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Enriched Microbial Community in Bioaugmentation of Petroleum-Contaminated Soil in the Presence of Wheat Straw

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Abstract The bioaugmentation of petroleum-contaminated soil using *Enterobacter cloacae* was profiled from the evolution of microbial community, soil dehydrogenase activity, to the degradation of petroleum contaminants. The seeding and proliferation of inoculant and the consequential microbial community were monitored by denaturing gradient gel electrophoresis analysis of the amplification of V3 zone of 16S rDNA. Degradation process kinetics was characterized by the degradation ratio of nC17 to nC18. The dehydrogenase activity was also determined during the degradation process. An abrupt change in the microbial community after inoculation was illustrated as well as successive changes in response to degradation of the petroleum contaminants. Seeding with *E. cloacae* stimulated the growth of other degrading stains such as *Pseudomonas* sp. and *Rhodothermus* sp. The application of wheat straw as a representative lignin waste, at 5% (w/w), induced an increase in the total dehydrogenase activity from 0.50 to 0.79, an increase in the microbial content of 130% for bacteria and 84% for fungi, and an increase of the overall degradation ratio from 44% to 56% after 56 days of treatment. The above mentioned results have provided a microbial ecological insight being essential for the design and implementation of bioaugmentation processes.

Keywords Bioremediation · Bioaugmentation · Microbial ecology · Petroleum-contaminated soil · *Enterobacter cloacae* · Wheat straw

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Introduction

Soil contamination with petroleum represents a critical environmental issue due to the hazardous properties of the contaminant in terms of its toxicity, mutagenicity, and other harmful consequences. Recent years have witnessed the growing efforts in applying microbes in the degradation of petroleum. Compared with the established physical and chemical methods, bioremediation techniques hold the attractiveness in terms of high efficiency, being environmentally friendly, and cost effectiveness [1, 2]. According to the way of applying microbes, the current bioremediation techniques can be classified into the following categories: (1) biostimulation, (2) bioaugmentation, and (3) natural attenuation. Bioaugmentation is the one that applies exogenous microbes to enhance the degradation of hazards [3–9]. Liu and co-workers reported the application of a microbial consortium of *Enterobacter cloacae* and *Cunninghamella echinulata* in the remediation of petroleum- and salt-contaminated soil [10, 11]. They further confirmed in both laboratory and field studies that the application of wheat straw enhanced salt leaching and subsequent petroleum degradation by above mentioned consortium [12]. It is noted here that Callaham et al. [13] have attributed enhanced biological activity in response to the application of wheat straw to an increased soil porosity, which facilitates oxygen transfer. On the other hand, degraded wheat straw has been shown to serve as quality carbon and energy source for growth of microorganisms over an extended remediation period [14, 15]. Given these results, our understanding of the function of wheat straw, a plentiful agricultural waste, remains inadequate, particularly from the viewpoint of microbial ecology.

Recent advances in molecular microbial ecology enabled a genetic level display of bioaugmentation. Li et al. [16, 17] have shown the enrichment of an aerobic heterotrophic bacterial community in the process of the degradation of diesel. Wilfred et al. [18] profiled the bacterial community using denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR)-amplified 16S rDNA and observed a rapid change in the bacterial community of plots treated with oil and slow-release fertilizer. Marc et al. [19] monitored bacterial community dynamics during the remediation of creosote-contaminated soil and observed shifting dominant strains once nutrients were added. Coppotelli et al. [20] observed an immediate and significant change in the soil bacterial community in response to inoculation with *Sphingomonas paucimobilis* and the degradation of phenanthrene. Katsivela et al. [21] monitored changes in both the petroleum composition and the microbial community using terminal restriction fragment length polymorphism (T-RFLP) and PCR-amplified 16S rDNA. Denaro et al. [22] applied T-RFLP for on-site tracking of the microbial community responsible for petroleum degradation and demonstrated that there was the potential to use community variation to aid the screening of functional microorganisms.

The objective of the present study was to profile the evolution of microbial community in bioaugmentation of petroleum-contaminated soil using *E. cloacae* as an inoculant. Special efforts were directed to the examination of the effect of wheat straw on development of microbial community during the degradation of oil. Degradation process kinetics was investigated, in which the process efficiency was characterized by the degradation ratio of nC17 to nC18. The seeding and proliferation of inoculants and the variations in the microbial community were then monitored by DGGE analysis of the amplification of 16S rDNA. With these a microbial ecological insight into the bioaugmentation of petroleum with the aid of wheat straw, a plentiful agricultural waste, is given to assist the bioaugmentation practice.

