RESEARCH ARTICLE

Enrichment and immobilization of oil-degrading microbial consortium on different sorbents for bioremediation testing under simulated aquatic and soil conditions

Dong-Sheng Li[1], **Jun-Qiao Feng**[1], **Yi-Fan Liu**[1], **Lei Zhou**[1], **Jin-Feng Liu**[1], **Ji-Dong Gu**[2], **Bo-Zhong Mu**[1,3], **Shi-Zhong Yang**[1,3*]
[1] State Key Laboratory of Bioreactor Engineering and Institute of Applied Chemistry, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, P.R. China
[2] School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, P.R. China
[3] Engineering Research Center for Microbial Enhanced Energy Recovery, Shanghai 200237, P.R. China

Abstract: Microorganisms with high oil-degrading ability are essential for bioremediation of oil-contaminated environments and oil spills. In the present study, a microbial consortium was enriched from a long-term oil-contaminated soil by acclimatization with crude oil, and was cultured with sucrose as a carbon source. Immobilization of the microbial consortium cells was prepared onto sodium alginate (SA) beads. To enhance the mass transfer of the immobilized microspheres, activated carbon, biochar, corn stalk and sawdust were used, respectively, to accelerate the degradation of petroleum hydrocarbons. The immobilized beads and the distribution of microbial cells inside the immobilized beads were examined using scanning electron microscopy (SEM). The degradation efficiency of total petroleum hydrocarbon (TPH) by different immobilized beads in aquatic systems (mineral salt medium, artificial seawater) was evaluated by gravimetric method after 7 d of incubation. Results showed that TPH degradation efficiency of the immobilized beads was higher than that of the microbial culture, and that of the immobilized beads containing adsorbent carriers was higher than the SA immobilized beads. The highest TPH degradation efficiencies of SA-CS immobilized beads in mineral salt and artificial seawater were up to 54.2% and 50.5%, respectively, and the highest TPH degradation efficiency of biostimulation + SA-AC immobilized beads treatment in oil-contaminated soil was up to 63.7% after 10 weeks of incubation. Our results suggest that the immobilized microorganism is a promising approach for a wide range of bioremediation applications in different petroleum-contaminated environments.
Keywords: Bioremediation, immobilization, petroleum hydrocarbon, oil pollution, biodegradation

*Correspondence to: Shi-Zhong Yang, State Key Laboratory of Bioreactor Engineering and Institute of Applied Chemistry, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China; E-mail: meor@ecust.edu.cn

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1 Introduction

Petroleum is an important energy source in modern society and also a raw material in refineries and petrochemical industries (Varjani and Upasani, 2017). Petroleum pollution has a detrimental effect on natural and anthropogenic environments (Lin et al., 2014) as it may result in a direct or indirect health risk to all forms of life (Varjani, 2017). Current treatment methods to eradicate oil pollution include mainly physical, chemical and microbial approaches. Compared with physical and chemical treatments, bioremediation is a more cost-effective, eco-friendly and efficient one (Angelim et al., 2013). In addition, bioremediation can be easily applied to a wide range of different environments (Lim et al., 2016; Mapelli et al., 2017). Bioremediation relied on the biodegradability of microorganisms to mineralize contaminants into water, carbon dioxide and biomass under aerobic condition (Chandran and Das, 2011; El Fantroussi and Agathos, 2005). Bioremediation can be divided into two major categories: i) biostimulation, addition of nutrients such as nitrogen and phosphorus to stimulate the growth of indigenous microorganisms to accelerate the biodegradation of contaminants by microorganisms; and ii) bioaugmentation, introduction of exogenous or indigenous microorganisms to accelerate biodegradation of contaminants by microorganisms (Dellagnezze et al., 2016). Since bioaugmentation and biostimulation have limitations when applied alone, the combination of the two becomes a better strategy (El Fantroussi and Agathos, 2005; Tyagi et al., 2011).

In order to achieve an effective bioremediation on any oil-contaminated sites, bioaugmentation microorganisms must have excellent degradation ability for the petroleum hydrocarbons. Enrichment of microbial consortium or isolation of pure microbial strains from petroleum contaminated sites is considered as a prerequisite to obtain the inocula for use in bioaugmentation (González et al., 2011; Mao et al., 2012; Roy et al., 2014; Song et al., 2018; Zhao et al., 2011). Considering crude oil is a complex mixture containing more than 17,000 compounds in the crude oil (Head et al., 2006), the microbial consortium obtained after enrichment pro-

