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# Use of biosurfactant to remediate phenanthrene-contaminated soil by the combined solubilization–biodegradation process

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**Abstract**

The applicability of the combined solubilization–biodegradation process was examined using soil-packed column. In the solubilization step, 50 pore volumes of 150 mg/l biosurfactants solution was injected and the percentage removal of phenanthrene (mg) was 17.3% and 9.5% from soil with pH 5 and 7, respectively. The highest solubility was detected at pH 5 and this result confirmed that adjusting the pH of the biosurfactants solution injected could enhance the solubility of phenanthrene. Following this, soil samples were completely transferred to batches and incubated for 10 weeks to monitor phenanthrene degradation. The phenanthrene concentration in the soil samples decreased significantly during the biodegradation step in all soil samples, except for the soil sample that was flushed with biosurfactants solution with pH 4. This indicated that the degradation of contaminants by specific species might not be affected by the residual biosurfactants following application of the solubilization process. Moreover, these results suggested that the biosurfactant-enhanced flushing process could be developed as a useful technology with no negative effects on subsurface environments and could be combined with the biodegradation process to increase the removal efficiency.


*Keywords:* Solubilization; Biodegradation; Biosurfactants; PAHs; Soil

## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread in the environment and are 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 adsorbed onto the surface of particles and be deposited in soil environments [1]. Thus, the efficiency of their removal is limited in low mass transfer phases such as PAH-contaminated soils, since most chemical and biological remediation technologies require transfer from geosorbents and NAPLs into the mobile phase [2,3].

Many authors employed successfully surfactant aided washing for remediation of soils contaminated with hydrophobic contaminants [4–11]. In addition, recent research has examined the possibility of enhancing the bioavailability of low solubility and highly adsorptive compounds by the addition of solubilization agents such as surfactants to the system [12–15].

The introduction of surfactants into the soil environment can lead to contamination concerns. Consequently, the toxicity of the surfactant and its potential degradation products needs to be carefully considered prior to the selection of a surfactant for the purposes of soil clean up [16]. Biologically produced surfactants occur naturally in soil, and use of these surfactants in remediation processes may be more acceptable from this point of view.

Variable results have been shown concerning the utility of using biosurfactants in hydrocarbon solubilization and biodegradation [17–19]. Bai et al. [19] used an anionic mono-rhamnolipid biosurfactant from *P. aeruginosa* to remove residual hydrocarbons from sand columns. They recovered ca. 84% of residual hydrocarbon (hexadecane) from sand columns packed with 20/30-mesh sand, and 22% of hydrocarbons were recovered from 40/50-mesh sand. In another study, biosurfactants (5 g/l, pH 7) enhanced the solubility of naphthalene by more than 30 times its aqueous solubility [20]. Oberbremer et al. [21] used a mixed

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soil population to assess hydrocarbon degradation in a model oil system. They reported a statistically significant enhancement in hydrocarbon degradation when sophorose lipids were added to the system containing 10% soil and a 1.35% hydrocarbon mixture of tetradecane, pentadecane, hexadecane, pristane, phenyldecane and naphthalene in the mineral salt medium. In the absence of surfactant, 81% of the hydrocarbon mixture was degraded within 114 h, while in the presence of biosurfactant up to 90% of the hydrocarbon mixture was degraded within 79 h.

Although lower toxicity is expected from the use of most biosurfactants, concern still remains [22,23]. In the case of microorganisms degrading hydrophobic hydrocarbons, the presence of surfactants, especially in concentrations above the CMC, has had an inhibiting effect [24–26]. Although this phenomenon may be partly explained by the reduced availability of micellar hydrocarbons [27,28], inhibition was also observed for hydrophilic substrates. Compared to synthetic surfactants [2,24,27,29,30], the use of biosurfactants has been associated with less frequent inhibitory effects on biodegradation [31–34]. To date, research concerning bio/surfactant and bio/surfactant-enhanced contaminant toxicities has been sparse.

It is to be expected that some of the flushing agent such as surfactant, will remain in the treated zone after the flushing event. The potential impact of residual flushing agent on microbial processes is a question of concern. For these reasons, it is important to evaluate the potential impact of enhanced-flushing operations on microbial processes for systems wherein they will be used in conjunction with bioremediation [35].