Materials and Methods

Microbes and Culture

E. cloacae was isolated from an oil-polluted area in the Zhongyuan oil field, Henan Province, China, as reported elsewhere [12]. The organisms were then incubated at 37 °C in LB medium that contained (per liter) tryptone (10 g), yeast extract (5 g), and NaCl (10 g), and agar (16 g/L) was added as required.

Soil, Microcosm, and Inoculation Conditions

Soil samples were collected from an uncontaminated site in the Zhongyuan oil field, sieved (2 mm), and then stored in the dark at 4 °C until used. The physicochemical properties were determined at Beijing Academy of Agricultural and Forestry Sciences and were found to be as follows: particle size distribution; $\Phi<0.002$ mm: 17.88%, 0.002 mm$\leq\Phi<0.05$ mm: 61%, 0.05 mm$\leq\Phi<2.00$ mm, 21.12%; pH, 8.58; organic matter, 9,360 mg/kg; total nitrogen, 0.0595%; ammonium, 1.90 mg/kg; nitrate, 19.6 mg/kg; available phosphorus, 8.69 mg/kg; conductivity, 296 μS/cm. The crude oil concentration of the soil samples above was determined to ensure the lack of hydrocarbon pollutants contamination using microwave extraction.

Next, 1.2 kg of sieved soil were placed in a 30 cm×20 cm×10 cm container to form a 5-cm-depth microcosm and then contaminated with 3% (w/w) petroleum hydrocarbon, which was the average contaminated level of the crude oil polluted area in Zhongyuan oil field. To accomplish this, crude oil was delivered in a petroleum ether solution and mixed into the soil manually using a spatula. The samples were then used for the bioremediation experiments until the petroleum ether had completely volatilized.

Three remediation treatments were conducted in duplicate. The first treatment was an uninoculated control, for which all samples were incubated at 25±2 °C for 56 days in microcosm that were mixed every 2 days to enable aeration. Twenty-five degrees Celsius was chosen as a working temperature according to the average temperature of whole annual in the field pilot. During the incubation period, the moisture, defined in terms of water content of the soil (w/w), was maintained at 25±2% by adding distilled water. The second treatment consisted of bioaugmentation with *E. cloacae*. Specifically, an inoculum was cultured in nutrient broth at 37 °C for 24 h. The cells were then harvested by centrifugation, after which the pellet was washed and resuspended with 0.85% NaCl solution, and the absorbance at 600 nm was adjusted to 1 using sterilized physiological saline (amount to 10^8 CFU/mL). Thirty milliliters of microbial cell suspended solution above was added to this treatment. The third treatment consisted of bioaugmentation with *E. cloacae* and wheat straw (5%, w/w) under the same inoculation and incubation conditions as above. Before wheat straw was added, the pretreatment process of wheat straw was necessary for bioaugmentation effect. The wheat straw samples used in this experiment were smashed to 1 cm length, which was useful to biodegradation. The microcosms in all treatment groups were sampled at days 0, 2, 5, 9, 14, 20, 27, 35, 44, and 56 to subsequent analysis.

DNA Extraction

Soil samples of 5 g were mixed with 13.5 mL of DNA extraction buffer (100 mM, pH 8.0, Tris–HCl; 100 mM, pH 8.0, sodium EDTA; 100 mM, pH 8.0, sodium phosphate; 1.5 M

NaCl, 1% CTAB) and 150 μL of lysozyme (50 mg/mL), after which they were incubated at 37 °C for 30 min. Proteinase K (10 mg/mL) of 100 μL were added and the samples were then incubated at 37 °C for 30 min in a horizontal shaker. After adding 1.5 mL of 20% SDS, the samples were incubated at 65 °C for 2 h with gentle end-over-end inversions every 15 to 20 min. The supernatants were collected after centrifugation at 6,000×*g* for 10 min at room temperature and then transferred into 50-mL centrifuge tubes. Next, the soil pellets were extracted two or more times with 4.5 mL of extraction buffer and 0.5 mL of 20% SDS, after which they were vortexed for 10 s, incubated at 65 °C for 10 min, and centrifuged at 6,000×*g* for 10 min. The supernatants of the extractions were then collected and mixed with an equal volume of chloroform/isoamylol mixture (24:1, vol/vol). The aqueous phase was recovered by centrifugation and precipitated with 0.6 vol isopropanol at room temperature for 1 h. A pellet of crude nucleic acids was obtained by centrifugation at 16,000×*g* for 20 min at room temperature, washed with cold 70% ethanol, and resuspended in TE buffer to give a final volume of 500 μL [23].

