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A novel bioremediation strategy for petroleum hydrocarbon pollutants using salt tolerant *Corynebacterium variabile* HRJ4 and biochar

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ARTICLE INFO

Article history:
Received 20 August 2015
Revised 10 October 2015
Accepted 3 December 2015
Available online 4 March 2016

Keywords:
Bioremediation
Biochar
Corynebacterium variabile HRJ4
Immobilization
Petroleum hydrocarbons

ABSTRACT

The present work aimed to develop a novel strategy to bioremediate the petroleum hydrocarbon contaminants in the environment. Salt tolerant bacterium was isolated from Dagang oilfield, China and identified as *Corynebacterium variabile* HRJ4 based on 16S rRNA gene sequence analysis. The bacterium had a high salt tolerant capability and biochar was developed as carrier for the bacterium. The bacteria with biochar were most effective in degradation of *n*-alkanes (C16, C18, C19, C26, C28) and polycyclic aromatic hydrocarbons (NAP, PYR) mixture. The result demonstrated that immobilization of *C. variabile* HRJ4 with biochar showed higher degradation of total petroleum hydrocarbons (THPs) up to 78.9% after 7-day of incubation as compared to the free leaving bacteria. The approach of this study will be helpful in clean-up of petroleum-contamination in the environments through bioremediation process using eco-friendly and cost effective materials like biochar.

Introduction

Exploitation, production and transportation activities in oil industries had resulted in environmental contamination with substantial risk to the living flora and fauna. Conventional remediation techniques, including physical, chemical and thermal treatments in most of the cases are not economically feasible and often generate secondary contamination. Biological methods have been proved to be economical, versatile and efficient for the clean-up of petroleum pollutants with many advantages (Pandey et al., 2009). Likewise, the indigenous microflora present in soil or water can degrade a wide range of hydrocarbons, but their activities may be reduced when the concentration of toxic contaminants was high (Barathi and Vasudevan, 2003). Bioaugmentation and biostimulation can be used to overcome such obstacles, and natural attenuation remains a popular topic of study (Chauhan et al., 2008). Bioaugmentation is thought to be a feasible strategy for petroleum pollutants to be remediated on site (Jacques et al., 2008). In this process, the active microbial populations containing

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http://dx.doi.org/10.1016/j.jes.2015.12.023

specific enzymes for hydrocarbon degradation is important for the field application of the bioremediation technique.

Isolating the petroleum hydrocarbon degrading microorganisms and immobilizing them on a suitable carrier and then relocating them back to the contaminated site is an important strategy. For example, fungi targeting on petroleum hydrocarbon degradation were isolated and used for remediation of petroleum contaminated saline–alkaline soil (Qin et al., 2012). Recently, sodium alginate has been excessively applied for immobilizing of oil degrading microorganisms in bioremediation process (Sarma and Pakshirajan, 2010). Nevertheless, the dense gel layer structure in the sodium alginate beads hindered the exchange of gases, which hampered the aerobic microbial degradation process (Zhang et al., 2008; Yanez-Ocampo et al., 2009). Therefore, there was a need to find materials which can be as carriers in order to improve the microbial degradation efficiency. The carrier used in remediation should be ecofriendly, because it is not easy to recover the immobilized agents after they are input into the soil or water in *in-situ* bioremediation (Mohammadi and Nasernejad, 2009; Cunningham et al., 2004).

Many materials have been applied as carriers for enhancing microbial degradation. Chitosan was used as carriers for *Bacillus pumilus* and immobilized cells showed a better degradation of hexadecane in liquid medium (Costa et al., 2014). Furthermore, plant residues like wood chips and wheat straw were also commonly used as conventional carriers owing to their high affinity with microorganisms (Chen et al., 2011). However, the sorption properties of these materials could act on other organic matters in water or soil more than petroleum hydrocarbons (Chen et al., 2012). Therefore, exploration of a novel carrier having high rigidity and stability with less impact on environment was necessary. Biochar, produced by thermal decomposition of biomass under oxygen-limited conditions, is carbon-enriched and porous with high specific surface area and biodegradability (Tang et al., 2013). Biochar has higher mass transport of oxygen and nutrients and has been less focused as carrier material in immobilization of the microflora (Beesley et al., 2010).

