Enhanced Bioremediation of Fuel-Oil Contaminated Soils: Laboratory Feasibility Study

T. T. Tsai[1]; C. M. Kao[2]; Rao Y. Surampalli[3]; and H. Y. Chien[4]

Abstract: In this study, microcosm experiments were conducted to evaluate the effectiveness of (1) nutrients, hydrogen peroxide (H_2O_2), and cane molasses addition; (2) soil washing by biodegradable surfactant [Simple Green (SG)]; and (3) soil pretreatment by Fenton-like oxidation on the bioremediation of fuel-oil contaminated soils. The dominant native microorganisms in the fuel-oil contaminated soils after each treatment process were determined via polymerase chain reaction, denaturing gradient gel electrophoresis, and nucleotide sequence analysis. Results show that approximately 32 and 56% of total petroleum hydrocarbon (TPH) removal (initial concentration of 5,000 mg kg^{-1}) were observed in microcosms with the addition of nutrient and cane molasses (1,000 mg L^{-1}), respectively, compared to only 9% of TPH removal in live control microcosms under intrinsic conditions (without amendment) after 120 days of incubation. Addition of cane molasses would cause the increase in microbial population and thus enhance the TPH degradation rate. Results also show that approximately 61% of TPH removal was observed in microcosms with the addition of H_2O_2(100 mg L^{-1}) and nutrient after 120 days of incubation. This indicates that the addition of low concentration of H_2O_2(100 mg L^{-1}) would cause the desorption of TPH from soil particles and increase the dissolved oxygen and subsequent bioremediation efficiency in microcosms. Approximately 95 and 69% of TPH removal were observed in microcosms with SG (100 mg L^{-1}) and higher dose of H_2O_2(900 mg L^{-1}) addition, respectively. Moreover, significant increases in microbial populations were observed and two TPH biodegraders (*Pseudomonas* sp. and *Shewanella* sp.) might exist in microcosms with SG or H_2O_2 addition. This indicates that the commonly used soil remedial techniques, biodegradable surfactant flushing, and Fenton-like oxidation would improve the TPH removal efficiency and would not cause adverse effects on the following bioremediation process.

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CE Database subject headings: Soil pollution; Oils; Petroleum; Biological treatment; Soil treatment.

Introduction

Soil and groundwater at many existing and former industrial areas and disposal sites are contaminated by petroleum hydrocarbons that were released into the environment. Among those petroleum hydrocarbons, fuel oil is more difficult to treat compared to gasoline and diesel fuel due to its characteristics of low volatility, low biodegradability, high viscosity, and low mobility. Bioremediation of petroleum hydrocarbons has been proposed as an effective, economic, and environmentally friendly technology (Gallego et al. 2001; Bundy et al. 2002; Mulligan and Yong 2004; Bento et al. 2005; Joo et al. 2008), although bioavailability of hydrophobic organic compounds to microorganisms could be a limiting factor during the biodegradation process.

The term "enhanced bioremediation" encompasses a broad continuum of technologies that differ with respect to their inputs (Margesin and Schinner 2001; Sarkar et al. 2005; Chien et al. 2008). These technologies may involve the addition of electron acceptors or electron donors to stimulate naturally occurring microbial populations (biostimulation) or could be the introduction of specific microorganisms to enhance the biodegradation of the target compound (bioaugmentation). Biodegradation efficiencies of petroleum hydrocarbons in soils are limited by many factors (e.g., microorganism types, nutrients, oxygen, and contaminant concentrations) (Namkoong et al. 2002; Kaplan and Kitts 2004; Atlas and Philp 2005; Kulik et al. 2006; Kao et al. 2008). Thus, pollutant degradation rates can be enhanced by the addition of nutrients, oxygen, and primary substrates into the contaminated systems. This could increase the populations of indigenous microorganisms and thus improve the efficiency of pollutant biodegradation.

In the method of biostimulation, nutrient supplementation for petroleum hydrocarbon degradation has traditionally focused on addition of nitrogen (N) and phosphorus (P), either organically or inorganically. Because carbon (C) is a major constituent of petroleum fuels, its traditional role in bioremediation research has typically been an index to determine the amount of N and P that need to be added to reach the optimal C:N:P ratio. More recently, the role of C supplementation in contaminant biodegradation has been investigated with the use of cane molasses, surfactant, glucose, biosolids, and composts (Shulga et al. 2000; Mulligan et al. 2001; Kao et al. 2003; Molina-Barahona et al. 2004). Cane molasses are wastes from sugar industry. It has the following characteristics that make it good for this type of application: (1) it is

[1]Postdoctoral Fellow, Institute of Environmental Engineering, National Sun Yat-Sen Univ., Kaohsiung 804, Taiwan.

[2]Professor, Director, Institute of Environmental Engineering, National Sun Yat-Sen Univ., Kaohsiung 804, Taiwan (corresponding author). E-mail: jkao@mail.nsysu.edu.tw

[3]Engineer Director, USEPA, Kansas City, KS.

[4]Ph.D. Candidate, Institute of Environmental Engineering, National Sun Yat-Sen Univ., Kaohsiung 804, Taiwan.

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rich in carbon, an essential energy source for biodegradation; and (2) it is relatively inexpensive (Kao et al. 2003).