PCR Amplification

Prior to PCR amplification, crude DNA samples were purified using a DNA purification Kit (TianGen). The V3 region of the 16S rDNA from the soil bacterial community was amplified by PCR with a primer set, 907r (5′-CCGTCAATTCCTTTRAGTTT-3′) and 341f-GC (5′-CCTACGGGAGGCAGCAG-3′), which had a 40-bp GC clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′) linked to the 5′ end of the primer to improve the separation of the DNA fragments. PCR was conducted in a mixture with a total volume of 50 μL that contained 2.5 μL of each primer (10 μM), 1 μL of deoxynucleoside triphosphates (10 mM each), 2.5 U of Taq DNA polymerase, 5 μL of the buffer supplied with the enzyme (Takara Bio. Inc., Shiga, Japan), 2 μL of BSA (10 mg/mL), and 0.5 μL of template DNA. Thermocycling was performed using a Mastercycler EP Gradient S Thermocycler (Eppendorf Inc., Germany) under the following conditions: initial denaturation at 95 °C for 5 min, followed by 20 cycles of 95 °C for 1 min, 65 °C for 1 min, 72 °C for 3 min with a touchdown of 1 °C per cycle, and then a final extension at 72 °C for 7 min [24].

Denaturing Gradient Gel Electrophoresis

The Bio-Rad D-Code Universal Detection Mutation System (Bio-Rad, Hercules, CA, USA) was used for DGGE using a setup similar to that used by Muyzer et al. [25]. Briefly, approximately 10 μL of amplified PCR products was loaded onto 1-mm-thick 6% (w/v) polyacrylamide (37.5:1 acrylamide to bisacrylamide) gels containing a 40% to 60% linear denaturing gradient. The denaturant was comprised of 7 M urea and 40% (v/v) deionized formamide. To prepare the system, a denaturing gradient gel was prepared and allowed to polymerize for 1.5 h. A top gel [2 mL 0% denaturant, 16 μL 0.1% ammonium persulfate and 3 μL *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) solution approximately 10 mm in height] containing a 20-well comb was applied on top of the denaturant gel to minimize denaturant gradient disturbance during comb insertion and allowed to polymerize for 1–2 min. The gels were then run in 1× TAE buffer at 60 °C and 120 V for 5 h. The resulting gel was stained with 1× TAE buffer containing SYBR Green I (diluted 1:10,000; Sigma, St. Louis, MO, USA) after which a digital image of the gel was obtained using the Gel Doc 2000 gel documentation system (Bio-Rad).

DGGE Band Excision and Sequencing

DGGE bands were excised under a blue light using a sterile scalpel, and the excised band was then briefly washed in 0.5 mL sterile Millipore H_2O. The gel sliver was then crushed with a pipette tip in 0.1 mL fresh sterile Millipore H_2O and incubated for 48 h at 4 °C to allow passive diffusion of the band DNA. Next, the released DNA was reamplified under the aforementioned conditions without the GC clamp [25]. Sequencing was subsequently performed using an Applied Biosystems 3100 capillary sequencer and the Fluorescent Dye Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems). The obtained sequences were then compared to similar sequences in the public domain databases using the BLAST and CLUSTALW algorithms. Phylogenetic analyses of the DGGE bands were performed using a CLUSTALW multiple sequence alignment program and the resultant trees were processed using the MEGA 3.1 software.

Chemical Analysis, Microbial Enumeration, and Biological Activity

The total petroleum hydrocarbon (TPH) in the soil samples was extracted using a Microwave Reaction System (CEM, USA). Briefly, a dried soil sample (2 g) was mixed with hexane/acetone (1:1, vol/vol) solution and then extracted for 30 min at 120 °C. Next, the TPH concentration in the soil samples was quantified using a gas chromatography–flame ionization detector. Specifically, a Shimadzu GC-2010 with a flame ionization detector was employed using nitrogen as the carrier gas. The injection port was maintained at a temperature of 300 °C and the detector temperature was maintained at 300 °C. The analysis protocol was set to 40 °C for 2 min, after which it was raised to 290 °C at a rate of 15 °C/min, where it was maintained for 5 min. An Ultra Alloy capillary column DX-30 (30 m×0.53 mm inside diameter) was used.

