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Short communication

Bench-scale *ex situ* diesel removal process using a biobarrier and surfactant flushing

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ABSTRACT

Ex situ diesel removal was demonstrated using a biobarrier with immobilized cells and surfactant flushing in a bench-scale system. Four strains (two *Acinetobacter* sp., one *Gordonia* sp., and one *Rhodococcus* sp.) isolated from a diesel-contaminated site were immobilized onto a matrix to act as a biofiller. Peat moss, bentonite, and alginate were used as a hybrid support, and a procedure for the use of a bench-scale biobarrier was also employed. According to a microbial counting assay used for the biobarrier, the total amount of bacterial cells increased from approximately 2×10^9 to 8×10^9 (colony forming unit (CFU)/g) and the amount of inoculated diesel-degrading bacteria slightly increased from $\sim 2 \times 10^6$ to $\sim 5 \times 10^6$ (CFU/g) in the same period (over 30 days). This increase resulted in the reduction of diesel from 6000 ± 45 mg/kg to below 5 mg/kg. While 99.9%, i.e. up to below 5 mg/L of the diesel in soil was treated during 30 days of operation, diesel biodegradation accounted for 24.3% of the reduction of diesel.

1. Introduction

Diesel oil leaks from underground storage tanks, army depots, and oil-contaminated sandy beaches pose a serious threat to aqueous bodies of water, soil ecosystems, and human beings [1]. Physical and chemical processes, such as adsorption, thermal treatment, soil washing [2], and many biological treatments, have been reported to treat diesel-contaminated sites [3–7]. Among them, bioaugmentation by mixed microbial cultures has appeared to be effective [4,5].

Especially, physical immobilization of cells has been suggested although some releasing of cells is emerged. As a candidate of matrix for immobilization of cells, peat moss is promising due to a high and fast removal of hydrophobic contaminants [2]. The hybrid support (here, denoted as PBA) consisting of Peat moss combined with bentonite and alginate provides higher diesel adsorption and promotes cell immobilization due to a higher surface area [9,10].

Herein, diesel removal has been demonstrated using immobilized cells on the PBA as a biofiller in the biobarrier for the treatment of diesel oil-contaminated soil, where the biobarrier was supported by flushing with a biodegradable surfactant solution. This biobarrier and surfactant flushing system offers the possibility to use the biobarrier until achieving the clean-up goal without spreading of diesel as an *in situ* remediation technology in diesel-contaminated sites, becoming a cost efficient process. Moreover, the clean-up aim by immobilized microorganisms in the diesel-contaminated soil ends up remediating sites without exchange of biofiller to below 5 mg/kg of target diesel. If remediation project has been done, the biobarrier can function to avoid problems for additional events of diesel spreading. When the biobarrier is necessitated no more, biofiller materials can be treated easily such as burning or use of fertilizer, and so forth.

Prior to pilot experiment, a bench-scale study grants important information because applications from the pilot-scale to field-scale have led to different results, meaning that prediction is difficult in scale-up case studies [8]. This information includes the amount of flushing agent for diesel treatment, biofiller loading (biobarrier thickness), and the microorganism injection period. We have a goal to do *ex situ* bench and pilot systems by using diesel-contaminated soil to be excavated diesel, but the ultimate remediation of field application is focused on *in situ* system. Therefore, the specific

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purposes of this work in the bench-scale system were the following: (1) evaluation of the *ex situ* diesel oil degradation, (2) counts of the total and diesel-degrading bacteria in the biobarrier, and (3) qualitative analyses of the cells in the biobarrier by denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) during 30 days of operation.

2. Experimental

2.1. Materials

Peat moss was purchased from TERRA-TORF, DEMETRA (Yuzhno-Sakhalinsk Timovskoye, Russia). Bentonite, alginate, and Tween 80® were obtained from Sigma–Aldrich (St. Louis, USA). Dichloromethane was purchased from Merck (Darmstadt, Germany). The biosurfactant used as a flushing agent in this study was used with SWA 1503® (H-Plus Eco., Daejeon, Korea; see Table S1 in supporting information). The frames of the bench-scale system were fabricated from stainless steel with mesh-type barrier fabrics.

