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Effect of Biosurfactant Addition on the Biodegradation of Phenanthrene in Soil-water System

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Abstract

The extent of solubility enhancement by biosurfactant was examined at various pHs prior to the biodegradation experiments. The molar solubilization ratio (MSR) was calculated from the batch solubilization experiments and the highest MSR was detected at pH 5. The effect of the biosurfactant, rhamnolipids, on the phenanthrene mineralization in soil-water system was investigated. The strain 3Y was selected for the mineralization assay and large amounts of phenanthrene were degraded at neutral pH in soil-water system without the biosurfactant. The addition of 150 mg/L rhamnolipids showed no effect on mineralization of phenanthrene in soil-water system, and total mineralization rates after 6 weeks incubation at each pH showed no differences in presence and absence of rhamnolipids. Our result indicated that the toxic effect of rhamnolipids can disappear when soil particles exist, and also the enhanced solubility of phenanthrene does not work for mineralization enhancement in this soil-water system.

Keywords: Rhamnolipids, Soil, Solubility, Mineralization, Phenanthrene

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are wide-spread in the environment and hydrophobic; as the number of rings in the molecular structure increases, water solubility decreases and the octanol/water partition coefficient (K_{OW}) increases. Owing to their high partition coefficients, these compounds can be strongly sorbed onto the surface of particles and be deposited to the soil environments. With respect to their suspected carcinogenicities and mutagenicities, PAHs pose threats to aquatic organisms and human health.[1)] In addition, their removal efficiency can be limited by low mass transfer phases, such as PAH-contaminated soils, because most chemical and biological remediation technologies require transfer from geosorbents and non-aqueous phase liquids (NAPLs) into the mobile phase.[2,3)] Researches have examined the possibility of enhancing the bioavailability of low solubility and highly sorptive compounds by the addition of solubilization agents, such as surfactants, to the system.[4-7)]

Biosurfactants have several advantages over chemical surfactants, including lower toxicity,[8,9)] higher biodegradability,[10-13)] better environmental compatibility, and the ability to be synthesized from renewable feedstocks.[14)] They occur naturally in soil, which makes them acceptable from a social and ecological point of view. Thus, complete removal after treatment may not be necessary.[15-17)] These points make biosurfactants ideal for environmental applications.

The use of surfactants may be one way to enhance the bioavailability of sorbed PAHs. Surfactants can increase bioavailability by increasing contaminant aqueous solubility, thereby increasing the fraction of soluble compound and hence the amount available for microbial uptake. However, there is still controversy regarding their effects on contaminant biodegradation. Several studies have shown enhanced biodegradation of sorbed and NAPL contaminants in the presence of surfactants.[18-22)] On the contrary, inhibitory effects on biodegradation in the presence of surfactants have been seen in other cases.[2,23-30)] For example, Guha and Jaffe[27)] reported that the rate of degradation of phenanthrene in the presence of surfactant was dependent on the type of surfactant used and phenanthrene present in micellar phase is resistant to be bioavailable. Therefore, improved understandings of interactions between contaminant, surfactant, microorganisms, and soil/sediment in different environments are required.

The morphology of biosurfactant aggregates can also be significantly impacted by changes in pH, surfactant concentration, temperature, and ionic strength of the solution.[31-32)] Previous

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work[19] has shown that the effect of a rhamnolipid biosurfactant on surface tension and solute dispersion is a function of pH. The pH effect on organic dispersion appears to be related to the type of rhamnolipid structure present.[31] Ishigami et al.[31] and Champion et al.[31] have shown that the morphology of rhamnolipid biosurfactants is a function of pH, changing from lamellar, to vesicular and ultimately to micellar as the pH increases.

The objective of this study was to examine the effect of biosurfactant addition on the mineralization of phenanthrene in soil-water system at various pH conditions.

