Enhancing Performance of *Sphingobacterium spiritivorum* in Bioremediation Phenanthrene Contaminated Sand

**INTRODUCTION**

The most difficult issue in the application of bioremediation by inoculation of isolated bacteria at contaminated sites is the survival of the bacteria. Studies show that some of the isolated bacteria population decreased shortly after soil inoculation (Mrozik & Piotrowska-Seget, 2010). This may be due to the lack of certain nutrients that are needed for the bacteria growth, the environment in the contaminated site is toxic to the isolated bacteria, or the bacteria have less potential to degrade the contaminant. If survival of the strain can be improved and increase the bacteria population, the efficiency of soil bioremediation also can be increased.

A few studies have reported on the use of biocarriers, such as activated carbon and zeolite (Liang et al., 2009) or chitin and chitosan (Gentili et al., 2006), to convey nutrients to the bacteria for growth. Biocarriers enhanced oxygen diffusion, nutrient mass transfer, and water retention (Liang et al., 2009). Besides biocarriers, Tortora et al. (2007) reported that the use of bioenhancers, such as nitrogen and phosphorus plant...
fertilizers, with contaminated soil increased the number of degrading bacteria, as compared to an unfertilized control soil. In addition, the bioenhancers subsequently improved the bioremediation process.

In line with improving survival of degraders in new environments, Karamalidis et al. (2010) reported on a technology that provides protection for isolated bacteria using an encapsulation method. Sodium alginate encapsulated Pseudomonas aeruginosa strain spot was used to remediate soil contaminated with polycyclic aromatic hydrocarbons (PAHs). However, the study showed that inoculation with or without the encapsulation had no significant effect on PAH biodegradation.

Furthermore, microbiologists have been working on improving the efficiency of degrading bacteria by altering the bacteria with recombinant DNA technology. The alteration is performed artificially by incorporating DNA from two or more sources into a single recombinant molecule (Tortora et al., 2007). Meyer et al. (2005) incorporated xylene monooxygenase enzyme from Pseudomonas putida into Escherichia coli. This recombinant oxidized m-nitrotoluene to the corresponding alcohols, aldehydes, and acids.

These studies focused on enhancing the survival of isolated bacteria either through increasing or maintaining the bacteria population to enhance the efficiency of soil bioremediation. Inoculation with new bacteria increased the contaminant degradation. But, the performance of the inoculated bacteria in the presence of existing bacteria remains relatively unknown. The performance may be similar or less to that of the existing bacteria.

Reinoculation is simple, but the important effect of increasing the bacteria population itself through inoculation of the same strain is unknown. Inoculation with higher bacteria counts may increase the percentage removal, but the escalation of removal efficiency remains unknown. In this study, Sphingobacterium spiritivorum, isolated from municipal sludge, was used as the PAH-degrading microorganisms while phenanthrene served as the PAH surrogate.

The primary aim of this study is to enhance the performance of S. spiritivorum in phenanthrene degradation through reinoculation. Reinoculation with a similar bacteria count was evaluated based on the growth curve in two reactors. The potential of a single colony shows the preliminary result of high potential strain. The enhancement was evaluated based on degradation rate and percentage removal for all reactors.

METHODOLOGY

Materials

All chemicals used for extraction, preparation of minimal media, and bacteria culture were of analytical grade and supplied by Merck, Germany. The phenanthrene standard for the gas chromatography mass spectrometer (GCMS) analyses was also obtained from Merck, Germany. Ultra-pure water (UPW) used in this study was produced from Alga Purelab Ultra (18.2 MΩ, United Kingdom).
Sample Preparation

Preparation of Contaminated Sand

Silica sand was sampled and washed three times in a sand:water mixture of 1:2 (w:v), air dried in the dark, and passed through a 1 mm sieve. The sand samples were stored in a 500 mL glass jar and dried at 60 °C until a constant mass was recorded. Phenanthrene was dissolved in hexane to produce phenanthrene/hexane solution. The prepared sand samples were autoclaved (Hirayama, HVE-50, Japan) for 20 minutes at 121 °C and spiked with the phenanthrene/hexane solution. Hexane was evaporated under continuous mixing to ensure homogenous distribution of phenanthrene in the sand samples. The samples were stored at 4 °C until to be used in the biodegradation studies. The initial concentration of phenanthrene was verified in triplicate before being used for biodegradation study.

