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Ex situ bioremediation of oil-contaminated soil

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ABSTRACT

An innovative bioprocess method, Systematic Environmental Molecular Bioremediation Technology (SEMBT) that combines bioaugmentation and biostimulation with a molecular monitoring microarray biochip, was developed as an integrated bioremediation technology to treat S- and T-series biopiles by using the landfarming operation and reseeding process to enhance the bioremediation efficiency. After 28 days of the bioremediation process, diesel oil ($TPH_{C10–C28}$) and fuel oil ($TPH_{C10–C40}$) were degraded up to approximately 70% and 63% respectively in the S-series biopiles. When the bioaugmentation and biostimulation were applied in the beginning of bioremediation, the microbial concentration increased from approximately 10^5 to 10^6 CFU/g dry soil along with the TPH biodegradation. Analysis of microbial diversity in the contaminated soils by microarray biochips revealed that *Acinetobacter* sp. and *Pseudomonas aeruginosa* were the predominant groups in indigenous consortia, while the augmented consortia were *Gordonia alkanivorans* and *Rhodococcus erythropolis* in both series of biopiles during bioremediation. Microbial respiration as influenced by the microbial activity reflected directly the active microbial population and indirectly the biodegradation of TPH. Field experimental results showed that the residual TPH concentration in the complex biopile was reduced to less than 500 mg TPH/kg dry soil. The above results demonstrated that the SEMBT technology is a feasible alternative to bioremediate the oil-contaminated soil.

1. Introduction

Soil and groundwater contamination with petroleum hydrocarbon compounds causes environmental and health concerns. This has led to increased attention to develop innovative technologies for remediation [1]. Bioremediation of petroleum hydrocarbons is an effective, economical, and environmentally friendly technology, which is considered a feasible method for treating petroleum hydrocarbon-contaminated soils [2,3]. Bioremediation is generally achieved via bioaugmentation or biostimulation or both, depending on soil conditions and the microbial community structure. The guidelines of the US EPA suggest that bioremediation is feasible when there is about 10^3 CFU/g soil of the microbial population. However, a low microbial population and insufficient microbial diversity affect bioremediation efficiency. According to Alexander [2], bioremediation efficiency is a function of the ability of the inoculated microbial degraders to remain active in the natural environment. Therefore, increasing the ability of the inoculated microbial degraders by bioaugmentation or promoting the activity of indigenous microbial degraders by biostimulation could improve bioremediation efficiency. Microbial communities should thus be monitored to promise the efficiency of bioremediation. Bioaugmentation is the introduction of exogenous microorganisms into environments to accelerate bioremediation [4]. Bioaugmentation can increase pollutant removal rates by increasing the bacterial population [5,6]. In biostimulation, the soil is amended with nutrient mainly containing nitrogen and phosphorous source or biosurfactant known to enhance the TPH bioavailability at the site, thereby increasing the bioremediation efficiency [1,7]. Hence, the application of bioaugmentation and biostimulation is needed to improve bioremediation efficiency which is affected by the concentration and component of hydrocarbon pollution [8,9].

The oil removal efficiency in a bioremediation process is mainly determined by microbial activity, which can be monitored by using molecular tools or rapid assessment packages [10]. Molecular techniques for identifying hydrocarbon-degrading bacteria have been widely used in environmental studies, especially for microarrays that rapidly grow in number. Microarray biochips, a novel technology that has been applied in the environmental field, could offer great accuracy and sensitivity for analysis of microbial diversity [10].

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The landfarming method used in the bioremediation of oil-contaminated soil is an effective, economic and promising technology for cleaning up hydrocarbon-contaminated soil [3]. Turning the soil regularly, provides oxygen transportation needed for biostimulation and increases the opportunity of contact by mixing microbes with oil-pollutants and water. Since microorganisms play a vital role in bioremediation process, they should be monitored with an accurate molecular biotechnology. Therefore, in this study an innovative bioprocess technology, Systematic Environmental Molecular Biotechnology (SEMBT), was developed for field applications in treating petroleum oil-contaminated soil. This bioprocess included bioaugmentation and biostimulation using the landfarming procedure with the operational strategy of reseeding previous biopile soils in series. Molecular microarray biotechnology was used for monitoring during the bioremediation process. The integrated operational strategy of SEMBT improves the biodegradation of hydrocarbon as well as bioremediation efficiency.

2. Materials and methods

2.1. KH-100 site description

The KH-100 site is located near the harbor of Kaohsiung City, southern Taiwan. The site has a storage tank station that has had an oil leak from the past ten years, mainly diesel oil and fuel oil. The mean daily temperature of the operational time was $30 \pm 10\,^{\circ}C$ during bioremediation. Total average rainfall was 1800 mm in per year, and mostly concentrated from May to August. The annual mean air humidity was approximately 77%.