2. Materials and Methods

Biosurfactant. The biosurfactant used in this study was a glycolipid and is the most commonly isolated type of biosurfactant. Members of the genus *Pseudomonas*, which is a common soil microorganism, produce various rhamnolipids.[19] The rhamnolipid solution was purchased from the Jeneil Biosurfactant Company (Saukville, WI, USA). Specifically, the Jeneil product JBR 425, with a mono- to di- rhamnolipid ratio of 1:1, was used, and supplied as a 25% aqueous solution. The two major rhamnolipid components in this solution are a monorhamnolipid (α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate), and a dirhamnolipid (2-o-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate), with molecular weights of 504 and 650, respectively. The critical micelle concentration (CMC) of this biosurfactant is 0.1mM (57.7 mg/L).[34]

Chemicals. The mineral salt medium (MSM) was composed of (per liter) 0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 1 g each of KH_2PO_4, $(NH_4)_2HPO_4$ and KNO_3 and 0.05 g $FeCl_3$. The Yeast Extract-Polypeptone-Glucose (YEPG) medium (pH 7.0) contained (per liter) 0.2 g yeast extract, 2.0 g polypeptone, 1.0 g glucose and 0.2 g NH_4NO_3. The YEPG was used as a 50% strength multipurpose growth. Phenanthrene (purity >98%) was purchased from Aldrich Chemical Company (Milwaukee, WI, USA). For mineralization study, [9-^{14}C]-phenanthrene was purchased from Aldrich Chemical Company (Milwaukee, WI, USA) and specific activity was 8.2 mCi/mmol. The methanol (High Performance Liquid Chromatography (HPLC) grade), acetonitrile (HPLC grade) and water (HPLC grade) were all purchased from Fisher Scientific Company (Pittsburgh, PA, USA).

Microorganism. Two phenanthrene-degrading strains, 3Y and 4-3 were isolated from a diesel-contaminated site in Korea, using the spray plate method.[35] The strains, 3Y and 4-3, were identified as *Sphingomonas* sp. and *Paenibacillus* sp., respectively, based on the partial sequencing of their 16S rDNA.[36] In this study, growth of 3Y and 4-3 on biosurfactant was checked, with no growth confirmed. Biosurfactants production by these strains was also checked by measuring surface tension during incubation of precultures and no production was confirmed in our experimental condition.

The *Pseudomonas putida* CRE 7 was obtained from Dr. Raina M. Maier of the Department of Soil, Water and Environmental Science, University of Arizona. This strain has previously been reported not to produce or utilize biosurfactants during growth on mineral salts medium (MSM) containing phenanthrene.[30] In this study, the growth of CRE 7 on rhamnolipid was also checked and no growth was observed.

Molar solubilization ratio (MSR). The effect of pH on the solubility of phenanthrene in 25, 50, 100, 150, 200, and 250 mg/L rhamnolipid solution was determined at various pHs. In each experiment, 200 mg of phenanthrene was added to an autoclaved 25-mL Teflon tube (Nalgen Company, Rochester, NY) along with 20 mL of each rhamnolipid solution. The rhamnolipid solutions with various concentrations were prepared and the rhamnolipid solutions' initial pHs were adjusted to 4, 5, 6, 7, and 8 by the addition of 0.1 N HCl or 0.1 N NaOH solution, as necessary. Triplicate samples at each pH were placed on a rotary shaker (Scientific Industries, Bohemia, NY) for 48 hours at room temperature (24±1°C). The pH did not change significantly during this period. Afterwards, samples were then centrifuged at 4000 rpm for 10 minutes, and the supernatants were analyzed for phenanthrene by HPLC. The HPLC instrument equipped with a Waters model 717 Plus autosampler, two Waters model 510 pumps, a Waters model 490 E programmable multi- wavelength detector, and a Novapak column C18 (Waters, Milford, MA, USA). The HPLC analyses were performed isocratically using a mobile phase of 35% water and 65% acetonitrile, at a flow rate of 1 mL/min, using UV detection of the phenanthrene at a wavelength of 254 nm. The sample injection volume was 10 μL.

Characterization of phenanthrene degraders. To obtain the precultures, cells were transferred from a plate culture to YEPG medium. After 2 days incubation, the cells were harvested by centrifuge and washed twice with MSM medium before inoculation.