The microbial enumeration was conducted according to Ref. [26]. During a run, a 1-g (wet weight) soil sample suspended in 9 mL of sterilized physiological saline water (0.85% NaCl solution) was homogenized for 1 h on a rotary shaker (250 rpm). Next, 0.1 mL of seven-fold dilutions was spread onto LB plates for bacteria growth and Martin plates for fungi, which were subsequently incubated at 37 °C for 2 days (bacteria) and 28 °C for 5 days (fungi).

The dehydrogenase activity was determined according to the reduction of 2,3,5-triphenyl-2H-tetrazoliumtrichloride (TTC) to triphenyl formazan (TF). During each assay, 1 g of soil samples (wet weight) was mixed with 2 mL of Tris–HCl buffer (0.05 M, pH 8.5), 2 mL of glucose solution (0.1 M), and 2 mL of TTC (0.5%), and then incubated at 30 °C for 12 h in tubes that were sealed with a rubber plug. Sulfuric acid was used to suspend the reaction. The supernatants were then added to 5 mL of acetone and mixed well, after which the samples were centrifuged at 4,000 rpm for 5 min. Finally, the absorbance of the supernatants was measured at 486 nm.

Statistical Analysis

The mean and standard deviation of three replicates of the TPH degradation values, population sizes of microorganisms, and biological activity analyses were obtained. The mean values were compared by one-way analysis of variance at a level of $P \leq 0.05$. All statistical analyses were conducted using SPSS 12.0.

Results and Discussion

Degradation of Total Petroleum Hydrocarbon (TPH)

The concentration of TPH during treatment in all microcosms is shown in Fig. 1. After 56 days of treatment, all microcosms showed a significant reduction in TPH. Specifically, the degradation ratios were determined to be 25% for the control, 44% for the treatment group in which *E. cloacae* was used as an inoculant and 56% for the groups in which *E. cloacae* was used as an inoculant in the presence of 5% (w/w) wheat straw.

E. cloacae is capable of using crude oil as the sole source of carbon and energy and has thus attracted great interest for use in bioremediation projects [12, 27]. Here we analyzed the degradation products of samples treated with *E. cloacae* by gas chromatography. Pristane (nC_{17}) and phytane (nC_{18}) were used as the standards for calibration of the GC analysis. The nC_{17}/nC_{18} can serve as an index of the degradation of petroleum, in which a high nC_{17}/nC_{18} indicates a low biodegradation ratio [28].

Figure 2a shows the chromatographic analysis of crude oil while Fig. 2b–d shows degradation intermediates obtained at 2, 9, 14, and 20 days. It can be seen from Fig. 2b–d the reductions in both the magnitude and the popularity of the measurable hydrocarbons during the degradation experiments, particularly in the presence of wheat straw 5% (w/w). Improved degradation was obtained using *E. cloacae* in the presence of wheat straw, which gave the lowest nC_{17}/nC_{18} (0.72), while ratios of 1.01 and 0.89 were obtained for the control and the *E. cloacae* treatment, respectively.

Dehydrogenase Activity

Figure 3 shows the soil dehydrogenase activity determined according to the reduction of TTC to TF. As shown in Fig. 3, the dehydrogenase activity remained low in all three treatment groups for the first 5 days. The highest dehydrogenase activity was obtained in the presence of wheat straw, 0.79, followed by the I treatment, 0.50. The enzyme activity of the control treatment was maintained at low level, 0.36. These findings, in conjunction with the improved TPH degradation shown in Fig. 1, demonstrate that the application of wheat straw increases the total dehydrogenase activity and contributes to enhanced TPH degradation.