cess could have a wider range of substrate specificity for degradation of petroleum hydrocarbons in contrast to single microbial strain or the oil-degrading microbial consortium obtained by reconstituting individual microbial strains. In addition, bioaugmentation microbes need to survive in oil-contaminated environment to further carry out degradation of petroleum hydrocarbons. Carrier materials are used to retain sufficient biomass and maintain high microbial activity for a long period of time in cell immobilization, thereby improve bioremediation effectiveness (Partovinia and Rasekh, 2018). One of the main roles of the carrier materials in soils is to provide protective micro-habitats for the cells from various environmental stresses negatively, predation by protozoa, and competition with autochthonous microorganisms and toxic substances (Li et al., 2016). Adsorption of microbial cells on a carrier and entrapment of such cells into a polymeric network of carrier are common methods of immobilization. Cellulose-containing materials are commonly carriers used for physical retention of microbial cells, particularly agricultural and forestry residues, e.g., walnut shell, sawdust, corncob powder, wood chips, wheat bran and peanut hull powder, biochar, cotton fibers, polyethylene plastic pellets, activated carbon, and polyurethane foam (Alessandrello et al., 2017; Chandran and Das, 2011; Liang et al., 2009; Lin et al., 2014; Mishra et al., 2001; Nopcharoenkul et al., 2013; Shen et al., 2015; Zhang et al., 2016). However, a major disadvantage of sorbent carriers is that the sorbed microbial cells are easily leaked out from the carriers (Li et al., 2016). Carriers used for entrapment immobilization mainly include sodium alginate, polyvinyl alcohol and chitosan (Costa et al., 2014; Kuyukina et al., 2013; Simons et al., 2013). Among these polymer carriers, the immobilization process of sodium alginate is the simplest, and the activity of the microbial cells is not impaired. Alginate is a natural polymer extracted from algae and is degradable, and low cost. The dense gel layer of alginate microspheres hinders the mass transfer of substrates, contaminants and degradation products, limiting their potential applications in bioremediation. Some sorbent carriers could be added to the alginate beads to promote the migration of contaminants to the surface and internal regions (Wang et al., 2012).

In this study, the microbial consortium for petroleum hydrocarbon bioremediation was enriched from long-term oil-contaminated soil. The degradation efficiencies were investigated with the microbial consortium immobilized using sodium alginate mixed with activated carbon, biochar, corn straw and sawdust respectively in this paper.

2 Materials and Methods

2.1 Materials

Petroleum was obtained from Jiangsu Oilfield, Jiangsu, China. Sodium alginate (SA) was of analytical grade and purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China). Sawdust (S) and biochar (B) were purchased from Henan Yuzhongao Agricultural Technology Co., Ltd. (Anyang, China). Corn stalks (CS) were kindly provided by Dr. Jie Bao (School of Biotechnology, East China University of Science and Technology). Sawdust, corn stalks and biochar were crushed and screened by using a 40 mesh sieve. Powdered activated carbon and *n*-hexane were of analytical grade and purchased from SinoPharm Chemical Reagent Co., Ltd. (Shanghai, China). Squalane and 1-chlorohexadecane were of analytical grade and purchased from Meryer Chemical Technology Co., Ltd. (Shanghai, China).

2.2 Culture Medium

Mineral salt medium (MSM) consisted of: 0.2 g of $MgSO_4$, 0.02 g of $CaCl_2$, 1.0 g of KH_2PO_4, 1.0 g of Na_2HPO_4, 2.0 g of $(NH_2)_2HPO_4$, 1.0 g of NH_4Cl, 1.0 g of $NaNO_3$ and 0.05 g of $FeCl_3$ were added into 1000 mL of deionized water with a pH adjusted to 7.0 (Zheng et al., 2018).

Artificial seawater (AS) contained: 24.0 g of NaCl, 7.0 g of $MgSO_4 \cdot 7H_2O$, 2.0 g of KH_2PO_4, 3.0 g of Na_2HPO_4, 2.0 g of $(NH_4)_2HPO_4$, 1.0 g of NH_4Cl, 1.0 g of $NaNO_3$ and 0.7 g of KCl were added into 1,000 mL of deionized water, with a pH adjusted to 7.5. The trace elements solution contained (per litre) the following: 2.0 mg $CaCl_2$, 50 mg $FeCl_3 \cdot 6H_2O$, 0.5 mg $CuSO_4$, 0.5 mg $MnCl_2 \cdot 4H_2O$, 10 mg $ZnSO_4 \cdot 7H_2O$. AS was immediately supplemented with 2% (*v/v*) trace elements solution to simulate marine environment before use (Hou et al., 2013).

Crude oil medium: 0.5% (*w/v*) of crude oil was added into MSM.

Fermentation medium: 2% (*w/v*) of sucrose were added into MSM. All of culture media were sterilized in an autoclave at 121°C for 20 min before use.

2.3 Soil Sample

Soil contaminated with crude oil for a long period of time was collected from Daqing oilfield, Daqing, China. After being air-dried and sieved through 2 mm mesh, and then the soil was homogenized thoroughly. The physicochemical characteristics of the soil are listed in Table 1.

Table 1. Physicochemical characteristics of the soil used in this study.

Parameter	Value
pH	10.26
Total salt content (mg kg^{-1})	19
Ammonium nitrogen (mg kg^{-1})	10.6
Nitrate nitrogen (mg kg^{-1})	8.8
Total phosphorus (mg kg^{-1})	2.34
Organic matter (%)	9.88%
Moisture (%)	1.40%
Water holding capacity (%)	34.94%
TPH (mg kg^{-1})	36294

TPH: total petroleum hydrocarbon

2.4 Microorganisms

The soil (5 g) was inoculated into a 250 mL glass triangular flask containing 100 mL of crude oil medium and the enrichment culture was incubated at 30°C for 7 d on a shaking incubator at 150 rpm in the dark. Then the enrichment culture was transferred into fresh crude oil medium (10% *v/v*) for further 7 d of incubation under identical conditions. This transfer process was repeated for eight times successively. The final enrichment microbial enrichment consortium was designated MCA. The MCA culture was transferred into the fermentation medium (10% *v/v*) for on other 7 d of incubation, and the new microbial enrichment consortium was designated MCB.

