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Microbial Community Changes of Crude Oil Polluted Soil During Combined Remediation

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The changes of microbial community structure are important indicators which indicate the effect of remediation of the oily soil. In this study, winter wheat and a high-efficiency degradation strain *Pseudomonas* sp. DG17 isolated from oil-contaminated soil were joined up to degrade petroleum hydrocarbon. The bacteria were in two forms: immobilized bacteria and bacteria inoculum. After 70 days, the combination of winter wheat and immobilized bacteria DG17 could degrade up to 18.09 % petroleum hydrocarbon, showing great potential in repairing oiled soil. The diversity of rhizosphere microorganisms which included the microorganism function diversity and the genetic diversity was analyzed by some emerging molecular biology methods. It was found that with the degradation of the petroleum hydrocarbon, the carbon source utilization types and dominant bacteria varied a lot in all treatments. During the late period of the experiment, functional bacteria which could degrade petroleum hydrocarbon better appeared.

Keywords: Microbial community structure, Combined remediation, Microorganism function diversity, Biolog analysis.

INTRODUCTION

Bioremediation is considered to be the most cost-effective and environmental friendly technology to treat oil-contaminated soil. Meanwhile, many studies have shown that the introduction of plants could enhance the effect of remediation[1,2]. The combination of microbe and plants would be practical and effective. Furthermore, change of microbial community structure is an important indicator to identify the effect of remediation. The information of microbial functional diversity is of great significance to identify microbial community in different samples. However, the traditional plate count method could just detect a part of microorganisms of the samples, making it difficult for microbiologists to quantitatively describe microbial community. Currently, analyzing rRNA(rDNA) of the microorganisms and phospholipid fatty acid PLFAin the samples are two universally accepted techniques to illustrate microbial community functional diversity[3]. But these two methods are labour-intensive, time-consuming and of high technicality, making them difficult to analyze more samples in a short period of time. The Biolog redox technology is evaluated as a rapid and community-level method to characterize and classify heterotrophic microbial communities at present.The biolog plates which include GN, GP and ECO microplates are originally designed to identify bacterial isolates. In addition to that role, however, the ECO microplates have been found useful in microbial community studies and have been widely used to characterize bacterial communities from various environments, including soil[4], freshwater[5], sediments[6], activated sludge[7] and seawater[8]. Biolog analysis based on carbon source utilization on community level rather than sole carbon source utilization provides a quicker and easier method to describe metabolic function of microbial community[5]. With the development of molecular biology, more molecular biological techniques are employed to get insight into modifications of bacterial populations in soils during the remediation processes. The application of amplified ribosomal DNA restriction analysis (ARDRA) could provide a way for examining the successionand convergence/divergence of microbial communities[9]. In this study, DNA of different soil samples are extracted and purified. Then soil bacterial 16S rRNA clone libraries areconstructed. This research aims to get insight into the changes of microbial community structure using ARDRA and analyze the effect of the introduction of plants and exogenous bacteria on community structure.

EXPERIMENTAL

Experimental treatments and the determination of total petroleum hydrocarbons concentration: The experimental soils used for this study were collected from an agriculture field in Beijing, China. And the bacterium *Pseudomonas* sp. DG17 (CGMCC: NO. 5052; NCBIaccession No. JN 216878) used in this study was isolated from petroleum contaminated soil (Da Gang oilfield, China). The bacterias were in two forms: immobilized bacteria and bacteria inoculum. *Pseudomonas* sp. DG17 was immobilized with 2.5-3.5 % sodium alginate, 0.5-1.5 % attapulgite clay and 2-4 % $CaCl_2$. Soils and bacteria were both preserved in the refrigerator at 4 ºC. Plant used here was winter wheat. Meanwhile, no plant and bacteria was regarded as the control group. All treatments were incubated in the greenhouse for 126 days.

Biolog analysis: 10 g air-dried soilwas added to a conical flask with 100 mL sterilized water. The flask was shaken at 250 r min^{-1} for 1 h and quite for 15 min. The soil suspension was diluted to ten folds. The supernatant liquid was removed to a sterilized centrifuge tube and the microplate was preheated at 25 ºC. Then automated liquid handlerwas used to pipette soil extractable solution and inoculated to microplates. The plates were cultured at 30 ºC and after 1, 2, 3, 4, 7, 10 days colour formation in microplate wells (absorbance at 590 nm) and analyzed by using the microplate reader.

