Available online at www.sciencedirect.com
ScienceDirect
Journal of Hazardous Materials 151 (2008) 155–163
Journal of Hazardous Materials
www.elsevier.com/locate/jhazmat

Application of biosurfactants, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil

Liang-Ming Whang [a,b,*], Pao-Wen G. Liu [c], Chih-Chung Ma [a], Sheng-Shung Cheng [a,b]

[a] *Department of Environmental Engineering, National Cheng Kung University, No. 1, University Road, Tainan 701, Taiwan, ROC*
[b] *Sustainable Environment Research Center (SERC), National Cheng Kung University, No. 1, University Road, Tainan 701, Taiwan, ROC*
[c] *Department of Safety Health and Environmental Engineering, Chung Hwa University of Medical Technology, No. 89, Wenhua 1st Street, Rende Shiang, Tainan County 71703, Taiwan, ROC*

Received 15 March 2007; received in revised form 22 May 2007; accepted 22 May 2007
Available online 26 May 2007

Abstract

This study investigated potential application of two biosurfactants, surfactin (SF) and rhamnolipid (RL), for enhanced biodegradation of diesel-contaminated water and soil with a series of bench-scale experiments. The rhamnolipid used in this study, a commonly isolated glycolipid biosurfactant, was produced by *Pseudomonas aeruginosa* J4, while the surfactin, a lipoprotein type biosurfactant, was produced by *Bacillus subtilis* ATCC 21332. Both biosurfactants were able to reduce surface tension to less than 30 dynes/cm from 72 dynes/cm with critical micelle concentration (CMC) values of 45 and 50 mg/L for surfactin and rhamnolipid, respectively. In addition, the results of diesel dissolution experiments also demonstrated their ability in increasing diesel solubility with increased biosurfactant addition. In diesel/water batch experiments, an addition of 40 mg/L of surfactin significantly enhanced biomass growth (2500 mg VSS/L) as well as increased diesel biodegradation percentage (94%), compared to batch experiments with no surfactin addition (1000 mg VSS/L and 40% biodegradation percentage). Addition of surfactin more than 40 mg/L, however, decreased both biomass growth and diesel biodegradation efficiency, with a worse diesel biodegradation percentage (0%) at 400 mg/L of SF addition. Similar trends were also observed for both specific rate constants of biomass growth and diesel degradation, as surfactin addition increased from 0 to 400 mg/L. Addition of rhamnolipid to diesel/water systems from 0 to 80 mg/L substantially increased biomass growth and diesel biodegradation percentage from 1000 to 2500 mg VSS/L and 40 to 100%, respectively. Rhamnolipid addition at a concentration of 160 mg/L provided similar results to those of an 80 mg/L addition. Finally, potential application of surfactin and rhamnolipid in stimulating indigenous microorganisms for enhanced bioremediation of diesel-contaminated soil was also examined. The results confirmed their enhancing capability on both efficiency and rate of diesel biodegradation in diesel/soil systems.

Keywords: Biosurfactant; Surfactin; Rhamnolipid; Diesel; Bioremediation

1. Introduction

Soil and groundwater contamination with petroleum hydrocarbon compounds brings up critical issues regarding environmental and health concerns, resulting in an increased attention with respect to development of innovative and sound technologies for its remediation. Bioremediation of petroleum hydrocarbons has been proposed as an effective, economic, and environmentally friendly technology [1–3], although bioavailability of hydrophobic organic compounds (HOCs) to microorganisms could be a limiting factor during the biodegradation process [4–8]. Application of surfactants to contaminated soil and water, at concentrations above their critical micelle concentration (CMC) values, can potentially reduce the interfacial tension, increase the solubility and bioavailability of HOCs, and thus, facilitate their biodegradation [8–10]. Studies with respect to enhanced bioremediation by surfactant addition have greatly focused on chemically synthetic surfactants. Addition of synthetic surfactants to environments contaminated with HOCs, has been studied as a means by which, however, their inhibitory effects on biodegradation were recognized, especially in concentrations above their CMC values [5,6,11–18].

In comparison to synthetic surfactants, relatively little information is available for biologically produced surfactants

* Corresponding author. Tel.: +886 6 2757575x65837; fax: +886 6 2752790.
E-mail address: whang@mail.ncku.edu.tw (L.-M. Whang).

0304-3894/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2007.05.063

(biosurfactants), but their application in bioremediation processes may be more acceptable from a social point of view due to their naturally occurring property. Potential advantages of biosurfactants include their unusual structural diversity, that may lead to unique properties, the possibility of cost-effective production, and their biodegradability [8,10,19–21]. These properties make biosurfactants a promising choice for applications in enhancing hydrocarbon bioremediation. Partially purified biosurfactants, applied for the purpose of enhanced bioavailability and biodegradation of different HOCs have been reported. These studies, however, mostly dealt with pure chemicals of HOCs such as tetradecane, pentadecane [22], hexadecane [22–25], octadecane [26,27], and naphthalene [19,22,28]. Currently, available information regarding the effects of biosurfactant addition on enhanced biodegradation of petrochemical mixtures, such as diesel fuel is limited [21]. The present study was primarily motivated to investigate potential application of two biosurfactants, rhamnolipid (RL) and surfactin (SF), to increase solubility and bioavailability of a commercial petrochemical mixture, diesel, and thus, enhance its biodegradation in diesel-contaminated water and soil systems.

The rhamnolipid, a commonly isolated glycolipid biosurfactant, used in this study was produced by *Pseudomonas aeruginosa* J4 [29], while the surfactin, a lipoprotein type biosurfactant, was produced by *Bacillus subtilis* ATCC 21332 [30]. In this article, we present the experimental evidence of the capability of rhamnolipid and surfactin in lowering surface tension, increasing diesel solubility, and most importantly, their ability to enhance diesel biodegradation in diesel/water systems. Additionally, their potential application in stimulating indigenous microorganisms for bioremediation of diesel-contaminated soil was also examined.

2. Materials and methods

2.1. Production of biosurfactants

The surfactin applied in this study was produced using *B. subtilis* ATCC 21332 grown on an iron-enriched mineral salt medium at 30 °C. Produced surfactin was purified and its concentration was determined before application in diesel-degradation batch tests. The details of surfactin production can be found in Yeh et al. [30]. The rhamnolipid used in this study was produced with *P. aeruginosa* J4, an indigenous bacteria isolated from petrochemical wastewater. The isolate was grown in the Luria-Bertani (LB) medium at 30 °C for rhamnolipid production. Produced rhamnolipid was purified and its concentration was determined before application in diesel-degradation batch tests. The details of rhamnolipid production can be found in Wei et al. [29].