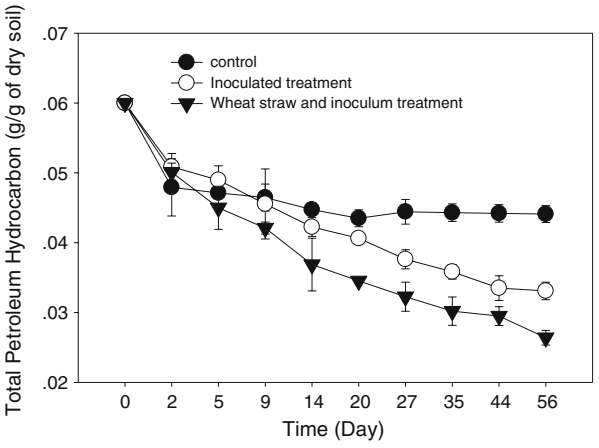

Fig. 1 Degradation of total petroleum hydrocarbon

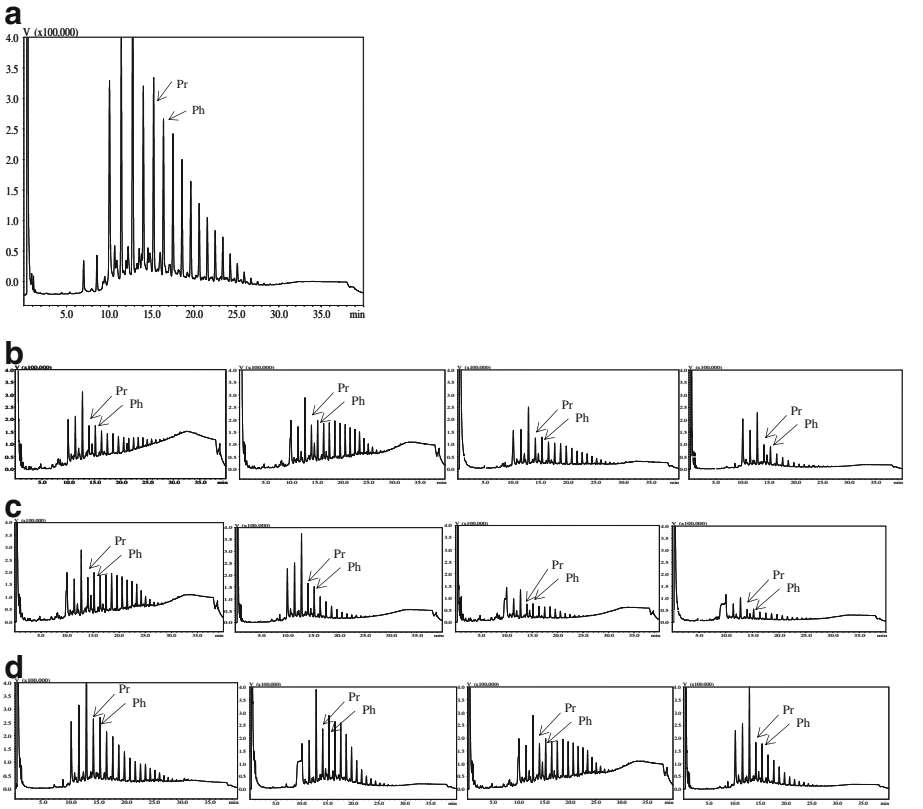

Fig. 2 GC analysis: **a** crude oil (control); **b** samples from the I treatment (2, 9, 14, and 20 days); **c** samples from the WI treatment (2, 9, 14, and 20 days); **d** samples from the control treatment (2, 9, 14, and 20 days)

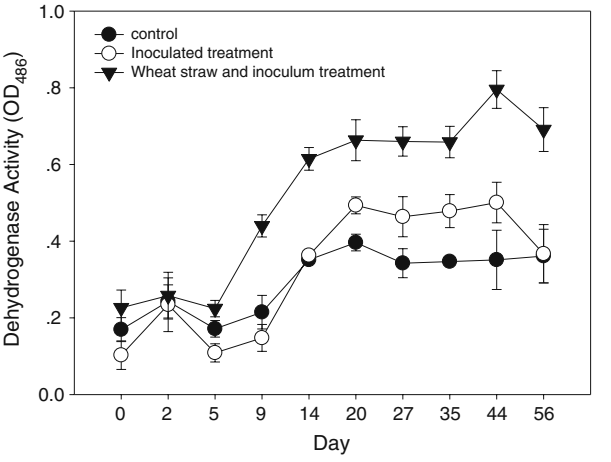

Fig. 3 Determination of the dehydrogenase activity of soil samples

Growth of Microorganisms

As shown in Figs. 4 and 5, which illustrates the microbial growth during the degradation process, the overall number of microbes was relatively low in the control group, which accounts for the low degradation observed in Fig. 1. For the *E. cloacae* treatment group, significant growth was observed during the first 9 days, while the total number of bacteria was approximately 2×10^7 CFU/g (dry soil). Specifically, the presence of wheat straw (5% w/w) enabled an increase in the total number of bacteria, being averaged at 3.5×10^7 CFU/g (dry soil) throughout the 56-day operation (Fig. 4).