Application of surfactants to contaminated soil can potentially reduce the interfacial tension, increase the solubility and bioavailability of petroleum hydrocarbons, and thus facilitate their biodegradation (Mulligan et al. 2001; Franzetti et al. 2008). Addition of synthetic surfactants to environments contaminated with petroleum hydrocarbons has been studied as a means by which their inhibitory effects on biodegradation were recognized, especially in concentrations above their critical micelle concentration (CMC) values (Boopathy 2002; Torres et al. 2005). In comparison to synthetic surfactants, relatively little information is available for biodegradable surfactants (e.g., Tween 80, Triton X-100, and Brij 30). Their application in bioremediation processes may be more acceptable from a social point of view due to their naturally occurring property (Kim et al. 2001). Potential advantages of biodegradable surfactants include their unusual structural diversity that may lead to unique properties, the possibility of cost effective production, and their biodegradability. Currently, available information regarding the effects of biodegradable surfactants' addition on enhanced biodegradation of petroleum hydrocarbons (e.g., fuel oil) is limited (Wang and Mulligan 2004; Yu et al. 2007).

As most of the bioremediation technologies are aerobic processes, due to the greater efficiency of aerobic biodegradation of petroleum hydrocarbon, both the oxygen generated and delivery of oxygen to soil in situ bioremediation technologies are crucial to success. The most obvious route is the introduction of pure oxygen rather than air, as this can significantly increase the dissolved oxygen (DO) concentrations. Injection of hydrogen peroxide (H_2O_2) into the groundwater is one of the methods to increase the DO concentrations. Characteristics of H_2O_2 that make it a desirable source of oxygen include (1) it is relatively cheap and readily available; (2) oxygen from its disproportion is available for use by microorganisms; and (3) it can be added to the environment at high concentrations (from 100 to 500 mg L^{-1}), providing an oxygen supply several orders of magnitude more concentrated than possible from saturating water with pure oxygen; and (4) it does not persist in the environment [American Petroleum Institute (API) 1987; Pardieck et al. 1992; Kulik et al. 2006].

Fenton-like oxidation is one of the oxidation processes which is used successfully, as it is comparatively cheap. A significant portion of contaminants in soils was found to be oxidized by H_2O_2 without any addition of soluble iron and the mineral-catalyzed Fenton-like reaction was proposed to describe the oxidation occurring in the natural soils. The main objective of most previous researches using Fenton-like reaction for the treatment of petroleum hydrocarbons was to remove a significant amount of total petroleum hydrocarbon (TPH) via the desorption and oxidation processes (Watts and Dilly 1996; Yeh et al. 2003; Ferguson et al. 2004; Yeh et al. 2008). One of the advantages of applying Fenton-like oxidation for contaminant oxidation is that the produced oxygen during the decomposition of H_2O_2 would increase the DO concentration. This would enhance the aerobic biodegradation rate of contaminant (Lodha and Chaudhari 2007). However, the inherent toxicity of H_2O_2 toward microorganisms is the major concern when H_2O_2 is used as an alternative source of oxygen. However, populations of higher cell density are able to tolerate higher concentrations of H_2O_2 than in less dense populations (Carberry and Benzing 1991; Kulik et al. 2006).

The main objectives of this study were to evaluate the effectiveness of bioremediation of fuel-oil contaminated soils under the following conditions: (1) supplement of nutrients H_2O_2 or cane molasses; (2) pretreating by soil washing process with biodegradable surfactants [Simple Green (SG)]; (3) pretreating by Fenton-like oxidation; and (4) determine the dominant native microorganisms in treated soils for further application.

Materials and Methods

Soil Characteristics

The tested soil was sampled from a background and uncontaminated area of a petroleum hydrocarbon (fuel oil) spill site in Taiwan. The tested soils were collected from the surface layer of the vadose zone (15–30 cm below land surface). The collected soils were air dried, passed through a 2-mm sieve, and kept at room temperature for further analysis. Soil samples were analyzed to determine their characteristics including parameters of particle size, soil pH, total C, total N, and bioavailable P. Particle size distribution was determined by the pipette methods (Gee and Bauder 1986). The soil pH was measured in water using a 1:5 soil/water ratio with a glass electrode. The amount of total C and N was determined using a dry combustion method with a PE 2400 Elemental Analyzer. Bioavailable P was determined using the Mehlich-3 extraction technique (Schoumans 2000).

Chemicals

The fuel oil (No. 6 fuel oil) was purchased from Chinese Petroleum Corp. (Taiwan) and used as received. The fuel oil was characterized by the presence of hydrocarbons ranging from $C_{12}-C_{40}$ [Taiwan Environmental Protection Administration (TEPA) 2003a]. The H_2O_2 (30%) was purchased from Merck and Co., Inc. (United States) and used as received. This study employed a biodegradable nonionic surfactant SG (manufactured by Sunshine Makers, Inc., United States), which has an average molecular weight of 88 and a molecular formula as $HOCH_2H_2O\text{-}(CH_2)_3CH_3$. The CMC value of surfactant SG was 1,000 mg L^{-1} that was comparatively higher than those of commonly used nonionic surfactants.

Microcosm Study

Collected soils were mechanically homogenized in a stainless steel container. The soil was spiked with fuel oil dispersed in 1.5 L of *n*-hexane/acetone (v/v, 1:1) solution. The soil was then further homogenized. The solvents were allowed to evaporate from the soil by placing the container of spiked soil in a fume hood, thus leaving behind the fuel oil in the soil at a theoretical initial TPH concentration of approximately 5,000 mg kg^{-1} of soil (after equilibration).