2.2. Diesel solubilization experiment

Batch experiments of diesel solubilization into deionized water by the addition of biosurfactants were performed following a batch solubilization technique modified from previous studies [31–33]. Commercially available diesel used in this study was refined by Chinese petroleum corporation of Taiwan, with a weight percent composition: 75.2% of aliphatic hydrocarbon, 24.77% of aromatic hydrocarbon (0.7% of poly aromatic hydrocarbon), and 0.004% of sulfur [34]. For each experiment, 25 mL of diesel and different concentrations of biosurfactant solutions were added to a glass-stoppered 250 mL pyrex separatory funnel with Teflon-plugged stopcocks positioned approximately 5 cm from the base. The funnel was placed in a funnel shaker agitated at 200 rpm and 25 °C for 36 h. A 12-h settling period was allowed, followed by withdrawal of the aqueous phase from the bottom of the funnel with minimum disturbance. Samples were stored in autoclaved amber vials with Teflon-lined screw caps until further analysis.

2.3. Enrichment of diesel-degrading consortia

In order to enrich diesel-degrading consortia, a 50 g soil sample collected from a 3 m deep sandy loam soil (84% of sand) of a diesel-contaminated site was inoculated into a 2 L conical flask containing 1.5 L of growth medium. The Bushnëll and Haas medium (BH medium) [35,36] containing 1 g/L of $(NH_4)_2NO_3$, 1 g/L of KH_2PO_4, 1 g/L of K_2HPO_4, 0.2 g/L of $MgSO_4{\cdot}7H_2O$, 0.05 g/L of $FeCl_3$, and 0.02 g/L of $CaCl_2$ was used to enumerate diesel-degrading consortia with addition of commercially available diesel as the main carbon source. The components in the flask was well mixed with a magnetic stir machine and the temperature was maintained at 26 °C using an incubator. Air was supplied through diffusers to provide sufficient oxygen and additional mixing during microbial growth. Every other day, 15 g of commercial diesel was added to 1.5 L of mixed liquor to provide a carbon source at an initial concentration of 1%. Every other week, 500 mL of the mixed liquor was transferred to 1 L of fresh BH medium to provide sufficient nutrient for microbial growth. The enriched diesel-degrading consortium was used in batch diesel/water experiments to evaluate the potential application of rhamnolipid and surfactin for enhanced diesel biodegradation.

2.4. Biosurfactants-enhanced biodegradation in batch diesel/water systems

Series of batch diesel/water experiments were conducted using enriched diesel-degrading consortia to evaluate the effects of rhamnolipid and surfactin concentrations on diesel biodegradation. From the flask used for enriching the diesel-degrading consortia, 30 mL of mixed liquor was removed and centrifuged at $10{,}000 \times g$ for 10 min. The supernatant was discarded and the solids were resuspended in a 1 L flask batch reactor containing 300 mL of a buffered BH medium with an addition of diesel (1%) and varied amount of biosurfactants. The buffered BH medium was prepared using the BH medium with modification on concentrations of K_2HPO_4 (10.7 g/L) and KH_2PO_4 (5.2 g/L) in order to maintain a pH of 7.0 ± 0.2 in diesel/water systems during the experiments [37,38]. The batch reactor was vigorously shaken at 150 rpm using a reciprocating shaker to keep the dissolved oxygen (DO) concentration above 2 mg/L and the shaker was placed in an incubator maintained at 26 °C. Sam-

ples were taken for the determination of mixed liquor volatile suspended solids (MLVSS), pH, DO, ammonia nitrogen, and total petroleum hydrocarbon-diesel (TPH_d) throughout the batch experiment for a period of about 200 h.

2.5. Biosurfactants-enhanced diesel degradation biopile tests

Three bench-scale biopiles, including control (no biosurfactant addition), addition with 50 mg/kg soil of RL, and addition with 40 mg/kg soil of SF, were conducted to evaluate biosurfactant-enhanced biodegradation of diesel-contaminated soil. Each pile, containing 700 g of diesel-contaminated sandy loam soil sample (84% of sand) with an initial TPH_d concentration of 7000 mg TPH_d/kg soil, was prepared in an aluminum foil covered stainless pot with diameter and height of 0.30 and 0.15 m, respectively. For the biopiles with addition of 50 mg/kg soil of RL and 40 mg/kg soil of SF, 35 mL of RL and 28 mL of SF solution, with a concentration of 1000 mg/L, were evenly added with gentle agitation to the RL and SF biopiles, respectively, at Day 0. The temperature of the biopiles was maintained at 26 °C using an incubator. The biopiles were gently agitated once a week and soil samples were frequently taken for the determination of TPH_d, moisture, pH, and bacteria plate count throughout the experiments. Regarding the soil sampling, during agitation, we collected five soil samples from different locations of the biopiles and then mixed the five samples into one. Small amount of water was added to maintain soil moisture between 15 and 20%.

2.6. Analytical methods

Surface tension of mixtures with addition of different concentrations of surfactin and rhamnolipid was determined with a FACE surface tensiometer (model CBVP-3, Tokyo, Japan) following the method described in Wei et al. [29]. MLVSS and ammonia nitrogen in batch diesel/water experiments were measured according to standard methods 2540-E and 4500-B, respectively [39]. Measurements of soil moisture and pH followed procedures in methods of soil analysis [40]. Analysis of bacteria plate count for soil samples followed the methods described in Gallego et al. [36].

2.7. Determination of total petroleum hydrocarbon-diesel

TPH_d in diesel/water and diesel/soil systems were extracted with methylene chloride as extraction solvent, following the procedure recommended in U.S.EPA test methods 3510C [41] and 3550B [41], respectively. Following the procedure recommended in U.S.EPA test methods 8015B [41], the quantity of TPH_d in extract was determined using a gas chromatograph with a flame ionization detector (GC-FID, Varian CP-3800, Palo Alto, CA, USA) equipped with a 30 m capillary column (Supelco SPB^{TM}-5, 0.53 mm I.D., 1.5 μm film thickness). The temperature conditions of GC-FID were operated at 250 °C for injection port, 300 °C for detector, and an oven temperature program of 45 °C (held for 3 min) to 300 °C (held for 10 min) at a rate of 12 °C/min. Nitrogen was used as the carrier gas at a flow rate of 5 mL/min.

