Use of bacteria-immobilized cotton fibers to absorb and degrade crude oil

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**A B S T R A C T**

Using enrichment culture technique, two isolates that brought a significant degradation and dispersion of crude oil were obtained from contaminated sediments of the Bohai Bay, China. 16S rRNA gene sequencing and phylogenetic analysis indicated that the two bacterial strains affiliated with the genera \textit{Vibrio} and \textit{Acinetobacter}. Subsequently, the bacterial cells were immobilized on the surface of cotton fibers. Cotton fibers were used as crude oil sorbent as well as a biocarrier for bacteria immobilization. Among the two isolates, the marine bacteria \textit{Acinetobacter} sp. HCB-3S showed a strong binding to the cotton fibers, possibly enhanced through extracellular dispersant excreted by \textit{Acinetobacter} sp. HCB-3S. Both planktonic and immobilized bacteria showed relatively high biodegradation (>60\%) of saturated hydrocarbons fraction of crude oil, in the pH range of 5.6--8.6. The degradation activities of planktonic and immobilized bacteria were not affected significantly when the NaCl concentration reached 70 g/L. The immobilized bacterial cells exhibited an enhanced biodegradation of crude oil. The efficiency of saturated hydrocarbons degradation by the immobilized bacterial cells increased about 30\% compared to the planktonic bacterial cells.

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1. Introduction

Oil pollution originated from both natural and anthropogenic sources can have dramatic detrimental effects to the environment. Large amount of crude oil entering marine, groundwater, soil and other environment could cause significant damages to resident organisms. Recent disastrous oil spills, e.g., Deepwater Horizon oil spill in Gulf of Mexico in 2010 and Penglai 19-3 oil spills in Bohai Bay (China, 2011), become one of the major sources of oil pollution in the ocean. Cost-effective and environmentally benign strategies are urgently demanded for cleaning up spilled oil. Many techniques are utilized to mitigate and cleanse crude oil pollution in the environment (Obuekwe and Al–Muttawa, 2001). Conventional physical and chemical methods could rapidly remove the majority of leaked oil, but in most cases, the removal just transfer contaminants from one environment medium to another, even produce toxic by-products. More importantly, crude oil could not be completely cleaned up by physical and chemical methods (Gavrilescu, 2010).

Bioremediation is a technique utilizing biological organisms to aid in removal of hazardous substances from polluted area (Head et al., 2006). Biological treatments have been used to treat crude oil spills (Gentili et al., 2006), microorganisms are key players in the process (Röling et al., 2004). Compared to the planktonic bacteria, immobilized bacteria could not only stay away from predators and natural competition with the indigenous microorganisms, but also shield perturbations of environmental conditions, such as toxic compounds (Wang et al., 2012). Otherwise, in an open water system, immobilization also prevents bacteria from being washed away (Rahman et al., 2006). Many immobilized microorganisms have been successfully used for bioremediation of crude oil (Liang et al., 2009). Considering the broad choices, low cost, simple process and less impact on microbial activity (Oh et al., 2000; Lee et al., 2010), immobilization have been proved to be an effective strategy to apply functional microorganism for bioremediation (Wang et al., 2007; Mollaie et al., 2010).

Herein, untreated cotton fibers were selected as a crude oil sorbent as well as a biocarrier for bacteria immobilization. Two high efficient crude oil-degrading bacterial strains were immobilized on the cotton fibers under mild conditions. Oil degrading efficiency of immobilized and planktonic bacterial cells was compared subsequently. The biodegradation rate of saturated
hydrocarbons by planktonic and immobilized bacterial cells was analyzed using gas chromatography (GC).