Three major objectives of this microcosm study included (1) evaluation of the effects of supplement of nutrients, H_2O_2, and cane molasses; (2) evaluation of the effects of SG washing on the biodegradation of TPH; and (3) evaluation of the effects of Fenton-like oxidation on the biodegradation of TPH. In the microcosm study, soils from a background and uncontaminated areas of the petroleum hydrocarbon (fuel oil) spill site were used as the inocula. The tested groundwater at depth from 6 to 7 m was collected by bailers after the well purging process following the standard groundwater sampling procedures [Taiwan Environmental Protection Administration (TEPA) 2003b]. The site groundwater contained the following components at the specified concentrations (units are in milligram per liter of water): nitrate,

Table 1. Characteristics and Components of Microcosm Experiments

Group	Reaction mechanism	Constituents	Objective
A1 (control)	Desorption/intrinsic biodegradation	Fuel-oil contaminated soils+groundwater	Evaluate the effects of natural bioremediation process on the efficiency of natural TPH bioremediation
A2	Desorption/enhanced biodegradation	Fuel-oil contaminated soils+groundwater+nutrient [a]	Evaluate the effects of nutrient, H_2O_2 and cane molasses addition on the efficiency of TPH bioremediation process
A3	Desorption/oxidation/aerobic biodegradation (low O_2 condition and no nutrient addition)	Fuel-oil contaminated soils+groundwater with 100 mg L^{-1} of H_2O_2 solution	
A4	Desorption/oxidation/aerobic biodegradation (low O_2 condition and nutrient addition)	Fuel-oil contaminated soils+groundwater with both 100 mg L^{-1} of H_2O_2 solution and nutrient[a]	
A5	Desorption/carbon supplementation of biodegradation	Fuel-oil contaminated soils+groundwater with 1,000 mg L^{-1} of cane molasses	
B1	Desorption/solubilization /carbon supplement of biodegradation	Fuel-oil contaminated soils+groundwater with 100 mg L^{-1} of SG	Evaluate the effects of biosurfactant SG addition on the efficiency of TPH removal
B2		Fuel-oil contaminated soils+groundwater with 1,000 mg L^{-1} of SG	
C1	Desorption/oxidation/aerobic biodegradation (high O_2 condition)	Fuel-oil contaminated soils+groundwater with 900 mg L^{-1} of H_2O_2 solution	Evaluate the effects of high H_2O_2 addition and Fenton-like oxidation on the efficiency of TPH removal
C2	Desorption/oxidation/aerobic biodegradation (high O_2 condition and nutrient addition)	Fuel-oil contaminated soils+groundwater with both 900 mg L^{-1} of H_2O_2 solution and nutrient[a]	

[a]Nutrients solution=$(NH_4)_2SO_4$(0.124 M)+Na_2HPO_4(7.75×10^{-3} M).

6.2; total iron, 1.9; ferrous iron, 0.1; sulfate, 31; sulfide, 2; methane; 0.02; pH, 7.2; ammonia nitrogen, 4; phosphate, 1.16. Table 1 shows the components of nine groups of microcosms. To evaluate the potential of TPH biodegradation by intrinsic microorganisms under different site conditions, nine groups of microcosms were prepared under the following conditions: no amendments (natural bioremediation) (Group A1), nutrient addition (Group A2), addition of 100 mg L^{-1} of H_2O_2 (Groups A3), addition of 100 mg L^{-1} of H_2O_2 and nutrients (Groups A4), addition of 1,000 mg L^{-1} of cane molasses (Group A5), addition of biodegradable surfactant SG [Groups B1 (100 mg L^{-1}) and B2 (1,000 mg L^{-1})], 900 mg L^{-1} of H_2O_2 (Group C1), and 900 mg L^{-1} of H_2O_2+nutrients (Group C2). Each microcosm contained 75 mL of site groundwater and 100 g of fuel oil contaminated soil in a 250-mL serum bottles sealed with thick butyl rubber septa. Then a 75-mL premixed groundwater and amendments solution (e.g., nutrient, H_2O_2, cane molasses, and biodegradable surfactant SG) was added to the microcosm to achieve the desired concentration.

Soil samples were collected and analyzed for TPH and culturable bacterial populations periodically at each time point (e.g., 0, 30, 60, 95, and 120 days). Triplicate microcosms were scarified at each time point during the experiment. Samples were spun on a Hettich-Zentrifugen EBA 21 Centrifuge for 30 min at 10,000 rpm and stored until analysis at room temperature in the dark. Removal of TPH was monitored by shake extracting the vial contents for 24 h with mixture of *n*-hexane/acetone (v/v, 1:1). Analysis of TPH extract was performed using a Hewlett-Packard 6890 gas chromatography (GC) equipped with a flame ionization detector and a DB-1 capillary column (0.32 mm×30 m) (Hewlett-Packard, U.S.A.). The injector temperature was 300°C and the detector temperature was 350°C. The oven temperature was programmed to increase from 50°C (5 min) to 350°C (10 min) at 10°C/min. Total plate counts were conducted using plate count agar (Himedia) to assess the approximate size of the culturable bacterial populations in the soil sample using the spread-plate technique (American Public Health Association (APHA) 2001). Prepared plates were incubated, inverted at 37°C for 48 h, then counted for colony forming unit. The detection levels for TPH, H_2O_2, and SG were approximately 100, 15, and 10 mg/L, respectively.