The objective of this study was to immobilize the petroleum hydrocarbon degrading microorganisms for bioremediation of petroleum hydrocarbons in saline alkaline condition using biochar. A novel method combining bacterium and biochar was developed and applied in enhancing degradation of the alkanes and polycyclic aromatic hydrocarbons (PAHs).

1. Materials and methods

1.1. Chemicals and culture media

The *n*-hexadecane (*n*C16), *n*-octadecane (*n*C18), *n*-nonadecane (C19), *n*-hexacosane (C26), *n*-octacosane (C28), naphthalene (NAP) and pyrene (PYR) were purchased from J and K organic Co., Inc. (China). All the above mentioned hydrocarbons were of the highest purity and analytical grade available.

The Luria-Bertani (LB) medium contained (g/L): 10-tryptone, 5-yeast extract, 10-NaCl. The minimal medium (M9MM) consisted of the following components (g/L): 8.5-Na_2HPO_4, 3.0-KH_2PO_4, 0.5-NaCl, 1.0-NH_4Cl, 0.49-$MgSO_4$, 0.011-$CaCl_2$ and 1.0 mL of trace element solution (Karamalidis et al., 2010).

1.2. Isolation and identification of petroleum hydrocarbons (PHs) degrading bacteria

1.2.1. Isolation of bacteria

Soil sample (5 g) contaminated with oil was collected from Dagang Oilfield, China and suspended in 250 mL Erlenmeyer flask containing 100 mL M9MM. This medium was supplemented with 0.5% (W/V) crude oil and incubated in 30°C at 180 r/min for 7 days in dark. The enrichment cultivation was repeated with increasing crude oil concentration to 1% (W/V). After consecutive transfer, hydrocarbon degraders were isolated by plating on M9MM agar plates containing 1% crude oil as sole carbon sources. Colonies showing clear colony zones were selected as petroleum-degrading microorganisms, and were purified and stocked in LB medium containing 25% glycerol and stored at –80°C. Morphological characters of the bacteria were observed using microscope OLYMPUS CX31 (OLYMPUS Corporation, Tokyo, Japan) at 1000× resolution.

1.2.2. Molecular identification of the isolated bacteria

The 16S rDNA genes of the isolated strains were amplified using Taq DNA polymerase (TransGen Co., Ltd., Beijing, China) under the standard reaction conditions using 27F and 1492R primers (Zhang et al., 2011). The reaction mixture consisted of 5 μL 10× EasyTaq® Buffer, 4 μL dNTPs (2.5 mmol/L), 1 μL EasyTaq® DNA polymerase, 1 μL each of 27F and 1492R primers, 35 μL ddH_2O, and 3 μL DNA extracted from the culture. The PCR conditions: initial denaturation for 5 min at 94°C; denaturation for 1 min at 94°C, then annealing from 65°C to 55°C (decreased by 1°C in each cycle), extension for 1 min at 72°C (10 cycles); followed by denaturation for 1 min at 94°C, annealing for 1 min at 55°C, extension for 1 min at 72°C (25 cycles). Final extension was performed for 7 min at 72°C.

The products of amplification were sequenced from the BGI Life Tech Co., Ltd. (China). Similarity search was performed with the BLASTn program at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST.html). The 16S rDNA gene sequence was submitted to the GeneBank under the accession number KP140842. Phylogenetic tree was generated from alignments by the neighbor-joining method and the reliability of inferred tree was checked with bootstrap test using the MEGA4 program (www.megasoftware.net). The reference sequences from the GenBank were used to construct the phylogenetic tree.

1.2.3. Salinity and pH tolerant of the bacteria

In order to explore the pH and salinity tolerance, isolates were cultured at different concentrations of sodium chloride (g/L) of 5, 10, 20, 30, 50, 70, 90 in LB medium having pH (7 ± 0.2). Similarly, optimum pH required for growth was determined by varying media pH to 5, 6, 7, 8, 9, 10, 11 and the salinity was kept at a constant value (10 g/L). The growth of bacteria was documented in terms of the optical density at 600 nm at different time intervals.

