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Monitoring of Microbial Diversity and Activity During Bioremediation of Crude Oil-Contaminated Soil with Different Treatments

BAEK, KYUNG-HWA[1], BYUNG-DAE YOON[1], BYUNG-HYUK KIM[1], DAE-HYUN CHO[1], IN-SOOK LEE[2], HEE-MOCK OH[1], AND HEE-SIK KIM[1]*

[1]*Environmental Biotechnology Research Center, KRIBB, Daejeon 305-806, Korea*
[2]*Department of Life Science, Ewha Womans University, Seoul 120-750, Korea*

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Abstract The present study compared the microbial diversity and activity during the application of various bioremediation processes to crude oil-contaminated soil. Five different treatments, including natural attenuation (NA), biostimulation (BS), biosurfactant addition (BE), bioaugmentation (BA), and a combined treatment (CT) of biostimulation, biosurfactant addition, and bioaugmentation, were used to analyze the degradation rate and microbial communities. After 120 days, the level of remaining hydrocarbons after all the treatments was similar, however, the highest rate (k) of total petroleum hydrocarbon (TPH) degradation was observed with the CT treatment ($P<0.05$). The total bacterial counts increased during the first 2 weeks with all the treatments, and then remained stable. The bacterial communities and alkane monooxygenase gene fragment, *alkB*, were compared by denaturing gradient gel electrophoresis (DGGE). The DGGE analyses of the BA and CT treatments, which included *Nocardia* sp. H17-1, revealed a simple dominant population structure, compared with the other treatments. The Shannon-Weaver diversity index (H') and Simpson dominance index (D), calculated from the DGGE profiles using 16S rDNA, showed considerable qualitative differences in the community structure before and after the bioremediation treatment as well as between treatment conditions.

Key words: *alkB* gene, bioaugmentation, biostimulation, biosurfactant, denaturing gradient gel electrophoresis, microbial community

The use of microbes to clean up polluted environments through *in situ* bioremediation is particularly attractive for both environmental and economic reasons [28]. There are two major bioremediation techniques among the numerous available treatment technologies that can be used to maximize efficiency: biostimulation, where the activity of the indigenous microbial populations is increased through the addition of nutrients and/or a terminal electron acceptor, and bioaugmentation, where the potential degradation of the pollutant is increased by the addition of exogenous degrading microbial strains. Biosurfactants produced from microorganisms have also been successfully used to enhance the apparent solubility of organic contaminants in soil [21]. However, among the various bioremediation processes, it is difficult to determine the best approach to remediate contaminated soil.

For the success of any bioremediation process, it is important to clarify the behavior of the microbial populations responsible for the degradation of the contaminants. An understanding of the whole microbial community is also essential, including microbial populations not responsible for the degradation, as they can affect the behavior of the degrading bacteria through microbial interactions. However, information on changes in microbial communities during bioremediation is limited. Recently, molecular approaches based on analyzing the 16S rDNA have been applied to detect and identify microbial diversity in environmental samples [29], where denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), and single-strand conformational polymorphism (SSCP) are the most commonly used [1, 8, 10, 15, 16]. In particular, DGGE, which is frequently used to determine microbial community fingerprints, is also widely used to detect population shifts over time and under different environmental conditions [17, 20]. DGGE provides sequence data on the dominant species from individual bands. Recent reports have also shown that PCR-amplified fragments of catabolic genes can be separated from environmental DNA by DGGE [10, 19]. Furthermore, an exciting new direction within the field

*Corresponding author
Phone: 82-42-860-4326; Fax: 82-42-860-4594;
E-mail: hkim@kribb.re.kr

of molecular microbial ecology is the use of functional genes as molecular markers to understand the dynamics of specific bacteria in a given community.

Although several recent studies have focused on the comparative degradation kinetics associated with the bioremediation of environmental contaminants [4, 22, 23], relatively few studies have focused on a detailed description of the bacterial community dynamics during this process [12, 17]. Accordingly, the present investigation was undertaken to monitor the changes in the microbial populations and degradation activity, when different bioremediation processes were applied to crude oil-contaminated soil. The applied processes included natural attenuation, biostimulation (addition of N and P), bioaugmentation (inoculation with oil-degrading bacterium), and bioavailability enhancement (addition of biosurfactant), where *Nocardia* sp. H17-1 [2, 3] was inoculated as the oil-degrading bacterium, and MEL (mannosylerythritol lipid) produced by *Candida antarctica* SY16 [13, 14] was added as the biosurfactant.