2.2. Isolation, identification, and culturing of bacteria

Four strains of bacteria (*Acinetobacter* sp. MJ1, *Rhodococcus* sp. MJ2, *Gordonia* sp. MJ3, and *Acinetobacter* sp. MJ4) were isolated from a diesel oil-contaminated site in Daejeon, Korea [1]. The 16S rRNA gene sequences of the four strains were deposited in the NCBI GenBank with the accession numbers GU991530, GU991529, GU991528, and HQ650820, respectively. The phylogenetic tree (clustering) showing similarities within groups and between groups for the four strains is shown in Fig. S1 (supporting information). Detailed isolation and culture methods of the bacteria from the soil sites are included in Ex. S1 (supporting information).

2.3. Preparation of biofiller

Biofiller to pack inside of biobarrier was prepared: the each cultured bacteria (~10^9 CFU) was centrifuged, and then the collected biomass was redispersed with PBA. Afterwards all materials were mixed in the container vigorously by stirrer (3000 rpm and 30 min), resulting in a slurry, i.e. biofiller. The composition of the PBA was sterilized peat moss, bentonite, and alginate (ratio of 80/19/1, w/w/w) whose recipe was designed to give enough permeability for the biobarrier [1]. The ratio of the PBA and double-distilled (DI) water was 1:1 (w/w). Based on previously reported studies, the PBA was immobilized with the four bacterial strains prior to packing in the column. For the bacteria immobilized into the PBA, the cultured microorganisms for *Acinetobacter* sp. MJ1, *Rhodococcus* sp. MJ2, *Gordonia* sp. MJ3, and *Acinetobacter* sp. MJ4 were determined to be 3×10^9, 7×10^9, 5×10^9, and 2×10^9 CFU/g, respectively. To quantify the immobilized cells, a 1 mL aliquot of biofiller was placed into a 15 mL conical tube (BD Falcon™) with 9 mL sterilized DI water. After vigorous vortexing for 5 min to dislodge the immobilized cells, an aliquot of 100 μL was plated onto a R2A agar plate and serially diluted until colonies appeared at 37 °C. The number of colonies was expressed as CFU per 1 g of biofiller on a wet weight basis. The quantification experiments were conducted in triplicate.

2.4. Description of bench-scale set-up

As shown in Fig. 1, the volume of the equipment was 0.11 m^3 (45 kg) with dimensions 0.3 m × 0.6 m × 0.6 m (width/length/height). The volume of the barrier was 0.01 m^3 (1.6 kg) with dimensions 0.3 m × 0.05 m × 0.6 m; the barrier connected to each of the three injection and outlet points at the top, middle, and bottom of set-up. The biobarrier composed of mesh-type stainless steel is contacting the position (A) and (B) without a gap. The flushing flow rate was determined to be 1 mL/min and flushing

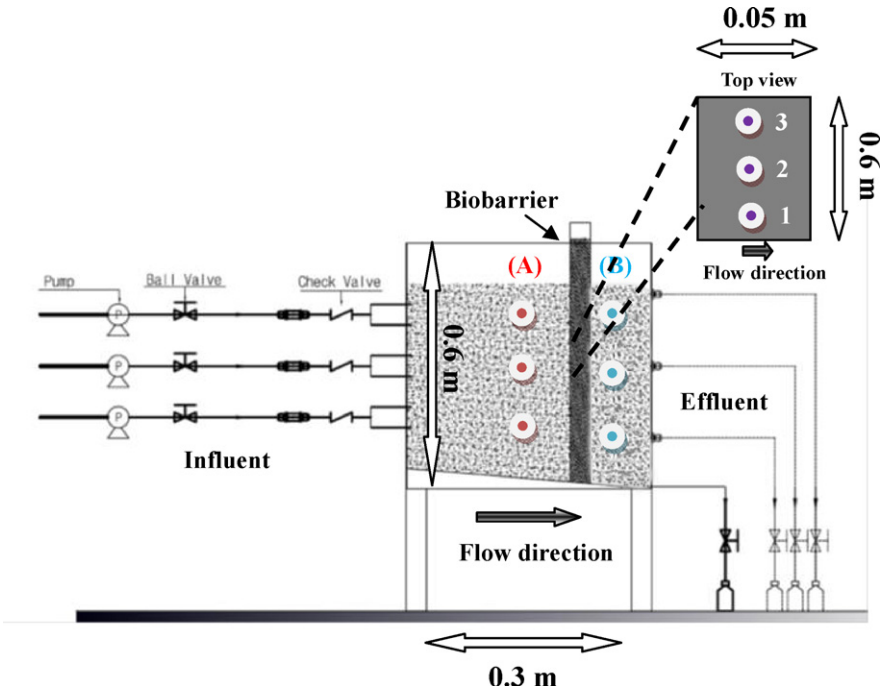

Fig. 1. Schematic presentation of the bench-scale set-up horizontally. *Note*: Three sampling points at positions (A) and (B) represent the diesel analysis at the left/right of the biobarrier. Three sampling points of position (1–3 points) in the biobarrier represent microbiological analysis.

