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Optimization Strategy of Polycyclic Aromatic Hydrocarbon Contaminated Media Bioremediation through Biosurfactant Addition

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A microbial surfactant was investigated for its potential to enhance bioavailability and, hence, the biodegradation of PAHs contaminated soil. Phenanthrene, a 3-ring polycyclic aromatic hydrocarbon (PAH), was chosen as a model target compound. The bioavailability and biodegradation tests were performed in aqueous and soil-slurry microscosms .The rhamnolipid biosurfactant used in this study was extracted from culture supernatants after growth of Pseudomonas aeruginosa BP9 strain in nitrogen-limited mineral salts medium. Solubilisation of phenanthrene in aqueous solution was enhanced by 400 mg of the rhamnolipid per litre increasing more than 19 folds. Phenanthrene aqueous phase biodegradation experiments were done with an initial concentration of 200 mg/L and showed 92 % mineralization in 6 days with a rhamnolipid concentration of 400 mg/L, in comparison to the 27 % mineralization of the other microcosm with no rhamnolipid amendment. Which accelerated the biodegradation rate, by increasing the bioavailabilty and by shortening the lag phase. This shows the potential application of the rhamnolipid in stimulating in-situ and ex-situ enhanced bioremediation of polycyclic aromatic hydrocarbon contaminated media.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are stable aromatic hydrocarbon molecules with two or more fused benzene and/or pentacyclic rings in linear, angular or cluster formation (Chauhan et al., 2008). The release of PAHs into the environment is wide-spread since these compounds are ubiquitous products of incomplete combustion. PAHs have been detected in a wide variety of environmental samples, including air, soil, sediments, water, oils, tars and creosote and foodstuffs. The widespread occurrence of PAHs is largely due to their formation and release in all processes of incomplete combustion of organic materials. The last century of industrial development caused a significant increase of PAH concentrations in the natural environment (Maliszewska-Kordybach, 1999).

PAHs have generated considerable interest, not only because of their wide distribution in the environment, but also their carcinogenic and mutagenic potential. (Baird et al., 2005).Due to their hydrophobic nature, most PAHs in aquatic and terrestrial ecosystems bind to particulates in soil and sediments, rendering them less available for biological uptake, and they also bioaccumulate in food chains. They are persistent organic pollutants, their molecular stability and hydrophobicity are two primary factors which contribute to the persistence of PAHs in the environment (Kanaly and Harayama, 2000).

Bioremediation has attracted great interest for treatment of soils and sediments contaminated with polycyclic aromatic hydrocarbons (PAHs). However, bioremediation is limited by the bioavailability of soil-bound PAHs due to their low aqueous solubility and strong sorption to soil. Bioavailability is of extreme importance because it frequently accounts for the persistence of compounds that may be biodegradable and that might otherwise be assumed to be readily decomposed (Weissenfels et al., 1992). Strategies to improve bioavailability can enhance the biodegradation of PAHs, although the biodegradation of high-molecular-weight (HMW) PAHs can be limited by factors other than bioavailability as well (Zhu and Aitken, 2010). Biosurfactants are surface-active molecules that have both hydrophobic and hydrophilic domains

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and are capable of lowering the surface tension and the interfacial tension of the growth medium. They can also emulsify hydrophobic compounds, form stable emulsions and increase PAH solubility and consequently bioavailability in the environment (Cameotra and Bollag, 2003).They can enhance hydrocarbon bioremediation by two mechanisms. The first includes the increase of substrate bioavailability for microorganisms, while the other involves interaction with the cell surface which increases the hydrophobicity of the surface allowing hydrophobic substrates to associate more easily with bacterial cells (Mulligan and Gibbs, 2004). The bioavailability of hydrophobic pollutants can be enhanced by biosurfactants through the following mechanisms: emulsification of non-aqueous phase liquid contaminants (Volkering et al., 1997) and enhancement of the apparent solubility of the pollutants (Volkering et al., 1995). Biosurfactants produced by bacteria, fungi and yeasts include glycolipids, lipoaminoacids, lipopeptides, lipoproteins, lipopolysaccharides, phospholipids, monoglycerides and diglycerides. Amongst these, the rhamnolipids produced by Pseudomonas strains have received much attention due to their remarkable tensioactive and emulsifying properties (Maier and Sober´on-Ch´avez, 2000). These rhamnolipids are able to reduce the water/air surface tension from 72 mNm^{-1} to as low as 25 mNm^{-1}, as well as the water/oil interface tension from 43 mNm^{-1} to values below 1 mNm^{-1} (Lang and Wullbrandt, 1999).