MATERIALS AND METHODS

Experimental Design

The soil type used for the biodegradation of crude oil was sandy loam (3.8% organic matter, pH 7.1), which was passed through a 2-mm sieve and air-dried. An aliquot (10 kg) of the soil was then artificially contaminated with 5% (w/w) Arabian light oil, which has 54.9% aliphatic hydrocarbons, 10% aromatic hydrocarbons, 21.2% polar materials, and 13.4% asphaltene (SK Chem., Korea), and they were thoroughly homogenized by hand-mixing to distribute the oil through the soil particle and then kept outdoors for 1 month. Thereafter, the treatments were as follows: (1) natural attenuation (NA; no treatment), (2) biostimulation (BS; addition of nutrients), (3) addition of biosurfactant (BE; addition of MEL), (4) bioaugmentation (BA; inoculation with *Nocardia* sp. H17-1), and (5) combined treatment [CT; with (2), (3), and (4)]. The MEL supplement was 1 g/kg-soil. The added nutrients were 0.1% (w/w) KNO_3 and K_2HPO_4. The water content of all treatments was adjusted with sterile water to 50% of the maximum water holding capacity. Each treated soil was thoroughly homogenized by manual mixing, and duplicate soil treatments (1 kg contaminated soil) were then kept in 2.25-l polyethylene containers. All the soil samples were kept at room temperature during the experiments. Containers were regularly aerated to provide sufficient air and oxygen, and were moistened by addition of 20 ml of sterile water until the end of the experiment.

Inoculum

To prepare the inoculum for the BA and CT, the *Nocardia* sp. H17-1 was allowed to grow to the late exponential phase in Luria-Bertani medium. The cells were then collected by centrifugation at 10,000 ×*g* for 20 min, and the cell pellets were washed twice in 20 ml of sterile water. The bacterial suspension was applied in droplets to the soil surface at the level of approximately 1×10^6 cells/g-soil, and the soil sample was then thoroughly mixed manually. The growth of microorganism was estimated based on periodic plate counts.

Analysis of Total Petroleum Hydrocarbons (TPH)

At every two weeks, 5 g (dry wt) of soil samples that were removed from duplicate batch tests was transferred to 25-ml vials, and mixed with anhydrous sodium sulfate. Twenty ml of dichloromethane was added to the vial. The vials were tightly capped, thoroughly mixed for 10 min with a vortex mixer, and sonicated for 1 h in a water bath (4°C). The supernatant was passed through a 0.45-μm teflon filter. One μl of extract was analyzed on a gas chromatograph (Varian 3400CX, CA, U.S.A.) equipped with a flame ionization detector using a DB-1 column (30 m×0.32 mm and 0.25 μm film thickness, J&W Scientific, IL, U.S.A.). During the analysis, the injector and detector temperatures were maintained at 250°C and 300°C, respectively. The column temperature was programmed to increase from 40°C to 170°C in 6°C/min increments, maintain at 170°C for 3 min, rise from 170°C to 300°C in 8°C/min increments, and finally hold at 300°C for 10 min.

DNA Extraction, PCR, and DGGE Analysis

The total DNA was extracted from the soil samples (0.5 g, dry wt) using a FastDNA SPIN kit for soil (Bio101, CA, U.S.A.). The DNA extracts from the soil were then amplified using primers 341f-GC (5'-CGCCCGCCGCGC-GCGGCGGGCGGGGCGGGGGCACGGGGGGCCTAC GGGAGGCAGCAG-3') and 907r (5'-CCGTCAATTCAT-TTGAGTTT-3') for the bacterial 16S rDNA sequences [18]. The reactions were carried out in a 50-μl volume containing 1× PCR buffer, 2 mM dNTP mixture, 1 μM each of primer, template DNA, and 5U of *Taq* polymerase. The temperature cycle for the PCR was 45 s of denaturation at 94°C, 45°Cs of annealing (see below), and a 90 s extension at 72°C. During the initial touchdown cycle, the annealing temperature was lowered from 63°C to 51°C in 2°C intervals per two cycles, and then 20 additional annealing cycles were performed at 51°C. The final primer extension was for 10 min. The *alkB* gene was amplified with primers AlkBF-GC (5'-CGCCCGCCGCGCGCGGCGGGCGGG-GCGGGGGCACGGGGGCCTGCTCCCGATCCTCGA-3') and AlkBR (5'-TCGTACCGCCCGCTGTCCAG-3') [3]. The PCR for the *alkB* gene was carried out based on 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 50°C, a 1 min extension at 72°C, and final primer extension of 7 min at 72°C. The DGGE was performed using a D-Code