The mineralization assays of ^{14}C-phenanthrene were performed as follows. Four milliliters of MSM was added to a 25-mL EPA vial (Wheaton Sci., Millville, NJ) and CO_2 trap (8-mL vial) containing 0.5 mL of 0.5 M NaOH was placed inside the EPA vial. Before inoculating with 0.1 mL of preculture, 10,000 dpm ^{14}C -phenanthrene in methanol was added. The vial was sealed with a Teflon-lined cap (Wheaton Sci., Millville, NJ) and the assays were performed in triplicate. Precultures were grown in 20 mL of MSM with non-labeled phenanthrene for 2 days. The vials were incubated at 30°C and 150 rpm for 7 days.

Mineralization of ^{14}C-phenanthrene was followed by periodically removing the 0.5 mL NaOH trap and was replaced with a new trap solution. The trap solution (0.5 mL NaOH) was transferred to a 6-mL polyethylene scintillation vial (Wheaton Sci., Millville, NJ) and 5 mL scintillation cocktail, Ultima Gold (Perkin Elmer, Boston, MA), was added. Vials were sealed, mixed by shaking, held in the dark overnight, and the amount of $^{14}CO_2$ counted using Beckman LS 6500 Scintillation Counters (Beckman coulter, Fullerton, CA).

Mineralization in soil system. To examine the phenanthrene mineralization in soil-water system, ^{14}C-phenanthrene mineralization assays were introduced. For this experiment, only 3Y, *Sphingomonas* sp. was selected as this strain was isolated from diesel contaminated site in Korea. That diesel contaminated soil samples were collected directly from a gas station in Gwangju, Korea at depth of 5-7 m using a core sampler.[36] The solid phase used in this study to provide a simplified system mimicking the

effect of soil particles was Chumunjin standard sand, which was purchased from Dongyang Science Co. (Gwangju, Korea). Before each experiment, the sand was rinsed five times with deionized water to remove possible residual salts or impurities and air-dried. In addition, sand was 3 times autoclaved prior to the experiments. In sterile biometer flasks (modified 250-mL Erlenmeyer flasks containing a 3×5 cm glass vial fused to the cap), 15 g of autoclaved sand was added.[25] For an unlabelled phenanthrene addition, 75 μL of 1% (w/v) phenanthrene in methanol was added and then, 1.0×10^5 dpm ^{14}C-phenanthrene in methanol was added. This resulted in 50 mg phenanthrene per 1kg dry soil. Before inoculation, flaks were kept for 24 hours at room temperature (20±2°C). After 24 hours, soils receive the inocula and to obtain the precultures, strains were transferred from a plate culture to an Erlenmeyer flask, which had been prepared as described above. MSM's pH was adjusted to 4, 5, 6, 7, and 8 with 1 N NaOH and 1 N HCl and 4 mL of pH-adjusted MSM was added, that makes saturated soil-water condition.

Five milliliters of sterilized NaOH (2N) was injected into the central glass vial as a trap of $^{14}CO_2$ to measure phenanthrene mineralized. Flasks were incubated for 6 weeks at room temperature and each treatment was performed in triplicate. Mineralization of ^{14}C-phenanthrene was followed by periodically removing the whole trap and central vial was replaced with a new trap solution. The trap solution (1 mL) was transferred to a 6-mL polyethylene scintillation vial (Wheaton Sci., Millville, NJ) and 5 mL scintillation cocktail, Ultima Gold (PerkinElmer, Boston, MA), was added. Vials were sealed, mixed by shaking, held in the dark overnight, and the amount of $^{14}CO_2$ counted using Beckman LS 6500 Scintillation Counters (Beckman coulter, Fullerton, CA).

3. Results and Discussion

3.1. Effect of pH on Molar Solubilization Ratio of Rhamnolipids

The enhancement of bioavailability of PAHs is related with the solubilization capacity of surfactant and the solubility of PAHs is strongly affected by pH values if the surfactant is an ionic surfactant such as biosurfactant, rhamnolipids.