The aim of this study is to test the capability of rhamnolipid in increasing PAH solubility and hence their ability to enhance PAH biodegradation in aqueous and soil slurries. The rhamnolipid, a glycolipid biosurfactant, used in this study was produced by Pseudomonas aeruginosa strain designated BP 9 isolated from petroleum contaminated soil. The effect of the biosurfactant on the biodegradation of phenanthrene was investigated using aqueous and soil slurry systems. Phenanthrene (a 3-ring aromatic hydrocarbon) was used as a model target compound to analyze the effect of rhamnolipid on the biodegradation of PAHs.

2. Materials and Method

2.1 Microorganism (Microorganism isolation)

Samples of contaminated soil were collected from crane service station sites which have been contaminated by spilt oil for decades. The enrichment and isolation of the PAH-degrading microbial consortium was performed by using naphthalene as the sole carbon and energy source.

Initially, the bacterial consortium was enriched by adding 1 g of soil sample to 250 mL of minimal salt medium [MSM was composed of 6.0 g/L $(NH_4)_2SO_4$, 0.4 g/L $MgSO_4$ x 7 H_2O, 0.4 g/L $CaCl_2$ x 2 H_2O, 7.59 g/L Na_2HPO_4 x 2 H_2O, 4.43 g/L KH_2PO_4, and 2 mL/L of trace element solution. The trace element solution consisted of; 20.1 g/L EDTA (disodium salt), 16.7 g/L $FeCl_3$ x 6 H_2O, 0.18 g/L $CoCl_2$ x 6 H_2O, 0.18 g/L $ZnSO_4$ x 7 H_2O, 0.16 g/L $CuSO_4$x 5 H_2O, and 0.10 g/L $MnSO_4$ x H_2O] in a 250-mL Erlenmeyer flask. The flask was shaken in an orbital shaker (150 rpm) at 30 °C for 5 days for microbial enrichment. After 5 days, an aliquot of 10 mL enriched culture was inoculated into another 250-mL conical flask containing 200 mL MSM with 250 mg/L PAH (Naphthalene) for the first enrichment. Five consecutive enrichments were carried out under the same condition to enrich a PAH-degrading microbial consortium. The bacterial colonies were isolated by streaking the enriched consortium on nutrient agar plates containing naphthalene as a carbon and energy source provided in a vapour form put on the lid. Morphologically distinct colonies were re-isolated by transfer onto naphthalene containing agar plates at least three times to obtain pure cultures. 11 morphologically different colonies were isolated, and 5 of them were found to produce biosurfactant as checked by “drop collapse” and “oil spread” test and the best biosurfactant producing strain was identified as pseudomonas aeruginosa strain BP9. The phylogenetic identification of this strain was based on the nucleotide sequence obtained by using 16S rRNA gene sequencing analysis. The results showed that it was closely related to pseudomonas aeruginosa being the most closely related species with a similarity of 89.9 %. Strain BP9 culture was preserved in the MSM broth with 30 % (w/v) glycerol at −80 °C.

2.2 Chemicals and contaminated soil

2.2.1 Chemicals

Glycerol, Naphthalene (purity > 98 %), Phenanthrene (purity > 98 %), acetonitrile (HPLC grade), Hexane (HPLC grade), were all purchased from Sigma Aldrich Chemical Company (Aldrich, USA), Phenanthrene stock solution (2 mg/mL).

2.2.2 Soil contamination

Soil was collected from a pristine supply and was sieved to (< 2 mm) size. One hundred gram of sterile dry soil was placed in 1-L bottles and spiked with 80 mg of Phenanthrene dissolved in approximately 100 mL

of acetone to achieve a final soil contamination of 800 mg/kg of soil).The soil was shaken vigorously for 5 min to promote homogeneous distribution of phenanthrene in the soil. The amount of acetone added was sufficient to completely saturate the soil. Acetone was then evaporated by allowing the sample to rest for 3 days at 40 °C under a vacuum dryer. The contaminated soil will stay 15 days before each experiment starts. Texture of the soil % of Sand (2 …. 0.05 mm) is 26 %, % Clay (< 2 µm) is 33 % and % Silt (0.05 mm …2 µm) is 41 %. And organic carbon content of the soil was 1.6 % determined using Loss On Ignition test.

2.3 Culture Media for biosurfactant production

The organism was grown as described by (Trummler et al., 2003) to induce biosurfactant production. A two-step process was developed for producing rhamnolipids in high amounts using resting Pseudomonas aeruginosa cells. The actual rhamnolipid production was preceded by a culture for cell material production. This step was performed in shaking flasks with growth medium containing the carbon source.