16/16 cm gel system with a 0.7-mm gel width (Bio-Rad, CA, U.S.A.) maintained at a constant temperature of 60°C in 7 l of 1×TAE (40 mM Tris-acetate, 1 mM Na-EDTA, pH 8.0) buffer. The DGGE gels contained 6% polyacrylamide-bisacrylamide (37.5:1, w/w) and were poured with a urea and formamide (UF) gradient of 40 to 60%. Each lane of the gradient gel contained approximately 100 ng of the PCR product. Gels were run at 60 V for 15 h, and then stained with ethidium bromide and destained twice in 1×TAE buffer for 15 min each. To analyze the DGGE band patterns, the images were converted, normalized, and analyzed with the Kodak 1.0 software package (Eastman Kodak Co., NY, U.S.A.). A hierarchical cluster analysis, based on a Pearson correlation, was conducted on the transformed data using SPSS 11.5 (SPSS Inc., IL, U.S.A.). The Shannon-Weaver (H') index of microbial diversity [25] was calculated according to the formula $H'=-\sum Pi\log Pi$, $Pi=ni/N$, where ni is the intensity of band I in the lane, and N is the total intensity of all bands in the lane. The Simpson dominance index (D) [26] was calculated using the Pi value, $D=Pi^2$.

Sequencing of DNA from DGGE Gels

The strong DGGE bands were excised using a razor blade and soaked overnight in 20 μl of purified water. A portion (5 μl) was then removed and used as the template in a PCR reaction, as described above. The products were purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and sequenced with an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, U.S.A.). The sequences were then compared with the GenBank database using the BLASTN facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov) and classified using the Ribosomal Database Project (RDP) 16S rDNA database (release 8.1, 1 June 2001).

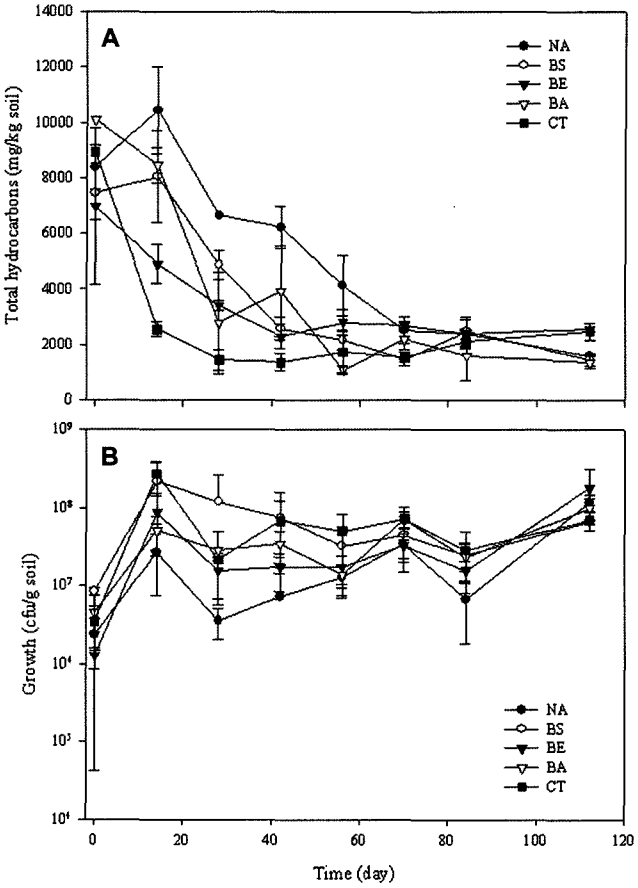

Fig. 1. Degradation of TPH (**A**) and growth (**B**) with different bioremediation treatments of crude oil-contaminated soil (n=4). After sampling, each container was aerated to provide sufficient air and oxygen and moistened by addition of 20 ml of sterile water until the end of the experiment. Natural attenuation (NA), biostimulation (BS), biosurfactant addition (BE), bioaugmentation (BA), and a combined treatment (CT) of biostimulation, biosurfactant addition, and bioaugmentation.