As shown in Fig. 5, treatment with *E. cloacae* also induced an increase in the population of fungal species to 3×10^3 CFU after 56 days of cultivation. In control treatment, the fungal concentration of the control experiments remained at 1×10^2. The application of wheat straw led to a greater increase in fungal species to 5.5×10^3 CFU/g (dry soil) after 56 days.

It is interesting to correlate the degradation of petroleum (Fig. 1) and the total dehydrogenase activity (Fig. 3) to soil microbial growth (Figs. 4 and 5), in which a lag phase in the growth of bacteria and fungi corresponding to the initial low degradation rate and dehydrogenase activity was observed during the first 2–3 days following inoculation. The growth of the degrading microbes contributed to the following successive degradation of petroleum. The presence of wheat straw resulted in a higher concentration of degrading microbes (Figs. 4 and 5) and consequently a more rapid degradation of petroleum (Fig. 1). In control treatment, no increase in the microbial quantity (Figs. 4 and 5) was observed, which accounted for the lowest dehydrogenase activity (Fig. 3) as well as the minimum degradation of petroleum (Fig. 1). Overall, these results confirmed that the augmented degradation that occurred in response to treatment with *E. cloacae* was further enhanced using wheat straw.

Evolution of the Microbial Community

The quantity and the density of DGGE bands offered direct evidence of variations in the bacterial community in response to contamination [25]. Analysis of the DGGE profiles of

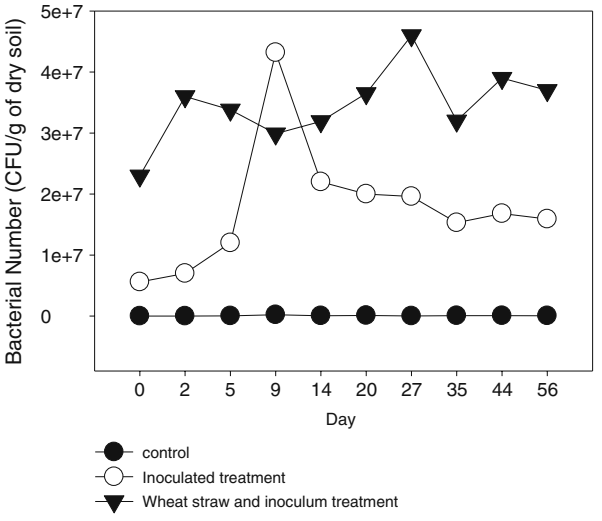

Fig. 4 The total number of bacteria in samples inoculated with *E. cloacae*

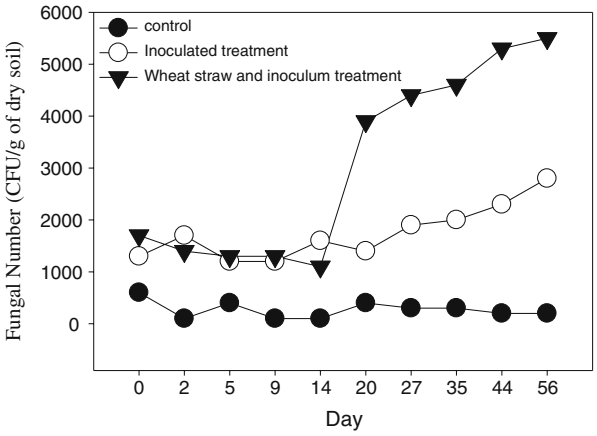

Fig. 5 The total number of fungi in samples treated with *E. cloacae*

the amplified 16S rDNA sequences was conducted using the methods described in "DGGE Band Excision and Sequencing" section. The results are shown in Fig. 6. Some bands on the gel were too weak to reamplify for subsequent analysis, especially in the control treatment. The dominant bands in the DGGE profiles of samples collected from the three

