The Journal of Microbiology, June, 2006, p.354-359 Vol. 44, No. 3

NOTE

Biodegradation of Hydrocarbon Contamination by Immobilized Bacterial Cells

Raja Noor Zaliha Abd. Rahman[1,*], Farinazleen Mohamad Ghazali[1], Abu Bakar Salleh[1] and Mahiran Basri[2]

Enzyme and Microbial Technology Research, [1]Faculty of Biotechnology and Biomolecular Sciences
[2]Faculty of Science, Universiti Putra Malaysia

(Received September 28, 2005 / Accepted February 24, 2006)

This study examined the capacity of immobilized bacteria to degrade petroleum hydrocarbons. A mixture of hydrocarbon-degrading bacterial strains was immobilized in alginate and incubated in crude oil-contaminated artificial seawater (ASW). Analysis of hydrocarbon residues following a 30-day incubation period demonstrated that the biodegradation capacity of the microorganisms was not compromised by the immobilization. Removal of *n*-alkanes was similar in immobilized cells and control cells. To test reusability, the immobilized bacteria were incubated for sequential increments of 30 days. No decline in biodegradation capacity of the immobilized consortium of bacterial cells was noted over its repeated use. We conclude that immobilized hydrocarbon-degrading bacteria represent a promising application in the bioremediation of hydrocarbon-contaminated areas.

Keywords: bacterial consortium, hydrocarbon-degrading bacteria, petroleum contamination, immobilized cell

Immobilized cells have been used and studied extensively for the production of useful chemicals (Ohta *et al.*, 1994; Chang and Chou, 2002), the treatment of wastewaters (Gardea-Torresday *et al.*, 1998; Chen *et al.*, 2000; Wang *et al.*, 2000), and the bioremediation of contamination from numerous toxic chemicals. Immobilization not only simplifies separation and recovery of the immobilized bacteria and the binding agent, but it also makes the application reusable, which reduces the overall cost. Immobilized materials, furthermore, have comparatively longer operating lifetimes due to an enhanced stability of the macromolecules or cells and, consequentially, protection from adverse conditions. Díaz and co-workers (2001) reported that immobilization of bacterial cells significantly enhanced the biodegradation rate of crude oil compared to free-living cells over a wide range of culture medium salinity. In addition, Tope and co-workers (1999) reported that immobilization doubled the bacterial viability compared to free cells in a 2,4,6-trinitrotoluene bioremediation study. These findings support that immobilizing agents offer cellular protection against adverse environmental conditions.

While immobilized cells have been successfully employed as biocatalysts in environmental protection as well as in chemical, pharmaceutical, and food industry processes, there are very few reports of their direct application in the bioremediation of contaminated areas. Treating large amounts of hydrocarbon-contaminated liquid or soil in bioreactors is technically challenging and costly. Bioaugmentation (i.e. seeding the soil with hydrocarbon-degrading microorganisms such as fungal and/or bacterial cells) can circumvent many technical difficulties, but to date, releasing free cells into a polluted environment has produced variable results. Failure of hydrocarbon degradation has often been attributed to the inability of the introduced bacteria to compete with the indigenous microorganisms that are better adapted to the rapidly changing environmental conditions (Alexander, 1999). Birnbaum (1993) therefore suggested to immobilize the bacteria in non-liquid inocula to achieve more homogenous conditions and better protection of the cells.
Immobilization would also have the added benefit of

* To whom correspondence should be addressed.
(Tel) 603-89466713/7592; (Fax) 603-89467593
(E-mail) rnzaliha@biotech.upm.edu.my

reducing the cells' exposure to the elements, thus preventing them from being washed away, particularly in an open water system. Interestingly, Radwan and co-workers (2002) have provided evidence that the immobilization principle is already found in nature, as microalgal samples collected along the Gulf coast were coated with biofilms of oil-utilizing bacteria that help degrade hydrocarbons found in seawater.

Earlier studies in our lab have shown that a bacterial consortium comprising hydrocarbon-degrading *Pseudomonas aeruginosa* (1 strain) and *Bacillus* sp (2 different strains) could effectively biodegrade crude petroleum oil in liquid cultures as well as in polluted soil and sand (Salleh *et al.*, 2003). The objective of this study was hence to develop a strategy for bacterial immobilization that is applicable in the biodegradation of crude petroleum.