Average well colour development (AWCD) method was used to analyzed Biolog data[5], soil microorganism community function diversity index which was also called Shannon index (H) and microorganism community richness (S). And AWCD was calculated as follows.

$$AWCD = \frac{\Sigma Ci - R}{n}$$

In this formula, Ci represented colour production within each well; R represented the absorbance value of the plate's control well and n represented the number of substrates (GN plates, n = 95; ECO plates, n = 31). Samples at the beginning of the experiment and after 70 days' degradation were analyzed by using Biolog technology and the value of AWCD at different incubation time (24, 48, 72, 96, 120, 168 and 240 h) was compared. The AWCD value of initial samples sharply increased at 24 h and got stable at around 168 h. As for 70 days' samples, the AWCD value also began to rise at 24 h but got stable at 240 h. So the value at 72 h of the initial samples while at 96 h of 70 days' samples was chosen to analyze.

$$H = -\Sigma(Pi \ln Pi)$$

$$Pi = \frac{Ci - R}{n}\Sigma(Ci - R)$$

$$\text{Evenness index (E)} = \frac{H}{n}\ln S$$

In this study, Shannon-Wiener diversity index (H), evenness index (E), carbon source utilization richness index (S) and AWCD were chosen to represent microbial population diversity and indicate the changes of microbial community composition. Data treatments were performed using Excel 2010 and SPSS 17.0 for windows.

16S rDNA identification and the constructing of 16S rRNAclone library: In this study, the DNA of strain DG17 was extracted using DNA extracted kit (Biomed, Beijing, China). The 16S rDNA fragments obtained with the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3')and1492R(5'-CGGTTACCTTGTTACGACTT-3') of the treatments selected were used to clone and construct 16S rDNA libraries. Details of cloning procedure could be found in the study of Valenzuela-Encinas *et al.*[10].

Amplified ribosomal DNA restriction analysis (ARDRA) and constructing phylogenetic tree: The sequencing results of every operational taxonomic unit (OTU) were submitted to GenBank database of NCBI using the Basic Local Alignment Search Tool (BLAST) algorithm to find their nearest neighbor in order to roughly determine their phylogenetic affiliation. Then phylogenetic trees were generated usingthe MEGA 4.1 software[11].

RESULTS AND DISCUSSION

Changes of total petroleum hydrocarbon (TPH) concentration in different soil samples with time: As shown in Fig. 1, winter wheat and immobilized bacteria *Pseudomonas* sp. DG17 had the best effect on petroleum biodegradation. After 70 days,the petroleum hydrocarbondecreased from 30520-25000 mg/kg in the soil which meant 18.09 % petroleum hydrocarbon was biodegraded. As for winter wheat and bacteria inoculum, 16.78 % petroleum hydrocarbon in the soil was biodegraded which also indicated that combined remediation had great potential in repairing crude oil polluted soil. While in the control group only 12.83 % petroleum hydrocarbon was biodegraded.

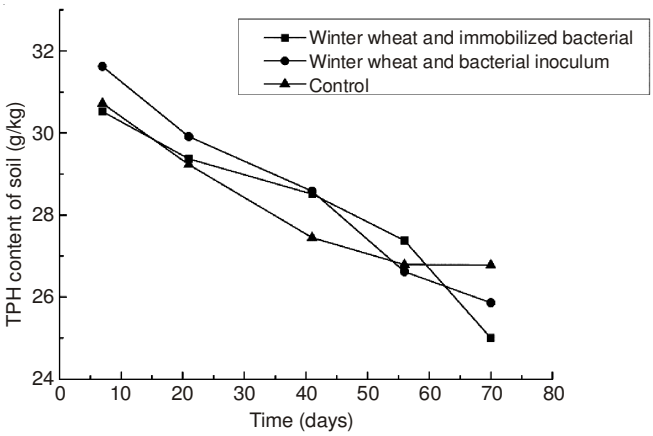


Fig. 1. Changes of TPH content of the soil during the remediation process

Microbial community changes during the combined remediation process: As shown in Table-1, four indicators in different treatments didn't present much difference. However, all indexes of all treatments in the initial samples were higher than that of the control group. Moreover, Shannon-Wiener diversity index, carbon source utilization richness as well as average well colour development in winter wheat and immobilized bacteria sample were the highest. As shown in Table-2, there were some changes in two treatments and the control group during the remediation process. Compared with the initial samples, the value of Shannon-Wiener diversity index