The slope of a straight line above CMC in Fig. 1 represents the degree to which micelles enhance phenanthrene solubilization. The MSR represents the capacity of specific surfactant in solubilization process. The MSR and log K_m values are listed in Table 1. Phenanthrene solubilized in the micelles increased linearly with increasing biosurfactant concentration for all pHs. The order of increasing solubility enhancement of phenanthrene was pH 8 < pH 7 < pH 6 < pH 4 < pH 5. The trends in MSR values was consistent with the solubility changes at various pHs in previous study[37] and they found that the apparent solubility at pH 5.5 was 3.8 times greater than at pH 7 in presence of 240 mg/L rhamnolipids. More recently, Shin et al.[38] confirmed the explanation that the greater solubilizing capacity of the biosurfactant at pH 5 and 6 may be differences in the structure and size of the biosurfactant aggregate. An anionic surfactant, such as rhamnolipid, can undergo changes in the diameter of the head

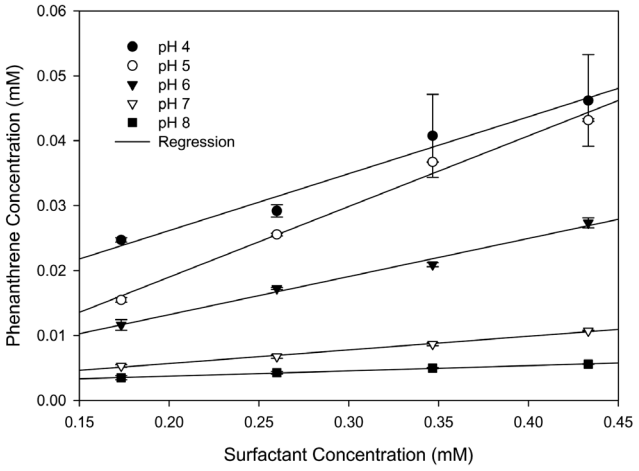

Fig. 1. The relationship between the solubility of phenanthrene and the concentration of rhamnolipid at various pHs. The slope of the solubilization curve is equal to the molar solubilization ratio. The lines represent linear regressions to the data and error bars represent ± one standard deviation.

Table 1. Calculated MSR values of rhamnolipid solutions at various pHs from batch solubilization experiments

pH	MSR	Log K_m	R^2
4	8.7×10^{-2}	5.79	0.9699
5	1.0×10^{-1}	5.87	0.9889
6	5.8×10^{-2}	5.63	0.9901
7	2.1×10^{-3}	5.20	0.9946
8	8.1×10^{-3}	4.79	0.9971

group depending on the protonation state of the carboxyl group.[31] To confirm the effect of pH on the rhamnolipid morphology, they introduced cryo-transmission electron microscopy (cryo-TEM) and micrographs showed that the rhamnolipid morphology changed from large lamellar sheets, to vesicle, and then to micelle as the pH increased. They concluded that the large and multilamellar vesicles at pH 5 were considered to be the most effective structure for the solubilization of phenanthrene.

Moreover, Shin et al.[38] examined the effect of pH on the solubility of phenanthrene in the sand-water system and they reported that a dramatic decrease in phenanthrene solubility at pH 4. The reason for this decrease was the loss of rhamnolipid molecules by the sorption into sand particles at pH 4. With the exception of solubility at pH 4, the trends in the solubility were similar to the results in aqueous system.

During experiment, precipitation of rhamnolipid was not apparent until the pH was decreased below 5.0. At pH 4.0, experimental error in the measurement of phenanthrene solubility became very high because of the precipitation of rhamnolipid (Fig. 1).

3.2. Growth and Degradability

The ^{14}C-phenanthrene mineralization assay was performed in MSM to validate the ring cleavage of phenanthrene. Three species which were previously tested phenanthrene-degraders was

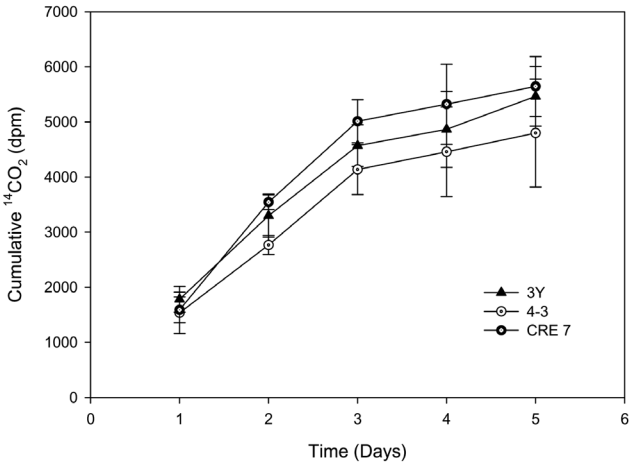

Fig. 2. Time course of [9-^{14}C] phenanthrene mineralization by 3Y and 4-3 in MSM. Data are given as mean ± standard deviation (n=3).