2.2. Soil biopile

Experimental soil was collected from two sites and divided into two series of biopiles (S- and T-series) with different levels of TPH concentration containing diesel oil and fuel oil. Each series consisted of four small-scale biopiles (S0, S1, S2, S3 and T0, T1, T2, T3), three treated biopiles, and one control or untreated biopile (S0 and T0). The biopile size was approximately 4 m (L) × 3 m (W) × 2 m (H) with a soil volume of approximately 20 m^3. Experimental soil biopiles were first analyzed and were then subjected to treatment using bioaugmentation with strains of *Gordonia alkanivorans* CC-JG39, *Rhodococcus erythropolis* CC-BC11, *Acinetobacter junii* CC-FH2 and *Exiguobacterium aurantiacum* CC-LSH4-1, as well as biostimulation with biosurfactant Rhamnolipid (RL) produced by *Pseudomonas aeruginosa*. The biopiles were sampled by using a composite sampling method. Measurement of soil moisture (%) and pH followed the procedures of soil analysis [11].

2.3. Microbial assay

A bioassay was carried out by using the total plate count as the quantitative estimation of enumeration, while a qualitative assay was accomplished by monitoring molecular DNA using microarray biochips with intergenic spacers (ITS) [12]. Enumeration of the bacterial plate count for soil samples followed the methods described in Gallego et al. [13]. The microarray biochips method consisted of the amplification by nested PCR of the ribosomal DNA intergenic spacers (ITS) regions of DNA extracted from contaminated soil. An oligonucleotide array was applied to directly detect bacteria in diesel and fuel oil-contaminated soil.

2.4. Total petroleum hydrocarbon analysis

Total petroleum hydrocarbons (TPH) were extracted from the soil samples by using dichloromethane following the procedure recommended in US EPA test methods 3550B [11]. The organic phase was passed through a cartridge filled with anhydrous sodium sulfate (Na_2SO_4, Sigma) to remove residual water and concentrated to near-dryness under a vacuum. The concentrate was re-dissolved with 2 ml dichloromethane and then concentrated to 1 ml by a N_2 purge. The samples were quantified by using a gas chromatograph with an Agilent DB-1 fused silica capillary column (type RTX-5; 30 m long, I.D. 0.53 mm, D.F. 1.5 μm; Restek, Bellefonte, USA) and flame ionization detector (GC-FID, PerkinElmer GC model no. 8310) as described by Mohn and Stewart [14].

2.5. Biogas analysis

Biogases were measured by using a gas chromatograph (model GC-8A, Shimadzu, Japan) equipped with a stainless steel column (3 m × 1/8 in. I.D.; stationary phase: Carbosieve SII) and a thermal conductivity detector (TCD). Gases were sampled by using 1 l collection bag (CAT#232-01, SKC) per week before turning over the biopiles periodically. After the biopiles were turned over, a 2 m porous pile was inserted into the bottom of each biopile to collect soil biogas randomly. The analytical method is referred to the literature [15]. Soil temperature in the soil was measured simultaneously with microbial respiration.

2.6. Bioremediation process design

The concept of the proposed bioremediation process was based on the combination of bioaugmentation and biostimulation with operational strategy using a landfarming procedure by reseeding previous 4 m^3 biopile soil input biopiles in the beginning to enhance the increase in bacterial population. Our experiment was conducted in the biopile, which is 1.8–2.0 m high at the center, by using landfarming strategy with a plough machine per week. The biopile soil was periodically turned over with approximately volume of 0.5 m^3 by landfarming. The bacterial community was monitored by a microarray biochip during the operational period.

2.7. Statistical analysis

An analysis of variance (ANOVA) was performed to test the difference of initial and final TPH concentrations between the treated experiment biopiles and untreated control biopiles.

3. Results and discussion

3.1. Characterization of contaminated soil

The characterization of soil is presented in Table 1. Two series of biopiles (S0, S1, S2, S3 and T0, T1, T2, T3) had different TPH levels, in which the THP concentrations in the S-series biopiles were half of that in the T-series biopiles. $TPH_{C10–C28}$ and $TPH_{C10–C40}$ are regulated under the Taiwan EPA guideline. According to the carbon number of hydrocarbons, the components of $TPH_{C10–C40}$ basically can be divided into low molecular weight as diesel oil ($TPH_{C10–C28}$) and high molecular weight as heavy oil ($TPH_{C28–C40}$), the data of $TPH_{C28–C40}$ can be approximately estimated by subtracting the concentration. The concentrations of diesel oil were similar in both the S- and T-series biopiles. Therefore, there were more fractions of high-molecular-weight heavy oil in the T-series biopiles. The microbial populations in both series of biopiles were about 10^5 CFU/g dry soil and the microbial diversities were similar. Among them, *Pseudomonas putida* only appeared in the T-series biopiles. Many of these are well known to be efficient fuel oil or diesel-degraders [4,6,16,17].

Table 1
Characterization of contaminated soils.