The rhamnolipid production step was performed in another 2 L flask with the production medium under nitrogen free (no nitrogen source) growth limited production medium. Two culture media produced one for growth of the organisms, and the other one nitrogen free media for the growth limited overproduction of biosurfactant.

Growth medium was composed of 6.0 g/L $(NH_4)_2SO_4$, 0.4 g/L $MgSO_4$ x 7 H2O, 0.4 g/L $CaCl_2$ x 2 H_2O, 7.59 g/L Na_2HPO_4 x 2 H_2O, 4.43 g/L KH_2PO4, and 2 mL/L of trace element solution. The trace element solution consisted of 20.1 g/L EDTA (disodium salt), 16.7 g/L $FeCl_3$ x 6 H_2O, 0.18 g/L $CoCl_2$ x 6 H_2O, 0.18 g/L $ZnSO_4$ x 7 H_2O, 0.16 g/L $CuSO_4$x 5 H_2O, and 0.10 g/L $MnSO_4$ x H_2O.

Growth limited / Nitrogen-free medium had the same composition as growth medium, except that no $(NH_4)_2SO_4$ was added. All media were adjusted to pH 7.

2.4 Culture of Pseudomonas aeruginosa for cell mass production

Shaking flasks, containing a total of 1.5 L of growth medium with 3 % carbon source, were inoculated with a total of 15 mL of the preculture and shaken for 60 h at 30 °C, 120 rpm. Cultures were adjusted to pH 6.5. Cell material was eventually harvested by centrifugation (10,000 rpm, 10 min). To be used for the subsequent production by resting or immobilized cells production strategy. The free-cell culture medium was checked for surface activity and oil displacement testing as well.

2.5 Production of rhamnolipids by Pseudomonas aeruginosa in shaking flasks

The desired cell mass was suspended in a suitable volume of production medium. Final concentration of wet biomass did not exceed 5 % (w/v). Glycerol and hexane carbon sources were added to the cell suspension, and incubated at 37 °C, 120 rpm and checked for optimum production.

2.6 Extraction and chemical characterization of biosurfactant

Biosurfactant Recovery (Pruthi and Cameotra, 2003)

Cells were removed from the culture broth by centrifugation at 10,000 rpm, for 15 min. After the removal of the bacterial cells by the centrifugation, to remove residuals it was filtered with Millipore membrane filter. The clear sterile supernatant served as the source of the crude biosurfactant. The biosurfactant was recovered from the cell free culture supernatant by cold acetone precipitation. Three volumes of chilled acetone was added and allowed to stand for 10 h at 4 °C. The precipitate was collected by centrifugation and Acetone was evaporated and biosurfactant was collected and weighted.

About 15 g of the crude biosurfactant was extracted per liter of culture medium.

2.7 Surface tension and emulsification index (E_{24}) measurements

The surface tension of the aqueous solution at different surfactant concentrations was measured by using a duNo¨uy ring-type tensiometer (KR¨ USS K6 Tensiometer) equipped with a 1.9 cm De No¨uy platinum. The surface tension measurement was carried out at 25 °C . All measurements were made on cell-free supernatant. A mixture of 10 mL supernatant and 10 mL of hexane was vortexed for 5 minutes and the height of emulsion layer was measured after 24 h to determine the emulsification index. The emulsion activity was investigated after 24 h and the emulsification index (E_{24}) was calculated by dividing the measured height of the emulsion layer by the total height of the mixture and multiplying by 100 (Cooper and Goldenberg,1987).

2.8 Biosurfactant-enhanced biodegradation in aqueous systems

For the substrate degradation experiments, which were conducted in triplicate, 10 mg of phenanthrene was dissolved in acetone and added to 100 mL flasks. The solvent was allowed to evaporate overnight, leaving a thin coating of phenanthrene covering the bottom of the flasks. MSM (50 mL) were added in each flask. Each set of samples was inoculated with 1 mL aliquots of the phenanthrene degraders from late-log precultures to achieve a final cell density of (10^7 CFU mL^{-1}) approximately optical density (OD) of

2 and different concentrations of rhamnolipids. The flasks were sealed with Aluminium foil caps and incubated at 30 °C on a rotary shaker at 150 rpm. Periodically, triplicate flasks were sacrificed to determine the amount of phenanthrene remaining using the following procedure.
The flasks were then extracted with 100 mL of hexane twice, centrifuged at 6,500 rpm for 6 min to remove emulsifications during solvent extraction. The solvent was pooled and evaporated. The residual phenanthrene was dissolved in equal volume 50 mL of acetonitrile (exchanged to mobile phase medium), and the amount of phenanthrene in the solution was analysed by reversed phase HPLC.