1.3. Biochar preparation and cell immobilization

Different biochars were prepared by pyrolysing the wood chips (WC) at different temperatures of 250°C (BC250), 400°C (BC400) and 700°C (BC700) under the oxygen-limited

conditions (Lou et al., 2013). The Brunauer, Emmett and Teller (BET) surface area of biochars at different pyrolysing temperatures was determined by a N_2 absorption apparatus (ASAP 2460, China). Biochar having maximum BET surface area was selected and used as carrier in bacteria immobilization.

HRJ4 was cultivated to the logarithmic phase in LB medium, then centrifuged the bacterium suspension at the rotating speed of 5000 r/min. The supernatant was discarded and cells were suspended in sterilized normal saline water. This process was repeated for two or three times. The biochar/wood chips and HRJ4 cell suspension were mixed in 5:100 (W/V) ratios for 12 hr in a shaker at the speed of 150 r/min in order to absorb the bacterium sufficiently to biochar. The mixture was placed in refrigerator at 4°C for further use.

1.4. Scanning electron microscopy analysis on the immobilized bacteria

Scanning electron microscopy of (1) biochar (BC), (2) wood chip (WC), (3) bacteria-biochar (B-BC), (4) bacteria-wood chip (B-WC) was performed using a scanning electron microscope (Model XC-30 ETAX, Philips, USA). All the samples were dried at 35°C and coated with 15 nm gold layer followed by scanning electron microscopy analysis (SEM, S-3000N, Hitachi Ltd., Tokyo, Japan).

1.5. Biodegradation of n-alkanes and PAHs

The petroleum hydrocarbon degradation study was performed using an artificial mixture by mixing the petroleum hydrocarbons belonging to different groups. The mole fraction of each component was maintained less than its fugacity ratio at room temperature (Mukherji et al., 1997). The ratio of *n*-alkanes and PAHs representing mass fractions of aliphatic/aromatic components was formulated as typically found in the crude oil. The mixture had the following composition: *n*-C16 (0.1%), *n*-C18 (0.1%), *n*-C19 (0.1%), *n*-C26 (0.05%), *n*-C28 (0.05%), NAP (0.05%), and PYR (0.05%). The solid components were added to the liquid components and heated to 100°C for 30 min to obtain a homogenous liquid mixture (0.5%) (Mohanty and Mukherji, 2012). The experiment was designed to perform for the degradation of the above formulated mixture in different sets as follows: (1) bacteria (B), (2) bacteria-biochar (B-BC), (3) biochar (BC), (4) only crude oil (CK). All the above combinations were cultured separately in 25 mL of M9MM containing 0.5% petroleum hydrocarbons mixture and incubated in darkness at 150 r/min at 30°C for 7 days. All the experiments were performed in triplicate.

Residual petroleum hydrocarbons were extracted with DCM and then quantified by a GC system with a method introduced in our previous study (Liu et al., 2015). All samples were analyzed in triplicate, and results were expressed as mean value with standard deviation.

2. Results and discussion

2.1. Isolating and characterization of the petroleum hydrocarbon-degrading strains

The enrichment of microbes was conducted in M9MM using crude oil as a carbon source. Then the bacteria were isolated and compared in their degrading ability on the hydrocarbon mixture (Data not shown). Among 10 different strains, strain HRJ4 showed the highest degradation ability which was further used in this study. The microscopic study of HRJ4 cells showed rod shape gram negative nature with the cell length about 1.5 μm. The 16S rRNA gene sequence (1377 bp) of the HRJ4 was determined and compared with the previously recorded sequences. According to the BLAST search, the 16S rRNA sequence of HRJ4 showed 100% similarity to the 16S rRNA sequence of *Corynebacterium* sp. RS665. Phylogenetic analysis suggested that the isolate belongs to the genus *Corynebacterium* and was named as *Corynebacterium variabile* HRJ4 and submitted to GenBank with accession number KP140842. At the same time, *C. variabile* HRJ4 was deposited in China General Microbiological Culture Center (CGMCC) with a number of CGMCC No.10134. The phylogenetic tree of strain HRJ4 is shown in Fig. 1.