Parameters	S-series soil	T-series soil
$TPH_{C10-C28}$ (mg/kg)	1020–2200	1800–2790
$TPH_{C10-C40}$ (mg/kg)	2200–4260	5850–7580
Soil texture	Sandy	Loamy sandy
Total N (%)	0.030 ± 0.002	0.052 ± 0.005
Total P (%)	0.046 ± 0.004	0.049 ± 0.007
Total organic matter (%)	1.9 ± 0.5	2.1 ± 0.4
Total organic carbon (%)	1.1 ± 0.3	1.3 ± 0.2
EC (dS/m)	0.54 ± 0.04	1.4 ± 0.3
pH	7.0 ± 0.3	7.2 ± 0.2
Total plate count (CFU/g dry soil)	$(2.2–6.3) \times 10^5$	$(3.3–6.7) \times 10^5$
Bacterial diversity	*Acinetobactor junii*, *Gordonia alkanivorans*, *Rhodococcu erythropolis*, *Acinetobacter* sp., *Gordonia desulfuricans*, *Pseudomonas* sp., *Pseudomonas aeruginosa*, *Ralstonia picketti*	*Acinetobactor junii*, *Gordonia alkanivorans*, *Rhodococcu erythropolis*, *Acinetobacter* sp., *Gordonia desulfuricans*, *Pseudomonas* sp., *Pseudomonas aeruginosa*, *Pseudomonas pudita*, *Ralstonia picketti*

3.2. TPH biodegradation

There are some differences between S- and T-series biopiles in the biodegradation curves of TPH including $TPH_{C10-C28}$ and $TPH_{C10-C40}$ as shown in Figs. 1 and 2. There seems to be two different biodegradation mechanisms that might involve the concentration and components of TPH [6,18]. In the S-series biopiles, two distinct phases are present in the bioremediation process, whereas a directly decreasing trend is present in the T-series biopiles. For both diesel and fuel oil, the biodegradation curves of TPH in the S-series biopiles rapidly decrease before 60 days in the first phase of bioremediation followed by a slow decrease phase, which remained stable from then on up to 240 days in the second phase of bioremediation. As shown in Fig. 1, the first phase occurred between days 0 and 60, and after day 60 the second phase was seen. There were two different degradation efficiencies in both the S1 and S2 biopiles. The degradation efficiency in the first phase was higher and the degradation curve in second phase became flat after day 60. This is due to low-molecular-weight diesel oil being easily biodegraded in the first phase, whereas high-molecular-weight heavy oil was difficult to biodegrade in the second phase. The percentages of diesel in the S-series biopiles were higher than those in the T-series biopiles, leading to fast biodegradation in the first phase of bioremediation. Due to an initially low TPH concentration in the S3 biopile, the biodegradation curve of TPH showed a directly decreasing trend similar to those of the T-series biopiles, in which the percentages of diesel were relatively low. Therefore, it is reasonable to assume that the biodegradation time and degree were effected by the fraction of TPH components and concentration [6]. Two phases of biodegradation efficiencies occurred in S-series biopiles which contain high fraction of diesel, whereas only single phase occurred

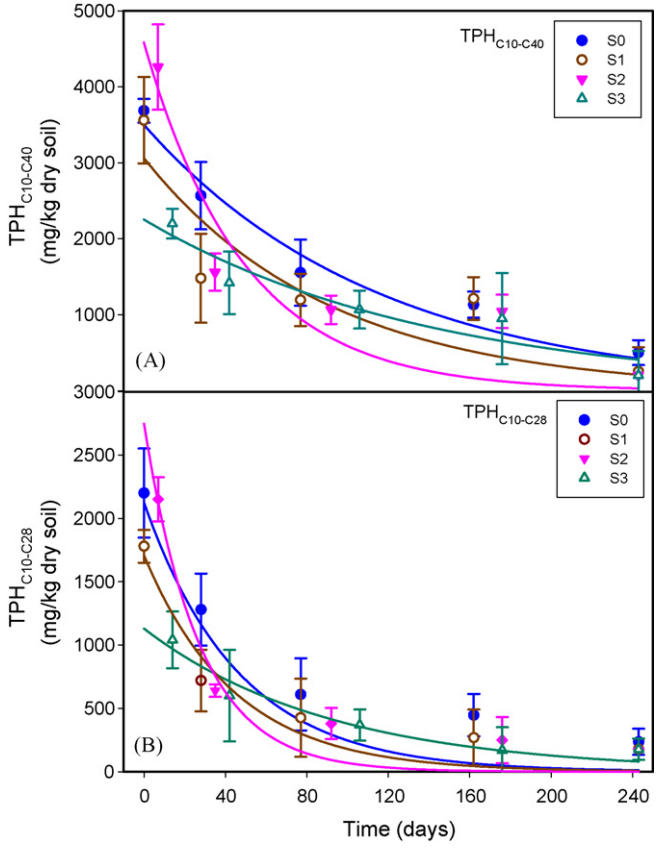

Fig. 1. The biodegradation curves of (A) $TPH_{C10-C40}$ and (B) $TPH_{C10-C28}$ in the S-series biopiles.