Table 1
Physical and chemical properties of soil (A) in the bench-scale system.

TPH concentration (g/kg)	Total N (g/kg)	Total P (g/kg)	pH (H_2O)	Bulk density (g/cm^3)	Porosity	Sand (%)	Silt (%)	Clay (%)	Fe	Cu	Ni	Pb	Zn
6000 ± 45 mg/L	0.09	373.23 mg/L	7.8–5.6	1.40	0.42	79.72	15.40	4.88	5075.78 mg/L	9.14 mg/L	2.78 mg/L	9.31 mg/L	29.51 mg/L

was conducted with as-prepared surfactant solution at 0.2 wt% above critical micelle concentration (CMC = 0.13 wt%).

The physicochemical properties of the soil are summarized in Table 1. The soil was packed in position (A) of the *ex situ* bench-scale set-up. Soil was considered from a diesel-contaminated site (37°25′11″N, 126°91′71″E, Hwa-Sung, Korea) with average hydraulic conductivity (K) of 2.0×10^{-4} cm/s that will be planned to remediate *in situ* as a field application after this study. Thus, the soil in position (A) was not sterilized to simulate a more realistic situation. Before packing the soil in position (A), diesel-contaminated soil was obtained from the 10 cm layer below the soil surface. The soil was homogenized and air-dried at 25 °C for 48 h under a hood after removing plants and stones. Position (B) was filled with sterilized sand (40–50 mesh, 20 kg, Junsei). Note that position (B) was used as uncontaminated zone for sampling points because this experiment is focused on the capacity of diesel removal by the biobarrier (coupling of surfactant flushing and biodegradation) to remediate position (A) soil. Subsequently, the prepared biofiller (as described in Section 2.3) was injected into the biobarrier by pushing with steel rod. The bench-scale reactor operation was performed for 30 days at room temperature. As a reference experiment, the same protocol was conducted where the filler was not immobilized of cultured microorganisms.

2.5. Plate count analysis of total and diesel-degrading microbes

The number of bacterial cells (cultured microorganisms) was estimated using the plate count method. Samples were prepared by shaking the biofiller (1 g) with 49 mL of 0.2% sodium phosphate for 30 min at 10 °C and 150 rpm. Appropriate dilutions, prepared in 0.9% NaCl, were spread onto agar plates. R2A agar plates (CFU/g) were used to enumerate the aerobic heterotrophic microorganisms (total microorganisms) for 30 days. To count the total number of microorganisms, the plate was incubated at 30 °C for 3 days.

Diesel-degrading microorganisms were quantified on agar plates that contained purified agar with minimum minerals in a phosphate buffer (10.0 mM, pH 7.0) with the following compositions: 0.13% NH_4NO_3, 0.05% $MgSO_4{\cdot}7H_2O$, 0.02% $CaCl_2{\cdot}2H_2O$, 0.5% KH_2NO_3, and 0.5% K_2HPO_4 with diesel (0.5%, v/v). Tween 80® (0.08%, v/v) was used to emulsify the diesel to easily disperse in the dishes. Diesel degraders were incubated at 30 °C for 7 days.