2. Experimental

2.1. Isolation, identification of oil-degrading bacteria

The crude oil contaminated sediment was collected from Bohai bay, China. The isolation of oil-degrading bacteria was conducted as follows. 10 g of sediment samples and 1 g of crude oil were co-incubated in 100 mL mineral solution (7.01 mM K2HPO4, 2.94 mM KH2PO4, 0.81 mM MgSO4·7H2O, 0.18 mM CaCl2, 1.71 mM NaCl) for 7 days with shaking at 180 rpm and 30 °C. One milliliter of liquid culture was tenfold sequentially diluted to 10−2 and 100 μL aliquots of each dilution were spread onto mineral agar which was prepared by adding 15% agar into mineral solution. The plates were Incubated at 30 °C for 48 h. Morphologically distinct colonies were streaked on mineral agar supplemented with crude oil as a sole source of carbon for purification. Single colony of each isolate was transferred to 10 mL of mineral solution. Aliquots (1.8 mL) of liquid culture were used for DNA extraction using ultra-clean microbial DNA isolation Kit (MoBio Laboratories, Carlsbad, CA) while 1 mL residual was cryopreserved at −80 °C with 1 mL of 60% glycerol. The 16S rRNA gene of each isolate was PCR amplified and sequenced using universal primers 27F (5'–AGAGTTT-GATCMTGGCTCAG–3') and 1492R (5'–CGYTACCTGTTAGCTT–3') as described previously (Enticknap et al., 2006). Sequences were analyzed using the BLASTn tool at the National Center for Biotechnology Information (NCBI) website. Isolates were presumptively identified according to the identity of the closest cultured relative in the top BLAST hits.

2.2. Pretreatment of cotton fibers

Cotton fibers (absorbent, Taizhou Xinkang Medical Materials Co., Ltd., Jiangsu, China) were selected as biocarrier because of their large surface area and high absorption capacity. In order to remove potentially toxic compounds, the cotton fibers were immersed into chloroform for 3 days, rinsed with distilled water and dried at room temperature.

Crosslinking cotton fabric containing carboxyl groups were prepared according to previous literature (Ibrahim et al., 2007). Cotton fibers were immersed into 100 g/L of 1,2,3,4-butanetetracarboxylic acid (BTCA) solution. After drying at 85 °C for 30 min, the treated cotton fibers were washed by 50 °C distilled water to remove excess reactants for 10 min. Carboxyl groups which from the crosslinked cotton fibers were activated by immersing into a 1-cyclohexyl-3-(2-morpholinoethoxy)-carbodiimide-p-toluen-sulfonate (CMTS) solution at 45 °C overnight. Followed by thoroughly rinsing, cotton fibers were immersed in an alkaline polyethyleneimine (PEI) and diethyamine (DEA) solutions for 24 h, respectively. Subsequently, the cotton fibers were dipped into a fixing agent containing glutaraldehyde solution (2%) for 2 h, washed with distilled water and dried.

2.3. Immobilization of oil-degrading bacteria

The functional bacteria were incubated in 50 mL Zobell 2216E medium at 30 °C, 180 rpm for 12 h first. Then, aliquots (1 mL) of two bacterial cultures were inoculated into 100 mL Erlenmeyer flasks, respectively. The PEI-treated, DEA-treated and untreated cotton fibers were used as biocarriers. The flasks containing 50 mL sterilized Zobell 2216E medium with 0.25 g biocarrier in a rotary shaker at 30 °C, 180 rpm for 24 h, which allowed the self-adhesion of the two bacteria strains. Cotton fibers immobilized with functional bacteria were dehydrated with a series of ethanol solutions (30–100%) and dried. Then, the bacteria were fixed with 2.5% (w/v) glutaraldehyde solution at 4 °C for 12 h and washed twice with phosphate buffered saline (PBS, pH = 7.4) for 10 min (Khondee et al., 2012). Scanning electron microscopy (SEM) was used to observe the immobilized bacteria on cotton fibers.

2.4. Biodegradation of crude oil by immobilized and planktonic bacteria

The crude oil which used in studying the aerobic biodegradation is from Shengli oil field, Dongying, China. Microbial degradation of crude oil was compared between planktonic and immobilized bacteria. The combination (0.25 g cotton fibers and 1.14 × 1010 cfu bacterial culture) were mixed with crude oil (0.5 g) placed into a 100 mL Erlenmeyer flask with 50 mL sterilized nutrient supplements. Control flask was set up with co-incubation of crude oil (0.5 g), bacterial culture (1.14 × 1010 cfu) and nutrient supplements without cotton fibers. The experiments were carried out at 30 °C in a rotary shaker (180 rpm). The reaction was maintained a pH of 7.6 throughout the entire experiment.

