm study. Data points represent means of determinations from triplicate experimental microcosms. Vertical bars correspond to 1 standard deviation about the mean

of the initial concentration of PHE had been degraded by the treatment with NERhBS + AC, which was significantly higher ($p < 0.05$) than the other treatments. It is interesting to note that the growth medium color changed depending on the treatment. This varied from colorless in the AC treatment, deep orange-yellow with NERhBS, to light orange-yellow with ERhBS. These changes in color are a result of the appearance of phenanthrene metabolites (Mueller et al.1990; Lal and Kanna 1996; Cuny et al. 1999).

4 Conclusions

The results of this study show that biodegradation activity of PHE-degrading bacteria is influenced by the bioavailability of the phenanthrene in the aqueous phase. By demonstrating that rhamnolipid biosurfactants enhance the emulsification of phenanthrene, it can inferred that the biosurfactants facilitate the transport of PHE into microbial cells, thus increasing its biodegradation. This positive effect on biodegradation is not a general response to the addition of biosurfactants, as some biosurfactants may inhibit biodegradation (Laha and Luthy 1991; Cuny et al. 1999).

This study also investigated the effectiveness of a previously developed microparticle system. Microparticles were observed to be a promising method of delivery of biosurfactants as results yielded by ERhBS + AC were considerably similar to those yielded by NERhBS + AC treatments. Further investigation of this system is underway. Once further testing is completed, this system could serve as an economically and environmentally friendly alternative for clean-up of residual PAH waste. This system could also be used as a method to deliver needed nutrients to indigenous bacteria, to increase their natural abilities to degrade such wastes.

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