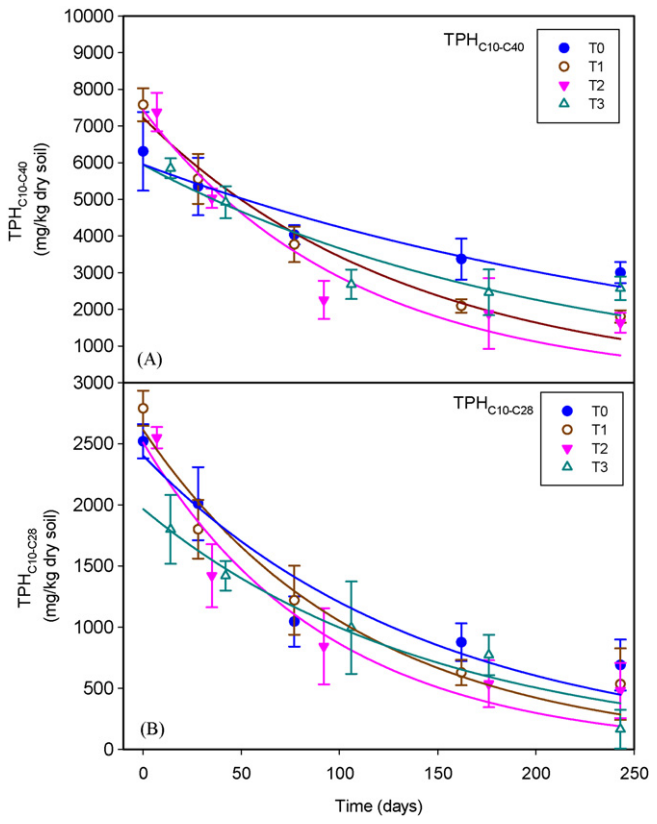

Fig. 2. The biodegradation curves of (A) $TPH_{C10-C40}$ and (B) $TPH_{C10-C28}$ in the T-series biopiles.

Table 2
Profiles of TPH removal in the S- and T-series biopiles on day 28 in the first phase of bioremediation.

Biopile	Day 0	Day 28	Removal	Removal	Removal rate
	mg/kg dry soil			%	mg/kg dry soil-day
$TPH_{C10-C40}$					
S0	3690	2570	1120	30	40
S1	3560	1480	2080	58	74
S2	4260	1560	2700	63	96
S3	2200	1420	780	35	28
T0	6310	5350	960	15	34
T1	7580	5560	2020	27	72
T2	7380	5030	2350	32	84
T3	5850	4920	930	16	33
$TPH_{C10-C28}$					
S0	2200	1280	920	42	33
S1	1780	720	1060	60	38
S2	2150	640	1510	70	54
S3	1020	600	420	41	15
T0	2520	2010	510	20	18
T1	2790	1800	990	35	35
T2	2550	1420	1130	44	40
T3	1800	1420	380	21	14

in the T-series biopile with low fraction of diesel. The TPH biodegradation of two phases in our experiments coincides with the level of the TPH concentration, as reported by Thomassin-Lacroix [6].

As shown in Table 2, during the first 28 days when about 60% of the total amount of $TPH_{C10-C40}$ was removed, the $TPH_{C10-C40}$ removal rates in the S1 and S2 biopiles were approximately 74 and 96 mg TPH/kg of dry soil per day, respectively. In contrast, the $TPH_{C10-C40}$ removal rate of the S0 control biopile was approximately 40 mg TPH/kg of dry soil per day during the first 28 days when approximately 30% of the total amount of TPH was removed. The results show that bioaugmentation and biostimulation with reseeding strategy performed well in the first month. The total amounts of $TPH_{C10-C40}$ removed (%) in the S-series biopiles were about twice compared with those in the T-series biopiles, while the TPH removal rates were similar. This shows that landfarming technology performed more efficiently in the S-series biopiles with a high fraction of diesel, than in the T-series biopile with a low fraction of diesel. The $TPH_{C10-C40}$ removal rate of S3 biopiles was approximately 28 mg TPH/kg of dry soil per day during the first 28 days, giving a 35% removal of TPH. Although the $TPH_{C10-C40}$ removal rate in biopile S3 was less than that in the biopile S0, the $TPH_{C10-C40}$ removals (%) of biopile S3 was higher than that in the biopile S0 during the 28 days. This might be due to the low biodegradation and high fraction of high-molecular-weight heavy oil in biopile S3 resisting to microbial attack. Consequently, the biodegradation curve of TPH shows a slowly decreasing trend as shown in Fig. 1 [19]. Due to both limited factors of the initial concentration and high fraction of high-molecular-weight heavy oil, the degradation efficiency of biopile S3 was found to be a little better than that of biopile S0. When the $TPH_{C10-C40}$ peak of S3 biopile at initial (no. S30727) and the 62th (no. S30927) day of bioremediation were compared, we observed that the diesel was biodegraded while the heavy oil was not. As shown in Fig. 3(B), the $TPH_{C28-C40}$ was hardly degradable and the biodegrading curve in the second phase of S-series became flat after 62 days (Fig. 1). A possible explanation for the phenomenon is the inability of inoculation to degrade the particular hydrocarbons present in the contaminated soil such as an unresolved complex mixture (UCM) [20]. Another reason is the inability of inoculation to attack the pollutant adsorption on the soil, because hydrocarbons bind strongly to humic substances and to clay minerals [21,22]. Therefore, the degradation efficiencies of biopiles S1 and S2 were better than that in the S3 biopile or in control biopile (S0). Hence, landfarming technology using the strategy with reseeding process can shorten treatment time and improve the bioremediation efficiency.