In an early study, Ishigami et al. [36] and Champion et al. [37] observed that the structure of rhamnolipid is strongly dependent on pH, and can undergo changes from large lamellar sheets, to vesicles, and to micelles. The reported p$K_a$ for rhamnolipid is 5.6 [36]. As the pH increases from 5.5 to 8.0, repulsion between the more negatively charged head groups effectively creates a larger head diameter, thus facilitating a change in the morphology from lamellar to vesicles, and finally to micelles [37]. Interestingly, Zhang and Miller [38] also observed that the surface tension and dispersion of octadecane was significantly affected by pH. These studies demonstrate that control of the pH needs to be considered in field applications for improved performance of anionic biosurfactant systems.

In this study, we examined the combined solubilization–biodegradation process with biosurfactants to remediate phenanthrene-contaminated soil. The removal efficiency of the process was compared at various pHs since the pH of biosurfactants solution could be an important factor in this system. The work presented herein will provide that the potential impact of biosurfactant-enhanced flushing operations on followed microbial processes and the possibility of combined remediation process.

## 2. Materials and methods

### 2.1. Biosurfactants and chemicals

The biosurfactant used in this study was a rhamnolipid. This biosurfactant was selected because it is a glycolipid, which is the most commonly isolated type of biosurfactant, and is produced by a member of the genus *Pseudomonas*, which represent common soil microorganisms that produce various rhamnolipids [38]. The rhamnolipid solution was purchased from the Jeneil Biosurfactant Company (Saukville, WI). The Jeneil product JBR425, 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 0.1 mM in deionized water [39].

The rhamnolipid solutions were prepared in mineral salt medium (MSM) and the initial pH was adjusted to 4, 5, 6, 7 or 8 by the addition of 0.1N HCl or 0.1N NaOH, as necessary. The rhamnolipid concentration used in this study was 150 mg/l (0.26 mM) and this concentration is higher than CMC in deionized water.

The MSM was composed (per liter) of 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 $NH_4NO_3$. YEPG was used as multipurpose growth medium at 50% strength. Phenanthrene (purity >98%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Chloroform, acetone and dichloromethane used to dissolve or extract the phenanthrene, in addition to methanol, acetonitrile and water, were all purchased from Fisher Scientific Co. (Pittsburgh, PA) and were of HPLC grade.

### 2.2. Microorganisms

The phenanthrene-degrading strain 3Y was isolated from a diesel-contaminated site in Korea using the spray plate method [40]. The strain 3Y was identified as *Sphingomonas* sp. based on partial sequencing of the 16S rDNA [41]. To examine the biosurfactants production or utilization by 3Y, surface tension was measured by NIMA tensiometer Model 9002 (NIMA technology, England). It was confirmed in this study that 3Y did not produce or utilize biosurfactants during growth on MSM containing phenanthrene.

### 2.3. Preparation of phenanthrene-contaminated soil

The clean field soil was collected near Yongsan River in Gwangju, Korea at a depth of 30 cm. After sampling, the soil was completely air-dried and sieved on a 10-mesh (<2 mm) screen prior to use in the experiments. The average soil pH was 6.7 and loss on ignition (LOI) was 3.8%. The sieved soil sample was analyzed for particle size distribution and soil texture was sandy loam (sand 55%, clay 15%, silt 30%). The soil was autoclaved three times and this could affect on phenanthrene sorption by modifying organic matter in soil. Nevertheless, the autoclaved soil was introduced because phenanthrene biodegradation by 3Y following solubilization step should be monitored.

The spiking method was employed to prepare artificial phenanthrene-contaminated soil, followed by several steps to ensure homogeneous conditions. In a 2-l beaker, 200 g of air-dried soil was added followed by 50 ml of spiking solution in dichloromethane. These steps were repeated for up to 1000 g of spiked soil until the approximate concentration of phenanthrene in the soil was 200 mg/kg. The contaminated soil was placed in a fume hood for 7 days to evaporate dichloromethane.