Petroleum hydrocarbon contaminants in the water or soil favor the acclimatization, adaptation and selection of native microorganisms carrying degrading genes involved in hydrocarbon decomposition. Therefore, isolating the bacterial strains native from hydrocarbon-contaminated environments is considered to be a significant strategy for bioremediation. Additionally, the autochthonous microorganisms in bioaugmentation increase the chances of survival and proliferation of cells after the reintroduction of pre-selected strains into the same environment. *C. variabile* isolated in this research was also found in oil contaminated sites such as in the Persian Gulf at Khorramshahr provenance, which was capable to degrade 82% of crude-oil after one week incubation (Hassanshahian et al., 2014). Different types of microorganisms and degrading genes may co-exist within one environment. For example, seven genotypes *alk*B, *alk*M, *alk*B1, *alk*B2, *xyl*E, *ndo*B, and *nid*A were identified from 4 microbial species of *Pseudomonas putida*, *Acinetobacter* spp., *Rhodococcus* spp. and *Mycobacterium* sp. in an oil contaminated area (Margesin et al., 2003). Compared with other well-known petroleum degrading bacteria, *C. variabile* is a new and interesting species and needs to be further studied.

As shown in Fig. 2a, the strain HRJ4 was found to be salt tolerant which showed maximum optical density of (1.81 ± 0.03) at NaCl concentration of 20 g/L. Further increase in the salt concentration up to 110 g/L declined the optical density to (1.32 ± 0.02). The result suggests that HRJ4 can tolerant a wide range of salinity. Similarly, pH of the medium required for the maximum growth was demonstrated in Fig. 2b. It was found that bacterium could effectively grow at the pH range from 6 to 9, with an optimum pH 7 (O.D. 1.49 ± 0.02). However, the cell growth was greatly restrained with pH higher than 9. Until now, information on molecular mechanisms and pathways of hydrocarbon degradation in high salinity is scarce and degradation of different types of hydrocarbons by halophilic and halotolerant microorganisms occur by pathways similar to those found in non-halophiles (Fathepure, 2014).

2.2. Morphological characterization of biochar and biochar-bacterium complex

The BET surface of biochar was determined after pyrolysis at different temperatures. The biochar of BC700 showed

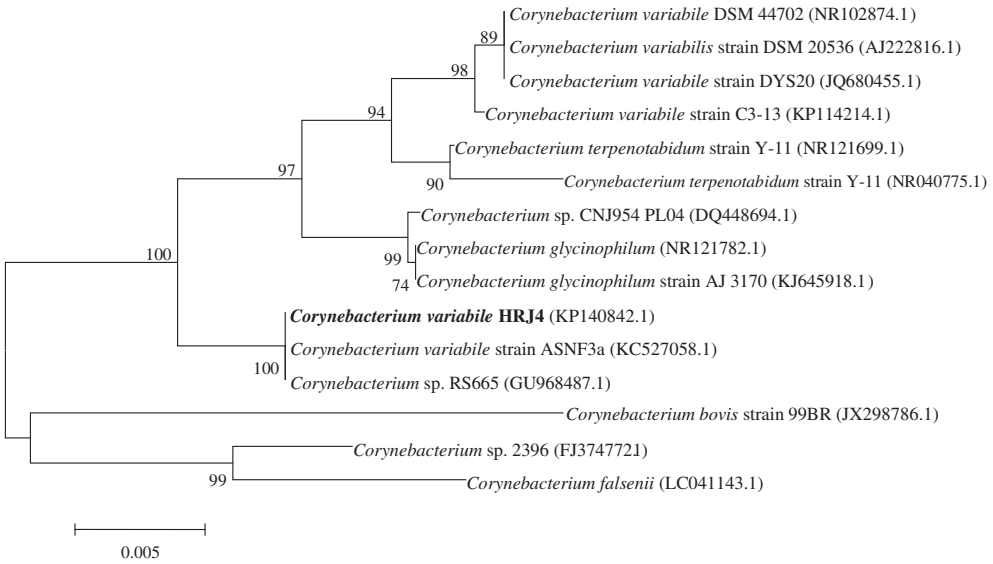

Fig. 1 – Phylogenetic position of strain HRJ4 within the genus *Corynebacterium* and allied bacteria. The branching pattern was generated by the neighbor-joining method. The number of each branch indicates the bootstrap values.