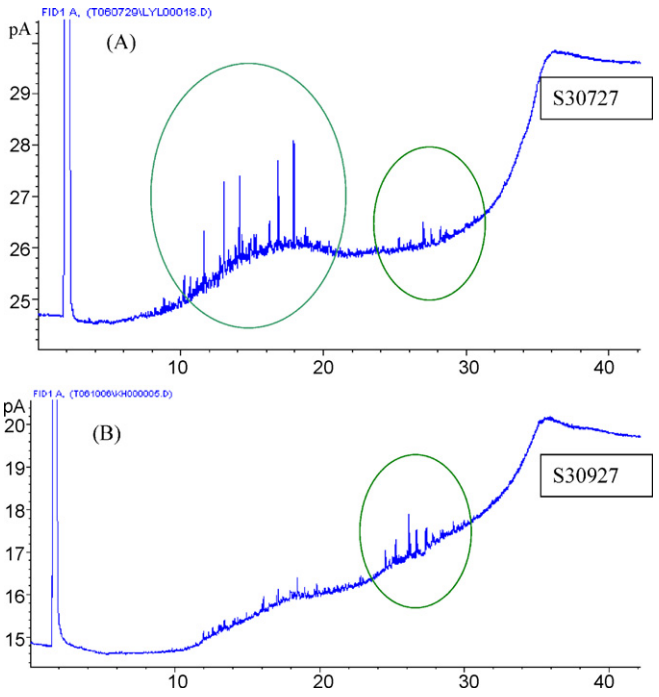

Fig. 3. Comparison of the TPH chromatogram showing $TPH_{C10-C40}$ portions in S3 biopile at (A) 0th day (no. S30727) and (B) 62th day (no. S30927). The $TPH_{C10-C28}$ peaks refer to low-molecular-weight hydrocarbons and the $TPH_{C28-C40}$ peaks refer to high-molecular-weight hydrocarbons.

ANOVA refers to an analysis of variance, which is frequently used in statistics. The removal rates were higher in the treated biopiles (S1 and S2) than the control S0 in the first phase of bioremediation at 5% level of significance. There were significant differences in the final $TPH_{C10-C40}$ concentrations between treated experiment biopiles and untreated control biopiles in both series of biopiles (ANOVA with $\alpha = 0.05$). There was a significant effect of bioaugmentation and biostimulation in the first phase of bioremediation. Although final $TPH_{C10-C40}$ concentrations of all treated S-series biopiles were in the range of 200–600 mg/kg of dry soil at the end of the treatment period (240 days), the level of $TPH_{C10-C40}$ in the treated S-series biopiles reduced below the legal TPH concentration (1000 mg/kg dry soil) regulated by the Taiwan government after 100 days. More than 150 days were needed for the untreated control of the S-series biopiles to reduce to less than 1000 mg/kg dry soil. This shows that SEMBT can shorten treatment time by half (Fig. 1). ANOVA was also applied to test variability among the all biopiles for $TPH_{C10-C40}$ biodegradation. Results of this statistical analysis indicated that there were significant differences in the final $TPH_{C10-C40}$ concentrations between treated experiment biopiles and untreated control biopiles in both series of biopiles ($\alpha = 0.05$) [6]. Hence, the achieved end point TPH in S-series biopiles of this experiment was within limitations of Taiwan EPA regulation.

3.3. Microbial investigations on bioremediation biopile

3.3.1. Enumeration of microbial population

As shown in Table 1, the populations were in the range of $2.2–6.7 \times 10^5$ CFU/g soil at the beginning of bioremediation in both the series of bipoles. The supplement of bioaugmentation and biostimulation at the first phase of bioremediation resulted in a higher count ($10^6–10^7$ CFU/g soil) in the experiment group, compared to that within the control group (10^5 CFU/g soil), as shown in Fig. 4.