2.6. DGGE analysis

DGGE was performed using PCR-amplified 16S rRNA gene fragments to monitor changes in the inoculated cell populations of biofiller. Samples of the PBA biofiller were acquired at day 0, 1, 2, 3, 5, 7, 15, and 30 after inoculation and used for PCR-DGGE. Attached cells were released from the PBA by vortexing vigorously for 5 min in a 50 mL Falcon tube after adding sterile 0.1% (w/v) sodium phosphate buffer (10.0 mM, pH 7.0). The cell suspension was then transferred to a new tube and was centrifuged to obtain the biomass. The FastDNA® Spin for soil kit (MP Biomedicals, LCC., OH, USA) was used, following the manufacturer's instructions. The extracted DNA was used as a template in the polymerase chain reaction (PCR) for DGGE analysis [10]. The bacterial 16S rRNA gene was amplified using primers 341f with attached GC-clamp and 536r. Cycling conditions were 15 min of denaturation at 95 °C, 5 cycles of 0.5 min at 95 °C, 53 °C, and 72 °C with a ramp of 0.1 °C/s, and a final 7 min extension step at 72 °C. PCR products were condensed to their final volume (30 μL) using a purification kit (TaKaRa, Japan) after confirmation of base pair (bp) size with electrophoresis (1.5 wt% agarose gel). Polyacrylamide gels (8%) with denaturing gradients ranging from 35% to 60% were used to separate PCR products. Gels were electrophoresed in 1X TAE buffer at 60 °C and 60 V for 15 h using a Dcode system (Bio-Rad, Hercules, CA, USA).

2.7. T-RFLP analysis

For T-RFLP analysis, the extracted bacterial 16S rRNA gene was amplified using primers 9f (FAM labeled) and 805r. *Hha* (Progmega, Madison, WI, USA) was used for restriction enzyme digestion. Cycling conditions were 1 min of denaturation at 95 °C, 30 cycles of 0.5 min at 95 °C, 0.5 min at 57 °C, and 0.75 min at 72 °C, and a final 10 min extension step at 72 °C. The PCR product (100–50 ng) with restriction enzyme, buffer solution and DI water were mixed to 10 μL of final volume and then incubated at 37 °C overnight. Digested samples were analyzed using a Genetic Analyzer (ABI PRISM 3100®, Applied Biosystems, USA), and the sizes of the generated fluorescence-labeled 5′-terminal fragments were analyzed based on the summation of the peak area of the bp using a Peak scanner™ (Version 1.0, Applied Biosystems, USA).

2.8. Diesel (total petroleum hydrocarbon, TPH) analysis of soils and effluent solutions

Measurement of the diesel concentration was conducted with soil samples taken from left (A) and right (B) positions as well as water samples from effluent of flushing solution outside of the biobarrier, compared to a control experiment that had an absence of immobilized cells. Each analyzed sample for each three positions in (A) and (B) in Fig. 1 was mixed with 100 mL of dichloromethane, sonicated for 30 min twice, and placed in a rotary shaker (150 rpm at 20 °C) overnight. After centrifugation (4000 × g and 20 min at 20 °C) to remove residual solid materials, anhydrous sodium sulfate was added due to the humidity in the dichloromethane solution and allowed to settle. The diesel extracted in dichloromethane was purified using silica gel and concentrated to a constant weight with rotary evaporation [11]. To quantify samples, an aliquot (2 μL) of the condensed and evaporated diesel samples was injected into a gas chromatography/flame ionization detector and mass spectrometry (GC/FID and MS) instrument (Hewlett Packard 7890, Palo Alto, CA, USA). The chromatographic capillary column was a HP-5 model (phenyl methyl siloxane; 30.0 m long × 320 μm diameter × 0.25 μm thickness). The GC conditions were as follows: injector temperature was 290 °C, column temperature was 50 °C for 5 min with a ramp to 290 °C at a rate of 10 °C/min, column temperature was maintained at 290 °C for 25 min, and the detector temperature was 300 °C. The carrier gas was helium, and the column flow rate was 1.5 mL/min without splitting. The diesel detection limit of GC/FID was 0.1 mg/L. And octane was used as an internal standard.

Each experiment was conducted in triplicate. The mean and standard deviations were plotted using SigmaPlot 10.0 (Systat Software Inc., USA).

