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The optimal combination of entrapped bacteria for diesel remediation in seawater

Pao-Wen Grace Liu [a, *], Jia Wen Liou [a], Yi Tsung Li [a], Wen Liang Su [a], Chih-Hung Chen [b]

[a] Department of Safety Health and Environmental Engineering, Chung Hwa University of Medical Technology, Tainan, Taiwan, ROC
[b] Department of Environmental Engineering, National Cheng Kung University, Tainan, Taiwan, ROC

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ABSTRACT

The objective is to determine an optimized combination of immobilized cell when remediating diesel-oil contaminated seawater. Experiments were conducted using the diesel-degrading bacteria: *Rhodococcus pyridinivorans* CC-HCCH11, *Gordonia alkanivorans* CC-JG39, and *Alcaligenes piechaudii* CC-ESB2. They were entrapped in calcium alginate gel beads. All of the combinations were evaluated with the total petroleum hydrocarbon-diesel (TPHd) degradability in modified Bushnell Haas medium by baffled flask tests. Design I (dominant CC-HCCH11) achieved the highest TPHd degradation percentage (78%), followed by Design II (75%), Design III (70%), and Design IV (61%). Design I reduced TPHd from initial 2342 mg/L to 504 mg/L, and also achieved the highest 1st order kinetic rate (0.0049/hr) followed by Design III (0.0046/hr), Design IV (0.0042/hr), and Design II (0.0022/hr). A parallel batch result showed that the free-cell Design I revealed only about half of the TPHd degradability. Nutrient test showed that Bushnell Haas medium was adequate for the remediation.

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1. Introduction

Seawater contaminations by petroleum products have been reported as disasters by the Exxon Valdez catastrophe (1989), the Amorgos cargo ship oil leaking accident in Taiwan (2001), and the BP oil spill in the Gulf of Mexico (2010). For removal or degradation of petroleum hydrocarbons from contaminated seawater, bioremediation has been proposed as an important means for effective, economic, and environmentally friendly advantages. Bioremediation for oil spill cleanup can be either carried out by bioaugmentation or biostimulation. Bioaugmentation has been attempted with varying degrees of success (Das and Chandran, 2011; Hou et al., 2013). However, bioaugmentation approaches could cause challenges when non-native microbes are introduced to the contaminated seawater. Micro-species may show high degradation rates in a laboratory, but when introducing to the real world, it may fail due to harsh environmental conditions. The hard conditions include potential competition with indigenous microorganisms and limited nutrients. Also, the dilution of the microbes is definitely a problem when the bioaugmentation is applied to an open-water system (Tagger et al., 1983). For a successful biostimulation, ideal concentrations of nutrients are required for maximum growth of the hydrocarbon degraders. Yet, due to wave and tidal movements, the applied nutrients could be dissolved and dispersed in the water, and then become less bioavailable.

Therefore, we are proposing an alternative approach: immobilization of diesel-degrading bacterial cells to enhance the bioremediation for diesel oil-contaminated seawater. According to existing studies, immobilized cells are superior to suspended free cells in different ways, including relatively high cell density, easy separation from the reaction medium, continuous operation without being washed out, reduction of lag phase, less inhibition by products, and control of cell replication (Duarte et al., 2013). Also, the characters of improved cell viability and higher tolerance to toxicants during a long period of time are emphasized (Banerjee and Ghoshal, 2011). Improved operational stability, and most importantly, potential for scaling-up (Banerjee and Ghoshal, 2011) attracted people's attention. In addition to diesel oil remediation, the whole cell immobilization techniques have been emphasized for diesel oil biodesulfurization with one bacterial strain (Li et al., 2010). Adhesive method such as cell immobilization by biofilms from two different bacterial consortia was used to improve degradation efficiency and maintain the stability of the microbes during the process (Ławniczak et al., 2011).

* Corresponding author.
E-mail address: grace_ncku@yahoo.com.tw (P.-W.G. Liu).

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However, developing a generic method for immobilization of microbial species for remediation of hydrocarbon contaminated seawater can hardly be done. For example, Chen (2012) applied polyurethane-polyurea copolymer (PPC), which was the same major proportion with our immobilization technique, to the indigenous microbes in seawater samples around Taiwan. The result shows that the degrading efficiency was 47.25% at virtual marine condition, without providing enough nutrients. Sun et al. (2014) conducted the same immobilization techniques for cell beads containing single bacterial strain individually: *Gordonia alkanivorans* CC-JG39, *Comamonas testosterone* CC-CF3, *Alcaligenes piechaudii* CC-ESB2, and *Rhodococcus pyridinivorans* CC-HCCH11, in batch tests for diesel remediation in seawater. However, the highest TPHd degradation achieved only 53%. Therefore, the optimal combination of some of the above entrapped bacteria was evaluated to enhance the total petroleum hydrocarbons-diesel (TPHd) degradability.

Polymeric gel beads of calcium-alginate have been well studied as supporting materials for efficient entrapment of microbial cells due to their advantages of good biocompatibility, easy preparation, less costly, and high availability (Banerjee and Ghoshal, 2011; Duarte et al., 2013). Polyvinyl alcohol (PVA) cryogelation as an entrapment technique to microorganisms indigenous degradation of diesel removal showed successful (Cunningham et al., 2004). Activated carbons have been deployed in certain soil and sediment for remediation purposes (Qin et al., 2013) and they were also observed in improving bacteria releasing from entrapped gel beads (Fang, 2007).