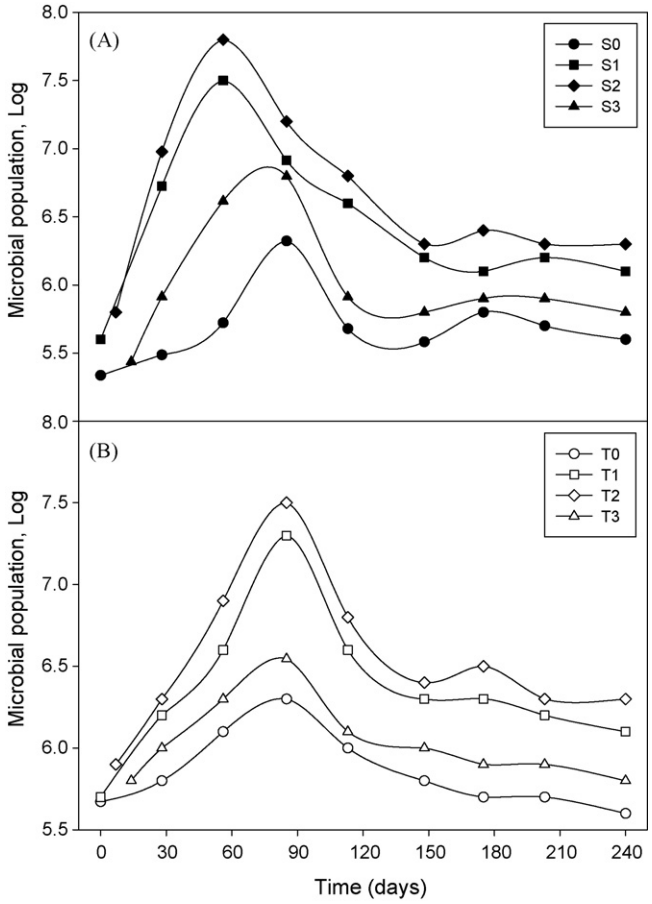

Fig. 4. Number profiles of microbial population in (A) S-series and (B) T-series soil during bioremediation.

In both series of biopiles, the initial population counts were about 2.2×10^5 CFU/g dry soil then increased to 6.3×10^7 CFU/g dry soil when supplemented with bioaugmentation and biostimulation at the first phase of bioremediation. The bacterial number in the S2 biopile was higher than that in the S1 biopile due to the reseeding approach at the beginning; the similar results were also obtained in T1 and T2 biopiles. The growth profiles of the microbial population reflected the TPH biodegradation compared to the control biopiles, as shown especially in S1, S2, T1, and T2 biopiles. Microbial population seems to be lower and constant in the S-series biopiles after 150 days of bioremediation, which is owing to the limited available carbon source in the soil. The fluctuation of microbial population was small after 150 days of bioremediation. Similar changes in microbial population were found in T-series biopile. The T0 biopile (control) showed the lowest bacterial number when compared with other biopiles, which corresponded to the TPH biodegradation efficiency.

Thus, an immediate increase in the population density of indigenous microbes could ensure rapid degradation of the pollutants [23]. Hence, the best bioaugmentation performance can be achieved by using pre-selected bacteria that increase in abundance. With the increase of a specific microbial community and biosurfactant addition, this approach could improve TPH biodegradation and reduce the cleanup time substantially. In the statistical analysis, the bacterial numbers in biopile S1 and S2 were one order of magnitude ($P < 0.05$) higher than that in biopile S0 after supplementation with bioaugmentation and biostimulation at the beginning. The microbial population of biopile S2 increased half an order of magnitude ($P < 0.05$) higher than that of biopile S1 mainly due to the reseeding of 4 m^3 soil from biopile S1 in the beginning. This phenomenon also occurred in the T-series biopiles T1 and T2. Therefore, the strategy with reseeding process performed well due to increasing microbial population. Hence, bioaugmentation and biostimulation increased the microbial population in the beginning, which resulted in rapid TPH biodegradation in the first phase of bioremediation [23,24].

With bioaugmentation and biostimulation, the population count was above 5.0×10^6 CFU/g dry soil in the first phase of bioremediation. In the second phase, however, it decreased to below 5.0×10^6 CFU/g dry soil due to the easily biodegradable diesel consumed and left the difficult biodegradable heavy oil. Microbial inoculation was deemed necessary since suitable HC-degrading bacteria were not found in sufficient numbers in the on-site samples prior to landfarming [6]. The TPH biodegradation was slower in the T-series because the microbial population could not utilize the lower quantity of diesel (32–37% of TPH) as a potential nutrient source; the S-series microbial population successfully utilized the more abundant diesel (46–60% of TPH) as a potential nutrient source. In the second phase, the TPH biodegradations were slower in all S-series biopiles. There might be a certain threshold for microbial populations to utilize $TPH_{C28-C40}$ [25]. For example, isoprenoids pristane, phytane, and cyclo-alkanes like resin composed of UCM ware partially or completely resistant to microbial attack [19,26].

3.3.2. Microbial community analysis with microarray identification

The microbial community was monitored by a microarray biochip and revealed the abundance of microbial diversity in the primitive soil in both series of biopiles. As shown in Table 3, five indigenous bacteria (i.e. *Acinetobactor* sp., *G. desulfuricans*, *Pseudomonas* sp., *P. aeruginosa*, and *R. Picketti*) and four augmented ones (i.e. *A. junii*, *G. alkanivorans*, and *R. erythropolis*) were initially detected in both series of biopiles. Microbial diversity was high during the first phase of bioremediation and microbial growth was prosperous due to bioaugmentation and biostimulation with the reseeding strategy. Therefore, TPH was rapidly removed by bacteria in the first phase of bioremediation. During the first 4 months of bioremediation, the five indigenous bacteria and four augmented bacteria monitored by the microarray biochip were still detected to a larger extent in the S-series biopiles, lasting to the ending of bioremediation, with the exception of S0 and S3 biopiles, in which *E. aurantiacum* and *G. desulfuricans* disappeared at the end. The strain *E. aurantiacum* being first screened from oil-contaminated soil, is here reported as a hydrocarbon assimilator capable of degrading heavy oil hydrocarbons, and disappeared in the S-series biopiles at last might be due to less fraction of heavy oil. Both bacteria with oil degrading activities disappeared at the final stage and this might affect the efficiency of bioremediation. As carbon is the key factor governing microbial growth in soil and produces functional diversity of soil microbes [27]. We found both bacteria with oil degrading activities disappeared at the final stage when the available carbon has depleted and this might affect the efficiency of bioremediation (Fig. 1). *Acinetobacter* sp. and *P. aeruginosa* were the predominant groups in indigenous consortia, while the augmented consortia were *G. alkanivorans* and *R. erythropolis* in the S-series of biopiles during bioremediation.