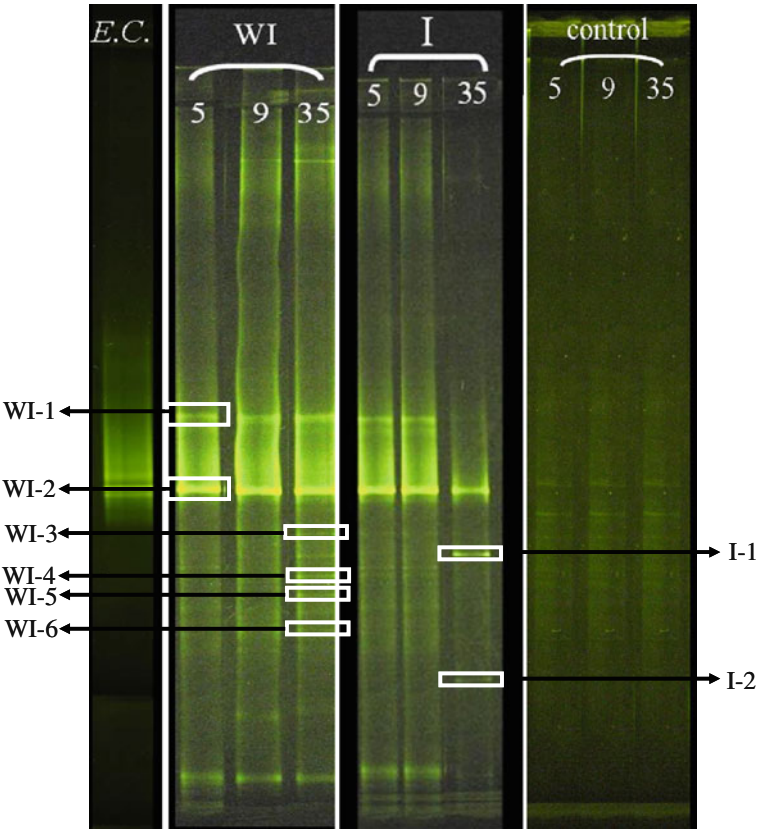

Fig. 6 PCR-DGGE analysis of bacterial populations of soil microcosms (WI, I, and CK)

treatments at 5, 9, to 35 days were excised, reamplified, and then the partial 16S rDNA sequences were determined. No detectable differences in the relative band intensity were observed within the control group, which indicated that the bacterial community in the control group was stable throughout the 56-day study period. For the bioaugmentation in the absence and presence of wheat straw, the profiles and phylogenetic analysis revealed the existence of bands that maintained the same density, which were much higher than that of the other bands in the soil sample. The identical positions of these bands, in comparison to their counterpart in the lane of *E. cloacae* sample, indicated the predominance of inoculated *E. cloacae* in the soil. On the other hand, more bands presented upon the inoculation, indicating the simulated growth of the native microbial upon inoculation of *E. cloacae*, i.e., bioaugmentation. Compared to those shown in the lane of CK and I, more dense bands, e.g., WI-3, WI-4, WI-5, and WI-6, appear in the lane of WI. This suggests the application of wheat straw gave more bands of 16S rDNA sequence. In other words, the application of wheat straw enriched the popularity of microbial community. Two bright bands were excised and sequenced (I-1 and I-2) in I treatment. These strains can utilize crude oil as carbon and energy matter like the introduced strain.

The distinctly different microbial community due to the application of wheat straw was determined by phylogenetic analysis, in which WI-2 was sequenced and identified being