2.8. Quantification of growth and biodegradation kinetics

Several mathematical models including Monod-type, first-order, second-order, and logistic equations have been evaluated to describe the kinetics of hydrocarbon degradation by microorganisms [1,42–45]. In this study, a rate expression, depending on the concentration of hydrocarbon (TPH_d) and the biomass (X_t), with a rate constant k_{bio} was applied to describe the hydrocarbon consumption rate

$$\left(-\frac{d[TPH_d]}{dt} = k_{bio}[TPH_d]X_t\right) \quad (1)$$

mainly due to its superb predictive capability against data collected in batch diesel/water experiments. In addition, a yield coefficient Y (mg of dry cells produced/mg TPH_d consumed) can be estimated simultaneously based on the proportional relationship between hydrocarbon consumption and biomass growth. Parameter estimations for k_{bio} and Y were performed using the AQUASIM software package [46].

3. Results and discussion

3.1. Effects of biosurfactants on surface tension and diesel solubilization

The dependence of surface tension on the biosurfactant concentrations studied are shown in Fig. 1. Surface tension decreased rapidly from 72 to 30 dynes/cm with increases in the RL concentration up to 40 mg/L. Further increases in the RL concentration, only slowly reduced the surface tension from 30 to 29 dynes/cm. Once the surface tension reached 29 dynes/cm, the further addition of RL had no effect. Two separate linear functions described a reasonably good fit ($r^2 = 0.97$) for the data at RL concentrations of less than 10 mg/L and greater than 50 mg/L. The close fit of the two linear functions implies that there are different behaviors for the RL in solution. Low RL concentrations (<10 mg/L) have a strong effect on surface tension, while high concentrations (>50 mg/L) have a negligible effect. A similar dependence pattern of surface tension on the SF concentration

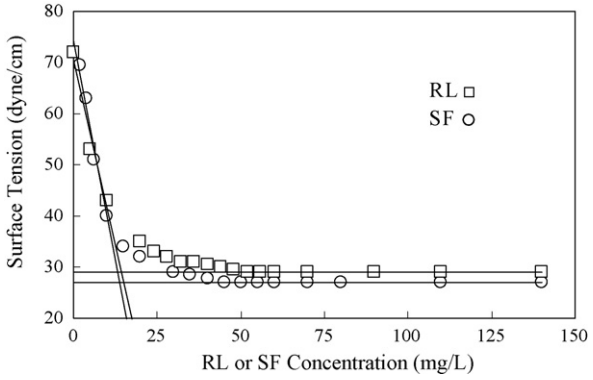

Fig. 1. Effect of increasing RL or SF concentrations on surface tension.

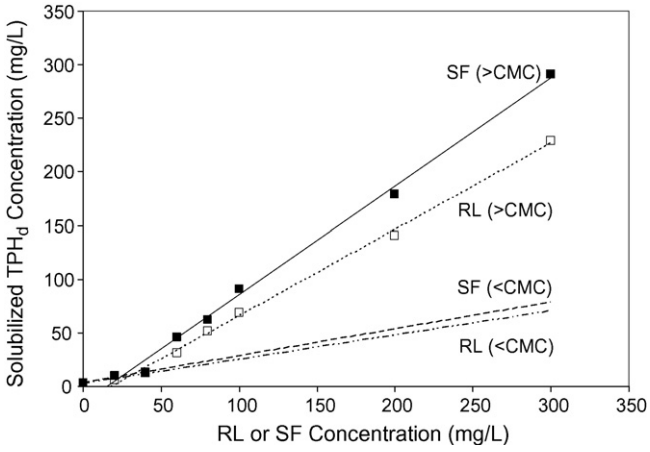

Fig. 2. Effect of increasing RL or SF concentrations on diesel solubilization.

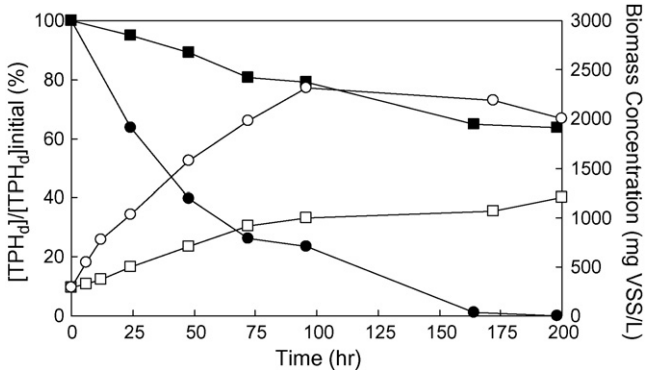

Fig. 3. The microbial growth (open symbols) and residual TPH_d percentage (solid symbols) profiles in batch diesel/water tests with 0 (square) and 80 (circle) mg/L of RL addition.

was also observed in Fig. 1. The added SF concentration, which was greater than 40 mg/L, however, was able to reduce the surface tension to 27 dynes/cm. The CMC values for RL and SF were determined to be 50 and 45 mg/L, respectively, from a semilog plot of surface tension versus their concentrations. Solubilization limits of diesel hydrocarbons were also examined for RL and SF, and the results are presented in Fig. 2. As indicated in Fig. 2, diesel solubilization could be predicted by two linear functions of surfactant concentrations in these systems where their CMC values lied on the intersection point of the two lines.

In this study, addition of biosurfactants, RL or SF, to deionized water at concentrations higher than the CMC values (50 and 45 mg/L for RL and SF, respectively), surface tension was greatly reduced to less than 30 dyne/cm, suggesting their ability in enhancing solubilization of petroleum components. The CMC values for RL and SF obtained in this study, were in agreement with those reported in Zhang and Miller [26] for RL and in Morán et al. [47] for SF. In addition, the CMC values for RL and SF were comparable to those for synthetic surfactants such as Tritone X-100, Brij 35, Brij 30, Tween 20, and Tween 80 (16–110 mg/L), and were significantly lower than those for tetradecyl trimethyl ammonium bromide (TTAB), Citrikleen, and sodium dodecyl sulfonate (1000–2300 mg/L) reported in literature [5,6,17,48]. Furthermore, according to Gustafson et al. [49], mean solubility of *n*-alkanes of C10–C15 is in the range of 0.052–0.007 mg/L, and C16–C20 ranged from 10^{-5} to 10^{-7} mg/L. The results of the diesel solubilization experiments shown in Fig. 2 confirmed the fact that a substantial increase in the solubility of petroleum hydrocarbon components can be achieved by RL or SF addition at concentrations higher than their CMC values, presumably due to their abilities to form micelles and to lower surface and interfacial energies [8,19,50].