Six indigenous bacteria (i.e. *Acinetobacter* sp., *G. desulfaricans*, *Pseudomonas* sp., *P. aeruginosa*, *P. pudita* and *R. picketti*) and four augmented bacteria (i.e. *A. junii*, *G. alkanivorans*, *R. erythropolis* and *E. aurantiacum*) monitored by the mircroarray biochip were found in the T-series biopile on sites (Table 3). Most of them have been reported as hydrocarbon degraders [28]. There were some differences between the S- and T-series biopiles. For instance, *P. putida* as a PAH-degrading bacterium [29], was a distinct species found

Table 3
Microbial diversity detected by microarray in both series of biopiles during bioremediation.

Bacteria	Day 0				Day 120				Day 240			
	S0/T00	S1/T1	S2/T2	S3/T3	S0/T00	S1/T1	S2/T2	S3/T3	S0/T0	S1/T1	S2/T2	S3/T3
Augmented bacteria												
A. junii	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/−	+/+	+/+	+/+
E. aurantiacum	+/+	+/+	+/+	+/+	−/+	+/+	+/+	−/+	−/−	+/+	+/+	−/+
G. alkanivorans	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
R. erythropolis	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Indigenous bacteria												
Acinetobacter sp.	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
G. desulfuricans	+/+	+/+	+/+	+/+	−/−	+/+	+/+	−/+	−/−	+/+	+/+	−/+
Pseudomonas sp.	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	−/+	+/+	+/+	−/−
P. aeruginosa	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
R. picketti	+/+	+/+	+/+	+/+	−/+	+/+	+/+	+/+	−/−	+/+	+/+	+/−
P. pudita	−/+	−/+	−/+	−/+	−/+	−/+	−/+	−/+	−/−	−/+	−/+	−/+

Note: +, Detectable; −, non-detectable.

in the T-series biopiles. Hence, we expected the biodegradation curves of TPH to be different for the biopiles since the fractions of TPH component and the microbial communities were different. The predominant groups of indigenous and augmented consortia in the T-series biopiles were the same as those in the S-series biopiles during bioremediation [30]. Among them, bacteria from the genus *Acinetobacter* are one of the most active strains in the assimilation of saturates and aromatics [19]. Bacteria from the genus *Gordonia* with the dioxygenase gene have been reported to degrade polyaromatic hydrocarbon compounds [29]. Bacteria of the genus *Rhodococcus* have been reported to assimilate n-alkanes and more than 90% of the branched alkanes [19]. These bacterial strains represent hydrocarbon (HC)-degrading genera [19].

3.4. Microbial respiration

The microbial respiration as influenced by the microbial activity reflects directly the microbial population and indirectly the biodegradation of TPH [30]. The observed oxygen concentration first decreased with time as oxygen was consumed by microbial respiration, while the carbon dioxide concentration increased with time as carbon dioxide was produced by the microbial respiration in soil (Fig. 5). The biogas analysis shows only a small different trend in both S- and T-series of biopiles. On day 60, the S-series biopiles were relatively higher in CO_2 concentration and lower in O_2 concentration, and this phenomenon was similar to that on day 90 in the T-series biopiles. These results reflect directly the microbial population and indirectly the biodegradation of TPH (Figs. 1, 2 and 4). The biodegradation model consisting of two phases in S1 and S2 biopiles leads to the highest CO_2 production at day 60, and it was proposed that higher fractions of diesel might be present in the pollutants in these two biopiles. Another biodegradation model consisting of only one phase was seen in S0 and S3 biopiles, which leads to the delay of the highest CO_2 production at day 90. This phenomenon can also be found in the CO_2 production patterns in T-series biopiles, owing to the lower fractions of diesel in the pollutants in these biopiles.

The entrapped air was utilized for oxygen uptake and CO_2 release due to microbial respiration during the period of landfarming. Landfarming method provided aerobic conditions to microbial consortium for TPH degradation. The degradation of TPH involves the oxidation of hydrocarbon by oxygenase, for which oxygen is required [31]. Therefore, the degradation of TPH was directly related to the respiration of microbial populations in the soil [32]. Only one phase was observed in the T-series during bioremediation, which might be due to a mass transfer limitation of the oxygen diffusion [33]. The effect of oxygen limitation on the microbial