2.5 DNA extraction and high-throughput sequencing

Bacterial chromosomal DNA of microbial consortia was extracted using AxyPrep™ bacterial Genomic DNA Miniprep Kit (Axygen Biosciences, USA) according to the manufacture's instructions.

The bacterial hypervariable regions V4–V5 of the 16S rRNA genes were amplified using the primers 515F (5'-GTGCCAGCMGCCGCGG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') (Li et al., 2017). Polymerase chain reaction (PCR) amplification was performed in a 20 μL reaction volume containing 5× FastPfu Buffer (4 μL), 2.5 mmol/L of dNTPs (2 μL), 5 μmol/L of each primer (0.8 μL), FastPfu Polymerase (0.4 μL), BSA (0.2 μL), and approximately 10 ng of template DNA in an ABI GeneAmp®9700. The amplifications were run under the following thermocycling conditions: initial denaturation at 95°C for 3 min; 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 45 s; and a final extension at 72°C for 10 min. The obtained PCR products were sequenced on Illumina Miseq platform (Majorbio, China).

2.6 Preparation of immobilized beads

On the basis of 2% concentration of sodium alginate, mixing ratios of sodium alginate with the sorbent carriers as follows: sodium alginate : activated carbon = 5 : 1, sodium alginate : biochar = 1 : 1, sodium alginate : corn straw = 1 : 1, sodium alginate : sawdust = 5 : 6. The preparation method of different immobilized beads was as follows. Firstly, 2.0 g of sodium alginate, 100 mL of MCB culture and corresponding sorbent carrier were added into a 300 mL beaker. The carriers and MCB culture were mixed using glass stirring rod. The mixture of carriers and MCB culture was delivered through a peristaltic pump to a calcium chloride solution (3%, *w/v*) for 16 h to form spherical beads. The immobilized microbial microspheres were rinsed with sterile deionized water. Finally, these immobilized microbial microspheres containing different carriers were preserved separately at 4°C for further use. The obtained five immobilized microbial microspheres of different carriers were named as SA immobilized beads (carrier: sodium alginate), SA-AC immobilized beads (carrier: sodium alginate with activated carbon), SA-B immobilized beads (carrier: sodium alginate with biochar), SA-CS immobilized beads (carrier: sodium alginate with corn stalk) and SA-S immobilized beads (carrier: sodium alginate with sawdust).

2.7 Microcosm Bioremediation Experiment

For bioremediation experiment in mineral salt medium, squalane was mixed into the crude oil at a ratio of 1:200 (*v/w*) to serve as the internal standard for GC–MS analysis (Szulc et al., 2014). Approximately 0.5 g of crude oil and 100 mL of MSM were introduced into a 250 mL Erlenmeyer flask, and the flask was then sterilized at 121°C for 20 min. The microbial culture of MCB and immobilized beads 3% (*w/v*) were added into the Erlenmeyer flasks, respectively. Sterilized Erlenmeyer flasks without immobilized beads were used as a control. The flasks were incubated at 30°C with shaking (150 rpm) for 7 d. All experiments were performed in triplicate. For bioremediation experiment in artificial seawater, squalane was mixed into the crude oil at a ratio of 1:100 (*w/w*). In artificial seawater with high concentration of inorganic salts, inoculum was set at 6%. Other experimental parameters were identical with bioremediation experiment conducted in mineral salt medium.

In order to test the bioremediation effect of immobilized beads on petroleum contaminated soil, 200 g of petroleum-contaminated soils described in Section 2.3 were put into 300 mL beaker. The following experimental set-ups were tested: (I) Control: air-dried petroleum-contaminated soil as control, (II) Biostimulation: air-dried petroleum-contaminated soil with 30 mL of mineral salt solution, (III) Biostimulation + SA-AC: air-dried petroleum-contaminated soil with 15% (*w/v*) SA-AC immobilized beads and 30 mL of mineral salt solution, (IV) Biostimulation + SA-S: air-dried petroleum-contaminated soil with 15% (*w/v*) SA-S immobilized beads and 30 mL of mineral salt solution. Mineral salt solution contained (per litre) the following compounds: 20 g of KH_2PO_4, 40 g of $NaNO_3$, 60 g of NH_4Cl, 0.6 g of $CaCl_2$, 6 g of $MgSO_4$ and 1.6 g of $FeCl_3$. All experiments were performed in triplicate. The beakers were incubated at a constant temperature of 30°C. Except to the control, soil moisture was maintained at 20.9% - 24.45% (60% - 70% of WHC) by adding water, and the soil were mixed once every two days to provide enough oxygen (Llado et al., 2013; Saviozzi et al., 2009).

2.8 Hydrocarbon Degradation Analysis

The petroleum hydrocarbons remaining in MSM and AS were extracted with *n*-hexane at the end of incubation period (Simons et al., 2012). The residual TPH in the soil was analyzed according to the national standard method (HJ 911-2017, China) with some modifications. Approximately 4.0 g soil were taken from beaker and dried in oven at 55°C. Then, the dried soils were triturated, and 3.0 g treated soil

was weighed and used for petroleum hydrocarbon analysis. Three mL of *n*-hexane were added into 3 g of treated soil samples that had been placed in a 7 mL of centrifuge tube. Subsequently, the sample was extracted with ultra-sonication at 300 W for 30 min and then centrifuged at 8000 rpm for 5 min. The supernatant was transferred into a 7 mL centrifuge tube, and the sediment was extracted twice more with *n*-hexane. Finally, the supernatants were combined together in a 7 mL centrifuge tube. After *n*-hexane was removed by air stripping, the TPH degradation efficiency in aquatic and soil environments was determined according to the gravimetric method (Gentili et al., 2006; Wu et al., 2017).