3.2. Biosurfactants-enhanced biodegradation in batch diesel/water systems

The microbial growth and residual TPH_d percentage profiles in batch tests with 0 and 80 mg/L of RL addition are presented in Fig. 3. The enriched diesel-degrading consortia were able to grow, with diesel as the sole carbon source, from 150 mg/L of MLVSS to 1200 and 2000 mg/L under the conditions with 0 and 80 mg/L of RL addition, respectively. In association with microbial growth, the residual diesel percentage in batch tests, expressed as $[TPH_d]/[TPH_d]_{inital}$, reduced from 100 to 64 and 0% under the conditions with 0 and 80 mg/L of RL addition, respectively. The patterns of microbial growth and diesel degradation were found to be similar with all added RL concentrations studied (0 to 160 mg/L), but with differences in total quantities of produced biomass and degraded diesel. The total produced biomass concentrations and degraded diesel percentages in batch tests with different RL concentrations studied are summarized in Fig. 4. There seemed to be a strong correlation between total produced biomass concentrations and degraded diesel percentages in these batch tests. In Fig. 4, the maximum biomass growth (2000 mg VSS/L) as well as maximum diesel biodegradation percentage (100%) was simultaneously achieved at 80 and 160 mg/L of RL addition, while the worst was resulted from no RL addition.

In addition to the results summarized in Fig. 4, estimated values of specific growth rate (μ), biomass yield coefficient (Y) and rate constants (k_{bio}) for different RL concentrations studied are presented in Fig. 5. The estimated μ values increased about fourfold from 0.0233 to 0.0828 and 0.078 h^{-1} as the added RL concentration increased from 0 to 80 and 160 mg/L, respec-

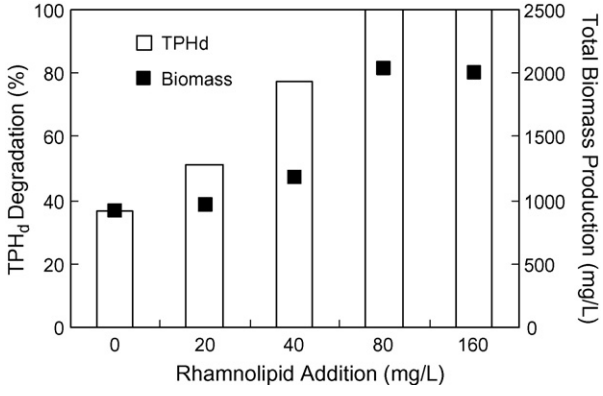

Fig. 4. Total produced biomass concentrations and degraded diesel percentages in batch diesel/water tests with different RL addition.

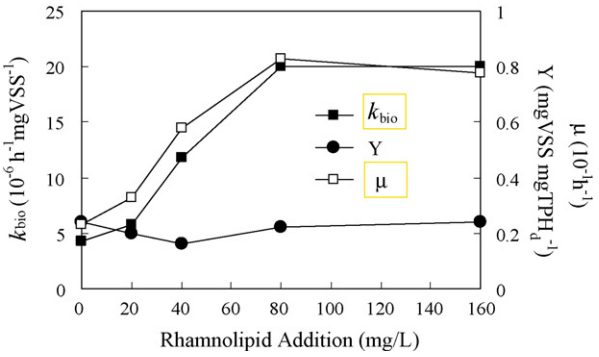

Fig. 5. Estimated values of specific growth rate (μ), biomass yield coefficients (Y) and specific diesel degradation rate constants (k_{bio}) in batch diesel/water tests with different RL addition.

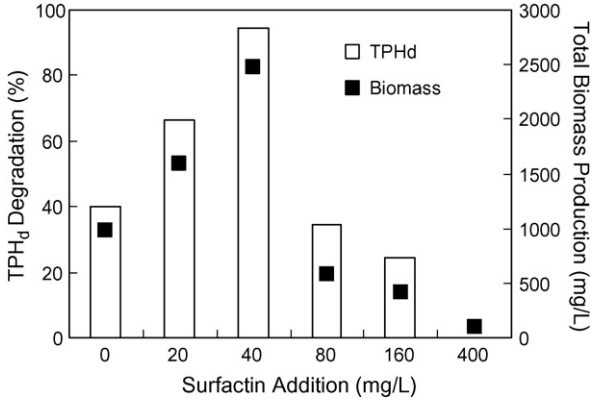

Fig. 7. The total produced biomass concentrations and degraded diesel percentages in batch diesel/water tests with different SF addition.

tively. The estimated Y values varied between 0.16 and 0.25 (mgVSS/mgTPH$_d$) with an average of 0.214 and it seemed that the values were not influenced by RL addition. The estimated k_{bio} values, sharing a similar trend with μ at different RL concentrations, increased fivefold from 4.24 to 20 (10^{-6} h^{-1}mgVSS^{-1}) as the added RL concentration increased from 0 to 80 and 160 mg/L, respectively.

For the cases of surfactin addition, the microbial growth and residual TPH$_d$ percentage profiles in batch tests with 0 and 40 mg/L of RL addition are presented in Fig. 6. The patterns of microbial growth and diesel degradation were found to be similar to those with RL addition shown in Fig. 3, but with differences in total quantities of produced biomass and degraded diesel. Additionally, the total produced biomass concentrations and degraded diesel percentages in batch tests with different SF concentrations studied are summarized in Fig. 7. Similar to the results for RL addition depicted in Fig. 4, a strong correlation between total produced biomass concentrations and degraded diesel percentages was also observed in batch tests with SF addition. In Fig. 7, the maximum biomass growth (2500 mg VSS/L) as well as diesel biodegradation percentage (94%) were simultaneously achieved at 40 mg/L of SF addition, while the worst diesel biodegradation percentage (0%) resulted from 400 mg/L of SF addition.

Estimated values of μ, Y, and k_{bio} for different SF concentrations studied are presented in Fig. 8. The estimated μ values increased from 0.0532 to 0.0794 h^{-1} as the added SF concentration increased from 0 to 40 mg/L, but decreased to 0.0696 and 0.0307 h^{-1} at SF addition of 80 and 160 mg/L, respectively. At SF addition of 400 mg/L, a μ value of 0.0124 h^{-1} was estimated using the biomass data obtained within first 24 h of experiment, but a much lower μ value of 0.0026 h^{-1} was obtained from the biomass data collected in 144 h. The estimated Y values for SF addition between 0 and 160 mg/L varied between 0.19 and 0.27 (mgVSS mgTPH$_d$)$^{-1}$ with an average of 0.22 and the values did not seem to be influenced by SF addition. The Y value for SF addition of 400 mg/L was not available since no noticeable diesel degradation was observed under that specific condition. The estimated k_{bio} values increased threefold from 5.7 to 17.75 (10^{-6} h^{-1}mgVSS^{-1}) as the added SF concentration increased from 0 to 40 mg/L, but decreased to 5.8 (10^{-6} h^{-1}mgVSS^{-1}) at SF addition of 80 and 160 mg/L. At SF addition of 400 mg/L, no significant diesel degradation, with an extremely low estimated k_{bio} value of 0.004 (10^{-6} h^{-1}mgVSS^{-1}), was observed as shown in Fig. 7.