Denaturing Gradient Gel Electrophoresis

On Day 120, total bacterial DNAs from 1 g of soil samples [A1 (control), B1, B2, and C1] were extracted with PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, California) for detecting the community dynamics in the process of TPH degradation. Bacterial 200-bp fragments of 16S rDNA V6-V8 region for subsequent denaturing gradient gel electrophoresis (DGGE) analysis were obtained with the primer combination of 968f with a GC clamp (40-nucleotide GC-rich sequence, 5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) and 1401r (5′-GCG TGT GTA CAA GAC CC-3′) (Felske et al. 1996; Kleikemper et al. 2005). The polymerase chain reaction (PCR) reacted mixtures containing 10 ng of DNA extract, 4 pmol of each primer, and *Taq* DNA Polymerase Master Mix RED (2.0 Master mix kit) in final concentrations of 1.5 mM of $MgCl_2$ and 0.12 mM of deoxyribonucleoside triphosphates in PCR buffer. The PCR amplification was conducted for 30 cycles: denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. The equal concentration of each amplified PCR products (2,500

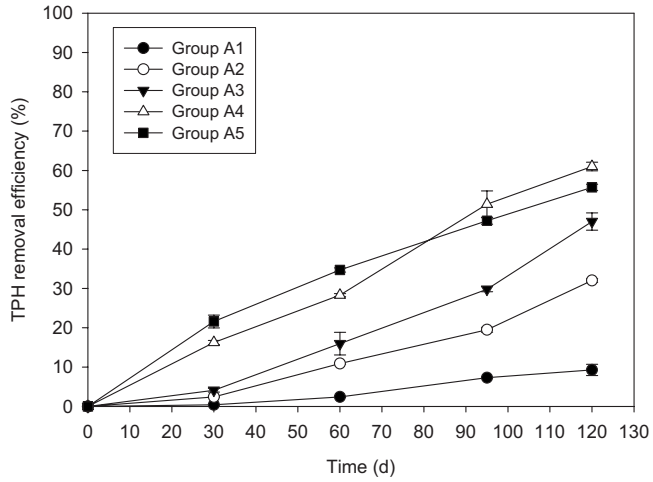

Fig. 1. TPH degradation pattern in various microcosm experiments with Groups A1, A2, A3, A4, and A5

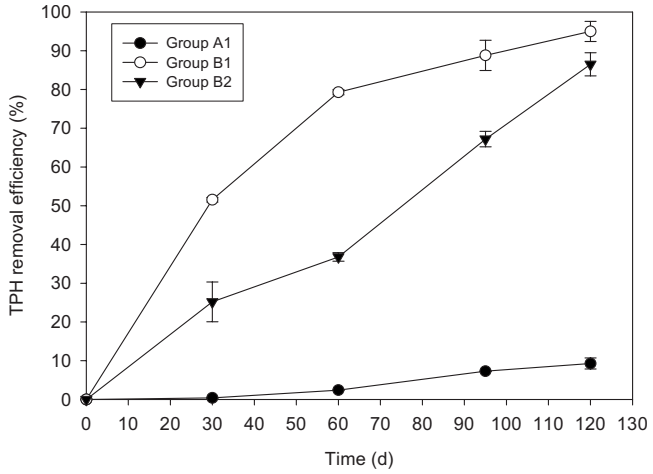

Fig. 2. TPH degradation pattern in various microcosm experiments with Groups A1, B1, and B2

ng) was further performed with DGGE using a Bio-Rad D.C.ode system (Bio-Rad, Hercules, Calif.), as described by the manufacturer. The 10% polyacrylamide gel with a 30–60% denaturant gradient was used and electrophoresis was performed at 60°C and 200 V for 3.75 h. The gels were then stained with SybrGreenI and photographed.

Banding Analysis and Phylogenic Analysis

The relative intensity of amplified bands in gels was analyzed with Quantity One software (Version 4.5, Bio-Rad Laboratories, Hercules, Calif.). The PCR-amplified products were electroeluted from gel and then sequenced by MdBio, Inc. in Taiwan. Those sequences were evaluated by using the basic local alignment search tool to determine the closest relatives in the GenBank databases (http://www.ncbi.nlm.nih.gov). Alignment of nucleotide sequences of PCR-amplified products generated a matrix of similarity coefficients with neighbor-joining method (Saitou and Nei 1987). The dendrogram based on these similarity coefficients was plotted with unweighted pair-group method with arithmetic mean (UPGMA) method for clustering. Clustal W software and PHYLIP (phylogenetic inference package, version 3.57) were applied for the Phylogeny Tree analysis (Watanabe et al. 2000; Watanabe 2001; Hall 2001).

Results and Discussion

Soil Characteristics

Results reveal that the tested soils had a sandy loam texture (60% sand, 35% silt, and 5% clay). The soil pH (1:5 H_2O) and total C were approximately 6.5 and 0.8%, respectively. Moreover, the tested soil had low concentrations of total N (0.03%), and bioavailable P (0.000 63%). Results indicate that the ratio of C:N:P (100:3.8:0.1) were lower than the commonly used C:N:P ratio of 100:10:1 for bioremediation (Molina-Barahona et al. 2004).

Supplement of Nutrients, H_2O_2, and Cane Molasses

Fig. 1 presents the observed TPH removal for Groups A1 (control), A2, A3, A4, and A5. Results show that approximately 9% of TPH removal was observed in Group A1 (control) microcosms while the TPH concentrations dropped from 5,000 to 4,536 mg kg^{-1} after 120 days of operation. However, in Group A2 with nutrients addition, approximately 32% of TPH degradation was observed after 120 days of incubation. Results suggest that nutrient addition can enhance the natural bioremediation rate and cause higher TPH removal. However, only 32% of TPH removal was obtained although nutrient was supplied. This might be due to the effect that fuel oil is more difficult to biodegrade compared to gasoline or diesel fuel due to its characteristics of low biodegradability (Perfumo et al. 2007). Nocentini et al. (2000) obtained similar results and concluded that the heavier compounds in fuel oils were depleted at slower rates than the lighter compounds. Insufficient oxygen source in the microcosms might also cause the limited TPH removal efficiency in the nutrient addition microcosms.