RESULTS

Biodegradation of Crude Oil

Fig. 1A shows the effects of each treatment on the time course of the crude oil degradation. During 16 weeks, the concentration of TPH was reduced from 8,378 to 1,608 mg/kg with the NA treatment, 7,452 to 1,469 mg/kg with the BS treatment, 6,978 to 2,563 mg/kg with the BE treatment, 10,111 to 1,362 mg/kg with the BA treatment, and 8,948 to 2,489 mg/kg with the CT treatment. The amount of remaining hydrocarbons after 100 days was similar for all the treatments; however, the TPH degradation rate constant (k) was the highest with the CT treatment. The value of k was 0.015 d^{-1} (r^2=0.85), 0.020 d^{-1} (r^2=0.86), 0.013 d^{-1} (r^2=0.76), 0.028 d^{-1} (r^2=0.89), and 0.058 d^{-1} (r^2=0.63) with the NA, BS, BE, BA, and CT treatments, respectively. The addition of *Nocardia* sp. H17-1, the biosurfactant, and nutrients caused a rapid reduction in the hydrocarbon content during the first 4 weeks of treatment, and the hydrocarbon degradation thereafter occurred only slowly.

Bacterial Growth

At time zero, the total viable cells were 1.27 to 8.4×10^6 CFU/g (Fig. 1B). The viable cell counts increased approximately 10 to 100 times during the first 2 weeks with all the treatments, and then decreased slightly or remained almost unchanged. In particular, the CT showed a 100-fold increase in the bacterial count during the first 2 weeks. The addition of the nutrients plus the biosurfactant and inoculant increased the number of indigenous microorganisms and inoculant in the contaminated environment.

PCR-DGGE Analysis of Bacterial Community Structure

The effect of the bioremediation treatments on the bacterial community structure was investigated using 16S rDNA-

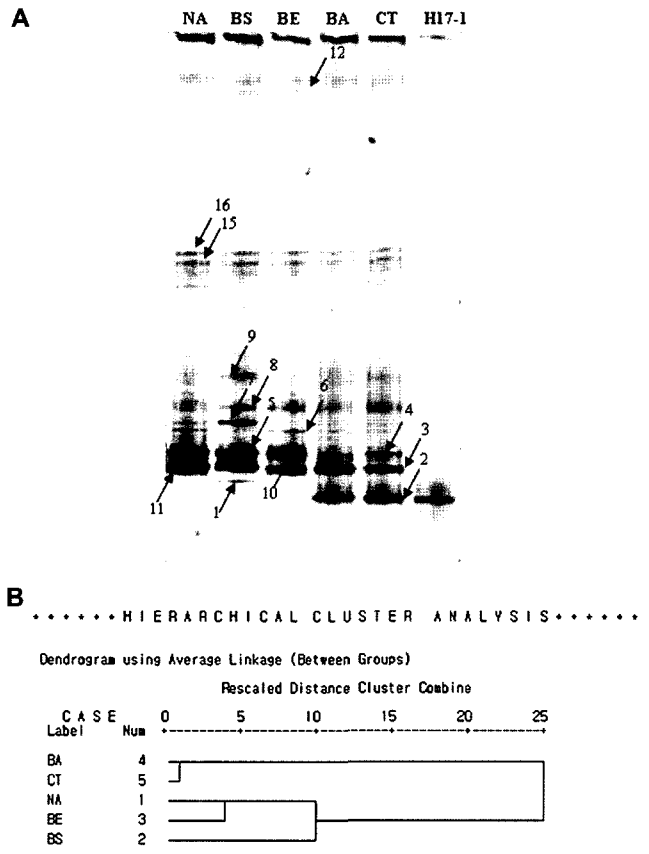

Fig. 2. A. DGGE analysis of PCR-amplified 16S rDNA fragments from soil bacterial communities after 16 weeks of treatment. **B.** Cluster analysis of DGGE profiles of the microbial community with five different treatments, based on position of bands.
The PCR fragments were separated on a DGGE gel using a denaturant gradient of 40–60%. The bands represented by arrows were successfully isolated and sequenced. Natural attenuation (NA), biostimulation (BS), biosurfactant addition (BE), bioaugmentation (BA), and a combined treatment (CT) of biostimulation, biosurfactant addition, and bioaugmentation.