Pseudomonas aeruginosa (1 strain) and *Bacillus* sp. were immobilized in calcium alginate according to Manohar *et al.* (1998). Briefly, bacteria were grown in separate cultures using 200 ml tryptic soy broth at 37°C and agitation of 150 rpm for 18 hours. The cells were harvested by centrifugation (15,000 × g, for 10 minutes), rinsed three times with sterile saline (0.85% NaCl), and collectively resuspended in 150 ml sterile sodium alginate (3%) which was also used as the inoculum for the immobilization matrix. Two different cell concentrations were tested by diluting 50 ml (size A) or 100 ml (size B) in 200 ml of 4% (w/v) sterilized alginate solution. The solutions were subsequently stirred and the resulting alginate/cell mixtures dripped into ice cold, sterile 0.2 M $CaCl_2$, which generated gel beads of approximately 2 mm diameter. The beads were then hardened in fresh $CaCl_2$ solution with gentle agitation for 2 h. The final beads reached 1.8×10^{10} cfu/g and 3.7×10^{10} cfu/g for sizes A and B, respectively.

The fermentation was done with crude petroleum oil (Malaysian Tapis reservoir) with freely suspended and immobilized bacterial cells in alginate.

Ten or twenty grams of the alginate-immobilized bacteria were added to 250 ml bottles containing 100 ml sterile artificial seawater (ASW, in g/L: 23.4 NaCl, 0.75 KCl, 7.0 $MgSO_4 \cdot 7\,H_2O$, 0.7 K_2HPO_4, 0.3 KH_2PO_4, and 1.0 NH_4NO_3) with 1% (v/v) crude oil substrate. All incubations were done in an orbital shaker at 37°C and 150 rpm. In comparison, freely suspended cells at 5% (v/v) were inoculated in ASW with 1% (v/v) crude oil and otherwise treated identically to the immobilized cells. Sterile alginate beads without any bacterial loading served as the control.

The fermentation was repeated several times to establish the long-term operational stability of the bacterial crude oil degradation. Twenty grams of size A (immobilized) alginate beads were added to 100 ml sterile ASW with 1% (v/v) crude oil in a 250-ml bottle. After 30 days of incubation, the alginate beads were washed three times with sterile ASW and the medium was refreshed. The degradation process was identical to the one used during the fermentation. All experiments were carried out in duplicates.

After each 30-day incubation period, residual crude oil levels were determined using a modified partition/gravimetry method (APHA Standard Methods) and as described by Huy *et al.* (1999). The extracting solvent *n*-hexane and 2-nonanone served as the internal standards.

A 1 µl aliquot of the hexane layer was withdrawn and injected into the GC/FID (Gas Chromatography/Flame Ionization Detector) apparatus. Hydrocarbon content was determined with a G3000 gas chromatograph attached to a D2500 Chromato Integrator (both Hitachi, Japan) using an RTX®-1 30 m × 0.25 mm methylsilicon column. The partial pressure for hydrogen was kept at 4 bar, while compressed air and helium were pressurized at 6 and 5.5 bar, respectively. The temperature was increased (8°C/min) up to 280°C from an initial hold (5 min) at 60°C, followed by a 5 min hold at (280°C). The injector and detector temperatures were kept at 290°C. Alkanes were chosen as the biodegradation reference, because they are susceptible to microbial attack and abundant in crude oil. Alkane levels were determined by comparing the sample peaks with those from internal standard 2 nonanone (Wang *et al.*, 2000). The degree of biodegradation was assessed by the alkane fraction C_{10} to C_{28} of crude oil.

Results are presented as percent aliphatic compounds remaining' after normalization to the initial concentration and factoring in the amount of abiotic or physical hydrocarbon breakdown in the beads.