In a previous study, Zhang and Miller [26] observed a similar result to the one shown in this study that the addition of RL (0–300 mg/L), produced by *P. aeruginosa* ATCC 9027,

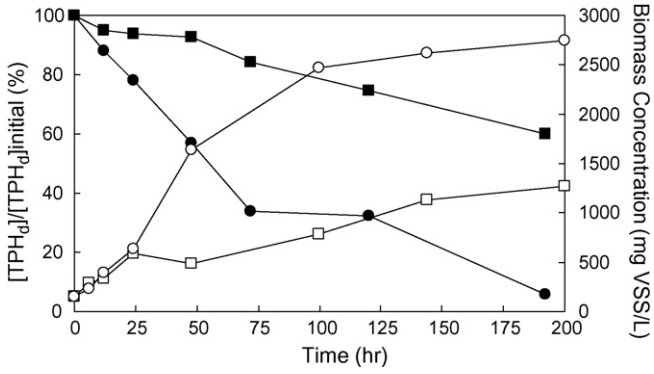

Fig. 6. The microbial growth (open symbols) and residual TPH$_d$ percentage (solid symbols) profiles in batch diesel/water tests with 0 (square) and 80 (circle) mg/L of RL addition.

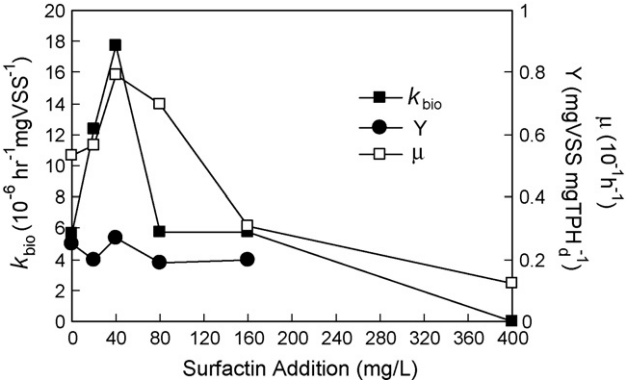

Fig. 8. The estimated values of specific growth rate (μ), biomass yield coefficients (Y) and specific diesel degradation rate constants (k_{bio}) in batch diesel/water tests with different SF addition.

enhanced biodegradation of octadecane in both efficiency and rate by the same culture. In their later studies, Zhang and Miller [23,27] reported that different forms of RL, in addition to their ability to increase solubility of octadecane, could affect the surface properties (such as hydrophobicity) of different degrading cells, in some cases leading to enhanced biodegradation and in other cases inhibiting biodegradation. They suggested that the bioavailability of octadecane in the presence of rhamnolipid is controlled by both aqueous dispersion of octadecane and cell hydrophobicity. In our study, addition of RL in batch diesel/water systems increased both efficiency and specific rate of TPH_d biodegradation without any inhibition up to 160 mg/L of RL addition. The results suggest that the RL used in this study is capable of increasing the bioavailability of sparingly soluble diesel for diesel-degrading bacteria, leading to an enhanced biodegradation. On the other hand, addition of RL, however, seemed to have a maximum capacity in enhancing specific growth and degradation rate once it reached 80 mg/L. This phenomenon confirmed the results reported in Bury and Miller [50], who observed specific growth rates of *P. aeruginosa* ATCC 15528 approaching a maximum value as solubilized *n*-decane concentrations increased with addition of a synthetic surfactant, Neodol 25-7, at various concentrations from 0.25 to 2%. The specific growth rates found in Bury and Miller [50] varied between 0.12 and 0.37 h^{-1}, which were higher than those (0.08 h^{-1}) observed in this study with 80 mg/L RL or 40 mg/L SF addition. There are two potential explanations of a lower specific growth rate obtained in our study. The first one is that the pure culture, *P. aeruginosa* ATCC 15528, used in Bury and Miller [50] has a better ability on utilizing decane, a pure petroleum hydrocarbon, leading to a higher growth rate than our enriched but mixed diesel-degrading cultures, which were grown in a mixture of petroleum hydrocarbon, diesel. The second one is that the surfactant concentrations (2500–20000 mg/L) applied in Bury and Miller [50] were significantly higher than ours (40 and 80 mg/L for SF and RL, respectively). Furthermore, reduced bioavailability and mineralization of HOCs due to their partitioning into the micellar phase of added surfactants at concentrations above their CMC values have been discussed in many studies [6,11,12,15,16], but this concern did not occur in our study with RL addition.

With regard to the case of SF addition in batch diesel/water systems, both efficiency and specific rate of TPH_d biodegradation increased as SF addition up to 40 mg/L, but decreased considerably as SF addition up to 80 mg/L and above. Vollenbroich et al. [51] found that SF was not cytotoxic to a variety of human and animal cells such as *Mycoplasma* species at a concentration below 10–25 mg/L. The presence of 30–66 mg/L SF, however, could lead to bursting of 50% of examined cells and no cells could survive in the presence of 70 mg/L of SF. These findings agreed well with the results presented in the current study and suggested that an addition of SF above 80 mg/L could damage the microbial membrane system due to its physical–chemical interaction property with cell membrane [51], leading to a considerably inhibitory effect on TPH_d biodegradation.