In this study, H_2O_2 and cane molasses were used as the oxygen source and carbon supplementation in this microcosm experiments. Fig. 1 presents that the effects of H_2O_2 and cane molasses addition on TPH degradation. Results show that the TPH (with initial concentration of 5,000 mg kg^{-1}) removal efficiency was in the order of Group A4(61%) > Group A5(56%) > Group A3(47%) > Group A1(9%) during the 120-day operation period. Results indicate that the highest TPH removal efficiency was observed in Group A4 with H_2O_2(100 mg L^{-1}) and nutrient addition. This indicates that the addition of low concentration of H_2O_2(100 mg L^{-1}) might cause the desorption of TPH from soil particles and increase the DO in microcosms. Results from this study reveal that the presence of H_2O_2 might be a feasible method to enhance the aerobic bioremediation efficiency of fuel-oil contaminated soils. Results also imply that the addition of H_2O_2 and nutrient is an applicable and effective biostimulation method to enhance the soil bioremediation rate. Furthermore, addition of cane molasses (Group A5) can also improve the efficiency of contaminant biodegradation.

Effect of SG Washing on the Biodegradation of TPH

The TPH removal efficiencies (initial concentration of 5,000 mg kg^{-1}) with the treatment of two different SG concentrations (100 and 1,000 mg L^{-1}) are shown in Fig. 2. The percentage of TPH removal for Group B microcosms was in the order of Group B1(95%) > Group B2(87%) > Group A1(9%) after 120 days of operation. Two dominant mechanisms in the

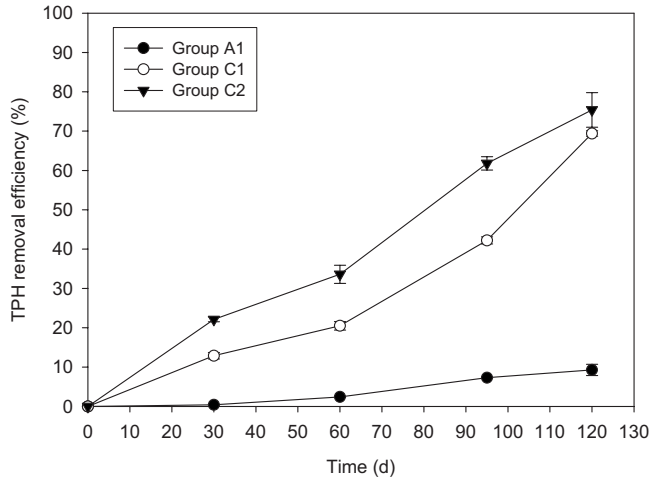

Fig. 3. TPH degradation pattern in various microcosm experiments with Groups A1, C1, and C2

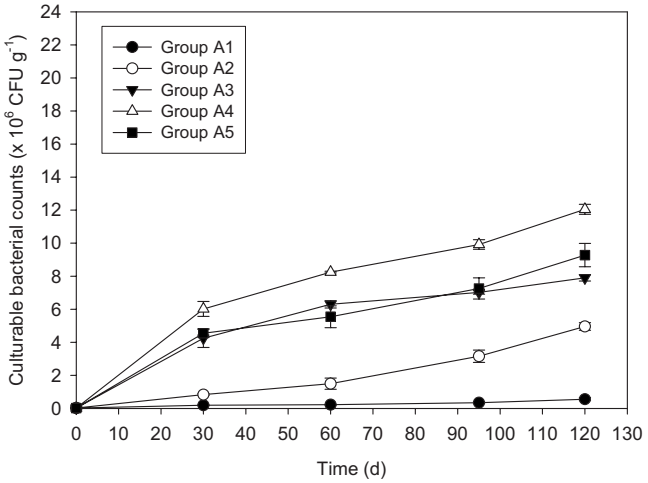

Fig. 4. Culturable bacterial counts in various microcosm experiments with Groups A1, A2, A3, A4, and A5

microcosms with SG addition included (1) enhance the solubility of contaminants; and (2) enhance the interactions between the microorganisms and contaminants. These mechanisms allowed contaminants (fuel oil) to contact more easily with microorganisms. Results also suggest that the addition of biodegradable surfactant SG would not cause adverse effect on the biodegradation efficiency of TPH.

Results indicate that the highest TPH removal efficiency was observed in the microcosm experiment with 100 mg L^{-1} of SG addition (Group B1). TPH removal efficiency was not further enhanced in Group B2 with SG concentration of 1,000 mg L^{-1}. Similar researches (Arostein et al. 1991; Laha and Luthy 1992) have pointed out that the biodegradation of hydrophobic hydrocarbons (e.g., petroleum hydrocarbons) was not increased proportional to the dosage of surfactant. The reasons were attributed to hydrocarbons entrapped within the micelle due to the increased surfactant addition might not be completely available for microorganism to utilize. With the addition of surfactant concentration at its CMC, the solubility of hydrocarbons was increased leading to the enhancement of removal efficiency due to the mass transfer from non-aqueous liquid phase to aqueous phase. However, the concentration of hydrocarbons truly solubilized in aqueous phase was not increased though the mass transfer has occurred. Therefore, the bioavailability of hydrocarbons was not enhanced due to the effect of surfactant increase dosing concentration.