2.9 Biosurfactant-enhanced biodegradation in soil slurry systems

Degradation trials in soil slurries were conducted in 100-mL Erlenmeyer flasks which were capped with aluminum foil. 5 gm of 800 mg/kg phenanthrene spiked soils were added to the flasks along with 50 mL of liquid media at 10 % (w/v). With and without biosurfactants at different concentrations (0, 300 mg/L, 500 mg/L). Each set of samples was inoculated with 1 mL aliquots of the phenanthrene degraders from late-log precultures to achieve a final cell density of (10^{7} CFU mL^{-1}) approximately optical density (OD) of 2 and different concentrations of rhamnolipids. On days 2, 4, 6, 8, 10, 12 and 14 samples were collected from the flasks to determine the amount of phenanthrene in solution. The aqueous phase was separated from the soil by centrifugation at 5,000 rpm for 20 min. A 10 mL of supernatant was sampled, extracted with hexane and the hexane was evaporated, the residue is resuspended in acetonitrile and filtered through 0.22 µm syringe filter. Phenanthrene in solutions was analyzed by HPLC Waters 2695 C_{18} reverse phase column (250 × 4.6 mm, 5 µm) .To determine the phenanthrene in the soil, the soil was air dried and extracted in ultrasonic bath (USEPA methods 3550C) 5 grams of soil was extracted with 30 mL of solvent hexane : acetone (1:1) trice and pooled. Acetone : hexane (1:1) was added to the sample which was then vortexed for 2 min and phenanthrene was extracted for 1 h at 55 °C in a Sonic Bath. The combined extracts obtained from the ultrasonic procedures were centrifuged for 10 min at 10,000 rpm the supernatant was drawn off and collected. The extraction solvent was evaporated then the residue is resuspended in 5 mL acetonitrile, filtered through 0.22 µm syringe filter and the phenanthrene in the solution was analyzed by HPLC system.

2.10 Analytical method

Phenanthrene in solutions was analyzed by HPLC system with a slightly modified EPA Method 8310 (EPA 1986). Chromatographic condition of A linear gradient elution condition from 70 % acetonitrile and 30 % ultra-pure-water to 100 % acetonitrile mobile phase over 10 min at a flow rate of 1 mL min^{-1} was set up. Phenanthrene is identified by its retention time and absorption spectrum and quantified by its absorbance compared with calibration curve prepared with the phenanthrene standards. The detection limit of the HPLC system was 0.01 mg/L. All tests were conducted in triplicate with uninoculated controls to monitor the volatilization losses and total recovery of contaminants.

3. Results and discussion

The critical micelle concentration (CMC) of the nonpurified biosurfactant was 80 mg L^{-1}, and occurred at a surface tension of ±35 mNm^{-1}. The emulsification index (E_{24}) of the non-purified biosurfactant was determined after 24 h. After 24 h, E_{24} was 60 % with hexane and 70 % with toluene.

Biodegradation test

Aqueous phase biodegradation of phenanthrene in the presence of rhamnolipid

The effect of rhamnolipid on the biodegradation of phenanthrene is presented (Figure 1). There was 15 % biodegradation at the unamended one; 20 % at 100 mg/L rhamnolipid amendment; 55 % at 400 mg/L of rhamnolipid and 6 % biodegradation at 700 mg/L rhamnolipid amendments in the first 72 h. Rhamnolipid amendment of 700 mg/L inhibited Phenanthrene biodegradation as only 6 % of phenanthrene was degraded during the first 72 h which is lower than the unamended microcosm which degraded 15 % during the first 72 h. At the sixth day there was 92 % mineralization by the 400 mg/L rhamnolipid amended culture where as there was almost 27 % degradation in the unamended microcosm. The inhibitory effect due to the rhamnolipid addition of 700 mg/L suggests that the rhamnolipid is toxic to the phenanthrene degrading community or alternatively the very high concentration of the substrate solubilized by the very high amount of rhamnolipid is toxic to the bacteria and inhibits their mineralisation activity, or the rhamnolipid is serving as a preferred carbon source. The effect of biosurfactants on the microorganisms depends on factors, such as: biosurfactant concentration and bioavailability, environmental and cultural conditions, and characteristic and properties of microorganisms as cellular ultra-structure (Van Hamme et al., 2006). Other studies showed an enhanced degradation with different pseudomonas strains; study with the strain, Pseudomonas marginalis, indicated that the biosurfactant produced by the strain solubilized polycyclic

aromatic hydrocarbons (PAHs) such as phenanthrene and enhanced biodegradation (Burd and Ward, 1996).