3.3. Biosurfactants-enhanced biodegradation in diesel/soil systems

The soil TPH_d and total plate count measurements for the diesel/soil systems of control (CT), 50 mg/kg of RL addition, and 40 mg/kg SF addition are presented in Fig. 8. During 88 days of experimental period, the soil TPH_d decreased from 6981 mg TPH_d/kg-dry soil at Day 0 to 3689 (control), 211 (RL addition), and 1655 (SF addition) mg TPH_d/kg-dry soil, respectively. Among them, the maximum TPH_d degradation efficiency of 97% was attained with RL addition, which was twice as efficient compared to the control one (47%). For the total soil plate count measurements, the CFU values increased from $10^{5.6}$ (CFU/g dry soil) at Day 0 to $10^{6.4}$–10^{7} (CFU/g dry soil) at Day 88 for the three biopiles studied. The maximum CFU value of 10^{7} was also achieved with RL addition, presumably attributed to its higher TPH_d degradation efficiency than the other two. A first-order kinetic analysis was performed on the TPH_d data collected from the three biopiles shown in Fig. 9 using the linear integrated form $\ln([TPH_d]/[TPH_d]ini) = -kt$ [52]. The k value for the control biopile in our study was estimated to be 0.007 d^{-1}, while the values for RL and SF addition biopiles were 0.043 and 0.023 d^{-1}, respectively. A wide range of first-order rate constant k values has been reported in many studies, including 0.005–0.01 d^{-1} [53–55], 0.01–0.02 d^{-1} [52–54,56], 0.05–0.09 d^{-1} [56]. Although the estimated k value for the control biopile seemed to be low compared to some values reported in literature, it is comparable to those in the lower range for k values reported in literature. The k values could vary widely depending on many factors such as soil characteristics, petroleum hydrocarbon composition, microbial characteristics etc., but it was obvious that, according to our observations, addition of RL or SF could be a beneficial measure on enhancing bioavailability and biodegradation of diesel.

Biodegradation of hydrophobic organic compounds in soil has shown that their slow release from the soil matrix to the aqueous phase is often the rate-limiting step in the process [4–8].

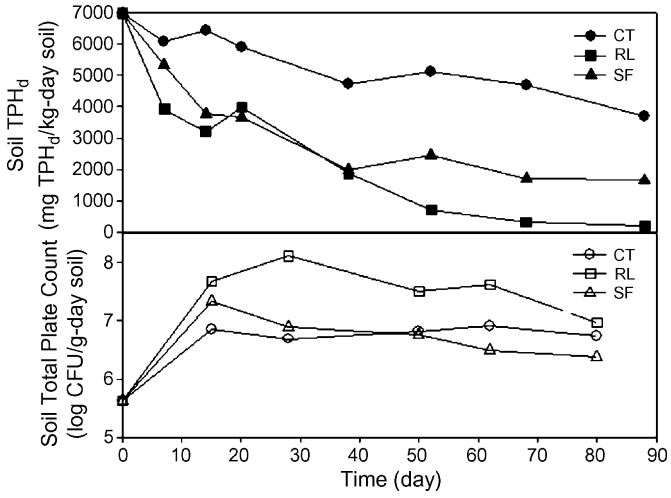

Fig. 9. TPH_d (solid symbols) and total plate count (open symbols) measurements for soil samples taken from biopiles of control (CT), 50 mg/kg of RL addition (RL), and 40 mg/kg SF addition.

In many studies, surfactants were applied to enhance bioavailability of hydrophobic compounds in contaminated soils, but their effects on bioremediation reported in previous studies were inconsistent, presumably due to the specificity of the interactions between target organic compounds, bacterial species and surfactants [8]. In addition to their characteristics in altering the cell-substrate-surfactant interactions that are relevant to biodegradation in contaminated soils [8,57], surfactant toxicity may alter the composition of the microbial populations responsible for hydrocarbon mineralization [14,58]. In comparison with synthetic surfactants, a lower toxicity can be expected from most biologically produced surfactants, although some biosurfactants can be as toxic as synthetic surfactants [59]. In soil slurry bioreactor experiments, several biologically produced glycolipids, including sophorose, cellubiose, and rhamnose lipids, promoted the onset and increased the extent of biodegradation [22]. Using a biosurfactant, with an aqueous CMC value of 18–19 mg/L, produced by *P. aeruginosa* UG2, Jain et al. [32] observed that, at a concentration of 100 mg/kg soil, the biosurfactant enhanced the degradation of a hydrocarbon mixture in soil. Furthermore, Morán et al. [47] examined the effects of SF, which was produced by an isolated *B. subtilis* strain, on bioremediation of soils polluted with crude oil. At a concentration above its aqueous CMC value (20 mg/L), addition of 80 mg/L SF could enhance the biodegradation of hydrocarbon, while SF addition below its CMC value at a concentration of 10 mg/L, did not increase hydrocarbon biodegradation. According to the biopile tests conducted in this study, either a 50 mg/kg soil of RL addition or a 40 mg/kg soil of SF addition, compared to the control biopile, greatly increased soil TPH_d degradation. The results confirmed the superb ability of RL and SF in enhancing bioavailability of hydrophobic compounds, as demonstrated in batch diesel/water systems and their potential application for bioremediation in diesel-contaminated soil. In addition, the biosurfactant concentrations applied in this study, which were very close to their aqueous CMC values but slightly lower than those applied in Jain et al. [32] and Morán et al. [47], respectively, seemed to be sufficient to provide a satisfactory effect on improving bioavailability and biodegradation of diesel compounds in diesel/soil systems. The surfactant dosages for soil bioremediation, however, require detailed evaluation before application since their optimization conditions can strongly depend on the characteristics of soils and contaminants [8–10].

In batch diesel/water systems, 40 mg/L of SF addition attained a maximum enhancement in both microbial growth and degradation efficiency, and no observable inhibitory effects were found under that specific condition. The same SF concentration applied in the diesel/soil system (40 mg/kg soil), indeed, improved the diesel bioavailability in the contaminated soil sample, but it also somewhat inhibited TPH_d biodegradation after 40 days of operation. It is presumed that SF, in addition to its surfactant property, presents its antibiotic properties as indicated in Vollenbroich et al. [51] and inhibits the bioactivity of certain indigenous bacteria in the diesel/soil system. The detailed explanations for this observation, however, require further investigation.

4. Conclusions

This study presents potential application of two biosurfactants, surfactin and rhamnolipid, for enhanced biodegradation in diesel-contaminated water and soil systems. Both biosurfactants are capable of reducing surface tension and increasing diesel solubility with increased biosurfactant addition. The following major outcomes can be drawn from this study.

In diesel/water batch experiments, addition of rhamnolipid to diesel/water systems from 0 to 80 mg/L significantly increases biomass growth and diesel biodegradation percentage from 1000 to 2500 mg VSS/L and 40–100%, respectively. Rhamnolipid addition at a concentration of 160 mg/L provides similar results to those of 80 mg/L addition. Similar trends are also observed for specific biomass growth rate and specific diesel degradation rate constant as rhamnolipid addition increased from 0 to 160 mg/L.

Addition of 40 mg/L of surfactin to diesel/water systems substantially enhances biomass growth (2500 mg VSS/L) as well as diesel biodegradation percentage (94%), compared to no surfactin addition (1000 mg VSS/L and 40% biodegradation percentage). Addition of surfactin, of more than 40 mg/L, however, decreases both biomass growth and diesel biodegradation efficiency, with a worse diesel biodegradation percentage (0%) at 400 mg/L of SF addition.