### 2.4. Combined solubilization–biodegradation process

#### 2.4.1. The solubilization step

A glass column (Spectra/Chrom, TX) with a diameter of 2.5 cm and a length of 10 cm was used. Prior to packing the column with phenanthrene-contaminated soil, the soil was inoculated with the 3Y species. Ten milliliters of preculture was added to a 250-ml media bottle with 100 g of soil and mixed using a rotary shaker for 24 h. This mixing was introduced to prepare 3Y-inoculated soil, where the initial cell concentration was ca. $5 \times 10^7$ CFU/g soil. The column was packed with phenanthrene-contaminated and 3Y-inoculated soil under vibration. The porous media was compacted using a stainless steel rod after every one-fifth of the column length was packed. The weight of the media packed in a column was measured to calculate the density and porosity. The bulk density and porosity of compacted soil were $1.426 \pm 0.020$ g/cm$^3$ and $0.357 \pm 0.009$, respectively.

The soil-packed column was then flushed with certain pore volumes of rhamnolipid solution at flow rate of 1 ml/min. During the flushing process, the effluent was collected using a fraction collector (Spectra/Chrom, TX) and the phenanthrene concentration and pH measured. These experiments were performed twice at each pH.

The cell density in the effluent was monitored during the flushing process by the spread plate method using YEPG agar plates according to experimental procedures based on standard methods [42]. Samples were serially diluted and duplicate plates were prepared at each dilution ratio. Colonies were enumerated following incubation for 2 days at 30 °C.

#### 2.4.2. The biodegradation step

After flushing at each pH, soil samples were layered into 500 ml beakers to a thickness of less than 2 cm. The beakers were then covered with polyethylene film and incubated at room temperature for 10 weeks. The beakers were aerated every 3 days by removing the covers during incubation [43]. In order to monitor phenanthrene degradation following solubilization step, soil samples were taken from the beakers and remaining phenanthrene in soil was extracted at the designated time intervals.

Moreover, the change of 3Y cell density in the soil samples after flushing was investigated. To count phenanthrene-degrading bacteria, 1 g of wet soil sample and 9 ml of dilution water were aseptically placed into a sterile dilution bottle and shaken for 10 min using a rotary shaker. After shaking, 1 ml of the suspension was aseptically transferred immediately to a test tube containing 9 ml of dilution water, resulting in a $10^{-2}$ dilution. The samples were serially diluted and duplicate plates were prepared at each dilution ratio. Colonies were enumerated following incubation for 2 days at 30 °C. Subsequently, the phenanthrene-degrading bacteria were checked using the clearing zone count method [44,45]. Briefly, a solution of phenanthrene in acetone (20 g phenanthrene/l) was sprayed onto the plates so that a thin film of phenanthrene remained on the surface of the agar. After spraying, the plates were incubated for an additional 4 days at 30 °C, after which time the number of colonies that produced clearing zones in the phenanthrene film were enumerated.

### 2.5. Analytical methods

The phenanthrene remaining in soil was extracted by pressurized fluid extraction (PFE), comparable to Method 3545, accelerated solvent extraction (SW-846, US EPA). In this study, One PSE system (Applied Separations, Allentown, PA) was introduced. Briefly, 3 g of soil sample was added into a stainless steel vessel fitted to the PSE. In this process, 3 min of a static state at 100 °C and 100 bar was followed by 30 s of gas flushing and 2 min of solvent flushing. The extraction solvent consisted of a mixture of acetone and dichloromethane (1:1, v:v). The percent recovery of phenanthrene in a control experiment by this extraction method was $93 \pm 3\%$.

The effluent and extract were analyzed for phenanthrene using an HPLC instrument equipped with a Waters model 717 Plus autosampler, Waters model 600 pumps, a M720 absorbance detector (Young-In, Korea) and a Novapak C18 column (Waters, MA). The HPLC analysis was performed isocratically using a mobile phase consisting of 35% water and 65% acetonitrile at a flow rate of 1 ml/min, and employing UV detection of phenanthrene at a wavelength of 254 nm. The injection volume used was 10 μl. A M474 fluorescence detector (Waters) was used to analyze low concentrations of phenanthrene. Fluorescence detector excitation and emission levels were set at 254 and 390 nm, respectively [46].