Preparation of Minimal Media

Minimal media solution was prepared by dissolving 8.5 g Na₂HPO₄, 3.0 g KH₂PO₄, 0.5 g NaCl, 1.0 g NH₄Cl, 0.5 g MgSO₄·7H₂O, 0.0147 g CaCl₂, 0.0004 g CuSO₄, 0.001 g KI, 0.004 g MnSO₄·H₂O, 0.004 g ZnSO₄, 0.005 g H₂BO₃, and 0.002 g FeCl₃ in 1 L UPW. The prepared minimal media were autoclaved (Hirayama, HVE-50, Japan) for 20 minutes at 121 °C.

Preparation of Bacteria Strain

The strain, S. spirituororum, was isolated from municipal sludge by Othman et al. (2009) preserved at 80 °C in microbeads (microbank™, Round Rock, TX). The strain was revived by transferring a few of the frozen beads into universal bottles containing 20 mL of Nutrient Broth that was incubated at 30 °C for three days. A series of dilution streaking was performed and the strain was subcultured three times to attain the active bacteria before it was used in biodegradation studies.

Growth Curve Test

The plate count method was used to establish the growth curve after the bacteria was inoculated in contaminated sand. This test was conducted in reactor 1 and reactor 2.

The bacterial number in the sand samples were quantified by mixing 1 g of sand with 9 mL of sterile phosphate buffered saline and homogenized at high speed for 1 minute using a vortex mixer. Successful 1/10 dilutions were made by adding 1 mL of the sand suspension to 9 mL of phosphate buffered saline. An aliquot (0.1 mL) of each dilution was transferred to nutrient agar on a Petri dish. The dishes were incubated at 30 °C at an inverted position. After 4 days, the numbers of bacterial colonies were counted using a plate counter. Plates with different dilutions were prepared and those with colonies in the range of 30 to 300 were used to estimate the number of bacteria. This number of colonies was then multiplied by the dilution factor to find the total number of bacteria per 1 g of the sand. The numbers of colonies are expressed as colony-forming units per gram of sand (CFU/g). All tests were conducted in triplicate.
Enhancing Performance of Sphingobacterium spiritivorum

A reactor contained 20 g autoclaved sand, 100 ppm phenanthrene, 5.6 mL minimal media, and 1.4 mL bacterial inoculums. 5.6 mL of minimal media constituted 40 percent of the total volume. This volume was selected based on the quantity of inorganic nutrient required for bioremediation of PAHs in soil (Li et al., 2008). A total 1.4 mL of bacterial inoculum occupied 10 percent of the total volume and this quantity was selected based on an optimization study conducted by Othman et al. (2009).

The bacteria inoculated into the samples were collected at the middle of the exponential growth phase based on established growth curves, that is, on day 4. This experiment was repeated three times and the average bacteria concentration on day 4 was 1 ± 1.2 × 10^7 cells/g soil. Then, the strain was inoculated into reactor 1 and reactor 2. For reactor 3, the bacteria strain was isolated from reactor 2 on day 9 and inoculated with almost a similar number as reactor 1 and 2. The method of isolation was adopted from Othman et al. (2010). Details of the experimental variations studies for all reactors are shown in Exhibit 1.

After inoculation, all flasks were shaken in an incubator shaker at 150 rpm in the dark at 30 °C. Sterile water was supplied at 2 percent remaining weight every day. All samples were analyzed in triplicate. Control samples consisted of 20 g autoclaved sand, 100 ppm phenanthrene, and 5.6 mL minimal media without bacterial inoculums. The plate count method was performed on the control samples and no colony was found.

### Biodegradation Study

Batch experiments were conducted in a 250 mL Erlenmeyer flask as the reactor. The reactor contained 20 g autoclaved sand, 100 ppm phenanthrene, 5.6 mL minimal media, and 1.4 mL bacterial inoculums. 5.6 mL of minimal media constituted 40 percent of the total volume. This volume was selected based on the quantity of inorganic nutrient required for bioremediation of PAHs in soil (Li et al., 2008). A total 1.4 mL of bacterial inoculum occupied 10 percent of the total volume and this quantity was selected based on an optimization study conducted by Othman et al. (2009).

The bacteria inoculated into the samples were collected at the middle of the exponential growth phase based on established growth curves, that is, on day 4. This experiment was repeated three times and the average bacteria concentration on day 4 was 1 ± 1.2 × 10^7 cells/g soil. Then, the strain was inoculated into reactor 1 and reactor 2. For reactor 3, the bacteria strain was isolated from reactor 2 on day 9 and inoculated with almost a similar number as reactor 1 and 2. The method of isolation was adopted from Othman et al. (2010). Details of the experimental variations studies for all reactors are shown in Exhibit 1.