Based on their superior enhancing capability on both efficiency and rate of diesel biodegradation in diesel/soil systems, potential application of surfactin and rhamnolipid in stimulating indigenous microorganisms for enhanced bioremediation of diesel-contaminated soil is confirmed. The possible inhibitory effects of surfactin on bioremediation, however, require careful determination before application.

Acknowledgements

This work was financially supported by the Ministry of economic affairs of Taiwan, Republic of China (92-EC-17-A10-S1-0013 and 93-EC-17-A-10-S1-0013). The authors gratefully acknowledge Dr. Jo-Shu Chang at the National Cheng Kung university and Dr. Yu-Hong Wei at the Yuan-Ze university for their generosity in providing surfactin and rhamnolipid during this study.

References

[1] M. Alexander, Biodegradation and Bioremediation, Academic Press, San Diego, CA, USA, 1999.
[2] B.E. Rittmann, P.L. McCarty, Environmental Biotechnology: Principles and Application, McCraw-Hill, New York, USA, 2001.
[3] M.T. Madigan, J.M. Martinko, J. Parker, Brock Biology of Microorganisms, 10th edition, Prentice-Hall, Upper Saddle River, NJ, USA, 2003.
[4] J.R. Mihelcic, D.R. Lueking, R.J. Mitzell, J.M. Stapleton, Bioavailability of sorbed- and separate-phase chemicals, Biodegradation 4 (1993) 141–153.
[5] S. Guha, P.R. Jaffe, Biodegradation kinetics of phenanthrene partitioned into the micellar phase of nonionic surfactants, Environ. Sci. Technol. 30 (2) (1996) 605–611.
[6] S. Guha, P.R. Jaffe, Bioavailability of hydrophobic compounds partitioned into the micellar phase of nonionic surfactants, Environ. Sci. Technol. 30 (4) (1996) 1382–1391.