maximum BET surface area of 380.20 m^2/g, followed by BC400 (0.84 m^2/g) and BC250 (0.67 m^2/g). The wood chips (WC) without heat treatment showed BET 0.30 m^2/g. The increase of the pyrolysis temperature led to the elevated surface area of biochar, which facilitated higher sorption and correlated with the previous report (Beesley et al., 2010). So the biochar BC700 having higher BET surface area was selected as biocarrier for the following immobilization study.

The scanning electron microscopic images of wood chips (WCs) and biochar (BC) without bacterial culture and with addition of HRJ4 culture is shown in Fig. 3. It was observed that BC showed additional porous nature with many crevices, huge surface area and special structure. This property was helpful in accommodating more bacterial cells with biochar (B-BC). A monolayer bacterial cell adherence pattern was observed both in B-BC and B-WC but more compact aggregation of cells forming a biofilm in B-BC due to its special structure and surface properties. In B-WC, wood chips and bacterium were poorly combined and less bacteria was embedded because that wood chips had less crevices and smaller surface area. Soil aggregate pore size played an important role on biodegradation of petroleum hydrocarbons that are biodegraded primarily at the oil–water interface (Akbari and Ghoshal, 2015). The addition of biochar in bacterium culture permitted excellent mass transportation of oxygen, nutrients, and hydrocarbons and formed a suitable micro-environment inside the system to colonize HRJ4. The SEM analysis results showed that BC was an excellent amendment than the WC for immobilization of HRJ4 in petroleum hydrocarbon degradation studies.

2.3. Biodegradation of n-alkanes and PAHs

The degradation of the *n*-alkanes (C16, C18, C19, C26, C28) and polycyclic aromatic hydrocarbons (NAP, PYR) mixture by *C. variabile* HRJ4 is shown in Fig. 4. Over 70% degradation of TPHs

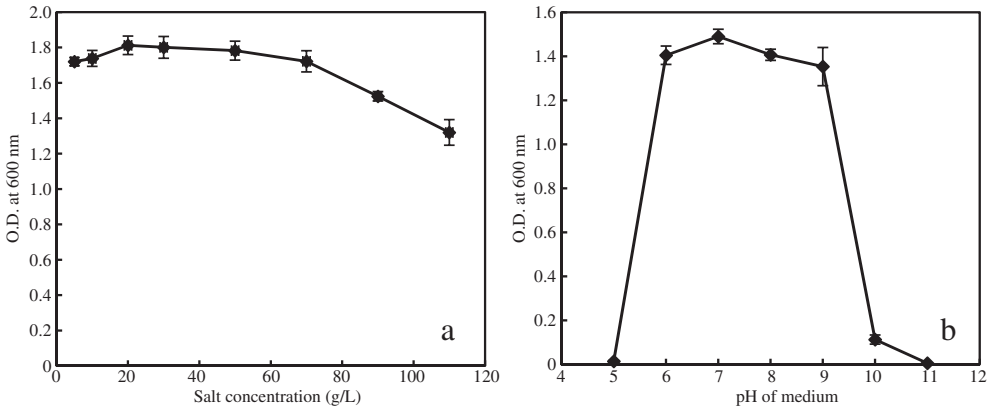

Fig. 2 – Effect of salinity and pH on the growth of HRJ4. (a) Salt tolerance of strain HRJ4, growth of bacterium was measured as optical density at 600 nm; (b) effect of pH on the growth of HRJ4 determined as optical density at 600 nm. Data shown as mean ± SEM, *n* = 3. SEM: scanning electron microscopy.