Effect of Fenton-Like Oxidation on the Biodegradation of TPH

Fig. 3 presents the TPH (initial concentration of 5,000 mg kg^{-1}) removal efficiency in Group C microcosms. The percentage of TPH for Group C microcosms was in the order of Group C2(75%) > Group C1(69%) > Group A1(9%) after 120 days of operation. Results indicate that higher TPH degradation rate was observed in Group C2 with both H_2O_2(900 mg L^{-1}) and nutrient addition. Results also imply that addition of higher dose of H_2O_2(900 mg L^{-1}) might activate that Fenton-like oxidation process and thus part of TPH removal was due to the oxidation mechanism. The produced oxygen via the decomposition H_2O_2 would enhance the following TPH biodegradation. Results from this study reveal that the addition of H_2O_2 might be a feasible method to enhance the aerobic bioremediation efficiency of fuel-oil contaminated soils. Results also suggest that Fenton-like oxidation with H_2O_2 concentration of 900 mg L^{-1} would not cause adverse effect on subsequent bioremediation process using intrinsic bacteria.

Microbial Populations

Figs. 4–6 present growth patterns of the culturable microbial populations in different microcosm experiments. Increases in microbial populations corresponded to the decreases in TPH concentrations during the operational period in Group A1 and other groups with amendments (Groups A2, A3, A4, A5, B1, B2, C1, and C2). Small increases in microbial population in Group A1 (control) was noticed compared to the increases in other groups with treatment. Results indicate that culturable bacterial counts increased from 2.3×10^4 to 5.6×10^5 CFU/g soil and TPH concentrations reduced from 5,000 to 4,536 mg kg^{-1} on Day 120 in Group A1 (control). Increases in microbial populations were observed in A2 microcosms (nutrient addition) from 2.4×10^4 to 5.0×10^6 CFU g^{-1} soil after 120 days of incubation. Significant increases in microbial populations in other seven groups of mi-

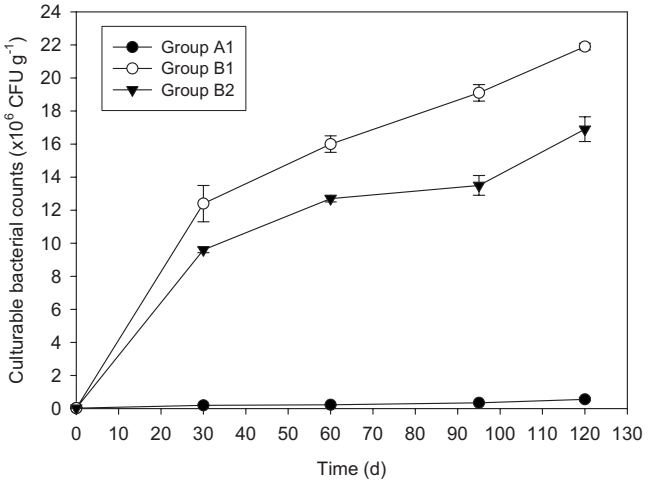

Fig. 5. Culturable bacterial counts in various microcosm experiments with Groups A1, B1, and B2

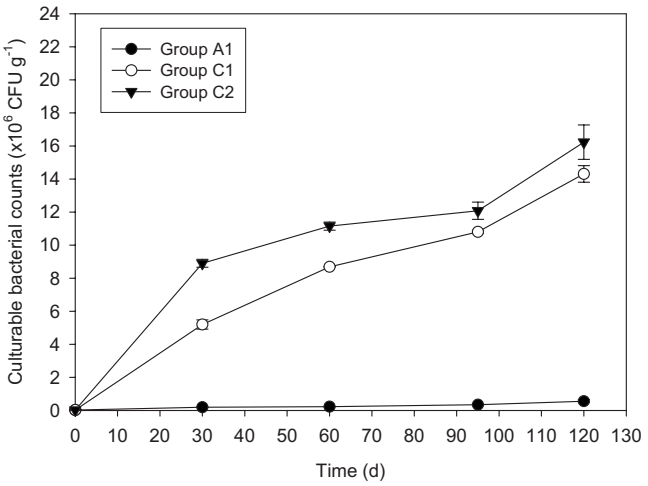

Fig. 6. Culturable bacterial counts in various microcosm experiments with Groups A1, C1, and C2

crocosms were also observed. The culturable bacterial counts in Groups A3, A4, A5, B1, B2, C1, and C2 microcosms were 7.9 $\times 10^6$, 1.4×10^7, 9.3×10^6, 2.2×10^7, 1.7×10^7, 1.2×10^7, and 1.6×10^7 CFU g^{-1} soil, respectively, after 120 days. The significant increase in microbial populations in microcosms with amendments could be due to the facts that (1) oxygen supplement by H_2O_2 stimulated the growth of aerobic and facultative bacteria and (2) carbon supplement by cane molasses or biodegradable surfactant SG enhanced microbial growth and subsequent biodegradation rate.

DGGE Analysis

PCR amplification of 16S rDNA and DGGE analyses was performed to determine the dominant microorganisms during the TPH biodegradation process. Fig. 7 presents the DGGE profiles of the PCR-amplified 16S rDNA for soils collected from various microcosms such as Groups A1 (control), B1 (100 mg L^{-1} SG), B2 (1,000 mg L^{-1} SG), and C1 (900 mg L^{-1} H_2O_2). Overall, the intensities of all microorganisms were located within an intensive range for strains 1, 2, and 3 during the incubation period.