## 3. Results and discussion

### 3.1. The solubilization step

Biosurfactant-enhanced soil flushing was carried out for phenanthrene-contaminated field soil by injecting ca. 50 pore volumes of rhamnolipid solution at various pHs. The phenanthrene concentration and variation in pH of the effluent are shown in Fig. 1. By comparing the maximum phenanthrene concentration in the effluent at various pHs, we can predict the effect of pH on phenanthrene solubility in the soil column system at constant flow. The maximum phenanthrene concentration was 1.5, 3.2, 2.2, 1.2 and 1.4 mg/l at pH 4, 5, 6, 7 and 8, respectively (Fig. 1(a)). The second set of solubilization experiment was performed and the trends were reproducible (data not shown).

A previous study [47] showed that the apparent solubility could be increased at pH 4, 5 and 6. Based on their results, the highest solubility was detected in the pH range 4.5–5.5, where the apparent solubility at pH 5.5 with a 240 mg/l rhamnolipid solution was 3.8 times greater than that at pH 7. Additionally,

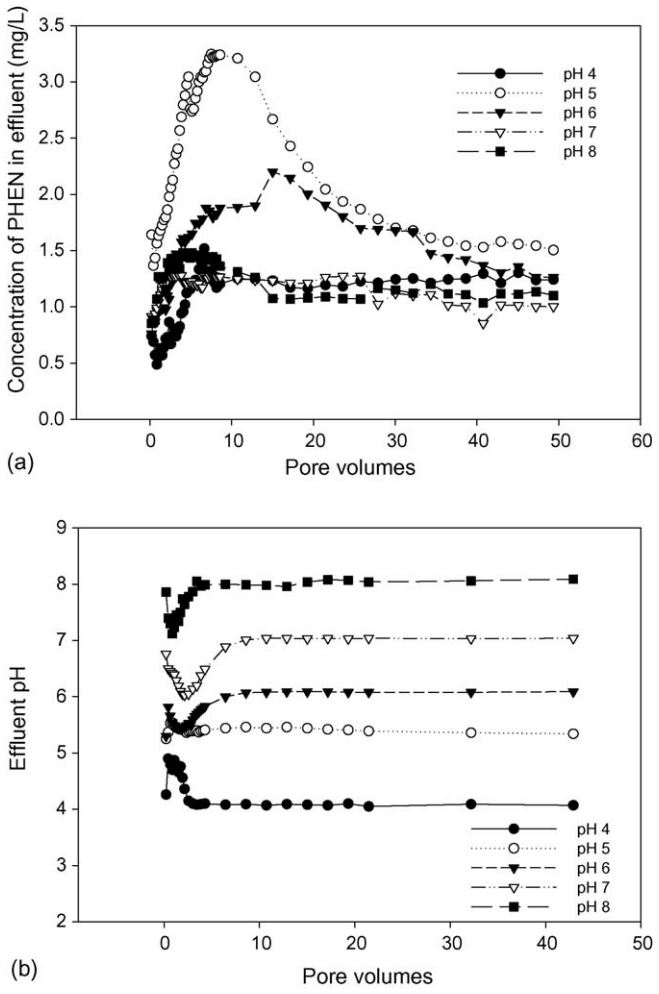


Fig. 1. Effect of pH on (a) phenanthrene solubility and (b) effluent pH during the solubilization step.

they showed that the aggregate structure of rhamnolipid was pH-dependent through the size distribution experiment. They concluded that changes in the apparent solubility with changes in pH were possibly related to the rhamnolipid, an anionic surfactant, forming various pH-dependent structures. Therefore, it could be concluded that adjusting the pH of the biosurfactants solution could enhance the solubility of phenanthrene in our soil system. However, the solubility of phenanthrene in the column experiment decreased comparing with the previous aqueous batch system [47]. The reason for this might be due to a rate-limited solubilization or surfactant sorption onto soil. The difference between the batch system and the column experiment could be attributable to the limited contact time in column experiment. Moreover, sorption of surfactant onto soil may result in a proportion of surfactant being unavailable for micellar solubilization of PAH [48]. A number of researchers have investigated the sorption of anionic and non-ionic surfactants onto soil. Grasso et al. [49] investigated the key factors that influence PAH desorption from contaminated soil and found that sorption of surfactant can prevent the effective desorption of PAHs.