For GC-MS analysis of oil alkanes, petroleum hydrocarbon samples extracted from aquatic environment were dissolved in 25 mL of *n*-hexane. Petroleum hydrocarbon samples extracted from soil were dissolved in 5 mL of *n*-hexane (containing internal standard 1-chlorohexadecane at 0.1 µl/mL) (Zheng et al., 2018).

The samples were analyzed using a GC-MS (Agilent 7890-5975c) equipped with an Hp-5 (30 m × 0.25 × 0.25 µm) capillary column. Carrier gas was the high purity helium (99.99%). The injector temperature was set at 280°C. The oven program was as follows: the initial temperature was 60°C and was maintained for 2 min. Then the temperature increased by 10°C/min until reaching 280°C and it was held for 25 min (Zheng et al., 2018). External standare were used to calibrate the response of output signal to the concentration.

2.9 Scanning electron microscopy (SEM)

Morphological observation of the five immobilized beads was performed on a scanning electron microscope (SEM S-3400N). Prior to being observed on SEM, the immobilized beads were freeze-dried. In order to observe the internal structure of immobilized beads, freeze-dried samples were sectioned cross-section wise and then coated with thin layer of gold (Deng et al., 2017).

3 Results and Discussion

3.1 Microbial community composition

Community composition of bacteria in MCA and MCB is shown in Fig 1. The dominant bacterial genera of the MCA were *Acinetobacter* (44.70%), *Pseudomonas* (12.25%), *Nubsella* (8.74%), *Stenotrophomonas* (6.50%) and *Sphingobacterium* (5.80%). *Acinetobacter* spp. constituted an important part of oil-degrading communities and was found in soil and sediment contaminated with crude oil, fuel oil or mineral oil (Ron and Rosenberg., 2010). *Acinetobacter* strains may degrade a variety of hydrocarbons, including short- and long-chain alkanes, branched-chain alkanes, e.g., pristine, and various aromatics. In addition, some *Acinetobacter* strains can produce potent high molecular weight emulsifiers (Ron and Rosenberg., 2010). *Pseudomonas* strains can degrade a large number of aliphatic, aromatic, poly-aromatic hydrocarbons and various derivatives, among a large variety of miscellaneous organic compounds (Palleroni et al., 2010). *Stenotrophomonas maltophila* was isolated from samples collected from oil-fields and could biodegrade *n*-alkanes (Varjani et al., 2015). Phenanthrene degrading bacteria strain was isolated from soil collected from area around petrochemical industries and oil refineries and was identified as *Sphingobacterium* sp. (Janbandhu and Fulekar, 2011). However, petroleum hydrocarbon-degrading bacteria belonging to *Nubsella* sp. were not isolated. The MCA originated from a soil polluted by petroleum for a long time and was cultured with petroleum as the sole carbon source for eight times of enrichment transfer culturing. Under the circumstances, the carbon needed for cell synthesis can only come from petroleum, therefore, the dominant bacteria genera must be related to degradation of petroleum hydrocarbons. Other minor genera such as *Chryseobacterium*, *Ochrobactrum* and *Gordonia* were often reported for degradation of petroleum hydrocarbons (Kummer et al., 1999; Peressutti et al., 2003; Szoboszlay et al., 2008). Crude oil was perhaps the most complex mixture of saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes (Head et al., 2006). Therefore, the enriched microbial consortium MCA should have a larger substrate degradation range than combination of isolated strains.

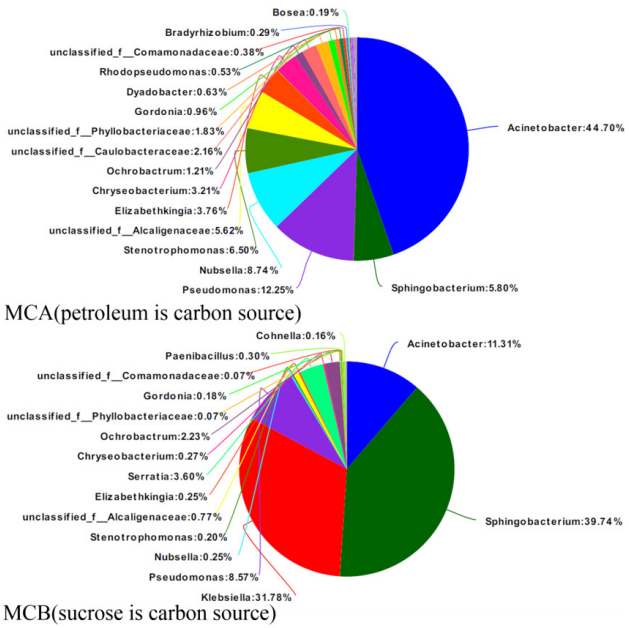

Figure 1. Taxonomic classification of bacterial reads retrieved from the two microbial consortia at the genus level via 16S rRNA Illumina gene sequencing.