TABLE-1
DIVERSITY INDICES FOR INITIAL SOIL MICROBIAL COMMUNITIES AND AWCD OF 72 h

Sample	Shannon-Wiener diversity index (H)	Evenness index (E)	Carbon source utilization richness index (S)	AWCD of 72 h
Winter wheat and immobilized bacteria	3.21 ± 0.02	0.97 ± 0.01	27.50 ± 0.71	1.29 ± 0.48
Winter wheat and bacteria inoculum	3.19 ± 0.03	0.97 ± 0.00	27.00 ± 1.41	1.14 ± 0.23
The control	3.00 ± 0.07	0.96 ± 0.01	22.50 ± 0.71	0.75 ± 0.09

TABLE-2
DIVERSITY INDICES FORSOIL MICROBIAL COMMUNITIES AND AWCD OF 96 h AFTER 70 DAYS'DEGRADATION

Sample	Shannon-Wiener diversity index (H)	Evenness index (E)	Carbon source utilization richness index (S)	AWCD of 96 h
Winter wheat and immobilized bacteria	3.28 ± 0.02	0.98 ± 0.01	28.00 ± 0.00	1.44 ± 0.13
Winter wheat and bacteria inoculum	3.03 ± 0.05	1.00 ± 0.01	21.00 ± 1.41	0.75 ± 0.02
The control	3.08 ± 0.08	1.00 ± 0.00	22.00 ± 1.41	0.89 ± 0.10

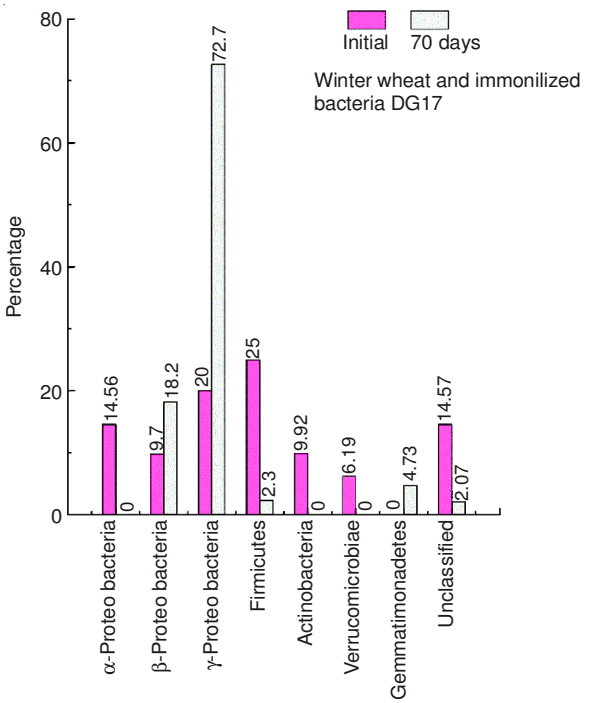
(H) in winter wheat and immobilized bacteria treatment increased from 3.21 to 3.28 after 70 days. While the value of H in winter wheat and bacteria inoculum treatment decreased from 3.19 to 3.03. Meanwhile, the carbon source utilization richness index (S) in this treatment also decreased from 27-21 during the combined remediation process.