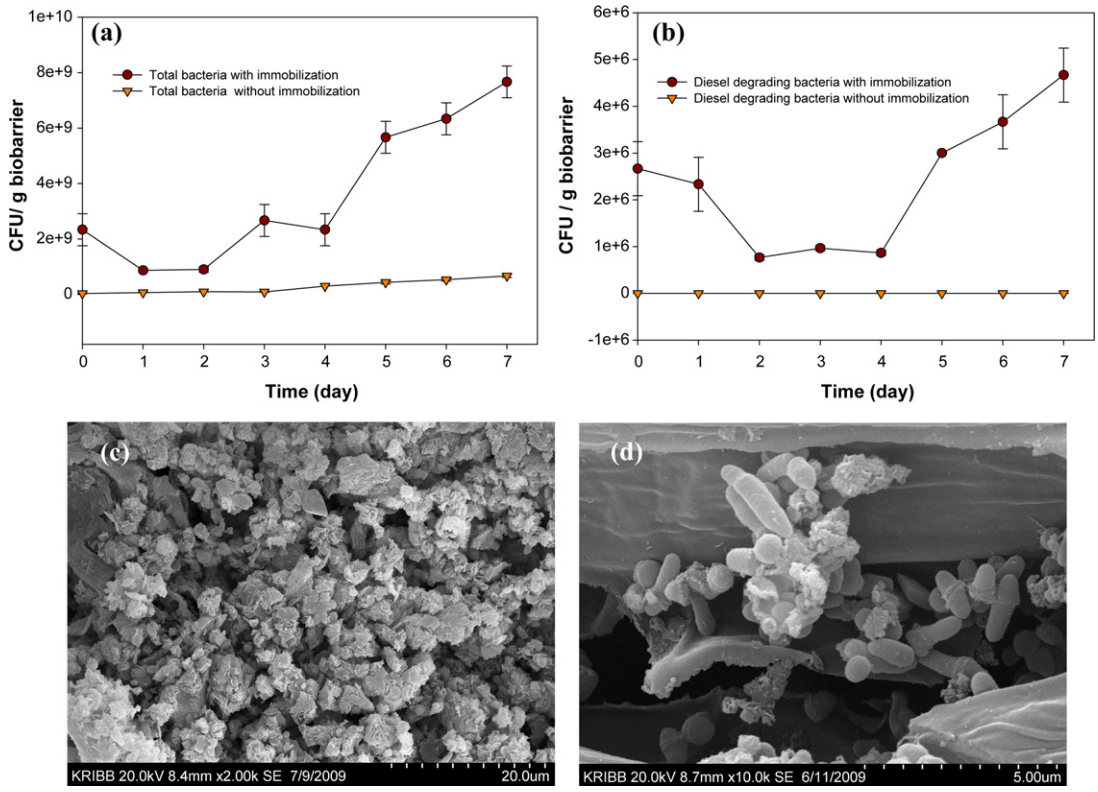

Fig. 2. Number of total and diesel-degrading bacterial cells in the biobarrier. Total bacteria (a) and diesel-degrading bacteria (b) with and without immobilization for 30 days. SEM images at low magnification (c) and high magnification (d) showed the bacteria immobilized onto the biofiller after 30 days.

3. Results and discussion

3.1. Variation of the number of total and diesel-degrading bacteria

Fig. 2a and b indicates the total and diesel-degrading cell numbers in the biobarrier; these were sampled and averaged from the centered spots (1, 2, and 3) horizontally in the biobarrier. In the control experiment (no cell immobilization), the total number of bacteria slightly increased, but the number of diesel-degrading bacteria were almost constant ($\sim 3 \times 10^2$ CFU/g). When the four bacteria were immobilized onto the biofiller, the total number of bacterial cells increased from $\sim 2 \times 10^9$ CFU/g to $\sim 8 \times 10^9$ CFU/g as well as the number of inoculated diesel-degrading bacteria increased from $\sim 2 \times 10^6$ CFU/g to $\sim 5 \times 10^6$ CFU/g by day 7. For 15 days and 30 days, the total number of bacteria and the number of diesel-degrading bacteria were maintained to similar counts at 7 days (data not shown). This result means that the inoculated cells required little adaption period and the total number of bacteria was not inhibited in the presence of the inoculated bacteria. Furthermore, it indicates that diesel flushed by washing with the surfactant solution could be properly degraded by microorganisms in the biobarrier [4]. Fig. 2c and d shows the SEM images of mixed bacteria immobilized in biofiller after 30 days of operation (details of the preparation of the SEM samples is in the supporting information, Ex. S2). The morphology of the bench-scale PBA biofiller was similar to that of the batch mode (Fig. 2c) [1], and Fig. 2d shows microbes on the PBA. However, these microorganisms were not considered immobilized bacteria like the *Rhodococcus* or *Gordonia* strains (even though they were compared to pure cultured bacteria) because the biofiller was not sterilized prior to the immobilization of cells and microorganisms in the soil (position A). As a result, the microorganism population introduced into the biofiller has been evaluated by observing the DGGE bands and T-RFLP.