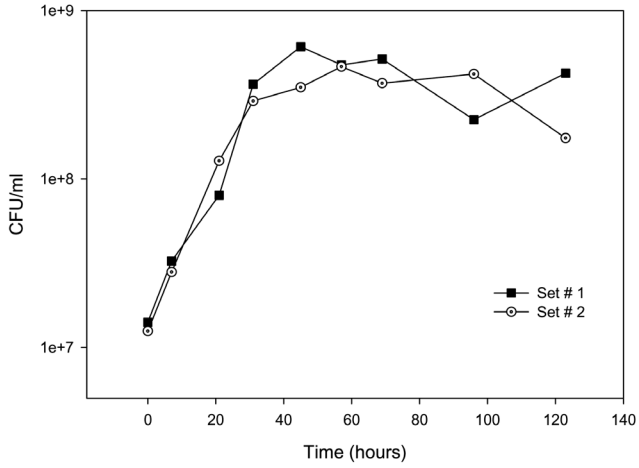

Fig. 3. Growth curve of 3Y in MSM with phenanthrene as a sole carbon source. Cell growth was monitored by plate count method and set #1 and #2 represent the duplicated experiments.

introduced in this study.[39)]

The result of $^{14}CO_2$ evolution (Fig. 2) from [9-^{14}C]-phenanthrene explained that three species could cleave the ring of phenanthrene. Three species showed the similar time course of mineralization during 5 days incubation. Although the degradability of 3Y was slightly lower than other species, 3Y, *Sphingomonas* sp., was selected for the further mineralization experiments in soil-water system because 3Y was isolated from a diesel-contaminated site in Korea and this strain was ubiquitous.

Growth of the phenanthrene degrader 3Y in MSM was monitored by observing the cell density (CFU/mL) by plate count method. In MSM, carbon and energy source was only phenanthrene and cell growth could be evidence of degradability of 3Y. Growth curve of 3Y at pH 7 and in absence of rhamnolipids is shown in Fig. 3. The cell growth was relatively slow. The cell growth of 3Y reached the stationary phase at 40 hours after incubation.

3.3. Phenanthrene Mineralization in Soil-water System

The mineralization of phenanthrene by 3Y was monitored in soil-water system in absence and presence of 150 mg/L rhamnolipid at various pHs. The mineralization experiments were conducted without rhamnolipids addition to determine the effect of only pH variation on the mineralization of phenanthrene in soil-waster system. The data are shown in Fig. 4 and maximum mineralization rate was detected at pH 6. This result indicates that neutral pH is more favorable for the mineralization of phenanthrene by 3Y in our experimental conditions. Relatively high mineralization rate was found at pH range 6~8.

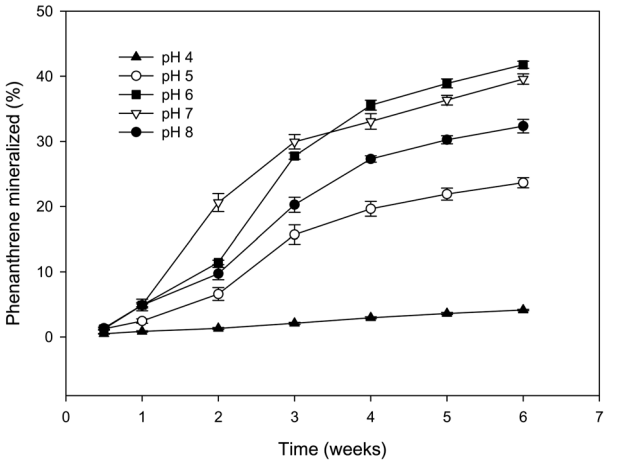

Fig. 4. The effect of pH on phenanthrene mineralization by 3Y in absence of biosurfactant in soil-water system. Symbols for phenanthrene (%) equal the average of triplicate flasks and error bars represent ± one standard deviation.