based PCR-DGGE. Although several bands were common between the treatments, the majority was unique for each treatment (Fig. 2A). As shown in Fig. 2A and Table 1, the nucleotide sequence for band 1 from the nutrient-treated soil was 98% similar to the 16S rDNA sequence of *Mycobacterium marinum* ATCC927 (AF456240). This band appeared only with the BS treatment. The 16S rDNA sequence for band 2 obtained from the H17-1-treated soil was identical to that found in *Nocardia* sp. H17-1 (AF487704). The intensity of this band, which predominated, increased with the treatment time. Most of the sequenced bands belonged to γ-proteobacteria.

The addition of nutrient increased the intensity of three prominent bands, 1, 7, and 9, during the experimental period. In particular, the prominent population band 1 with the BS treatment was not observed from any of the other treatments during the treatment period. Based on the DGGE band patterns after 16 weeks, no significant differences in the microbial diversities were observed between the NA and BE treatments (Fig. 2B). Compared with the other treatments, a DGGE analysis of the BA and CT treatments revealed a relatively simple and dominant population structure throughout the experimental periods.

The differences in the microbial diversity between the treatments were confirmed by the Shannon-Weaver diversity indexes (H') and Simpson dominance index (D) values calculated from the DGGE profiles (Table 2). Although the microbial diversity with the NA and BS treatments was low during the initial stage (4 weeks), compared with the other treatments, it increased with the passage of time. The microbial diversity with the BE and CT treatments was higher than those with the other treatments from the initial stage of treatment. The dominance of *Nocardia* sp. H17-1 with the BA treatment was higher than those with the other

Table 1. Sequence analysis of bands from DGGE gels, derived from bacterial 16S rDNA extracted from biotreated crude oil-contaminated soil.

Band	Bacterium with related bacterial sequence	% Similarity	GenBank accession no.
1	*Mycobacterium marinum* ATCC927	98 (402/410)	AF456240
2	*Nocardia* sp. H17-1	100 (400/400)	AF487704
3	γ-Proteobacteria strain JTB254	96 (347/363)	AB015253
4	Uncultured γ-Proteobacteria	90 (144/160)	AF440832
5	Uncultured γ-Proteobacteria	88 (139/158)	AF440850
6	*Ensifer adhaerens* (α-Proteobacteria)	98 (381/396)	AJ550288
7	*Thioalcalovibrio denitrificans*	91 (411/452)	AF126545
8	*Pseudomonas citronellosis*	98 (340/349)	Z76659
9	γ-Proteobacteria strain MN28	90 (248/275)	AJ555478
10	*Rhodonobacter* sp. D47	96 (353/364)	AF250416
11	Uncultured α-Proteobacteria	89 (133/150)	AF440849
12	Uncultured γ-Proteobacteria	88 (151/172)	AF548765
13	Sulfur-oxidizing bacterium OA112	94 (328/354)	AF170423
15	Uncultured bacterium	85 (153/175)	AJ640192
16	Uncultured bacterium	88 (180/210)	AY500281

Table 2. Shannon-Weaver index of diversity (H') and Simpson dominance index (D) calculated from PCR-DGGE analysis.

Time (days)	Natural attenuation (NA)		Biostimulation (BS)		Biosurfactant (BE)		Bioaugmentation (BA)		Combined treatment (CT)	
	H'	D	H'	D	H'	D	H'	D	H'	D
3	0.537	0.297	0.563	0.287	1.102	0.089	0.830	0.261	0.914	0.185
28	0.944	0.132	0.625	0.291	1.130	0.089	0.962	0.158	1.025	0.147
56	1.194	0.072	0.839	0.204	1.124	0.081	0.843	0.231	0.965	0.172
84	0.922	0.176	0.947	0.138	1.260	0.062	0.831	0.213	0.958	0.189
118	1.104	0.089	1.043	0.098	1.163	0.080	1.018	0.142	1.028	0.143

treatments, and the H' and D values for the BA treatment did not change significantly during the treatment period.