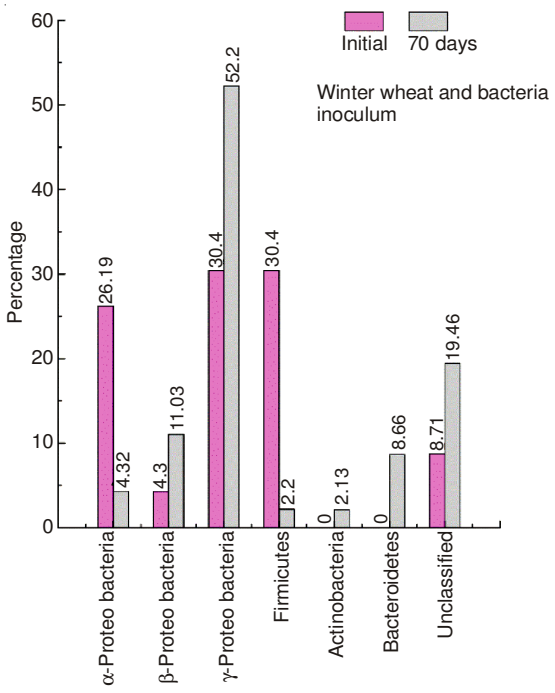
The reasons of the highest values in winter wheat and immobilized bacteria treatment both in initial samples and later samples might be that immobilized bacteria could resist adverse environment. Hence the microorganism in this treatment could take advantage of carbon source effectively and the diversity of microorganisms in soil was also more abundant with the degradation of petroleum hydrocarbon. As for the winter wheat and bacteria inoculum treatment, several microorganisms which didn't have the oil resistance or were unable to compete with the indigenous microorganisms couldn't survive from the oil. So the values of H and S decreased in this treatment.

Overall, it was apparent that the modifications of bacterial community structure were related with the petroleum hydrocarbon. And the existence form of the bacterial would also influence the community structure.

Change of microorganism genetic diversity during the combined remediation process: As shown in Fig. 2, the bacterial genus varied a lot during the remediation process. After 70 days, γ proteo bacteria dominated the community in two treatments as well as the control group. For instance, in winter wheat and immobilized bacteria treatment the ratio of γ-proteo bacteria to the whole microbial community increased from 20 to 72.7 %. In addition, after 70 days' degradation the amount of petroleum-degrading bacteria as well as the species in the clone library changed a lot while the number of microbial groups in wheat and immobilized bacteria treatment decreased and that increased in winter wheat and bacteria inoculum treatment. It might be because those bacteria which didn't have oil resistance couldn't survive or some microbes varied during the remediation process, which both meant the oil acted as a filter in the process.

And the presence of γ-proteo bacteria had been reported in studies which was carried out on beaches contaminated after the Nakhodka spillaccident in the Sea of Japan[12], on beach sediment microcosms contaminated with oil[13] and on microbial mats exposed to high nated with oil[13] and on microbial mats exposed to high pollution levels[14]. Moreover, different organisms





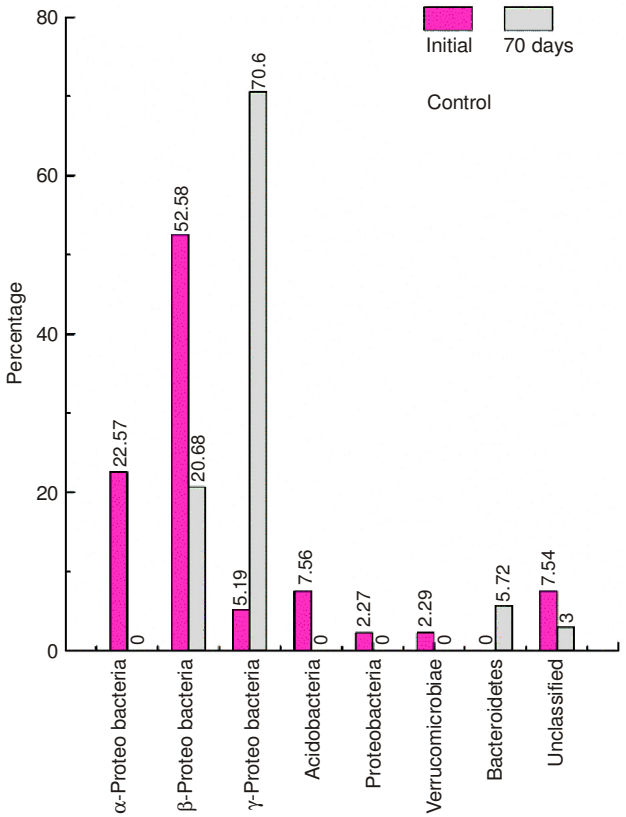


Fig. 2. Microbial community structure of different samplesrevealed by 16S rRNA cloning

such as Marinobacter, Halomonas and Alcanivorax, all belonging to the γproteo bacteria group were known as hydrocarbon-degrading bacteria and were frequently found in marine environments contaminated with oil[12]. It can be concluded that the dominance of γ aproteo bacteria was a characteristic of bacterial communities inhabiting environments contaminated with petroleum compounds and γproteo bacteria had the resistance of oil contamination on some level.