In order to obtain sufficient microbial isolates for further degradation experiments, petroleum or aromatic hydrocarbons were often used as carbon sources to continue enrichment of microbial consortium cells (Chagas-Spinelli et al., 2012; González et al., 2011; Mohajeri et al., 2010; Nikolopoulou et al., 2013; Vieira et al., 2007). But this culturing strategy is too slow to obtain cells comparing that

of culturing single strain. Therefore, glucose was also used as a carbon source to further enrich microbial consortium cells (Xu and Lu, 2010). In this study, sucrose was added as the sole carbon source into the MSM, and the medium was used as fermentation medium to enrich the microbial consortium cells for the subsequent degradation experiment.

The dominant bacterial genera of the MCB were *Sphingobacterium* (39.74%), *Klebsiella* (31.78%), *Acinetobacter* (11.31%) and *Pseudomonas* (8.57%). The top 20 bacterial genera in the two microbial consortia were the same (Fig 2). Compared with the MCA, the bacterial genera with a significant increase in the MCB were *Sphingobacterium*, *Klebsiella*, *Serratia*, *Ochrobactrum* and *Paenibacillus*. The major bacterial genera in the MCB were often related to the degradation of petroleum hydrocarbons. Table 2 summarizes the substrate range of major genera in the MCB from other studies. Therefore, the microbial consortium MCB should have wide substrate degradation range of petroleum hydrocarbons and would be suitable for further degradation experiments.

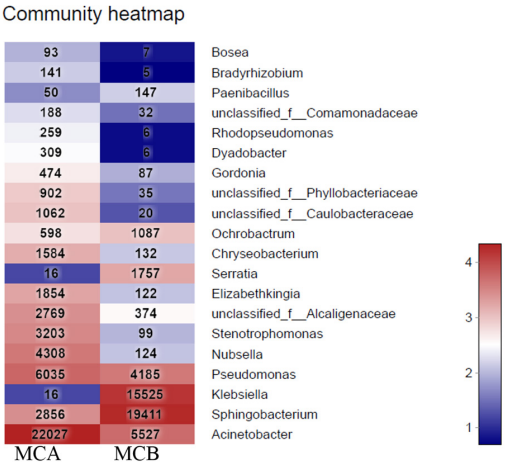

Figure 2. Heatmap of the distribution of dominant genus in the two microbial consortia based on the logarithm of the relative abundances of the top 20 genera. The colour scale showed the logarithm of the relative abundances.

Table 2. Summary of the substrate range of major genera in the MCB in other studies.

Strains	Substrate range	References
Acinetobacter sp. strains	short- and long-chain alkanes, branched-chain alkanes and various aromatic hydrocarbons	(Ron and Rosenberg, 2010)
Pseudomonas sp. strains	aliphatic and aromatic hydrocarbons	(Palleroni et al., 2010)
Sphingobacterium sp. strain	phenanthrene	(Janbandhu and Fulekar, 2011)
Klebsiella sp. strain	crude oil	(Odokuma and Dickson, 2003)
Serratia sp. strain	crude oil	(Odokuma and Dickson, 2003)
Ochrobactrum anthropic	crude oil	(Peressutti et al., 2003)
Paenibacillus sp. strain	phenanthrene	(Meyer et al., 1999)
Chryseobacterium sp. strain	crude oil	(Szoboszlay et al., 2008)
Stenotrophomonas sp. strain	C8-C35	(Varjani et al., 2015)
Gordonia sp. strain	Alkanes	(Kummer, et al., 1999)

3.2 Morphological Characterization of Immobilized Beads

The SEM images of the five immobilized beads are shown in Fig 3. The SA immobilized beads had a relatively dense structure, which was composed of few small pores and did not have larger porous channels (Fig 3A). This structure hindered the efficient transfer of oxygen, which limited the biodegradation ability of aerobic strains of microorganism community. Compared with SA immobilized beads, other immobilized beads containing adsorbent carriers had different interior porous structure (Fig 3).

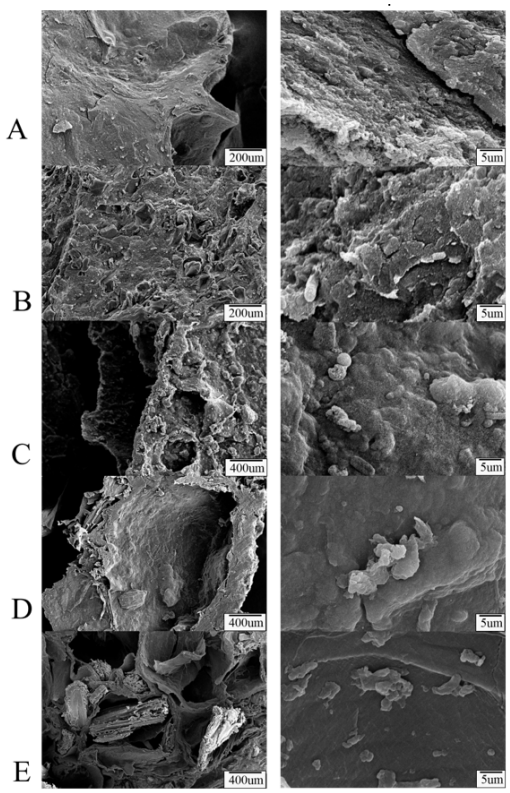

Figure 3. SEM images of different immobilized beads. (A): SA immobilized beads, (B): SA-AC immobilized beads, (C): SA-B immobilized beads, (D): SA-CS immobilized beads, (E): SA-S immobilized beads.