After inoculation, all flasks were shaken in an incubator shaker at 150 rpm in the dark at 30 °C. Sterile water was supplied at 2 percent remaining weight every day. All samples were analyzed in triplicate. Control samples consisted of 20 g autoclaved sand, 100 ppm phenanthrene, and 5.6 mL minimal media without bacterial inoculums. The plate count method was performed on the control samples and no colony was found.

### Extraction and Analysis

For sample analysis, 500 mg of the contaminated sand sample was dissolved in 25 mL of n-hexane and acetone 7:3 (v/v). The extractions were performed using the pressurized microwave-assisted extraction system (MAE) Multiwave 3000 (Rotor 8XF100 SOLV and solvent safety system; Graz, Austria). All samples that were placed in the MAE were extracted for 40 minutes under a pressure of 10 bars. When the extraction period was completed, the equipment was allowed to cool down to room temperature (20 minutes). Subsequently, the samples were filtered with a Whatman fiber filter with a pore size of 11 μm and stored in a 25 mL universal bottle. The samples were concentrated by means of a rotary evaporator to 1 mL. The extraction method had been pretested for recovery efficiency for phenanthrene and demonstrated an efficiency of 99 percent.

Phenanthrene concentrations were analyzed using a gas chromatography mass spectrometer (Perkin Elmer Clarus 600; Shelton, CT), equipped with Elite Column SMS of 30 m long × 0.25 mm internal dimension × 0.25 μm thickness. The injector was...
operated at 250 °C in the splitless mode with a 3-minute splitless period. Helium was used as the carrier gas with 1 mL/min constant flow rate. The column temperature was initially set at 50 °C for 1 minute, increased to 250 °C at a rate of 25°C/min, kept constant at 1 minute, and held constant until the end of the 22-minute total run time.

RESULTS AND DISCUSSION

Growth Curve

The strain grew rapidly after inoculation until day 1 with the growth rate of $3.9 \times 10^8$ CFU/g/day as shown in Exhibit 2. After day 1, the bacteria concentration in reactor 1 decreased throughout the experiment. For reactor 2, after reinoculation on day 5, the bacteria concentration increased until day 6 but decreased again until day 10. In reactor 2, the growth curve can be divided into two phases, that is, phase 1: from day 0 until day 4 and phase 2: from day 4 until day 10. Between these two phases, the growth of the strain was similar with the maximum bacteria concentration of $3 \times 10^8$ CFU/g.

Exhibit 2 shows the reduction of bacteria counts after inoculation into contaminated sand in both reactors. These results indicate that the bacteria were less adaptable to the new environment, which lead to mortality. This low survival may be due to low bioavailability of phenanthrene. Results from this study are contrasted from the study reported by Othman et al. (2009) where the bacteria showed encouraging growth rates in minimal media. Minimal media can be categorized as homogenous medium. There is unlimited bacteria access to phenanthrene in a homogenous medium. In heterogeneous media, such as contaminated sand, the phenanthrene is not homogeneously distributed and may be located in small pores or absorbed inside organic particles, which are

Exhibit 2. Growth curve of Sphingobacterium spiritovorum in reactor 1 and 2
inaccessible to bacteria (Johnsen et al., 2005). In addition, minimal media which is a liquid, mixes well and, thus, increases the bioavailability of phenanthrene to bacteria. Whereas, this is not the case for contaminated sand. Consequently, the physical separation between phenanthrene and the sand results in low bioavailability. Cao et al. (2009) also mentioned that the degradation process may be retarded if there is an insufficient amount of one of these important elements (i.e., phenanthrene as electron donor, oxygen as electron acceptor, or bacteria as biomass). In this degradation process, oxygen was not limited, as the cover for the flasks consisted of sterile cotton and the flasks were shaken throughout the experiment. Comparing this study to one by Kumar et al. (2009), which was conducted under anaerobic conditions and provided a degradation process driven by the unlimited availability of sulfide as the electron acceptor, resulted in similar reductions of bacteria counts.