PCR-DGGE Analysis of *alkB* Gene

To investigate the relationship between the catabolic activity and the microbial population during the bioremediation of the crude oil-contaminated soil, the *alkB* gene encoding alkane monooxygenase was analyzed by DGGE (Fig. 3). The DGGE band pattern for the NA treatment was similar to that for the BE treatment, and the band patterns for the BA and CT treatments were also similar, reflecting the results of microbial diversity analyzed by using the 16S rDNA. Band A1 was found from day 3 with the BA and CT treatments, and its intensity became stronger with time. The sequence analysis of band A1 indicated a 100% identity with the *alkB2* gene of *Nocardia* sp. H17-1 (AY625605).

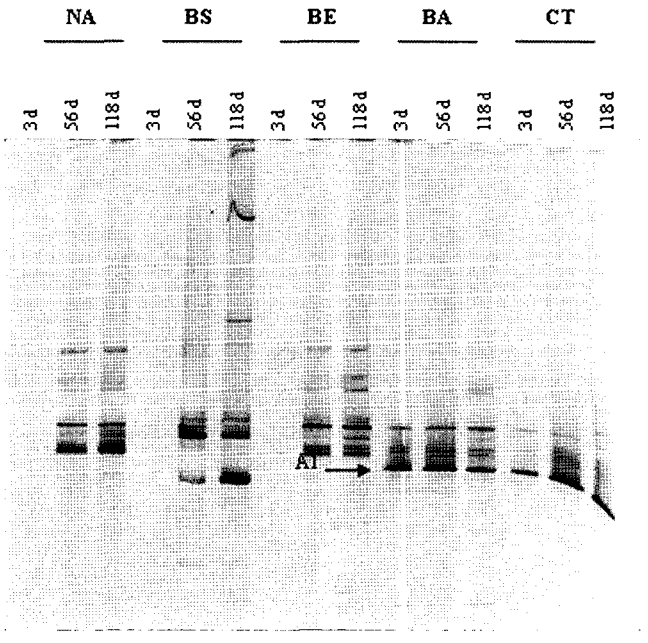

Fig. 3. DGGE analysis of PCR-amplified *alkB* gene fragments generated with DNA from soil bacterial communities.
The amplified products were separated on a denaturant gradient of 40 to 60%. Natural attenuation (NA), biostimulation (BS), biosurfactant addition (BE), bioaugmentation (BA), and a combined treatment (CT) of biostimulation, biosurfactant addition, and bioaugmentation.

DISCUSSION

This study comparatively evaluated natural attenuation, biostimulation, and bioaugmentation for the bioremediation of crude oil-contaminated soil. As shown in Fig. 1, the remaining TPH after 16 weeks was similar with all the treatments, the percent of total degradation was the highest for the bioaugmented soil, and the degradation rate constant was the highest with the combined treatment ($P<0.05$). In particular, the value of k for the combined treatment was three times higher than that for the natural attenuation. In a previous study, *Nocardia* sp. H17-1 was found to effectively degrade *n*-alkanes, ranging from C_{12} to C_{25}, however, the biodegradation of longer-chain hydrocarbons, C_{26} and above, was significantly low [3]. This was also confirmed in the present bioaugmented and combined treatments that included *Nocardia* sp. H17-1, where a rapid degradation of TPH was initially observed until day 28, followed by the slow degradation of the long-chain hydrocarbons until the end of the treatment.

The addition of nutrients to the contaminated site accelerated the growth of the indigenous microorganisms, and their increased biological activities had an effect on reducing the time for oil degradation. Many studies have already been described to increase microbial activity and the rate of biodegradation following the addition of inorganic nutrients, such as nitrogen and phosphorus [6, 16, 20]. Conversely, Seklemova *et al.* [24] found that the addition of nutrients had no effect on the remediation of a forest soil contaminated with diesel. In this study, the biostimulation treatment (addition of nitrogen and phosphorus) increased the bacterial growth and diversity as well as the degradation rate. The DGGE profile (Fig. 2A) showed the appearance of specific bacteria, *Mycobacterium marinum*, *Thioalcalovibrio denitrificans*, and *γ-Proteobacteria* strain MN28, which were not clearly observed with the other treatments. This might have been due to the activation of the bacterial community through the available nutrient input, favoring changes for adaptation, and enhancing the biodegradation capabilities of the indigenous microorganisms. It is already known that members of the genus *Mycobacterium* degrade straight-chain aliphatic and high molecular weight polycyclic aromatic hydrocarbons [5].