Analysis of hydrocarbon residues following a 30-day incubation demonstrated that the biodegradation capacity was not compromised by the immobilization of the cells. Alkane removal of C_{10} chains (42-57% remaining) was comparable to that of free cells (47%, Fig. 1). Reduction of C_{27} chains was also similar in immobilized and freely suspended cells (55-74 vs. 68% remaining, respectively). Hydrocarbon removal was then compared across different bacterial concentrations. Twenty grams of size A alginate beads (1.8×10^{10} cfu/g) resulted in the highest level of hydrocarbon removal (Fig. 1). By contrast, addition of 10 or 20 g size B alginate beads (3.7×10^{10} cfu/g) resulted in reduced aliphatic compound biodegradation. To test whether the biodegradation capacity was stable over time and repeated fermentations, we subjected immobilized hydrocarbon-degrading microbial cells to repeated fermentations. Twenty grams of size A

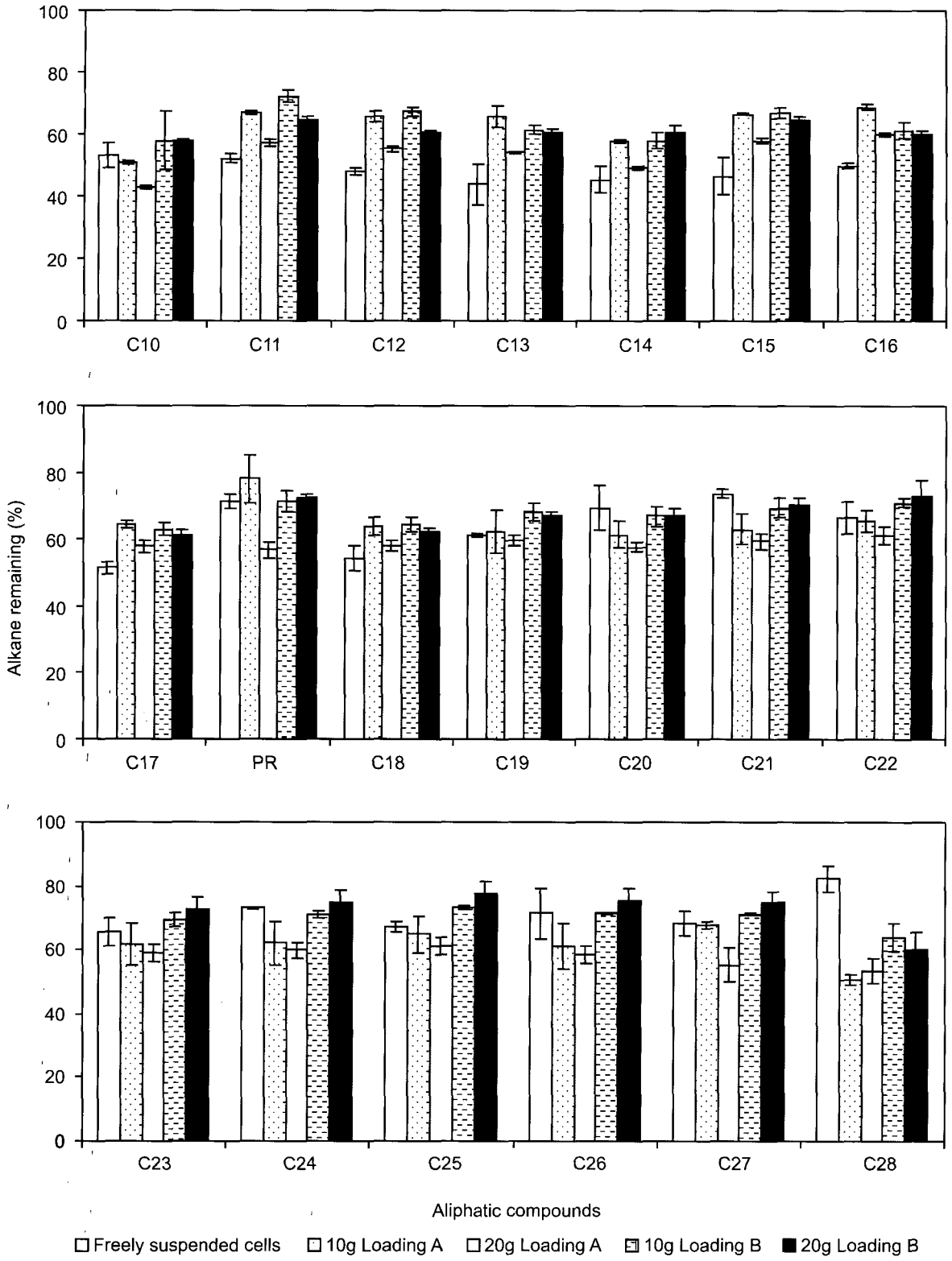

Fig. 1. Thirty-day degradation of crude oil aliphatic compounds by freely suspended cells and by Ca^{2+} alginate-immobilized cells in artificial seawater at 150 rpm (PR = pristine. Error bars = standard deviation).