As shown in Figs. 3-5, several bacteria which could degrade the petroleum hydrocarbon were identified by analyzing nucleotide sequences. It can be seen that there were a certain proportion of petroleum-degrading bacteria such as uncultured *Xanthomonas*sp (AM934771) and *Staphylococcus haemolyticus* (HQ699551) which could effectively degrade petroleum hydrocarbons at the beginning of the experiment. Then after 70 days' degradation, there were pyrene-degrading bacteria and anthracene-degrading bacteria in the samples. For example, uncultured *Lysobacter* sp. clone T311E2 (HM438538) and uncultured *Lysobacter* sp. clone T302F04 (HM438526) were both anthracene-degrading bacteria and they could be found almost in every treatment. Similarly, *Alcanivorax* sp. (DQ659451) was found in all treatments and it could effectively degrade pyrene, a kind of polycyclic aromatic hydrocarbons. Meanwhile, after 70 days' degradation the amount of petroleum-degrading bacteria as well as the species in the clone library changed a lot. And the ratio of the petroleum-degrading bacteria to the total number of bacteria also increased. It was because the petroleum was screening the petroleum-degrading bacteria to some extent during the reaction. Those bacteria which could degrade the petroleum could survive better.

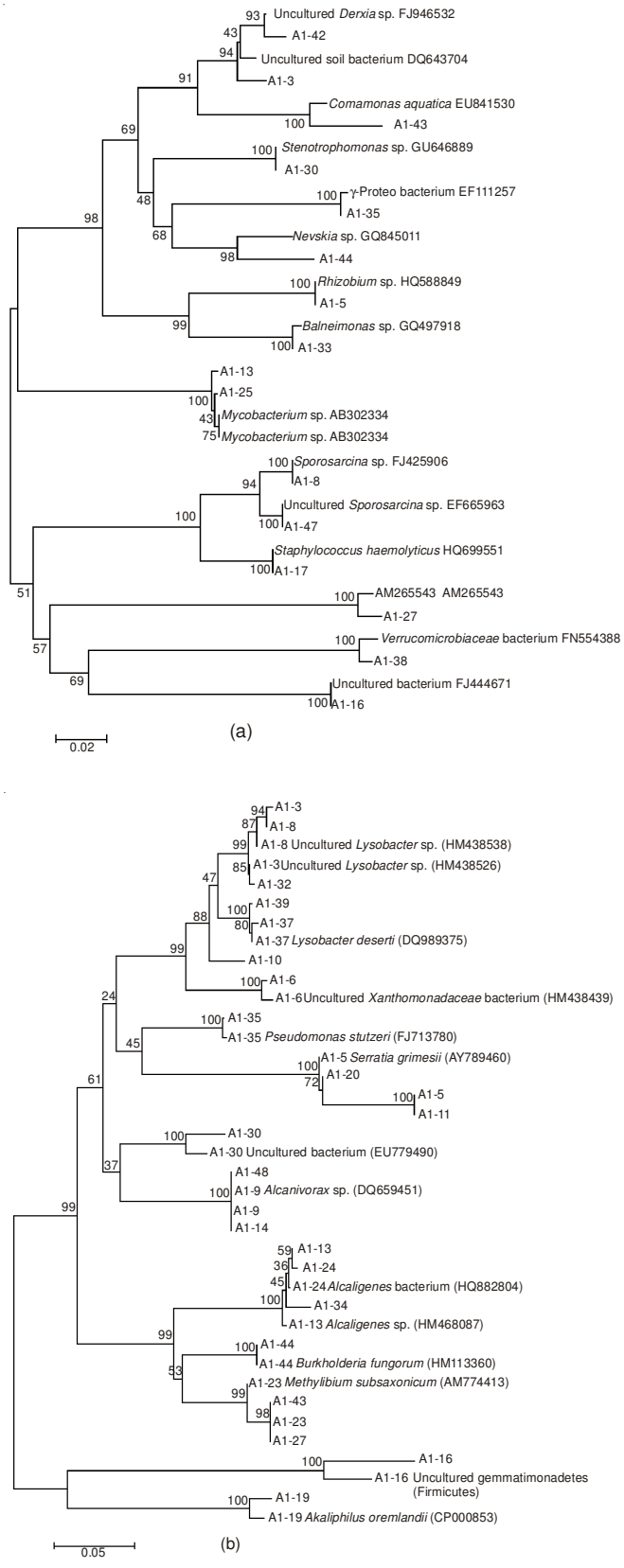


Fig. 3. Phylogenetic tree of the bacteria in wheat-immobilized bacteria system, (a) initial soil (b) soil after 70d degradation

Apart from the petroleum-degrading bacteria, there were also other functional microorganisms after 70 days. For instance, *Alcaligenes* sp. (HM468087) which was closely related to a strain isolated from atannery polluted by chromium was

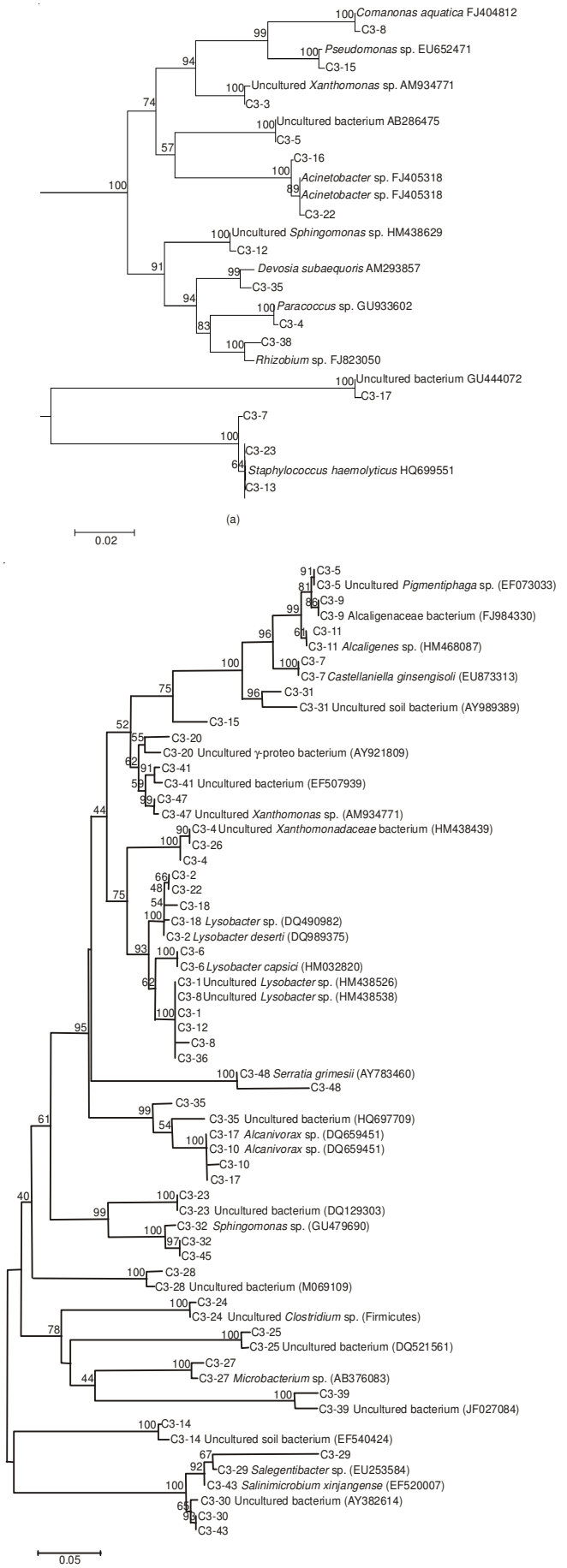


Fig. 4. Phylogenetic tree ofthe bacteria in wheat-bacteria liquid system, (a) initial soil (b) soil after 70d degradation