**Potential of a Single Colony**

The potential for one colony can be evaluated by dividing the degradation rate by the number of colonies. Exhibit 3 shows the degradation per colony in reactor 1 and reactor 2. In reactor 1, the degradation rate per colony for the first 2 days was lower than $10^{-7}$ mg/kg/day/CFU/g. The degradation rate per colony increased to $5.03 \times 10^{-6}$ mg/kg/day/CFU/g on day 5 and slowly increased to a value of $6.24 \times 10^{-5}$ mg/kg/day CFU/g on day 10. The degradation rate per colony in reactor 2 also decreased for the first 2 days and after reinoculation, that is, from day 5 until day 6, with almost a similar value in reactor 1, that is, less than $10^{-7}$ mg/kg/day CFU/g. Then, the degradation rate per colony quickly increased to $8.58 \times 10^{-6}$ mg/kg/day CFU/g on day 10. Reduction of the degradation rate per colony after reinoculation for the first 2 days may be due to the bacteria needing to adapt to phenanthrene as it is a new source of carbon. After 2 days, the bacteria appeared to have adapted and started using phenanthrene as a carbon source.
In addition, the bacteria may be utilizing other carbon sources contained in the nutrient broth. Agathos and Reineke (2002) reported on degradation of PAHs in soils, where bacteria were able to utilize other carbon-containing compounds instead of only the targeted PAHs. If other carbon compounds are relatively easier to utilize as compared to the targeted PAH, then the potential of the bacteria to degrade PAHs may be reduced. As shown in Exhibit 2, the bacteria number decreased after inoculation and reinoculation. However, the degradation activity of the colony in degrading phenanthrene had slowly increased for reactor 1. On the other hand, the degradation rate per colony in reactor 2 rapidly increased until the last day of the experiment.

The increment of the degradation rate per colony could be due to survival of high potential bacteria (bacteria with more adaptability under nonindigenous conditions). The samples were inoculated with a high bacteria concentration. However, the reduction of bacteria concentration occurred due to low bioavailability, as discussed above. Under this condition, the low potential bacteria may not survive due to lesser adaptability to a new environment and low bioavailability, resulting in fatality. Thus, the high potential bacteria have a greater ability to survive compared to low potential bacteria. In addition, the high potential bacteria may access and break the carbon chain of phenanthrene in the sand media. The high potential bacteria showed a higher degradation rate per colony.

**Degradation Rate and Percentage Removal**

In reactor 1, phenanthrene degradation can be divided into two phases, that is, phase 1: rapid degradation within five days with a degradation rate of 12.46 mg/kg/day followed by phase 2: slow degradation with a rate of 0.74 mg/kg/day as shown in Exhibit 4. In enhancing the slow degradation observed in phase 2, reactor 2 was reinoculated with similar bacteria on day 5. In reactor 2, the degradation rate for the first 5 days was
observed to be quite similar to reactor 1 with a degradation rate of 12.31 mg/kg/day. After reinoculation on day 5, the degradation rate was found to be 4.49 mg/kg/day, which was much higher than that observed in reactor 1. In evaluating the high potential bacteria, as previously discussed, bacteria in reactor 2 was isolated and inoculated in reactor 3. In reactor 3, the degradation rate was determined to be 13.61 mg/kg/day and 4.66 mg/kg/day in phase 1 and phase 2, respectively. As a whole, the degradation rate in phase 2 was lower than phase 1. As duration increased, concentrations of phenanthrene were reduced. Thus, it decreased the carbon sources to be degraded by the bacteria (Boopathy, 2000).

The percentage removals for reactor 1, 2, and 3 were determined to be 68.93 percent, 86.75 percent, and 95.36 percent, respectively. Reactor 3 showed the highest degradation compared to reactor 1 and 2. Thus, the bacteria produced from the reinoculation process is the best phenanthrene degrader in contaminated sand.

Exhibit 5 shows the enhancement methods of increasing bacteria performance in site bioremediation. Escalation of percentage removal from this study was slightly lower than that demonstrated in a study conducted by Gentili et al. (2006). However, the escalation was higher than in other studies conducted by Liang et al. (2009) and Ihah and Ukpe (1992). On the other hand, Karamalidis et al. (2010) showed no significant effect of the encapsulation method in PAH biodegradation.

The study conducted by Gentili et al. (2006) showed the highest escalation of percentage removal. The study was conducted in a liquid culture, that is, minimal salt medium. Thus, there was a higher bioavailability of contaminants compared to the soil medium (Liang et al., 2009). However, a study using a similar method of enhancement (Liang et al., 2009), that is, biocarrier, only showed 11.49 percent of escalation of percentage removal. The results from Liang et al. show that biocarrier may not be sufficient in soil medium.