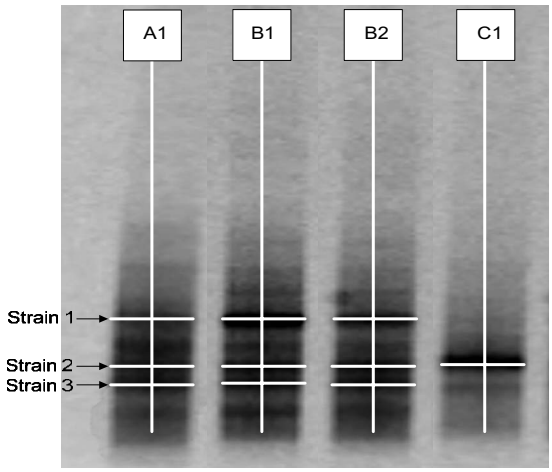

Fig. 7. DGGE profiles of the PCR-amplified 16S rDNA for soils collected from various microcosm experiments such as Groups A1, B1, B2, and C1 on day 120

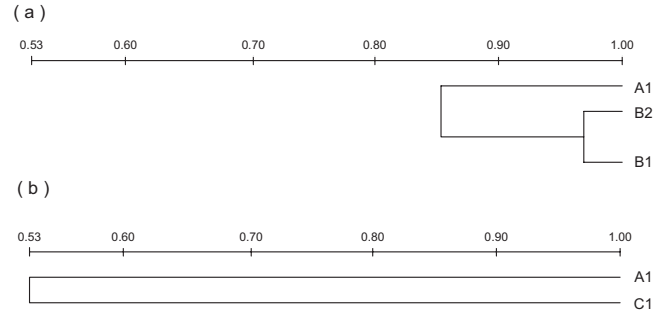

Fig. 8. UPGMA dendrogram for illustrating relationships among microorganisms collected from (a) Groups A1, B1, and B2; (b) A1 and C1 on day 120

Results indicate that the mixed bacterial consortia might be responsible for the biodegradation of TPH in this study. Consequently, these dominant native microorganisms could be further isolated and enriched to enhance the efficiency of the bioremediation process. Moreover, Strain 2 was the most significant microbe appeared on Day 120. Fig. 8 presents the UPGMA dendrogram for illustrating relationships among microorganisms collected from Groups A1, B1, B2, and C1 on Day 120. Results show that the microbial species in these four groups could be grouped into two major phylogenetic clusters. These microorganisms might be employed as indicators to evaluate the occurrence and completion of the bioremediation process of TPH at this contaminated soil.

Dendrograms Comparing of Bacterial Communities

As shown in Table 2, the identities of the nucleotide sequences of three dominant bands are shown to be in a range of 93–97% of specific microorganisms as compared to database of GeneBank (Table 2). This indicates that novel microorganisms, which might be sensitive to TPH and capable of degrading TPH, exist in this

Table 2. Comparison of the Nucleotide Sequences of 16S rDNA of Two Specific Microorganisms in Groups A1, B1, B2, and C1 with the Database from Gene Bank

DGGE band	Microorganisms (NCBI accession number)	Similarity (%)
Strain 1	*Pseudomonas* sp. Sc-R8 (DQ357697)	94
	Pseudomonas sp. CG2 (DQ357706)	94
	Pseudomonas sp. SDA21A (DQ323747)	94
	Pseudomonas sp. D6-9 (AM403197)	94
	Pseudomonas sp. D2-17 (AM403176)	94
	Pseudomonas sp. MBIC2027 (AB030085)	94
Strain 2	*Shewanella* sp. ES03 (AM286803)	93
	Shewanella sp. SQ10 (AF026460)	93
	Shewanella putrefaciens (AY321590)	93
	Shewanella kaireitica (AB201781)	93
	Shewanella sp. LMG 23025 (AJ967028)	93
	Shewanella schlegeliana (AB081761)	93
Strain 3	*Pseudomonas* sp. D5044 (DQ480134)	97
	Pseudomonas sp. G-R2A19 (EF554921)	97
	Pseudomonas sp. AB42 (EF554871)	97
	Pseudomonas pachastrellae (AB125367)	97
	Pseudomonas sp. MSI057 (EF428033)	97
	Pseudomonas sp. M2-1 (AY880297)	97