[7] H. De Jonge, J.I. Freijer, J.M. Verstraten, J. Westerveld, F.W.M. van der Wielen, Relation between bioavailability and fuel oil hydrocarbon composition in contaminated soils, Environ. Sci. Technol. 31 (3) (1997) 771–775.
[8] F. Volkering, A.M. Breure, W.H. Rulkens, Microbiological aspects of surfactant use for biological soil remediation, Biodegradation 8 (1998) 401–417.
[9] C.C. West, J.H. Harwell, Surfactant and subsurface remediation, Environ. Sci. Technol. 26 (12) (1992) 2324–2330.
[10] C.N. Mulligan, R.N. Yong, B.F. Gibbs, Surfactant-enhanced remediation of contaminated soil: a review, Eng. Geol. 60 (2001) 371–380.
[11] S. Laha, R.G. Luthy, Inhibition of phenanthrene mineralization by nonionic surfactants in soil/water systems, Environ. Sci. Technol. 25 (11) (1991) 1920–1930.
[12] S. Laha, R.G. Luthy, Effects of nonionic surfactants on the solubilization and mineralization of phenanthrene in soil-water systems, Biotechnol. Bioeng. 40 (11) (1992) 1367–1380.
[13] F. Roch, M. Alexander, Biodegradation of hydrophobic compounds in the presence of surfactants, Environ. Toxicol. Chem. 14 (7) (1995) 1151–1158.
[14] H.J. Tsomides, J.B. Hughes, J.M. Thomas, C.H. Ward, Effect of surfactant addition on phenanthrene biodegradation in sediments, Environ. Toxicol. Chem. 14 (6) (1995) 953–959.
[15] F. Volkering, A. Breure, J.G. van Andel, W.H. Rulkens, Influence of nonionic surfactants on bioavailability and biodegradation of polycyclic aromatic hydrocarbons, Appl. Environ. Microbiol. 61 (5) (1995) 1699–1705.
[16] S. Guha, P.R. Jaffe, C. Peters, Bioavailability of mixtures of PAHs partitioned into the micellar phase of a nonionic surfactant., Environ, Sci. Technol. 32 (15) (1998) 2317–2324.
[17] D.A.P. Bramwell, S. Laha, Effects of surfactant addition on the biomineralization and microbial toxicity of phenanthrene, Biodegradation 11 (2000) 263–277.
[18] P. Chen, M.A. Pickard, M.R. Gray, Surfactant inhibition of bacterial growth on solid anthracene, Biodegradation 11 (2000) 341–347.
[19] D.M. Falatko, J.T. Novak, Effect of biologically produced surfactants on the mobility and biodegradation of petroleum hydrocarbons, Water Environ. Res. 64 (2) (1992) 163–169.
[20] I.M. Banat, R.S. Makkar, S.S. Cameotra, Potential commercial applications of microbial surfactants, Appl. Microbiol. Biotechnol. 53 (2000) 495–508.
[21] C.N. Mulligan, Environmental applications for biosurfactants, Environ. Pollut. 133 (2005) 183–198.
[22] A. Oberbremer, R. Müller-Hurtig, F. Wagner, Effect of the addition of microbial surfactants on hydrocarbon degradation in a soil population in a stirred reactor, Appl. Microbiol. Biotechnol. 32 (1990) 485–489.
[23] Y. Zhang, R.M. Miller, Effect of rhamnolipid (biosurfactant) structure on solubilization and biodegradation of *n*-alkanes, Appl. Environ. Microbiol. 61 (6) (1995) 2247–2251.
[24] G.S. Shreve, S. Inguva, S. Gunnam, Rhamnolipid biosurfactant enhancement of hexadecane biodegradation by *Pseudomonas aeruginosa*, Mol. Mar. Biol. Biotech. 4 (4) (1995) 331–337.
[25] G. Bai, M.L. Brusseau, R.M. Miller, Biosurfactant-enhanced removal of hydrocarbon from Soil, J. Contam. Hydrol. 25 (1997) 157–170.
[26] Y. Zhang, R.M. Miller, Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant), Appl. Environ. Microbiol. 58 (10) (1992) 3276–3282.
[27] Y. Zhang, R.M. Miller, Effect of a *Pseudomonas* rhamnolipid surfactant on cell hydrophobicity and biodegradation of octadecane, Appl. Environ. Microbiol. 60 (6) (1994) 2101–2106.
[28] S.H. Kanga, J.S. Bonner, C.A. Page, M.A. Mills, R.L. Autenrieth, Solubilization of naphthalene and methyl-substituted naphthalenes from crude oil using biosurfactants, Environ. Sci. Technol. 31 (2) (1997) 556–561.
[29] Y.H. Wei, J.L. Chou, J.S. Chang, Rhamnolipid production by an indigenous isolate *Pseudomonas aeruginosa* J4 originating from petrochemical wastewater, Biochem. Eng. J. 27 (2) (2005) 146–154.
[30] M.S. Yeh, Y.H. Wei, J.S. Chang, Enhanced production of surfactin from *Bacillus subtilis* by addition of solid carriers, Biotechnol. Progr. 21 (2005) 1329–1334.
[31] S. Banerjee, Solubility of organic mixtures in water, Environ. Sci. Technol. 18 (8) (1984) 587–591.
[32] D.K. Jain, H. Lee, J.T. Trevors, Effect of addition of *Pseudomonas aeruginosa* UG2 inocula or biosurfactants on biodegradation of selected hydrocarbons in soil, J. Ind. Microbiol. 10 (1992) 87–93.
[33] C.A. Page, J.S. Bonner, P.L. Sumner, R.L. Autenrieth, Solubility of petroleum hydrocarbons in oil/water systems, Mar. Chem. 70 (2000) 79–87.
[34] Chinese Petroleum Company of Taiwan, Personal Communication.
[35] L.D. Bushnell, H.F. Hass, The utilization of certain hydrocarbons by microorganisms, J. Bacteriol. 41 (5) (1941) 653–673.
[36] J.L.R. Gallego, J. Loredo, J.F. Llamas, F. Vázquez, J. Sánchez, Bioremediation of diesel-contaminated soils: evaluation of potential *in situ* techniques by study of bacterial degradation, Biorem. J. 12 (2001) 325–335.
[37] G. Gomori, Preparation of buffers for use in enzyme studies, Methods Enzymol. 1 (1955) 138–146;
ASA, Methods of Soil Analysis, 2nd edition, American Society of Agronomy, Madison, WI., USA, 1982.
[38] J. Sambrook, D.W. Russell, Molecular Cloning A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2003.
[39] APHA, AWWA, and WEF, Standard Methods for the Examination of Water and Wastewater. 19th edition, American Public Health Association/American Water Works Association/Water Environment Federation. Washington DC, USA, 1995.
[40] ASA, Methods of Soil Analysis, second ed., American Society of Agronomy, Madison, WI, USA, 1982.
[41] U.S. EPA Test Methods for Evaluating Solid Waste Physical/Chemical Methods. Washington, DC, USA, 1996.
[42] S. Simkins, M. Alexander, Models for mineralization kinetics with the variables of substrate concentration and population density, Appl. Environ. Microbiol. 47 (6) (1984) 1299–1306.
[43] D.F. Paris, J.E. Rogers, Kinetics concepts for measuring microbial rate constants: effects of nutrients on rate constants, Appl. Environ. Microbiol. 51 (2) (1986) 221–225.
[44] B.R. Robertson, D.K. Button, Toluene induction and uptake kinetics and their inclusion in the specific-affinity relationship for describing rates of hydrocarbon metabolism, Appl. Environ. Microbiol. 53 (9) (1987) 2193–2205.
[45] B.A. Bekins, E. Warren, E.M. Godsy, A comparison of zero-order, first-order, and Monod biotransformation models, Ground Water 36 (2) (1998) 261–268.
[46] P. Reichert, J. Ruchti, W. Simon, Aquasim 2.0, Swiss Federal Institute for Environmental Science and Technology (EAWAG), CH-8600 Duebendorf, Switzerland, 1998.
[47] A.C. Morán, N. Olivera, M. Commendatore, J.L. Esteves, F. Siñeriz, Enhancement of hydrocarbon waste biodegradation by addition of a biosurfactant from *Bacillus subtilis* O9, Biodegradation 11 (2000) 65–71.
[48] R.A. Doong, W.K. Lei, Solubilization and mineralization of polycyclic aromatic hydrocarbons by *Pseudomonas putida* in the presence of surfactant, J. Hazard. Mater. 96 (1) (2003) 15–27.
[49] J.B. Gustafson, J.G. Tell, D. Orem, Selection of Representative TPH Fractions Based on the Fate and Transport Considerations, Amherst Scientific Publishing, Minocqua, Wisconsin, 1996.
[50] S.J. Bury, C.A. Miller, Effect of micellar solubilization on biodegradation rates of hydrocarbons, Environ. Sci. Technol. 27 (1) (1993) 104–110.
[51] D. Vollenbroich, G. Pauli, M. Özel, J. Valter, Antimycoplasma properties and application in cell culture of surfactin, a lipopeptide antibiotic from *Bacillus subtilis*, Appl. Environ. Microbiol. 63 (1) (1997) 44–49.
[52] S. Admon, M. Green, T. Avnimelech, Biodegradation kinetics of hydrocarbons in soil during land treatment of oily sludge, Biorem. J. 5 (2001) 193–209.
[53] I.D. Bossert, G.C. Compeau, Cleanup of petroleum hydrocarbon contamination in soil, in: L.Y. Young, C.E. Cerniglia (Eds.), Microbial Transformation and Degradation of Toxic Organic Chemicals, Wiley-Liss, New York, 1995, pp. 77–124.
[54] M.A. Troy, S.W. Berry, D.E. Jerger, Biological land treatment of diesel fuel-contaminated soil: emergency response through closure, in: P.E. Flathmann,

D.E. Jerger, J.H. Exner (Eds.), Bioremediation–Field Experience, Lewis publishing, Boca Raton, 1993, pp. 145–159.
[55] J.B. Carberry, Bioremediation of hydrocarbon-contaminated soils using indigenous microbes, in: D.L. Wise, D.J. Trantolo (Eds.), Remediation of Hazardous Waste Contaminated Soils, Marcel Dekker, Inc, New York, 1994, pp. 543–598.
[56] W. Namkoong, E.Y. Hwang, J.S. Paek, J.Y. Choi, Bioremediation of diesel-contaminated soil with composting, Environ. Pollut. 119 (2002) 23–31.
[57] P.L. Stelmack, M.R. Gray, M.R.M.A. Pickard, Bacterial adhesion to soil contaminants in the presence of surfactants, Appl. Environ. Microbiol. 65 (1) (1999) 163–168.
[58] G.M. Colores, R.E. Macur, D.M. Ward, W.P. Inskeep, Molecular analysis of surfactant-driven microbial population shifts in hydrocarbon-contaminated soil, Appl. Environ. Microbiol. 66 (7) (2000) 2959–2964.
[59] S. Lang, F. Wagner, Biological activities of biosurfactants, in: N. Kosaric (Ed.), Biosurfactants, Marcel Dekker, Inc., New York, USA, 1993.