3.2. Time course of diesel removal

The mass balance of the diesel removal was calculated as follows:

Total diesel (mg/kg) in position (A) = removed diesel (mg/kg) (sorbed onto the biobarrier + biodegradation + surfactant flushing) + residual diesel (mg/kg) in position (A). The diesel biodegradation was calculated by subtracting diesel in position (A) − diesel [position (B) + sorbed onto the biobarrier + residual in position (A)].

Fig. 3a shows the results of surfactant flushing into the biobarrier with and without the immobilization of mixed cells. As time elapsed, the apparent diesel concentration in position (A) was reduced gradually from 6000 ± 45 mg/kg to 2584 ± 17 mg/kg over 7 days. Further operation up to 15 days and 30 days led to 500 ± 30 mg/kg and below 5 mg/kg (data not shown), which is permitted Korean Waste Water Discharge Limit of TPH [12]. Diesel removal (surfactant flushing + biodegradation) (%) in position (A) as approximately 67.6%, 91.7%, and 99.9% for 7 days, 15 days, and 30 days, respectively. Due to the difference of diesel concentration in position (A) and (B), therefore, approximately 10.6%, 18.2%, and 24.3% of the diesel was biodegraded.

To examine the net amount of diesel biodegradation by the immobilized cells, the difference between the diesel concentration in position (A) and (B) was plotted (Fig. 3b).

At that time the effluent diesel outside of the biobarrier was negligible. At day 0, the diesel biodegradation was lowest because adsorption onto the biofiller occurred instead of the biodegradation process, but for the next days, biodiesel degradation occurred and degraded ~15 g of diesel. Biodegradation of diesel was close to values, 600 mg/kg, 200 mg/kg, and 5 mg/kg after 7 days, 15 days, and 30 days, respectively. As a result, the biodegradation rate could be expressed for the first-order and the second-order models as following equations, respectively [13]:

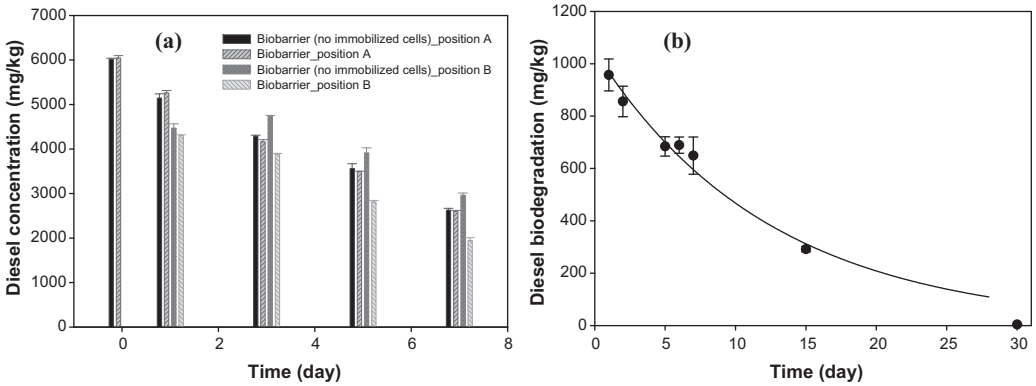

Fig. 3. (a) Time course of diesel removal in positions (A) and (B) of the biobarrier with soil flushing for 30 days. (b) biodegradation of diesel as measured based on the subtraction between the diesel concentrations in positions (A) and (B) in which curve is fitted model equation of the first-order kinetic.

$$C_t = C_i \exp(-k_1 t)$$

$$\frac{1}{C_t} = 2k_2 t + \left(\frac{1}{C_i}\right)$$

where C_t (mg/kg) is the biodegraded diesel concentration at time (day), C_i (mg/kg) is the initial diesel concentration, k_1 (day^{-1}) and k_2 (kg/mg day) is the rate constant for the first-order and second-order models. The rate constant (k_1) was 0.0821 with a relationship of $r^2 = 0.96$ while the rate constant (k_2) was 0.0848 with a relationship of $r^2 = 0.93$, showing that biodegradation kinetics of diesel is matched well with the first-order model than the second-order one.