Table 1
Mass balance of phenanthrene at the end of the solubilization step with filed soil-packed column

| | pH | | | | |
|---|---|---|---|---|---|
| | 4 | 5 | 6 | 7 | 8 |
| Effluent (mg) | 1.37 | 2.35 | 1.86 | 1.30 | 1.33 |
| Residual in soil (mg) | 11.52 | 10.21 | 13.21 | 12.10 | 11.09 |
| Total (mg) | 12.89 | 12.56 | 15.07 | 13.40 | 12.42 |
| Recovery (%) | 95.0 | 92.6 | 111.3 | 98.8 | 91.6 |

Initial mass of phenanthrene was $13.56 \pm 0.37$ mg for each experiment.

In general, the effluent pH variation was found during the initial pore volumes and this might have been due to the initial soil pH (Fig. 1(b)). However, after 2–3 pore volumes were injected, the effluent pH value was similar to the influent pH value.

The total phenanthrene mass removed was calculated by converting the phenanthrene concentration in the effluent to mass and by extracting the residual phenanthrene in the soil after flushing to determine the overall removal efficiency. Table 1 shows the mass balance of phenanthrene at the end of the solubilization experiment using 50 pore volumes of 150 mg/l rhamnolipid solution at each pH. The rhamnolipid solution removed 17.3% and 9.5% of phenanthrene from the soil at pH 5 and 7, respectively. This indicated that the removal efficiency was enhanced under acidic conditions and that phenanthrene solubility is highly dependent on the pH of the influent solution.

The cell density of 3Y in the effluent was monitored in this study to determine if any significant loss of cell density had occurred during the flushing process. The cell density (CFU/ml) of 3Y during the 50 pore volumes of flushing is shown in Fig. 2. The density of cells in the effluent was negligible (1.5–6.8%) compared to the initial cell density of 3Y in the soil.

### 3.2. The biodegradation step

The soil remediated by flushing was transferred to an autoclaved beaker and the phenanthrene concentration in the soil was

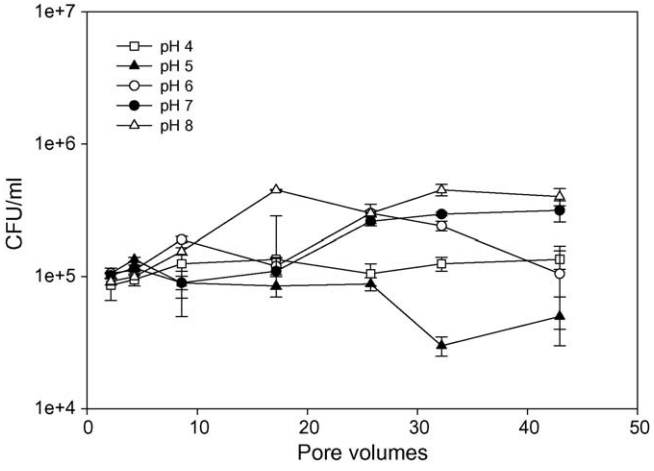


Fig. 2. Cell density (CFU/ml) of 3Y in the effluent during the solubilization step with rhamnolipid 150 mg/l at various pHs. Error bars represent the range of duplicate determinations.