The objective of the study is using a cell immobilization technology to effectively degrade diesel in seawater. Diesel-degrading bacterial cells were immobilized and entrapped with calcium alginate beads. The optimal combination of the entrapped diesel-degrading strains was decided, based on their TPHd degradability. Also, a parallel degradation test was carried out with the suspended free cells of the optimal bacterial strains, CC-JG39, in order to verify the superiority of the immobilization technique. Nutrient impacts were also analyzed in terms of the TPHd degradation.

2. Materials and methods

2.1. Experimental design

To the best of our knowledge, little information is available, particularly in entrapped forms, on the TPHd degradation behavior by our studied bacterial consortium. Three diesel-degrading bacterial strains: *Gordonia alkanivorans* CC-JG39, *A. piechaudii* CC-ESB2, and *Rhodococcus pyridinivorans* CC-HCCH11 were immobilized in calcium alginate beads and were tested for their diesel degradability, under different combinations. Four different combinations of the entrapments were tested, in order to emphasize dominant CC-HCCH11 (Design I), even distribution of CC-HCCH11 and CC-JG39 (Design II), dominant CC-ESB2 (Design III), and even distribution of CC-ESB2 and CC-JG39 (Design IV) (Table 1). Each of the strains was formed as individual cell beads and then mixed up with one another. TPHd degradability in modified Bushnell Haas medium was evaluated in all of the combinations.

Finally, the optimal immobilization combination was compared with suspended free cells which were produced with parallel operations and equivalent concentrations, to verify the success of the immobilization.

To find out the importance of the nutrient supplement, different nutrient levels were tested to the optimal free cell combinations on for their TPHd degradability (Table 2).

Table 1
Experimental design.

	CC-HCCH11	CC-ESB2	CC-JG39	Total bead number
Design I	50		10	60
Design II	30		30	60
Design III		50	10	60
Design IV		30	30	60
Bk	0	0	0	60

Note: numbers stand for the gel beads containing diesel-degrading bacteria.

2.2. Microorganisms

The bacterial strains CC-JG39, CC-HCCH11, and CC-ESB2 were isolated from sludge drainer of a gas station located in central Taiwan (Young et al., 2005; Lin et al., 2005), from soil located in a city of southern Taiwan, and from an oil contaminated site (Lin et al., 2009); respectively. CC-JG39 particularly showed floating activity and is able to utilize aromatic compounds such as benzene, toluene, xylene, naphthalene, which were frequently found in petroleum hydrocarbon oil (Lin et al., 2005). In addition, CC-HCCH11 and CC-ESB2 showed certain salinity tolerance. For example, CC-ESB2 could grow even at 7% (w/v) NaCl (Lin et al., 2009). Both CC-HCCH11 and CC-ESB2 were able to survive in the marine environment. CC-HCCH11 was identified from soil sampled from a scenic point at Pingtong Heng Cheng Chun, southern-west Taiwan. A commercial DNA extraction kit (UltraCleanTM; MO BIO, USA) was used to extract the genomic DNA for 16S rRNA gene amplification. The PCR was performed with bacterial universal primers 1F (5′-GAGTTTGATCATGGCTCAGA-3′) and 9R (5′-AAGGAGGTGATCCAACCGCA-3′), primers 3F (5′-CCTACGGGAGGCAGC-AG-3′), 5F (5′-AAACTCAAATGAATTGACGGGG-3′) and 4R (5′-TTACCGCGGCTGCTGGCAC-3′) were used in the sequencing reaction (Edwards et al., 1989). The DNA fragments (1475 bp) encoding for 16S rRNA were assembled using the Vector NTI 9.0 software (IBI, USA) and uploaded to EzBioCloud server (EzTaxon-e Database, Kim et al., 2012) and NCBI for identification. *Rhodococcus pyridinivorans* 1456/1456 (100%) was the identification result.

2.3. Growth media

All of the diesel-degrading bacterial cells were inoculated into 300 mL of Luria Broth (LB medium) in a 500 ml flask. LB medium was prepared as follows (per liter): 5 g NaCl, 10 g Casein enzymic hydrolysate, and 5 g yeast extract. The pH of the LB medium was adjusted to 7.0 ± 0.2. The flask was incubated for about 72 h at 35 °C with water bath on a rotary shaker operated at 100 rpm, to reach the desired inoculum density, which was approximately 1×10^8 CFU/mL. Then the resulting culture was centrifuged at 3000 rpm for 15 min in order to concentrate the culture from 300 ml to 10 ml, and ready for cell immobilization experiments. The tested free cells were made as the same concentration.

Table 2
Nutrient composition for the optimal suspended free cells.

BH medium (total nitrogen = 350.00 mg-N/L; total phosphor = 405.00 mg-P/L)			
Artificial seawater (TAAMR Inc.)	33.33 g/L	K_2HPO_4	1.0 g/L
NH_4NO_3	1.0 g/L	$MgSO_47H_2O$	0.2 g/L
KH_2PO_4	1.0 g/L	$CaCl_2$	0.02 g/L
GK medium (Total nitrogen = 261.00 mg-N/L; Total phosphor = 16.34 mg-P/L)			
Artificial seawater (TAAMR Inc.)	27.19 g/L	KH_2PO_4	4.3 mg/L
NH_4NO_3	746 mg/L	K_2HPO_4	86.2 mg/L

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