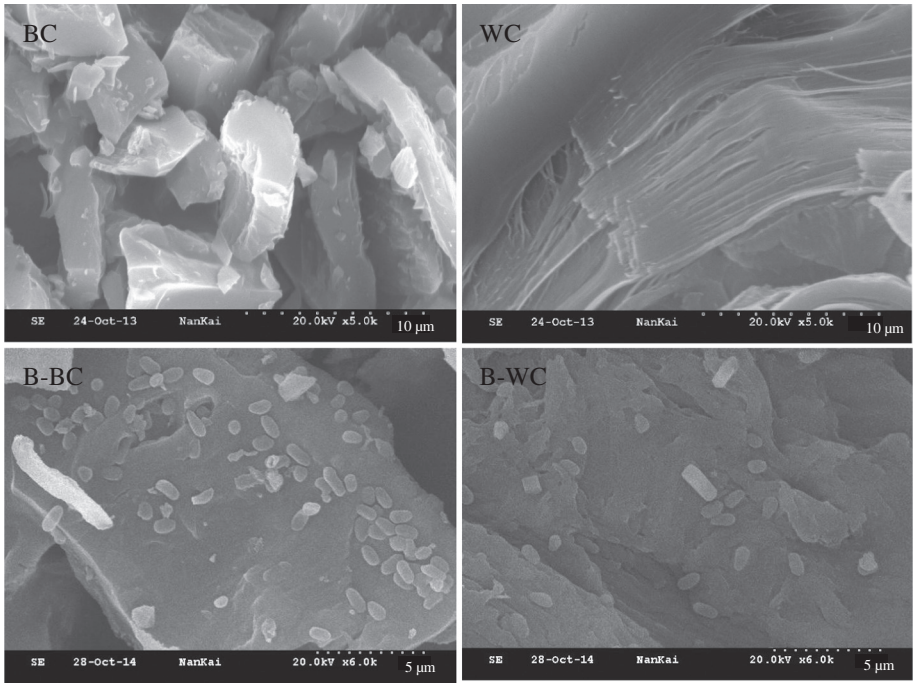

Fig. 3 – Scanning electron microscopic images of BC (biochar), WC (wood chips), B-BC (bacteria-biochar), and B-WC (bacteria-wood chips).

was found when the bacterium was used free (B) or in support with the B-BC. The highest reduction in THPs was observed in the B-BC treatment with a value of 78.9%, which demonstrated that immobilization of *C. variabile* HRJ4 on the carrier has enhanced degradation of THPs as compared to free leaving bacterium (71.9%). At the same time, a significantly higher degradation of *n*-alkane ($p < 0.05$) was found as compared to CK. The maximum reduction in the mid-length chain alkanes (C16–C19) was found in the B-BC treated hydrocarbons. After 7 days of incubation, the reduction of C16 and C18 was 94.75% and 82.29% in B-BC treatment. Similarly, the degradation of C19 was more effective when treated with B-BC. The biochar-microorganism system was used to remediate pollutants such as 4-bromodiphenyl ether contaminated soil and the removal rate increased by 63% and 83% compared with that with biochar and the strain individually, respectively (Du et al., 2015). The degradation of the long chain-alkanes (C26–C28) was not high as compared to free bacteria when the bacterium HRJ4 was supported or immobilized on biochar. The reason may be that the pollutants were absorbed to the biochar and the bioavailibity of some of the pollutants was reduced (García-Delgado et al., 2015; Ogbonnaya et al., 2014; Bushnaf et al., 2011). The Nap was evaporated due to mechanical agitation as shown from the disappearance of Nap in CK. However, biochar supported the Nap to stay in the medium without totally evaporated in treatment of BC and B-BC. It is also possible that a small amount of Nap may come from the pyrolysis process of biochar (Kloss et al., 2012; Keiluweit et al., 2012). All the treatments did not show any significant degradation of PYR with higher value in B-BC treatment (3.76%) after 7 days as compared to free bacteria (2.36%).