Other research by Garcia-Junco et al. (2001) indicated that addition of rhamnolipids led to attachment to the phenanthrene that enhanced bioavailability and hence degradation of the contaminant by P. aeruginosa.

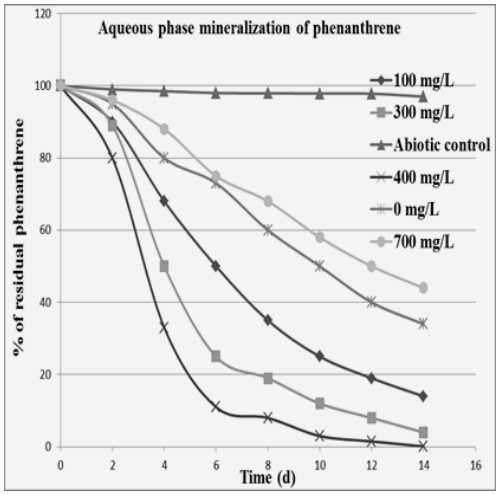

Figure 1: Aqueous phase mineralization of Phenanthren with different concentration of rhamnolipid

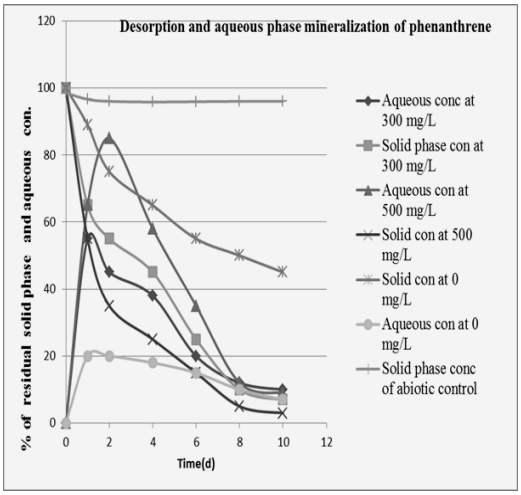

Figure 2: Desorbed and aqueous phase mineralisation graph of phenanthrene

The amount of PHE desorbed from the soil increased as the concentration of surfactant in the solution increased (Figure 2). In soils equilibrated with solution containing rhamnolipid at 500 mg/L, 75 % of the sorbed Phenanthrene was released after 5 d of equilibration from the artificially contaminated soil (800 mg/kg contamination level). The aqueous phase concentration rapidly increased during the initial fast desorption of the first 1 to 4 days, as the rate of desorption is greater than the rate of biodegradation bacterial metabolism is the rate limiting factor. Whereas for the unamended microcosm the aqueous concentration of phenanthrene is very low, because of the low desorption rate of the adsorbed substrate, there is low bioavailablity of phenanthrene to the degraders, and low mineralization too as a result, so desorption is the rate limiting factor. The data (Figure 2) shows, under the test conditions used in the experiments, that the rate of mineralization of phenanthrene is significantly slower than the rate of desorption for these compounds for the first days of fast desorption. But later on phenanthrene consumption by the isolates exceeded the phenanthrene desorption rate. Whereas the amendment of 500 mg/L resulted in inhibited degradation relative to the others at the outset but 4 days later rapid mineralization commenced, probably due to toxicity of increased solubilised contaminant concentration which took longer lag phase for the bacteria to adapt to the increased substrate concentration.

4. Conclusion

It can be concluded that from the studies conducted biosurfactants can generally enhance biodegradation in both aqueous and soil slurries by enhanced bioavailability of hydrophobic contaminants to microbial degradation. Even if the amended bio surfactant may temporarily cause extended lag phase, possibly due to toxicity of the excessively solubilized substrates, or the surfactants themselves there will be an enhancement of mineralization afterwards, though bacteria may take longer lag time to adapt to the excessive substrate concentration as long as the specific interaction between the surfactant and microorganisms is experimentally determined in advance and compatibility is confirmed. The other important and sustainable solution, insitu production of biosurfactant was observed in the biosurfactant unamended microscosm, as confirmed through drop collapse test and stable emulsification during hexane extraction of the residual phenanthrene. The insitu production of the biosurfactant, would be the most viable solution as exsitu application of biosurfactant would not be both economically feasible and would probably not be as suitable to the indigenous bacteria as that produced insitu. The insitu production can be stimulated through nutrient supplementation or bioaugmentation if the indigenous bacteria are not capable.

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