Petroleum hydrocarbon compounds bound to soil are not readily bioavailable, however, the addition of a biosurfactant can decrease the interfacial tension, thereby increasing the displacement of oily substances from the soil particles [21]. In the present study, the biosurfactant treatment produced a relatively high degradation rate constant during the initial stage of treatment. This was most likely caused by the biosurfactant, MEL-SY16, which increases the desorption of TPH from the soil, the hydrocarbon solubility, and the contact surface of the hydrophobic compounds with the microorganisms [14]. Hua *et al.* [9] showed that the addition of the biosurfactant, produced by *Candida antarctica*, to the process of biotreatment enhanced both the emulsification of hydrocarbon and the biodegradation rate. In this study, the microbial population during the first week (on day 3) was more diverse than the populations with the other treatments and even than the population in the same soil sample at a later time (Table 2).

Generally, a single species is capable of degrading only a limited number of compounds in crude oil. In addition, introduced microbes can lose their activity, and even fail to survive or thrive, owing to limited nutrients, predation, parasitism, and competition with indigenous bacteria under harsh environmental conditions [7]. In the present study, when *Nocardia* sp. H17-1 was added to the contaminated soil, it successfully degraded the TPH and survived through the treatment period, as evidenced by the increased band intensity in the DGGE. Consequently, the bioaugmentation with the H17-1 strain improved the bioremediation efficiency, when compared with the natural attenuation and biostimulation.

The Shannon-Weaver diversity index (H') and Simpson dominance index (D) were calculated from the DGGE profiles for each treatment. High H' and low D values indicate a high diversity in the microbial community. In the present study, considerable qualitative differences in the community structure were found before and after the bioremediation treatments, as well as between treatments. Initially (for 4 weeks), the natural attenuation and biostimulation treatments showed a relatively low microbial diversity, however, the indigenous microbial diversity increased about 2-fold after 16 weeks of treatment (Table 2). The dominance with the bioaugmentation treatment was higher than that with the other treatments, and furthermore, the H' and D values for the bioaugmentation with *Nocardia* sp. H17-1 did not change significantly during the treatment period. With the biosurfactant and combined treatments, the microbial diversity was higher than with the other treatments from the initial stage of treatment, indicating that the biosurfactant possibly enhanced the bioavailability of the hydrophobic petroleum hydrocarbons to the microorganisms.

It is important to directly monitor the target bacteria or genes related to the degradation of contaminants in the bioremediation process [30]. The catabolic genes encoding alkane monooxygenase (*alkB*), naphthalene dioxygenase (*nahH*), and catechol 2,3-dioxygenase (*xylE*) have been used to assess the prevalence of bacteria involved in petroleum hydrocarbon degradation [11, 27, 29, 30], and the use of a functional gene as a molecular marker is known to be a strong approach for detecting target bacteria [8, 10]. The DGGE analysis indicated possible shifts in the dominant population of alkane monooxygenase-containing bacteria during the treatment period. Since the DGGE profile for the *alkB* gene comprised fewer bands than that for the 16S rDNA, it is highly possible that not all the populations contributed to the alkane degradation, which is consistent with the assumption that the community of specialized TPH degraders is smaller than the total community of microorganisms in the contaminated soil [10].

Overall, the present results clearly showed that the combination of bioaugmentation, biostimulation, and biosurfactant addition might be a promising strategy to speed up the bioremediation of crude oil-contaminated soil. However, for process optimization and validation of the bioremediation, it is important to characterize the contaminated site before treatment. Information on the microbial community and environmental conditions, such as soil properties and oil concentration, is a key factor to be considered in the bioremediation of oil-contaminated areas. Moreover, monitoring the bacterial populations and functional genes responsible for the degradation of contaminants could be useful to increase the efficiency of the bioremediation process, and to help understand the dynamics of the microbial community.

Acknowledgments

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