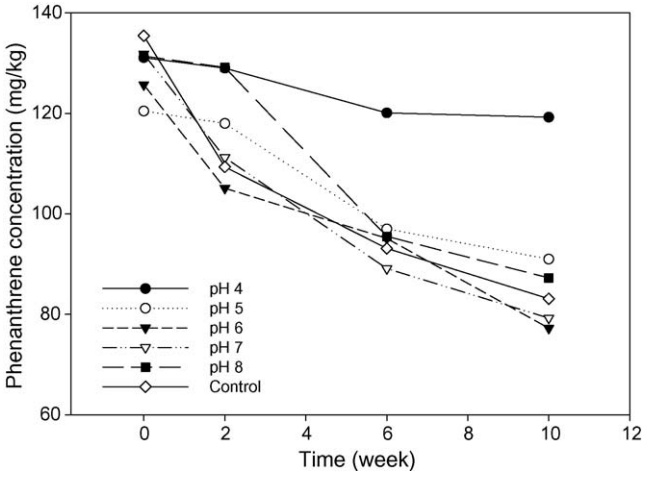


Fig. 3. Changes in phenanthrene concentration in soil after the solubilization step at various pHs (control sample was saturated with pH 7 MSM, without rhamnolipids).

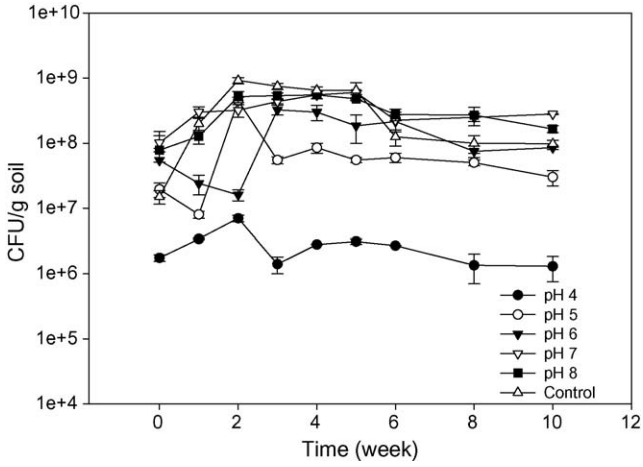


Fig. 4. Cell density of the phenanthrene degrader, 3Y, after the solubilization step at various pHs (control sample was saturated with pH 7 MSM, without rhamnolipids). Error bars represent the range of duplicate determinations.

monitored during a 10-week period in an effort to evaluate the rate of phenanthrene degradation after flushing and the effect of residual rhamnolipid on phenanthrene degradation. The variation in phenanthrene concentration (mg/kg) in the soil is shown in Fig. 3.

The amount of phenanthrene extracted by the PSE extraction method decreased as the incubation period increased. This suggested that microbial degradation of phenanthrene occurred during the biodegradation step. Except in the case of the soil sample that was flushed with a pH 4 rhamnolipid solution, the phenanthrene concentration decreased significantly in all the other soil samples. This suggested that residual rhamnolipid did not significantly inhibit the degradation, and that a negligible toxic effect was shown. In particular, the degradation rate of the control (pH 7, without rhamnolipid) and sample (pH 7, with rhamnolipid) were similar, and indicated the absence of any inhibitory effect.

These results suggested an important implication in flushing remediation technology for PAH-contaminated soil. The rhamnolipid-enhanced flushing process can be developed as a technology with no negative effect on the subsurface environment and can be combined with a biodegradation process to increase the removal efficiency.

Fig. 4 shows the changes in bacterial cell growth during the 10-week incubation period. The phenanthrene degrader, 3Y, was monitored and the colonies of phenanthrene degraders resembled 3Y species based on its characteristic yellow color and colony morphology. Prior to the flushing process being applied, the initial cell number of phenanthrene degraders was determined to be ca. $10^7$ CFU/g soil and the control soil sample, which is not flushed by rhamnolipid solution, showed the same cell density at 0 week. Within 2 weeks, the cell number of the control pH 7 and 8 soil samples reached up to $10^9$ CFU/g soil. This indicated that 3Y could degrade and use phenanthrene as a carbon source. In the case of pH 6, the cell number decreased until 2 weeks and then growth was detected. It is predictable that the pH range from 6 to 8 would be favorable for this phenanthrene degrader in this experimental system. At the stationary phase, the cell number of the pH 5 soil sample was $10^8$ CFU/g soil. This value was slightly lower than those values for the pH 7 and 8 soil samples. Although the phenanthrene degrader was not active when compared to the cases at pH 7 and 8, it seemed that the phenanthrene degrader could still degrade and use phenanthrene as a carbon source.

