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Immobilization of diesel-degrading consortia for bioremediation of diesel-contaminated groundwater and seawater

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ABSTRACT

This study investigated the potential application of immobilized diesel-degrading consortia for diesel removal in aqueous environments. The microorganisms were entrapped using polyurethane-polyurea co-polymers, alginate, and activate carbon, and total petroleum hydrocarbon (TPH) degradability were evaluated under freshwater and seawater conditions with diesel oil. The results indicated that immobilized cells remained active after entrapment, but rapid diesel degradation occurred after sufficient suspended cells growth in aqueous medium, suggesting that entrapped cells continuously released freely suspended cells and suspended cells degraded diesel as well. Under phosphorous-sufficient conditions (P/TPH>35%), TPH degradation efficiency was achieved at 80% even at low nitrogen condition (N/TPH<10%). Under phosphorous-insufficient conditions (P/TPH<10%), the better degradation efficiency was obtained only at high nitrogen content (TPH:N > 100:7). The stoichiometric relationship for diesel degradation, nitrogen consumption, phosphorous consumption, biomass production was obtained (100:5:0.9:35). The results of repeated batch indicated that immobilized cells could be repeatedly used for diesel degradation in simulated groundwater and seawater environments for more than 360 days of operation. With a combination of copolymer, alginate, and activate carbon, the entrapped matrix presented advantages on high surface area, high porosity, and high mechanical strength for a long-term operation for diesel bioremediation in aqueous environments.

1. Introduction

Soil and groundwater contaminated with petroleum hydrocarbon compounds brings up critical issues regarding environmental and health concerns (Prince, 1993; Yang et al., 2000), such as oil-spill in ocean and dispersed oil plume in the aquifers, discharge of oil-containing ballast water from ships and oil leakage from the explosion of ships in the ocean (Atlas et al., 2015; Doerffer, 1992), and underground oil tank leakage contamination of the aquifer system (Barbash and Roberts, 1986). Petroleum contaminants can be degraded through natural attenuation, following biodegradation with oil-degrading microorganisms. Oil-degrading microorganisms are diverse and pervasive, but enrichment of oil-degrading microorganisms in situ is one of the key factors for bioremediation of those petroleum contaminants (Atlas and Hazen, 2011).

Bioremediation of petroleum hydrocarbons has been considered as an effective, economic, and environmentally friendly technology to treat a large contaminated area with a potential to completely remove these contaminants (Frankenberger, 1992; Whang et al., 2008). Especially for treating oil plume spilled into subsurface, bioremediation is much suitable rather than collecting and removing through physical methods (Atlas and Hazen, 2011; Whang et al., 2009). During bioremediation, nutrients and hydrocarbon degraders are often added to enhance degradation rate. The Exxon Valdez spill is the famous case that applying bioremediation in marine environment, by combining physical flushing on shorelines and biostimulation with fertilizers in subsurface, and it takes 18 years for weathering most of spilled crude oil (Boehm et al., 2008). In such applications, the dilution effects on introduced

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nutrients and microorganisms, however, become a major issue during bioremediation for open systems such as oceans and underground sites (Swannell et al., 1996). Immobilization of microbial cells in biological processes is intended to provide a high microbial population density, a high contaminant-conversion rate, and a stable long-term operation. Not only encapsulating high density of healthy yeasts as probiotics in food industries via immobilization technology, various fermentation processes harvest their products much rapidly (such as solvents, enzymes, antibiotics) from microbes via applying cell immobilization (Gallo et al., 2016). The same idea was also considered to apply immobilization in wastewater treatment especially in some phenolic wastewater as well as the wastewater from petrochemical manufacturing (Das and Chandran, 2010; Kuyukina et al., 2009). In addition, it could prevent microorganisms from substrate inhibition via diffusional limitations, and biodegradation of toxic substances could be achieved in much higher loading rate (Callegari et al., 1986; Manohar and Karegoudar, 1998; Trevors et al., 1992). Among cell-immobilization methods, entrapment using polymeric gel materials such as polyvinyl alcohol, alginate, and agarose, has been introduced because of the characteristics of cost-effective and easy to apply (Klein and Wagner, 1983). However, the feasibility of selected entrapment matrix in various environments should be evaluated.

In this study, a novel immobilization technology for oil-degrading microorganisms was developed to minimize the loss of introduced oil-degrading microorganisms in open bioremediation sites such as oceans and groundwater. The diesel-degrading microorganisms were entrapped using polyurethane-polyurea copolymers, alginate, and activate carbon, and diesel degradation efficiency of immobilized cells were evaluated under freshwater and seawater conditions. The effects of nutrient levels on diesel degradation efficiency were evaluate and the stoichiometric relationship for diesel degradation, nitrogen consumption, phosphorous consumption, biomass production for seawater environment was obtained. Finally, the repeated diesel degradation by immobilized cells was examined for a long-term operation for diesel bioremediation in groundwater and sweater environments.