The pores of the carrier material provided protective niches for microbial cells, and the stable microenvironments of the pores may protect immobilized microbial cells from adverse environmental factors (Hou et al., 2013). The presence of pores is necessary to guarantee the viability of cells inside the beads (Angelim et al., 2013). Moreover, carrier material with large porosity could permit better mass transport of oxygen, nutrients and degradation substrates (Wang et al., 2012). SA-AC immobilized beads had many small pores and did not have larger porous channels. SA-B immobilized beads and SA-CS immobilized beads had large porous channels, but the distribution of these porous channels was not uniform. SA-S immobilized beads had uniform large pore structure, and this structure was similar with chitosan microsphere (Angelim et al., 2013). Our results showed that the four adsorbent carriers improved porosity and mass transfer properties. The cells in the SA and SA-AC immobilized beads were difficult to distinguish (Fig 3A and 3B). This result might be caused by the lack of larger porous channels inside these two immobilized beads, resulting in relatively uniform distribution of cells in

these two immobilized beads. But the distribution of cells in the other immobilized beads was not uniform, some cells gathered and others dispersed (Fig 3C, 3D and 3E), which was the same as the results obtained by others (Lin et al., 2010; Shen et al., 2015). The reason might be that there were bigger porous channels in these three immobilized beads, so the cells adsorbed on the adsorbent carriers and the form of cell adsorption on the carrier could be further divided into physical adsorption and adsorption by adhesion of extracellular secretions (Lin et al., 2010; Wang et al., 2015).

3.3 Biodegradation of Crude Oil by Different Immobilized Beads in Aquatic Environment

The TPH degradation efficiency of different treatments calculated by gravimetric method in aquatic system for 7 d is shown in Fig 4. For biodegradation of crude oil in MSM, the TPH degradation efficiencies of the six different treatments with MCB microbial culture, SA immobilized beads, SA-AC immobilized beads, SA-B immobilized beads, SA-CS immobilized beads and SA-S immobilized beads were 37.0%, 45.1%, 48.4%, 53.1%, 52.1% and 54.2%, respectively. The TPH degradation efficiency of these immobilized beads was higher than that of MCB microbial culture. The advantage of the free cells in MCB microbial culture was that the dispersion could make full contact with petroleum hydrocarbons, but the toxicity of petroleum may inhibit the growth of free cells. The toxic effect of most hydrocarbons was caused by general, non-specific effects on membrane fluidity due to their accumulation in the lipid bilayer (Heipieper and Martínez, 2010). The short-chain liquid *n*-alkanes such as hexane and octane were more toxic to bacteria. Compared to longer alkanes, they may have enhanced solvent action, and thus may have more pronounced deleterious effects on the phospholipid bilayers of biological membranes (Cunningham et al., 2004). For these immobilized beads, the carrier material provided a protective shelter against the toxicity of petroleum (Bao et al., 2012; Liu et al., 2015). In addition, immobilized microspheres had a higher cell density compared with microbial cultures. Compared with SA immobilized beads, the four immobilized beads with adsorbent carriers had higher TPH degradation efficiency. On the one hand, adding adsorbent carriers improved the mass transfer capacity of immobilized microspheres. On the other hand, with the biodegradation of petroleum hydrocarbons, the microorganisms entraped in the microspheres released slowly, and the microspheres gradually disintegrated, then the sorbent carriers dispersed into the culture medium. These sorbent carriers not only continued to adsorb the hydrocarbon-degrading microorganisms to provide the protective environment for microorganisms, but also adsorbed petroleum hydrocarbons to facilitate its degradation by the immobilized cells (Hou et al., 2013).

Representative samples for each conditions were analyzed

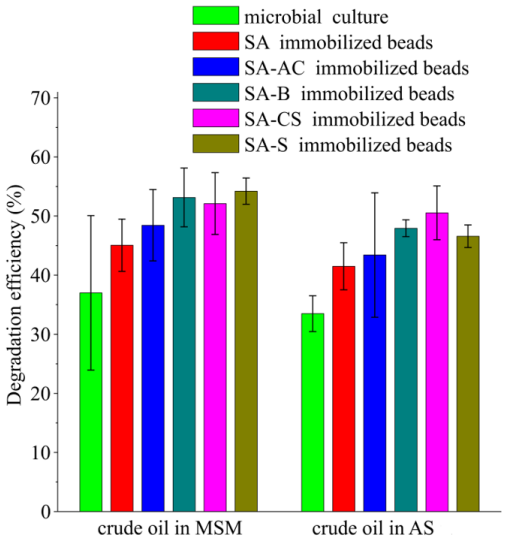

Figure 4. The TPH degradation efficiency of different treatments in aquatic environment for 7 d. Data correspond to means of triplicate values and error bars correspond to standard deviations.