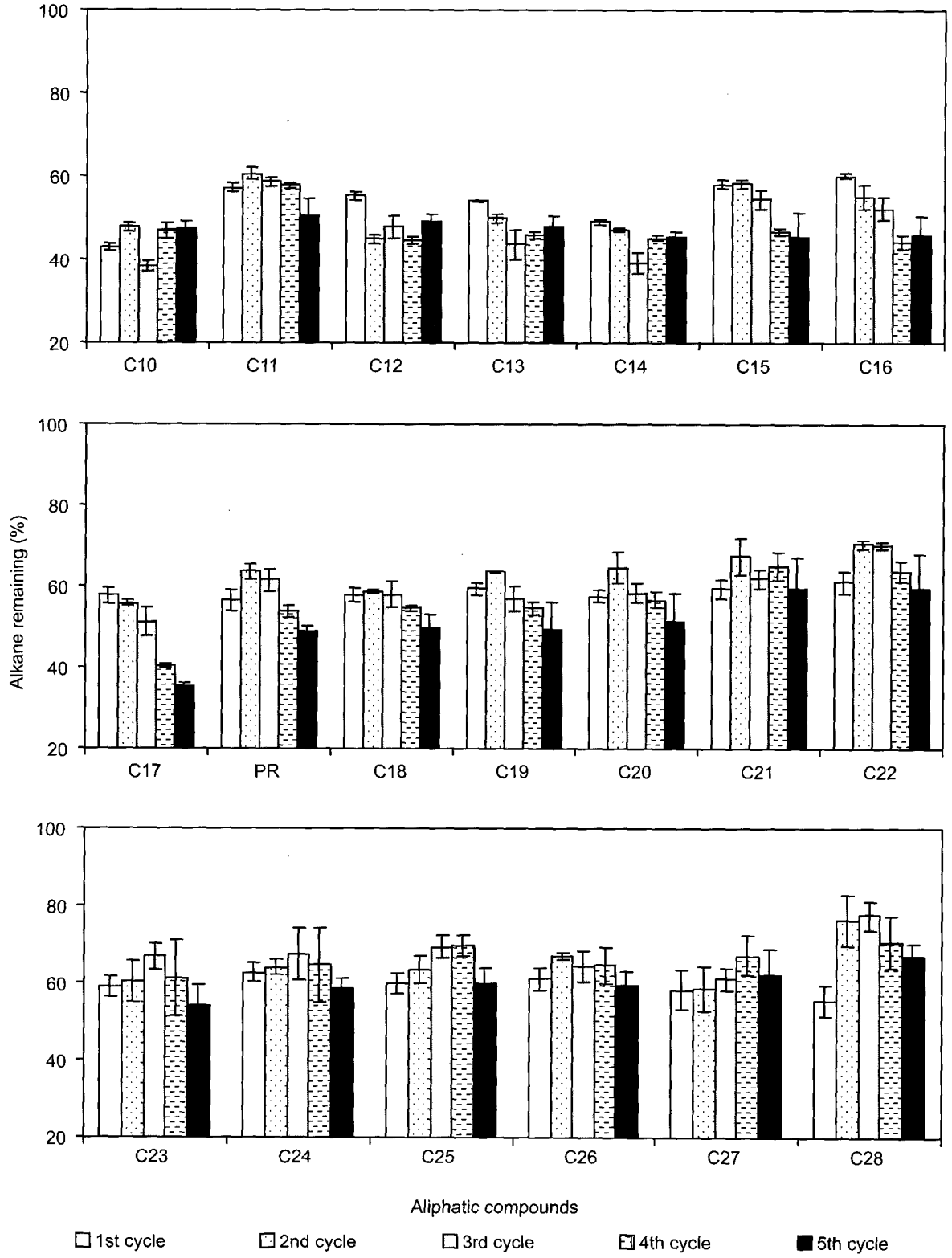

Fig. 2. Repetitive 30-day degradation of crude oil aliphatic compounds by alginate-immobilized bacteria in artificial seawater at 150 rpm (PR = pristine. Error bars = standard deviation).

alginate beads were agitated at 150 rpm for a series of five 30-day periods (150 days total), which resulted in no significant reduction in biodegradation capacity (Fig. 2). Reduction of C_{11} chains was representative for n-alkane degradation, leaving 57, 61, 59, 58, and 50% of the initial C_{11} content after the first, second, third, fourth, and fifth fermentation cycle, respectively. A similar reduction was noted in C_{27} chains, which were degraded to 67-58% of their original concentration when alginate-immobilized bacteria were used repeatedly. After five repeated uses, the alginate beads began to disintegrate due to shearing associated with the shaking of the incubation vessels, preventing us from examining biodegradation of crude oil by immobilized cells for longer (>150 days) durations.

The first section of this study examined the crude oil biodegradation capacity of immobilized bacteria using different cell and alginate bead loadings. It was found that the number of bacteria entrapped in the alginate was critical to the biodegradation effectiveness of the immobilized cells. Of the two different bacterial concentrations compared, beads loaded to 1.8×10^{10} cfu/g were more effective compared to their counterparts loaded at twice the cell concentration (3.7×10^{10} cfu/g). The decrease in degradation capacity could be the result of intercellular oxygen and nutrient competition commonly associated with high cell concentrations. Addition of 20 g immobilized microorganisms at 1.8×10^{10} cfu/g lead to the highest level of biodegradation among those measured. Even though the estimated total of bacterial cells present in 20 g of alginate beads at 1.8×10^{10} cfu/g approximated that for 10 g of alginate beads with a microbial loading of 3.7×10^{10} cfu/g, the former clearly lead to better biodegradation of n-alkanes. This may have been caused by a higher total surface area when lower concentration beads were used. The percentage of aliphatic compound removed from the crude oil was comparable in immobilized and free cells. We therefore conclude that the immobilization process does not decrease the biodegradation activity of the bacterial mixture.