Conclusively, in order to treat diesel by biodegradation process without above 5 mg/kg effluent, it can be modulated the biobarrier thickness, because not only diesel can remove completely in position (A) but also diesel concentration of position (B) can be detected below 5 mg/kg. Provided that all parameters of operation are constant, the size of the biobarrier should be increased at least 6 times with consideration of the capacity for diesel removal (surfactant flushing + biodegradation) at initial operation.

In order to check diesel degradation patterns, after 7 days of microbial diesel degradation at an intermittent time the levels of these various diesel components were uniformly and proportionally reduced because the *Acinetobacter* and *Rhodococcus* strains have the ability to degrade a wide range of TPHs (see Fig. S2 in supporting information) [14].

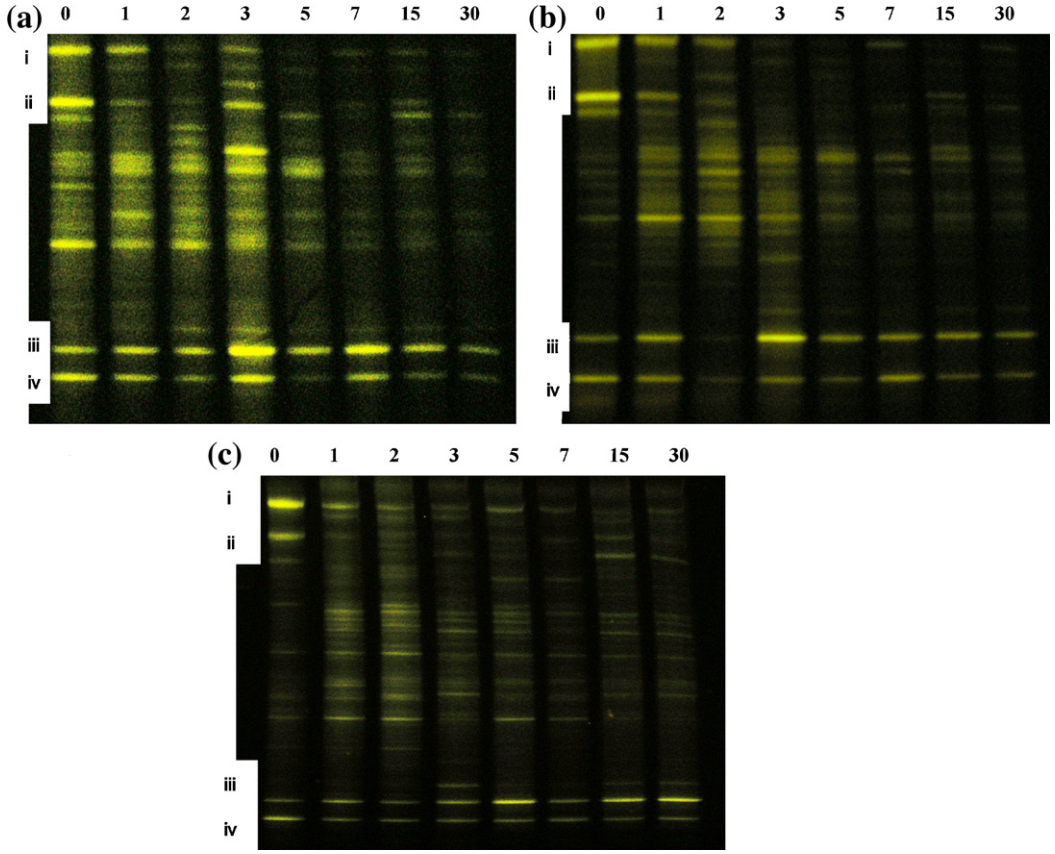

Fig. 4. DGGE profiles of the four bacterial strains sampled in three centered spots (1, 2, and 3) of the biobarrier are (a), (b), and (c). *Note*: Species (i, ii, iii, and iv) of each lanes are *Acinetobacter* sp. MJ1, *Acinetobacter* sp. MJ4, *Rhodococcus* sp. MJ2, and *Gordonia* sp. MJ3, respectively.