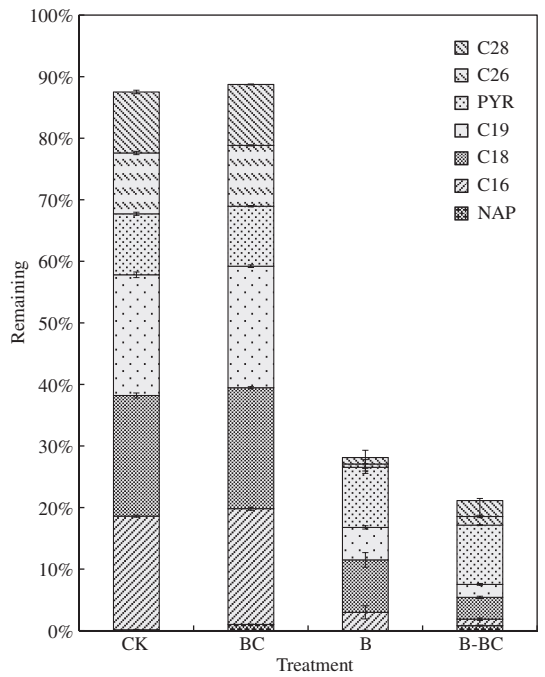

Fig. 4 – Degradradation of petroleum hydrocarbons after 7 days incubation with CK (only crude oil), BC (biochar), B (free bacteria), and B-BC (bacteria-biochar).

Biochar have been recently used to remediate the soil contaminated with both heavy metals and organic pollutants. The mechanism in case of heavy metals is electrostatic interaction and precipitation; whereas, in organic contaminants

the surface adsorption, partition and sequestration are the main processes (Beesley et al., 2010). It has been approved that biochars were able to support *Bacillus mucilaginosus* at population densities analogous to peat and also change the microbial community structure (Sun et al., 2015). Immobilization was used to make the TPHs bioremediation process more robust to the environmental factors and other competitors. Biochar as carriers can impose different effects on PAH bio-degradation by amending soil with immobilized bacteria, which can directly target on the carrier-associated PAHs (Chen et al., 2012). The advantages of cell immobilization were already demonstrated by other researchers in studying the degradation of phenol by a *P. putida* strain entrapped in chitosan beads (Hsieh et al., 2008). The efficacy of the immobilization techniques is under continuous discussion (Tyagi et al., 2011). The complexity of the field environment has prevented researchers from predicting the *in situ* microbial activity, adaptability and ecological competence of isolated microorganisms (Angelim et al., 2013). These factors are the primary limitations to the efficiency of bioremediation (El Fantroussi and Agathos, 2005). Similarly, biochar with strong sorption ability can enhance the biodegradation process as it could pre-concentrate the TPHs from water or contaminated soil (Chen and Yuan, 2011). Previous studies have demonstrated that biochars showed higher sorption capability with TPHs rather than other organic matter, and thus could accumulate a large number of TPHs (Cornelissen et al., 2005). Moreover, biochar is recalcitrant to decomposition and microbial mineralization. It was reported that the mineralization was negligible for biochar produced at 350°C with 120 days of incubation (Baldock and Smernik, 2002). Alginate was widely used to immobilize bacteria and combine with other materials to enhance the microbial degradation process. The degradation of phenanthrene was increased from 28.2% to 42.3% by inoculating bacteria to macroporous Ca-alginate (MCA) beads and macroporous Ca-alginate-lignin (MCAL) beads, respectively, after 4-day degradation (Zhang et al., 2008). Bioremediation strategy based on entrapping indigenous bacteria in biochar, which enabled bioaugmentation and biostimulation, should be considered as a new emerging bioremediation approach.

3. Conclusions

Biodegradation played an important role in reducing the effect of petroleum hydrocarbons in contaminated environments. This study demonstrated the successful entrapment of isolated bacterium *C. variabile* HRJ4 along with biochar. HRJ4 used in this study had significantly higher degradation rate of TPHs in the presence of biochar. The immobilization process will make the bioremediation process more robust against environmental factors and other competitors. Results of this research can be used to solve the pollution of petroleum hydrocarbons by eco-friendly and cost effective materials like biochar.

Acknowledgment

This work was supported by (1) the National Natural Science Foundation of China (No. 31270544, 41473070), (2) the Hi-Tech Research and Development Program (863) of China (No. 2013AA06A205), and (3) the 863 achievement transformation program in Tianjin (No. 14RCHZSF00144).

Note: The authors declare no competing financial interest.

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