Repeated degradation of crude oil by immobilized cells was tested with 20 g of alginate beads at 1.8×10^{11} cfu/g under incubation conditions optimized during the first section of this study. Manohar and Karegoudar (1998) reported that alginate beads containing entrapped *Pseudomonas* sp. (NGK 1 strain) could effectively degrade naphtalene eighteen times (cycle time: 2-3 days), before they lost their degrading activity. Contrary to our findings, the aforementioned study did not result in any visual disintegration of the alginate beads despite identical agitation procedures (150 rpm). This may be due to the fact that the total agitation time in the Manohar and Karegoudar experiments was considerably shorter than in this study.

Interestingly, the alginate beads containing immobilized crude oil-degrading bacteria showed no noticeable loss in their biodegradation capacity over the course of 150 incubation days. It has been suggested that immobilization provides a certain level of membrane stabilization and increased cell permeability, which is conceivably conductive to the bacteria's degradation capability on account of improved cell protection (Manohar and Karegoudar, 1998).

The ability to maintain the capacity for biodegradation for extended periods of time and during repeated use suggests that Ca^{2+}-alginate immobilization confers significant benefits to the here-tested bacteria such that application in waste water clean-up from oily substances seems possible. Agitation-associated sloughing of the alginate beads would likely be eliminated, if the beads were shaken in a packed-bed reactor where shearing is markedly reduced.

In summary, we developed an alginate-immobilized microbiological preparation comprising a mixture of bacteria capable of biodegrading petroleum crude oil for extended periods of time and with repeated use.