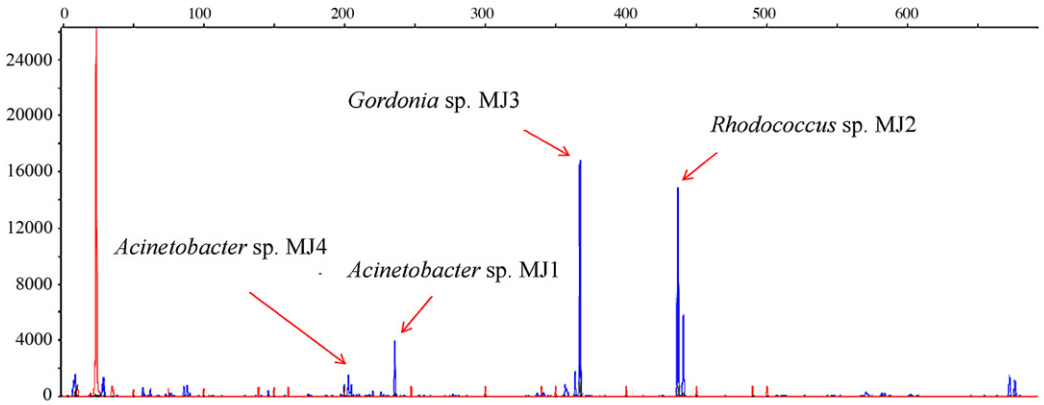

Fig. 5. T-RFLP profiles of the four bacterial strains sampled in the center spot (2) of the barrier. *Note*: *x*-axis is bp and *y*-axis is fluorescence intensity 0.05 m.

3.3. Analysis of the microbial population of the immobilized bacteria using DGGE and T-RFLP

To analyze the immobilized cell population after diesel degradation, DGGE profiles were obtained, and the sizes and intensities of characteristic bands were compared (Fig. 4). The DGGE band patterns reflected the presence of the four bacterial strains that had been inoculated in the biobarrier, as well as other naturally existing species in the supporting matrices and soil environment. After 30 days, the immobilized *Rhodococcus* and *Gordonia* strains displayed intense bands, while two other bands of the *Acinetobacter* strains became weak. To confirm the band positions of each strain, they were matched with the bands of pure cultured cells. Other microbial bands also became weaker; naturally occurring microorganisms in the biofiller were reduced during the bench-scale operation.

The ultimate purpose of the DGGE profiling in this study is to verify that the immobilized cells were successfully presented onto the biobarrier during the bench-scale operation. Thus, we did not try to identify the remaining DNA bands detected in the DGGE. DGGE results indicated that the *Rhodococcus* and *Gordonia* strains survived competitively with indigenous microorganisms and introduced two *Acinetobacter* strains; these results indicate that an increase in the number of diesel-degrading bacteria was predominantly due to the *Rhodococcus* and *Gordonia* strains when steadily supplying a diesel carbon source.

Due to limitations of qualitative analyses of the microbes with DGGE, T-RFLP analysis was needed to calibrate the DNA contents of each strain. Bacteria profiles were characterized using T-RFLP because both the sizes and the relative signal intensities of T-RFs of samples are well-known, highly reproducible, and rapidly comparable the microbial diversity method [15]. The standard peaks of each strain were in the supporting information (Fig. S3). As shown in Fig. 5, the resulting bacteria profiles were characterized by two distinct and intense peaks. Likewise, sampling data for the other two positions showed similar results (data not shown), indicating that the *Rhodococcus* and *Gordonia* strains were abundant individually after day 30 (in agreement with the DGGE patterns). Therefore, this information suggests that two *Acinetobacter* sp. should be supplied additionally into the biobarrier monthly during the operation, and furthermore, *Rhodococcus* sp. and *Gordonia* sp. are suitable for immobilizing cells in the biobarrier system for field applications.

4. Conclusions

This work described the effect of a PBA biofiller immobilized with mixed cells in combination with surfactant flushing on the removal of diesel in a bench-scale system. To treat diesel in soil, the amount of PBA loading and suitable microorganisms are the most important factors. Over 30 days of operation, the diesel concentration decreased gradually from 6000 ± 45 mg/kg to below 5 mg/kg, achieving 99.9% removal by surfactant flushing and biodegradation. For the immobilized microbial population study, counting analysis of mixed bacterial strains in the biobarrier resulted in approximately 8×10^9 CFU/g and 5×10^6 CFU/g for the total and diesel-degrading bacteria counts after 30 days, respectively. Importantly, DGGE and T-RFLP showed that two inoculated *Rhodococcus* and *Gordonia* strains were the competitively dominant species in the biobarrier for 30 days of operation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jiec.2012.01.020.

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