2. Materials and methods

2.1. Oil-degrading microorganisms

Oil-degrading microorganisms adaptable to freshwater and seawater environments were used for degrading petroleum hydrocarbons. Two strains, *Gordonia alkanivorans* CC-JG39 (JG39 as abbreviation) and *Rhodococcus erythropolis* CC-BC11 (BC11 as abbreviation), capable of utilizing diesel in freshwater environment (Lin et al., 2005), were isolated from petroleum contaminated sites in Taiwan and provided by Prof. Young Chiu-Chung of National Chung-Hsin University in Taiwan. The oil-degrading consortia used for high salinity environment experiments were enriched from seawater samples collected along the Taiwan coast. Two enriched samples, GLH and DWH, taken from Gelung Harbor and Dawu Harbor, respectively, were used for immobilized cell tests based on due the superior diesel degradation performance under seawater conditions. The indigenous species were cultured at room temperature in artificial seawater medium fed with 2000 mg l-1 of diesel as the sole organic carbon source.

2.2. Cultural media

Two different media were used to simulate freshwater and seawater environments, respectively. The medium for freshwater (FW as abbreviation), which was modified from the Bushnell and Haas medium, contained 0.2 g l-1 $MgSO_4 \cdot 7H_2O$, 0.05 g l-1 $FeCl_3$, 2.14 g l-1 K_2HPO_4, 1.05 g l-1 KH_2PO_4, 38 mg l-1 NH_4Cl, and 0.02 g l-1 $CaCl_2$ (Bushnell and Haas, 1941). The artificial seawater (ASW) medium consisted of 27.19 g marine salt, 10.55 mg NH_4NO_3, 0.38 mg KH_2PO_4, and 0.39 mg K_2HPO_4 per liter deionized water and nitrogen and phosphate concentrations were determined to simulate the real marine environment around the Taiwan coastal area. The simulated marine salt was a synthetic sea salt from RealOcean™ (TAAM Inc. USA) containing marine elements except phosphate and nitrate. The pH and salinity of the ASW medium were around 8.6 ± 0.2 and 2.61 ± 0.37%, respectively, measured by a pH meter (B-212, HORIBA, Japan) and a refractometer (Master S/MillM, Atago).

2.3. Cell entrapment

The modified cell entrapment technology combined alginate encapsulation (Abouseoud et al., 2008) and polyurethane-polyurea copolymer as entrapped matrix (Chou, 2005). The active carbon was added to increase the strength of matrix structure. The fabrication procedure of cell immobilization was as follows. The oil-degrading consortia were harvested by centrifugation from the enriched medium. Sodium alginate (0.44% w/w) and active carbon which was sieved 200 mesh before (0.44% w/w) were placed in deionized water (20% v/v) and labeled as solution A. The prepared polyurethane-polyurea copolymer (66.67% v/v) was labeled as solution B. Both solution A and solution B were then autoclaved at 121 °C, 1.21 atm for 20 min. The microorganisms (13.33% v/v) and solution B were added into solution A and mixed by stirring. This mixture was extruded through a tubing (Masterflux L/S 16, ID: 3.2 mm) by a peristaltic pump into 0.04 M sterile $CaCl_2$ solution (with additional 0.5% formic acid and 1% ethyl acetate inside), forming beads with a diameter of about 4 mm. After hardening for 30 min in a $CaCl_2$ solution, the beads were washed by 0.02 M phosphate buffer (18.8 mmol K_2HPO_4 and 1.2 mmol KH_2PO_4 per liter) for 30 min.

2.4. Batch experiments

After entrapment, entrapped cells were tested for their bioactivity and durability using the batch/fed-batch experiments to evaluate their bioremediation potential in aquatic environments. The premium diesel purchased from Chinese Petroleum Corporation (CPC) in Taiwan was used as the target pollutant in this study, and the diesel was filtered with 0.25 μm PVDF fiber filters to prevent microbial contamination. The same amount of enriched oil-degrading consortia was used for the batches of both suspended form and entrapped cell to compare the bioactivity. Also, the relationship between bioactivity and diesel degradation of entrapped cells were confirmed by time series batch tests. Furthermore, nutrients were also added as biostimulation to enhance the diesel removal efficiency, because the nutrient level has been considered to be the limiting factor of bioremediation of the oil spill (Atlas and Bartha, 1972). Besides, fed-batch experiments were conducted for entrapped cells to exam the long-term sustainability, in which diesel was again pulsed after degradation. Liquid samples were taken after batch/fed-batch experiments to determine mixed liquor volatile suspended solids (MLVSS) and diesel concentration. Gas samples were taken frequently during batch/fed-batch experiment to determine the gas composition (oxygen and carbon dioxide) in the headspace.

2.4.1. Diesel degradation by freely suspended and immobilized cells

Batch experiments were conducted in 500 ml flasks, in which a branch was installed for sampling. The flasks were placed on shaker

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