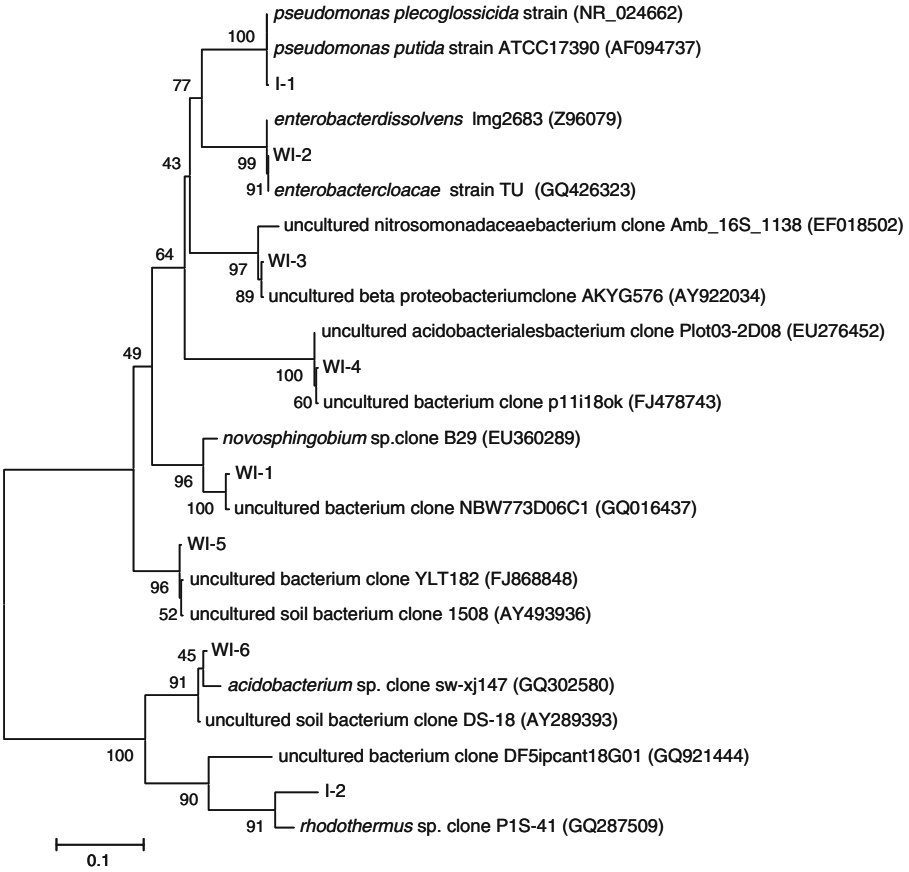

Fig. 7 Phylogenetic tree of sequences amplified from bands excised from DGGE gels

similar to the partial of 16S rDNA of *E. cloacae*. The result suggested that the inoculant existed in different soil microcosms. The other band was attributed to WI-1; the results of BLAST showed WI-1 belonged to *Novosphingobium* sp., which has a PAH-degraded gene [29, 30]. Compared to that obtained in the absence of wheat straw, four different bands appeared in the case of applying wheat straw. This indicates an enriched popularity and content of microbial community. Overall, the results of the study indicate that the application of wheat straw resulted in an enriched microbial population that underpinned the accelerated degradation of crude oil.

As shown in Fig. 7, phylogenetic analysis revealed that bands I-1 and I-2 belonged to *Pseudomonas* sp. and *Rhodothermus* sp., respectively, which are known to be involved in complicated pollution circumstances and are commonly used for the degradation of petroleum contaminants [31, 32]. The microbial diversity in the case of inoculation with *E. cloacae* was reduced during the process of bioremediation until it eventually reached a stable value. The strains that survived were those that utilize crude oil as a carbon source. These findings indicate that inoculation with *E. cloacae* could facilitate the selection of working strains for the degradation of petroleum contaminants.

Conclusions

The evolution of microbial community during the bioaugmentation of petroleum-contaminated soil using *E. cloacae* as an inoculant was monitored using DGGE and 16S rDNA analysis. Seeding with *E. cloacae* was found to stimulate the growth of other degrading stains such as *Pseudomonas* sp. and *Rhodothermus* sp. The application of wheat straw in conjunction with bioaugmentation enhanced the proliferation of *E. cloacae* and yielded a significantly enriched community. In all cases, *E. cloacae* was predominant in the microbial community during the early stages after inoculation, while the microbial community changed in response to the degradation of the petroleum contaminants. The growth kinetics of the microbial community correlated well with the total dehydrogenase activity and the petroleum degradation kinetics. These results have provided an insight into the microbial ecological processes that occur during bioaugmentation and indicated the potential of using microbial ecological tools to aid the design and implementation of bioaugmentation processes. The present study was just the first step toward the comprehensive elucidation of the wheat enhanced bioaugmentation from the viewpoint of microbial community. Determination and analysis of functional gene diversity including both those related to oil degradation and soil nutrient cycling should be included into the scope of following study to provide a microbial ecological insight into soil bioremediation.

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