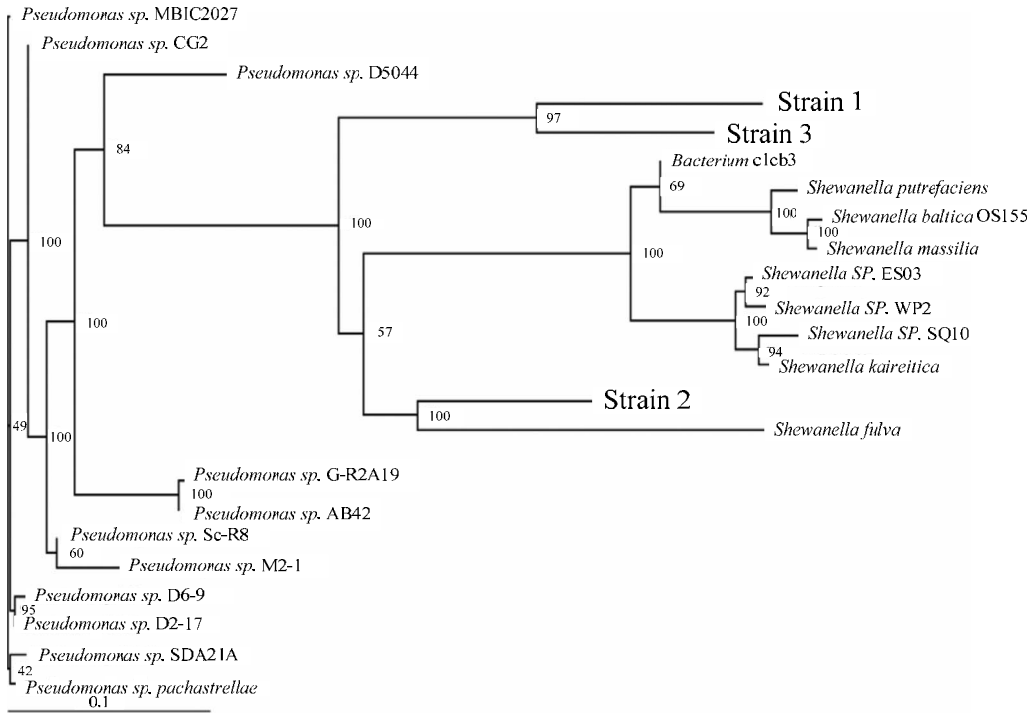

Fig. 9. Phylogeny tree for illustrating relationships among three microbial strains collected from Group A1, B1, B2, and C1. The bootstrap value, as determined from 1,000 bootstrap samples, is presented at each node (in percent).

ecosystem. Using the similarity coefficients in 16S cDNA gene sequences, a PHYLIP dendrogram allocated two specific microorganisms in this population into three separate phylogenetic clusters (Fig. 9). *Pseudomonas* sp., the possible species for Strain 1 and Strain 3, and *Shewanella* sp., the possible species for Strain 2, have been reported that they could biodegrade TPH under different experimental conditions (Saadoun 2002; Benoit et al. 2003; Gerdes et al. 2005; Chandler et al. 2006; Kerin et al. 2006).

If some microbes have similar genetic background in DNA and are closely related to some clusters, there is a possibility that they could utilize the same xenobiotic compounds (Roling et al. 2004; Bento et al. 2005). In this study, TPH biodegradability under different experimental conditions with the addition of H_2O_2 and various concentrations of surfactant SG was evaluated to determine native microorganisms in the treated soils. Results indicate that addition of H_2O_2 and SG would not inhibit the bacterial growth and biodegradation process could still occur. According to the results from GeneBank, two microorganisms, *Pseudomonas* sp. and *Shewanella* sp., which can biodegrade TPH, might exist in treated soils. Results also reveal that DGGE and nucleotide sequence techniques provide a guide for further microbial isolation and identification.

Results reveal that two or more established technologies may be used together in treatment trains to provide the necessary treatment and enhance the cleanup rate of the petroleum-hydrocarbon contaminated soils. Results from this study indicate that among these treatment methods, surfactant flushing, Fenton-like oxidation, and enhanced bioremediation have been considered as promising remedial technologies due to its potential for treating petroleum-hydrocarbon contaminated soils. When applied in situ for the remediation of fuel-oil contaminated soils, a more aggressive technology (e.g., surfactant flushing and Fenton-like oxidation) may be applied to remediate areas with high contaminant concentrations, followed by the application of a less aggressive technology (e.g., enhanced bioremediation) to remediate a larger area that includes the former hot spot area. This treatment train concept would be more applicable and also minimize the overall cost of the treatment.

Conclusions

In this study, microcosm experiments were conducted to evaluate the effectiveness of (1) nutrients, H_2O_2, and cane molasses addition; (2) soil washing by SG; and (3) soil pretreatment by Fenton-like oxidation on the bioremediation of fuel-oil contaminated soils. After 120 days of incubation, approximately 61% of TPH removal was observed in microcosms with H_2O_2(100 mg L^{-1}) and nutrient addition compared to only 9% of TPH removal in microcosms without amendments. Furthermore, addition of cane molasses can also improve the efficiency of contaminant biodegradation. Approximately 95 and 69% of TPH removal was observed in microcosms with SG (100 mg L^{-1}) and high concentration of H_2O_2(900 mg L^{-1}) addition, respectively. Moreover, significant increase in microbial populations in microcosms with the SG addition was also observed. Results indicate that the addition of biodegradable surfactant SG would (1) cause the increase in the concentrations of primary substrates (carbon sources) and microbial populations and (2) enhance the desorption and solubilization mechanisms of petroleum hydrocarbons. Thus, the TPH biodegradation rate would be significantly improved. Results also imply that addition of higher H_2O_2(900 mg L^{-1}) dose might activate that Fenton-like oxidation process and thus enhance the TPH removal efficiency via oxidation process. The produced oxygen via the decomposition H_2O_2 would enhance the subsequent TPH biodegradation. Results suggest that the two pretreatment techniques, SG washing and

Fenton-like oxidation (with H_2O_2 concentration of 900 mg L^{-1}), would not cause adverse effect on subsequent bioremediation process using intrinsic bacteria. Results from PCR/DGGE analysis also imply that two TPH biodegraders (*Pseudomonas* sp. and *Shewanella* sp.) might exist in the treated soils. Results indicate that enhanced bioremediation can be applied and combined with appropriate pretreatment techniques to effectively improve the bioremediation efficiency of fuel-oil contaminated soils.

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