by GC–MS and the results are shown in Fig 5. The alkanes in crude oil used in the experiment contained C14-C30 *n*-alkanes, as well as pristane (Pr) and phytane (Ph) ares shown in Fig 5A. Combining the mixture ratio of internal standard and crude oil, it could be estimated that the C14-C30 *n*-alkanes in crude oil accounted for about 20%. The TPH degradation efficiency of microbial culture was 18.6% (Fig 5B). The *n*-alkanes of C14-C18 were degraded completely, and the degradation efficiency of individual *n*-alkane ranging from C19-C30 decreased as the carbon number increased. Pristane and phytane were barely changed over the entire period of incubation. Overall, the degradation efficiency of total *n*-alkanes exceeded 80%, therefore, the degraded hydrocarbons in this sample should mainly be *n*-alkanes. The TPH degradation efficiency of SA microbial agent was 42.5% (Fig 5C). There was no significant difference between the chromatogram in Fig 5C and that in Fig 5B. However, the TPH degradation efficiency between the two samples varied greatly. It could be speculated that *n*-alkanes were first degraded by microbial cells, and the microbial cells in the immobilized beads began to degrade the other hydrocarbons in petroleum when the *n*-alkanes decreased significantly, such as cycloalkanes and aromatics. The TPH degradation efficiency of SA-S immobilized beads was 57.1% (Fig 5D). Almost all *n*-alkanes and pristane were degraded completely, and phytane degradation efficiency was also exceeded 70%, indicating a higher degree of overall biodegradation. In general, biodegradability of hydrocarbons could be ranked as: linear alkanes > branched alkanes > low-molecular-weight alkyl aromatics > monoaromatics > cyclic alkanes > polyaromatics>> asphaltenes (Varjani, 2017). However, in actual degradation experiments, the degradation rate of petroleum hydrocarbons may not fully match the biodegradability ranking. The trend in degradation rate of petroleum hydrocarbons in seawater followed the

pattern from high to low: C15 > C20 > C25 > (Pristane; Phytane) > C30 > (PAHs) (Nikolopoulou et al., 2013; Nikolopoulou et al., 2013). In this study, based on the results of the GC-MS analysis, it could be inferred that pristane and phytane began to be degraded after partial degradation of cycloalkanes and aromatics. The results might be related to the oil pollution environment, petroleum composition and microbial community composition.

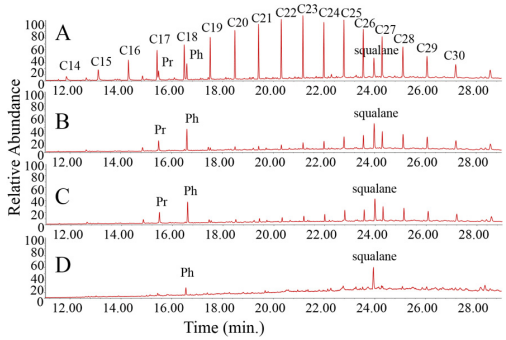

Figure 5. Gas chromatogram of extracted oil in MSM after biodegradation. Relative abundance: The internal standard (squalane) peak of (A) was assigned the value 30, and the heights of the other peaks were compared with the internal standard peak to give relative abundances. (A): control. (B): microbial culture treatment. (C): biodegeadation sample of SA immobilized beads treatment. (D): biodegeadation sample of SA-S immobilized beads treatment.

In order to verify the application potential of immobilized beads for bioremediation of marine oil spill pollution, the degradation effectiveness of different treatments for crude oil in artificial seawater were tested and the results are shown in the Fig 4. The TPH degradation efficiencies of the six different treatments of MCB microbial culture, SA immobilized beads, SA-AC immobilized beads, SA-B immobilized beads, SA-CS immobilized beads and SA-S immobilized beads were 33.5%, 41.5%, 43.4%, 47.9%, 50.5% and 46.5%, respectively. It was reported that when the salt concentration in medium reached a certain value, the degradation efficiency of microorganisms on petroleum hydrocarbons would significantly decreased. This result probably due to the osmotic stress induced by salt, which could cause loss of cytoplasmic water, and thus restrained degradation efficiency (Deng et al., 2017; Nie et al., 2016; Shen et al., 2015). Considering that the high salt concentration in seawater may affect the degradability by microorganisms, the inoculation amount was increased from 3% in MSM to 6% in AS. But the TPH degradation efficiency of each immobilized beads in AS was still lower than that in MSM. Moreover, the TPH degradation efficiency of these immobilized microbial agents was higher than that of planktonic microbial culture, and the immobilized beads added with sorbent carrier had higher TPH degradation efficiency than SA immobilized beads. This result was similar to the degradation in the MSM. In general, although the high salinity of seawater could adversely affect bioremediation, microbial immobilization technique could mitigate the adverse effects of high salinity to some extent. Therefore, the degradation results in AS proved the application potential of immobilized beads in marine oil spill.

3.4 Bioremediation of Petroleum-contaminated Soil by Immobilized Beads

The TPH degradation efficiency of different treatments calculated by gravimetric method in petroleum-contaminated soil during the first 10 weeks is shown in Fig 6. After the initial three weeks, the TPH degradation efficiencies of the three different treatments of biostimulation, biostimulation + SA-AC immobilized beads, and biostimulation + SA-S immobilized beads were 13.8%, 31.0% and 28.8%, respectively.

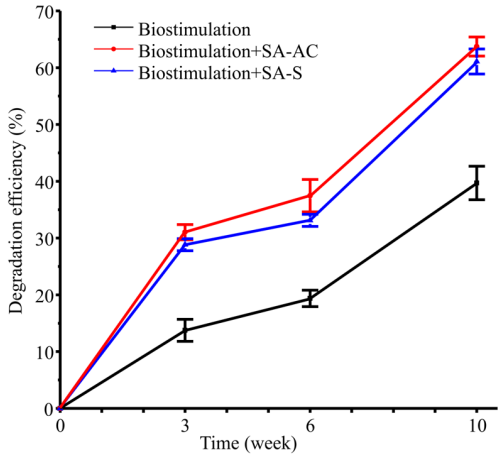

Figure 6. The TPH degradation efficiency of different treatments during the first 10 weeks. After 6 weeks, adding the same amount of inorganic salt solution as the initial amount to supply the nutrients. Data correspond to means of triplicate values and error bars correspond to standard deviations.