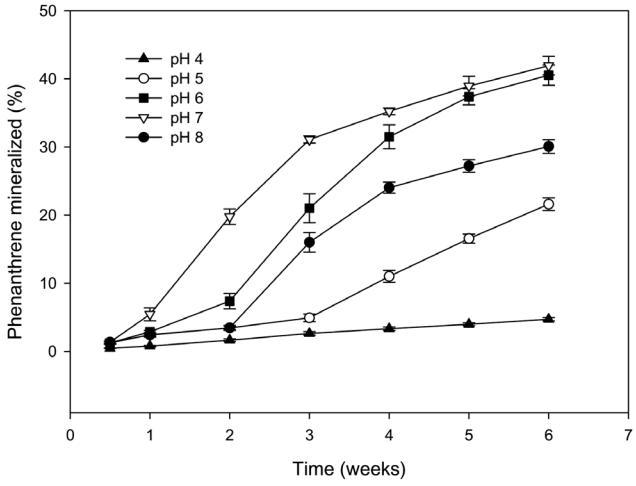

Fig. 5. The effect of pH on phenanthrene mineralization by 3Y in presence of biosurfactant (rhamnolipid 150 mg/L) in soil-water system. Symbols for phenanthrene (%) equal the average of triplicate flasks and error bars represent ± one standard deviation.

To determine whether the rhamnolipid addition could enhance the mineralization of phenanthrene by 3Y in soil-water system or not, the mineralization experiment was carried out in presence of 150 mg/L rhamnolipid. The result showed that the addition of rhamnolipid showed no effect on mineralization of phenanthrene in soil-water system (Fig. 5). Similar to the result of mineralization without rhamnolipids, relatively high mineralization rate was found at pH range 6~8. Even though the toxic eff-

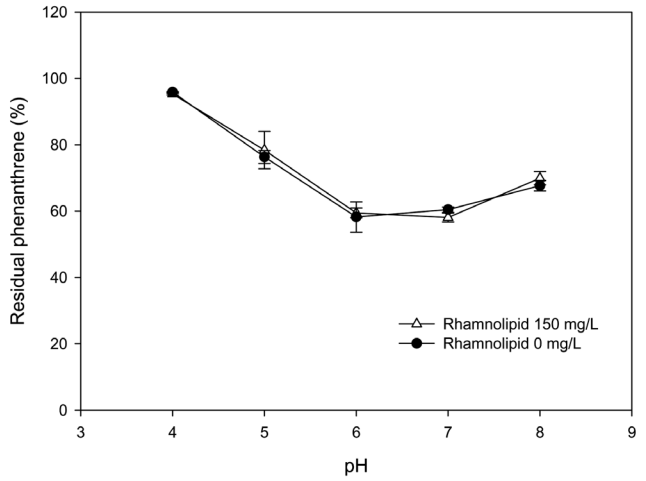

Fig. 6. The residual phenanthrene after 6 weeks mineralization by 3Y in absence and presence of biosurfactant (rhamnolipid 150 mg/L) in soil-water system. Symbols for phenanthrene (%) equal the average of triplicate flasks and error bars represent ± one standard deviation.

ect of rhamnolipids to 3Y species in aqueous biodegradation system was previously reported,[39] we expected the enhanced mineralization rate by rhamnolipid addition in soil-water system because the soil particle could complexly affect on surfactant sorption, phenanthrene solubilization and phenanthrene biodegradation.

As results, total mineralization rates after 6 weeks incubation at each pH showed no differences in presence and absence of rhamnolipids (Fig. 6). This result indicates that the toxic effect of rhamnolipids can disappear when soil particles exist and also the enhanced solubility of phenanthrene does not work for mineralization enhancement in this soil-water system.

More investigations are required to demonstrate how the toxicity of surfactant can be reduced or why enhanced solubility has no effect on the mineralization of PAHs in soil-water system. The complex interactions among biosurfactant, microorganisms, contaminants, and environmental conditions should be carefully investigated to get the effective biosurfactant-enhanced biodegradation process in soil-water system.

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