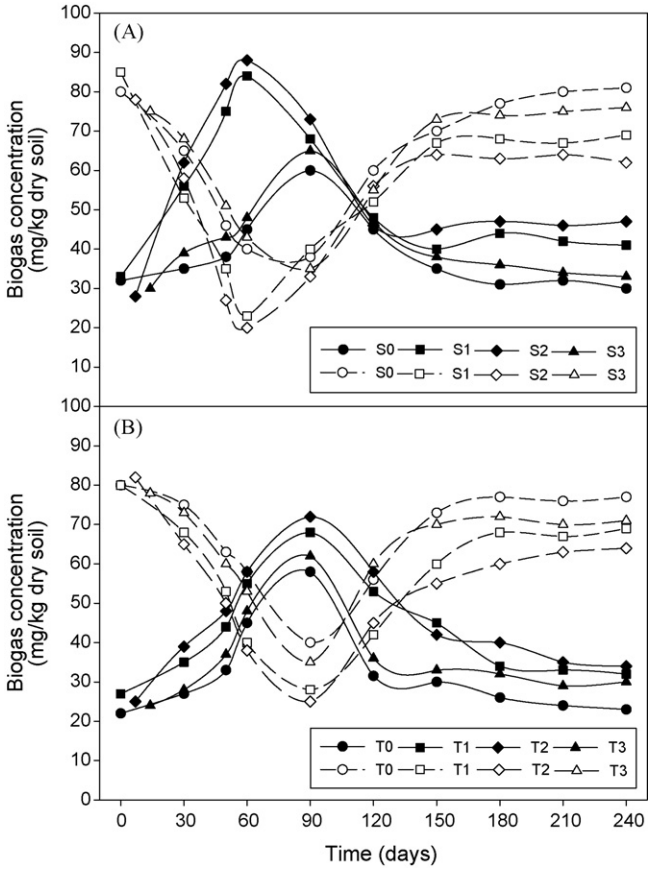

Fig. 5. Biogas profiles of microbial respiration in (A) S-series and (B) T-series soil during bioremediation. Symbol: (—), Carbon dioxide production; (- - -), oxygen consumption.

activity led to a slow biodegradation of TPH during the bioremediation. This indicates that the activities of bacteria were hindered in the T-series biopiles. At the middle stage of the landfarming process, when most of the easily biodegradable hydrocarbons present in the soil have been degraded by the microorganisms existing in the soil, the ratio of CO_2 concentration to O_2 concentration gradually decreased and then leveled off. This implies that the efficiency of the TPH minimization involves the component of TPH, and the microbial respiration reflects the bioremediation efficiency. Microorganisms prefer the more easily available component of TPH over the less easily degradable heavy oil [34]. Some differences

were observed between the S- and T-series biopiles during the initial stages of the TPH biodegradation. In the S-series biopiles, about 60 mg O_2/kg dry soil consumed and about 60 mg CO_2/kg dry soil produced were responsible for about 80% $TPH_{C10–C28}$ removal during the first 60 days of bioremediation. In the T-series biopiles, about 50 mg O_2/kg dry soil consumed and about 50 mg CO_2/kg dry soil produced accounted for about 60% $TPH_{C10–C28}$ removal during the first 90 days of bioremediation. Both the O_2 consumption and CO_2 production in the T-series biopiles were less than those in the S-series biopiles. This indicates that the biogas assay directly reflects the microbial activities in soil in accordance to the TPH degradation. Lee et al. [35] also monitored the O_2 utilization and CO_2 production pattern during biodegradation to measure the biodegradation rate of a diesel fuel in in situ bioremediation. It is essential to maintain an aerobic condition by optimizing the environmental condition for achieving improved results in biostimulation of TPHs in open field experiments [5].

Addition of the microbial consortia can increase the degrading microorganisms present in the biopiles, which will provide a short-term benefit. How to shorten the time for bioremediation is the main goal in such kinds of these experiments. By definition, bioaugmentation corresponds to an increase in the gene pool and genetic diversity of the site [36]. By using bioaugmentation strategy it was possible to reach better degradation when compared with natural attenuation or biostimulation during bioremediation process [23]. Although on some occasions it has been shown that only biostimulation works in the degradation of pollutants, the lack of microorganisms in the late phase (second phase) of bioremediation owing to unavailable or hard degradable hydrocarbons may lead long time to remediate the soil. Therefore bioaugmentation was needed when degrading microorganisms were in low number or diversity, or inadequate microbial populations were present in the oil-contaminated soils.

4. Conclusions

This study presented an innovative bioremediation method, the Systematic Environmental Molecular Bioremediation Technology (SEMBT), for biopiles by combining bioaugmentation and biostimulation with the reseeding strategy. The diesel-contamination was efficiently removed to about 70% by bioremediation of biopiles over a period of 28 days. The degradation and removal rates of TPH in the S-series biopiles were 10% higher than those in the T-series biopiles. During the initial stages of bioremediation, applied bioaugmentation and biostimulation increased the $TPH_{C10–C40}$ degradation removal by 16% on the average over the control. Monitoring the microbial population quantitatively and integrating microarray identification qualitatively during the bioremediation process proved to be beneficial. Biogases assay indicates that biodegradation of TPH is directly related to the microbial respiration. The microbial population size of 10^6 CFU/g soil with abundance of 8 different genera improved the TPH degradation in the experimental and control groups. Such microbial consortia with high and constant biodegradation ability can be used for industrial applications of bioremediation. Hence, the SEMBT shows potential applications in ex situ bioremediation.

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