Representative samples for each conditions were analyzed by GC–MS and the result is shown in Fig 7. The alkanes in crude oil used in the experiment contained C13-C30 *n*-alkanes, as well as pristane and phytane (Fig 7A). The TPH degradation efficiency of biostimulation treatment was 13.8% (Fig 7B). The total *n*-alkanes degradation efficiency exceeded 50%. Moreover, pristane and phytane were also partially degraded. The TPH degradation efficiency of biostimulation + SA-AC treatment was 32.8% (Fig 7C). And the TPH degradation efficiency of biostimulation + SA-S treatment was 30.0% (Fig 7D). The alkanes (*n*-alkanes, pristane and phytane) shown in the chromatograms were completely degraded (Fig 7C and 7D). As bioremediation continue, it could be speculated that microorganisms in soil added with immobilized beads would continue to degrade other petroleum hydrocarbons, such as cycloalkanes and aromatics.

The biodegradation rate of petroleum hydrocarbons during the fourth to sixth weeks was significantly slower compared with that in the first three weeks (Fig 6). There might be two reasons for this result. One was that the remaining petroleum hydrocarbons in the soil were more difficultly biodegradable than the alkanes experienced degradation, and the other was that the remaining nitrogen and phosphorus in the soil was in-

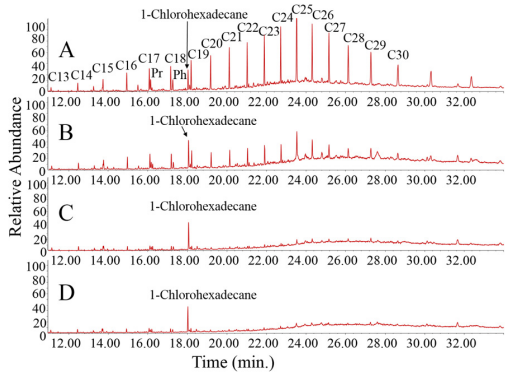

Figure 7. Gas chromatogram of extracted oil after bioremediation for three weeks. Relative abundance: The internal standard (1-Chlorohexadecane) peak of (A) was assigned the value 30, and the heights of the other peaks were compared with the internal standard peak to give relative abundances. (A): control. (B): biostimulation treatment. (C): biostimulation + SA-AC treatment. (D): biostimulation + SA-S treatment.

sufficient. Adding mineral salt solution to soil to supplement nutrients such as nitrogen and phosphorus, and the amount of mineral salt solution added in soil at this time was the same as the amount added at the initial time of the bioremediation experiment. After ten weeks of bioremediation, the TPH degradation efficiencies of the three different treatments of biostimulation, biostimulation + SA-AC immobilized beads, and biostimulation + SA-S immobilized beads were 39.7%, 63.7% and 61.7%, respectively. And the TPH degradation efficiency of the three treatments were significantly increased after four weeks of supplementing nutrients, which indicated that the slower rate of bioremediation during the fourth to sixth weeks was mainly due to the lack of nutrients in the soil. Therefore, microorganisms required far more nitrogen than phosphorus to proliferate, and different conditions at oilfields may modify the active microbial community (Liu et al. 2016a, 2016b).

The TPH degradation efficiency of SA-AC immobilized beads was always higher than that of SA-S immobilized beads during the first ten weeks, indicating that the improvement of biodegradation rate of activated carbon was better than that of sawdust. But the TPH degradation efficiency of SA-AC immobilized beads in aquatic environment was lower than that of SA-S immobilized beads. On the degradation, it shall be point out that the mechanisms of degradation processes shall be focused sharply to advance the knowledge on this topic and to provide substantial analysis of the stoichiometry (Gu, 2016).

4 Conclusion

In this study, the bacterial community structures of MCA and MCB, cultured with petroleum and sucrose as carbon sources were analyzed by high-throughput sequencing, respectively, which showed that MCB had a potential for a wide substrate degradation range of petroleum hydrocarbons. A series of immobilized beads were prepared by immobilizing MCB cells with the carrier, and the effect of the addition of adsorbent carrier on the internal structure of immobilized microspheres was analyzed by SEM. SEM results showed that immobilized microspheres had different porous structures by mixing different adsorbent carriers. Experiments on petroleum degradation of immobilized beads in aquatic environment showed that the TPH degradation efficiency of immobilized beads was higher than that of microbial culture. The addition of adsorbent carriers accelerated the degradation of petroleum hydrocarbons by immobilized beads. The SA-S immobilized beads showed highest TPH degradation efficiency in MSM up to 54.2% for 7 d of biodegradation. And the SA-CS immobilized beads showed highest TPH degradation efficiency in AS up to 50.5% for 7 d of biodegradation. The TPH degradation efficiency of Biostimulation + SA-AC immobilized beads treatment for petroleum-contaminated soil up to 63.7% after ten weeks of bioremediation. Microcosm bioremediation experiments in different oil-contaminated environments indicated that the degradation efficiency of immobilized beads was affected by the type of oil-contaminated environment. This work proposed a feasible bioremediation strategy and built a foundation for field application in different oil-contaminated environments.

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