Acknowledgement

This research was funded by The Ministry of Science, Technology and Environment Malaysia under the RM8 IRPA Grant 08-02-04-0096

References

Alexander, M. 1999. Biodegradation and Bioremediation, 2nd ed. Academic Press, San Diego.

Birnbaum, S. 1993. Immobilization of Macromolecules and Cells. p.23-35. *In* U.B. Sleytr, P. Messner, D. Pum, and M. Sára (eds) Immobilized Macromolecules: Application Potentials. Springer Verlag, Germany.

Bruheim, P., H. Bredholt, and K. Eimhjellen. 1997. Bacterial degradation of emulsified crude oil and the effects of various surfactants. *Can. J. Microbiol.* 43, 17-22.

Chang, Y.C. and C.C. Chou. 2002. Growth and production of cholesterol oxidase by alginate-immobilized cells of *Rhodococcus equi* No. 23. *Biotechnol. Appl. Biochem.* 35, 69-74.

Chen, K.C., J.J. Chen, and J.Y. Houng. 2000. Improvement of nitrogen removal efficiency using immobilized microorganisms with oxidation-reduction potential monitoring. *J. Ind. Microbiol. Biotechnol.* 25, 229-234.

Del'Arco, J.P., and F.P. França. 2001. Influence of contamination levels on hydrocarbon biodegradation in sandy sediment. *Environ. Poll.* 110, 515-519.

Díaz, M.P., K.G. Boyd, S.J.W. Grigson, and J.G. Burgess. 2002. Biodegradation of crude oil across a wide range of

salinities by an extremely halotolerant bacterial consortium MPD-M, immobilized onto polypropylene fibers. *Biotech. Bioeng.* 79, 145-153.

Gardea-Torresdey, J.L., J.L. Arenas, N.M.C. Francisco, K.J. Tiemann, and R. Webb. 1998. Ability of immobilized cyanobacteria to remove metal ions from solution and demonstration of the presence of metallothionein genes in various strains. *J. Hazardous Substance Research* 1(2), 1-8.

Huy, N.Q., S. Jin., K. Amada, M. Haruki, N.B. Huu, D.T. Hang, D.T.C. Ha, T. Imanaka, M. Morikawa, and S. Kanaya. 1999. Characterization of petroleum-degrading bacteria from oil-contaminated sites in Vietnam. *J. Biosci. Bioeng.* 88, 100-102.

Manohar, S., C.K. Kim, and T.B. Karegoudar. 2001. Enhanced degradation of naphthalene by immobilization of *Pseudomonas* sp. Strain NGK1 in polyurethane foam. *Appl. Microbiol. Biotechnol.* 55, 311-316.

Manohar, S. and T.B. Karegoudar. 1998. Degradation of naphthalene by cells of *Pseudomonas* sp. strain NGK 1 immobilized in alginate, agar and polyacrylamide. *Appl. Microbiol. Biotechnol.* 49, 785-792.

Ohta, T., J.C. Ogbonna, H. Tanaka, and M. Yajima. 1994. Development of a fermentation method using immobilized cells under unsterile conditions. 2. Ethanol and L-lactic acid production without heat and filter sterilization. *Appl. Microbiol. Biotech.* 42, 246-260.

Radwan, S.S., R.H. Al-Hasan, S. Salamah, and S. Al-Dabbous. 2002. Bioremediation of oily sea water by bacteria immobilized in biofilms coating microalgae. *Int. Biodet. Biodeg.* 50, 55-59.

Salleh, A.B., F. Mohamad Ghazali, R.N.Z. Abd Rahman, and M. Basri. 2003. Bioremediation of petroleum hydrocarbon pollution. *Ind. J. Biotechnol.* 2, 411-425.

Tope, A.M., K. Jamil, and T.R. Baggi. 1999. Transformation of 2,4,6-trinitrotoluene (TNT) by immobilized and resting cells of *Arthrobacter* sp. *J. Hazardous Substance Research.* 2(3), 1-8.

Wang, C.C., C.M. Lee, C.J. Lu, M.S. Chuang, and C.Z. Huang. 2000. Biodegradation of 2,4,6-trichlorophenol in the presence of primary substrate by immobilized pure culture bacteria. *Chemosphere.* 41, 1873-1879.