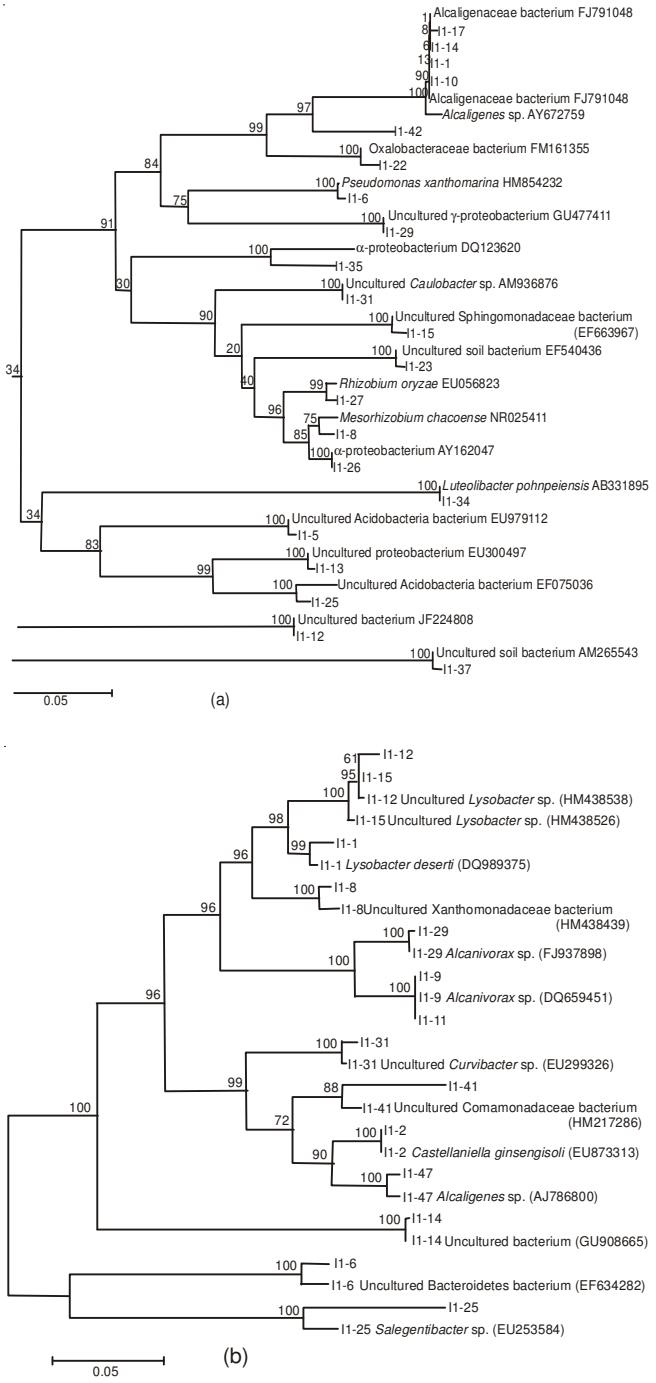


Fig. 5. Phylogenetic tree ofthe bacteria in control test soil, (a) initial soil (b) soil after 70d degradation

found in winter wheat and immobilized bacteria treatment. Obviously, it could resist a certain concentration of chromium. And in the conditions of winter wheat and bacteria inoculum, denitrifying-dephosphorized bacteria-comamonasaquatica strain DNPA9 (FJ404812) which was belonging to Betaproteo bacteria was found.

Conclusion

In winter wheat and immobilized bacteria treatment, the removal rate of total petroleum hydrocarbon and the value of Shannon-Wiener diversity index both was the highest, which indicated that the immobilized bacteria could resist the adverse environment better than the bacteria inoculum.Meanwhile,

bacteria species or strains in this treatment increased most. Moreover, the number and types of petroleum-degrading bacteria changed a lot after 70 days in all treatments. Anthracene degrading bacteria, pyrene degrading bacteria and other functional bacteria appeared after 70 days' degradation.

Based on the above research, it may be concluded that microorganism function diversity and gene diversity in all treatments changed a lot with the degradation of petroleum hydrocarbons. Further studies establishing links between different experimental conditions including soil types, moisture, soil nitrogen as well as phosphorus contents and community structure will provide more comprehensive insight into the microbial community structure during the combined remediation.

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