When the two-step remediation process was completed, the total removal efficiency at each pH was investigated and is shown in Fig. 5. Firstly, removal by solubilization during the 50 pore volume flushing is presented and the highest efficiency was detected at pH 5. In this study, total removal was relatively low compared to other flushing studies [50–52] since the concentration of the rhamnolipid solution was relatively low and only 50 pore volumes were injected. More significantly, this result showed that simple pH adjustment could enhance the removal efficiency in the biosurfactant-enhanced soil flushing process even when same concentration of surfactant was introduced. This flushing step was applied for less than 20 h.

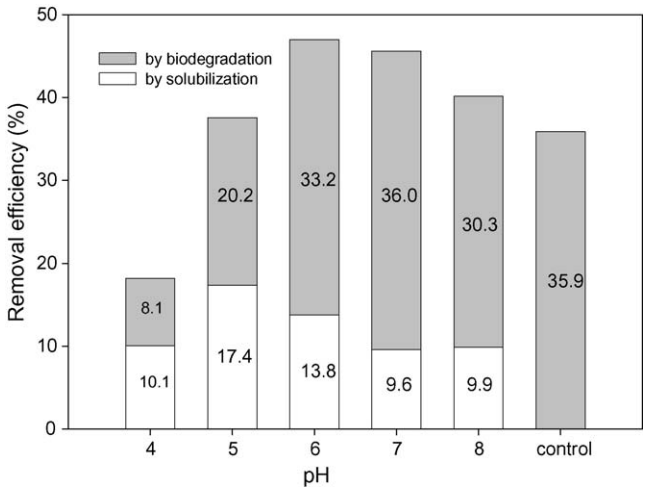


Fig. 5. The total removal efficiency of the combined solubilization–biodegradation process at various pHs (control sample was saturated with pH 7 MSM, without applying the flushing process).

Secondly, removal by degradation during 10 weeks is also shown in Fig. 5 and the highest degradation was found at pH 7. These results appear to be reasonable based on a previous study [53] using other *Sphingomonas* sp., where it was shown that the optimum pH for biotin production by fermentation of recombinant *Sphingomonas* sp. was 7.0. Nevertheless, the highest degradation rate was measured at pH 7, with a reasonable amount of phenanthrene being degraded at other pHs except pH 4. This indicated that the degradation of contaminants by specific species might not be affected by application of the flushing process. In other words, residual biosurfactants present after the flushing process terminated seemed to be non-toxic to this phenanthrene degrader.

These findings showed that the application of a combined flushing and biodegradation process could be an effective remediation tool for some field conditions. If greater pore volumes were injected during the flushing step to effect feasible removal efficiency, fewer residual amounts of contaminants would be degraded without inhibition by residual biosurfactants. Additionally, pH adjustment for the purposes of enhancing the solubility will not significantly affect the biodegradation rate specific for the 3Y population.

## 4. Conclusions

The combined solubilization–biodegradation process was examined by monitoring phenanthrene removal in the two steps.

In the solubilization step, relatively high removal efficiencies were found at pH 5 and 6. This confirmed that the pH-dependent rhamnolipid structure displayed varied solubilizing capacity in this flushing process.

In the biodegradation step, the phenanthrene mass in the soil decreased significantly, and suggested that residual rhamnolipid did not significantly inhibit the degradation, and that only a negligible toxic effect was shown. The cell density of the control pH 7 and 8 soil samples were up to $10^9$ CFU/g soil within 2 weeks. This indicated that they could degrade and use phenanthrene as a carbon source.

The total removal efficiency at each pH through two remediation steps was also investigated. In summary, the removal efficiency was highest at pH 5 for flushing and at pH 7 for biodegradation. Although the highest degradation rate was measured at pH 7, a reasonable amount of phenanthrene was degraded at other pHs except pH 4. This indicated that the degradation of contaminants by specific species might not be affected by application of the flushing process. In other words, residual biosurfactants present after the flushing process terminated seemed to be non-toxic to the phenanthrene degrader.

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