Utilization of activated carbon as a biocarrier by Liang et al. (2009) showed the highest increases in bacteria concentration, that is, $10^{10}$ cells/g. However, this study only

<table>
<thead>
<tr>
<th>Researchers</th>
<th>Medium</th>
<th>Method of enhancement</th>
<th>Performance</th>
<th>Escalation of percent removal</th>
<th>Increasing of Bacteria no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3]</td>
<td>Minimal salt</td>
<td>Bio-carrier: chitin and chitosan</td>
<td>With bio-carrier: 60 percent hydrocarbons removed; Without bio-carrier: 30 percent hydrocarbons removed</td>
<td>50 percent</td>
<td>$10^6$ cells/g</td>
</tr>
<tr>
<td>[4]</td>
<td>Soil</td>
<td>Bio-enhancer: organic nitrogen</td>
<td>Addition of organic nitrogen: 55 percent crude oil removed; Without organic nitrogen: 50.4 percent crude oil removed</td>
<td>4.6 percent</td>
<td>$10^9$ cells/g</td>
</tr>
<tr>
<td>[5]</td>
<td>Soil</td>
<td>Encapsulation: sodium alginate</td>
<td>Inoculation with or without encapsulation: method had no significant effect on PAH biodegradation</td>
<td>0 percent</td>
<td>NA</td>
</tr>
<tr>
<td>This study</td>
<td>Soil</td>
<td>Re inoculation: same strain</td>
<td>Without reinoculation: 68.91 percent; With reinoculation: 95.36 percent</td>
<td>26.43 percent</td>
<td>$10^9$ cells/g</td>
</tr>
</tbody>
</table>

Exhibit 5. Enhancement method in increasing site bioremediation efficiency
showed a 11.49 percent escalation of percentage removal, which is lower than Gentili et al. (2006) and this study. In addition, the study used more than one species of bacteria, resulting in the highest bacteria concentration. In this study, the bacteria concentration increased to 10^6 cells/g after reinoculation, which is the same order of magnitude as the study conducted by Gentili et al. (2006) and Ijah and Ukpe (1992). However, this study showed the highest escalation of phenanthrene removal of the three studies.

CONCLUSION

The performance of *S. spiriflorum* can be enhanced through reinoculation with a percentage removal of 95.36 percent. On the other hand, without reinoculation, 68.93 percent of phenanthrene was removed. It was also found that the strain cannot survive in sand. It was observed that the potential of a single colony increased after reinoculation. This result shows the early indication of survival on high potential bacteria. Even though the increased bacteria concentrations are of the same order magnitude as previous studies, the escalation of percentage removal from this study (26.43 percent) was higher. Elucidating the mechanism of high potential bacteria in PAH degradation will require further research.

ACKNOWLEDGMENTS

Financial support from the Ministry of Higher Education under a Long-Term Research Grant Scheme and the Universiti Teknologi Mara under an Excellent Fund Scheme is gratefully acknowledged. Assistance from the management of the Mawar Wastewater Treatment Plant in providing raw wastewater samples is greatly appreciated.

REFERENCES


Nor-Ami-Binti Mohd-Kamil, M.Eng, is a PhD student at Universiti Teknologi Mara, Shah Alam, Malaysia. Her research includes soil bioremediation and wastewater treatment. She received her MEng in environment management and BEng in civil engineering from Universiti Teknologi Malaysia, Johor, Malaysia.

Noor-Hana Hussain, PhD, is a senior lecturer at the faculty of applied science, Universiti Teknologi Mara, Shah Alam, Malaysia. Her research interests include the inducible stress responses in Gram negative bacteria, bacterial plant pathogens, antibiotic resistance, and quorum sensing. She is currently working on four projects funded by the Ministry of Higher Education. Dr. Hussain received her BSc in biological sciences from University of Essex, UK, and her PhD in microbiology from University College London, UK.

Meizareena Binti Mizad is a lecturer at the faculty of science, technology, and human development, Universiti Tun Hussein Onn Malaysia, Johor, Malaysia. She received her Bachelor of Human Sciences (English language & literature) from International Islamic University Malaysia, Selangor, Malaysia.

Suhaimi Abdul-Talib is a professor of civil engineering at Universiti Teknologi Mara, Shah Alam, Malaysia. His research interests include environmental management, pollution control, water and wastewater engineering, bioremediation, management of water supply, and wastewater services. He is currently working on ten projects funded by the Ministry of Higher Education and other agencies. Dr. Talib received his BE (Hons) civil from University of Melbourne, Australia, his MSc in water & environmental management (Distinction) from Loughborough University, UK, and his PhD in environmental engineering from Universiti Teknologi Malaysia, Johor, Malaysia.