2.5. Effect of pH and sodium chloride concentration on crude oil biodegradation

The effect of pH on biodegradation was assessed by modifying pH values in medium to 4.6, 5.6, 6.6, 7.6, 8.6 and/or 9.6 individually, and pH was adjusted using either NaOH or HCl solutions. The influence of NaCl concentration on crude oil biodegradation was investigated by using different NaCl concentrations set at 30, 40, 50, 70, 90, 120 g/L, respectively. After 5 days’ co-inoculation with crude oil at 30 °C and 180 rpm, the biodegradation efficiency of planktonic and immobilized bacteria was measured.

2.6. Crude oil extraction and components analysis

The 4 components (saturated hydrocarbons, aromatic hydrocarbons, the more polar, non-hydrocarbon components, and asphaltene) of crude oil were separated using typical column chromatography (Head et al., 2006). The residual oil was extracted from the cultures with 10 mL n-hexane for three times. The extracts were collected and dried in anhydrous sodium sulfate (Bost et al., 2001). After being concentrated with vacuum rotary evaporation, the extracts were fractionated by column chromatography. Multilayer column was carried out as following from the bottom: 3 g silica gel (activated at 110 °C for more than 4 h), 2 g alumina (activated at 400 °C for more than 4 h) and 1 g sodium sulfate anhydrous (activated at 400 °C for more than 4 h). Subsequently, the column was applied to separate components of the extracts crude oil. The saturated hydrocarbons were eluted under gravity with 25 mL n-hexane, aromatic hydrocarbons with 15 mL n-hexane and dichloromethane (v/v = 1:2) mixture, and polar compounds with 20 mL methanol (Bastow et al., 1999). Asphaltene was precipitated in n-hexane before washing. The saturated hydrocarbons of extracted oil hydrocarbons were analyzed by GC (Agilent 7890A) with a capillary column (HP 5 model, 30 m long × 0.32 mm diameter × 0.25 μm thick). The GC condition were as follows: injector temperature was 280 °C; column temperature was 80 °C for 2 min, with a ramp to 280 °C at a rate of 5 °C/min at 280 °C maintained for 20 min; detector temperature was 300 °C. The carrier gas (nitrogen) flow rate was 2 mL/min.
3. Results and discussion

A total of 20 bacterial strains were isolated from crude oil-contaminated sediment in Bohai bay by a conventional enrichment experiment using crude oil as a sole source of carbon. Among those, two strains, designated as HC8-3B and HC8-3S, showed dramatic degradation in 7 days. 16S rRNA gene sequences indicated that the two strains affiliated into the genera Vibrio (accession no. Cj11052) and Acinetobacter (accession no. KC210860), respectively.

Cell immobilization through adsorption provides a direct contact between nutrients and the immobilized cells (Klein and Ziehr, 1990). Adsorption capacity and strength of binding are believed to be two major criteria for the selection of suitable supporting material (Hsu and Lo, 2003). Fibrous materials offer advantageous properties, such as low cost, maximum loading, durability and nontoxicity (Hsu et al., 2004). The immobilization of HC8-3S in the PEI-treated, DEA-treated and untreated cotton fibers was confirmed by SEM and no visible bacteria of HC8-3B were appeared (data not shown). There was no significant difference between the amount of the immobilized bacteria through treated and untreated cotton fibers. Therefore, in our study, the untreated absorbent cotton fibers were selected for bacteria immobilization.

Fig. 1 shows SEM of the two bacterial strains immobilized onto the surface of cotton fibers after washing with phosphate buffer solution. Fig. 1a revealed that HC8-3B was attached on the bio-carriers, which was thought to be washed away by phosphate buffer solution. In comparison, a sufficiently strong interaction was detected between the bacterial strain HC8-3S and the cotton fibers, after washing with phosphate buffer solution. The coherent mass of HC8-3S grown on the carrier surface, and the inter-connection between individual bacterial cells, suggested that HC8-3S was likely to produce extracellular polysaccharides. The SEM images shown in Fig. 1b revealed that the bacteria synthesized strands of exopolysaccharide fibers with which they attach to the substrata. The incubation of the planktonic bacteria with the biocarriers was necessary for the visualization of exopolysaccharide synthesis, and the production of exopolysaccharide during incubation enhanced the adhesion of immobilized bacterial cells to carrier materials (Obuekwe and Al-Muttawa, 2001). In addition, the high surface roughness of cotton fibers was considered to be an important factor for cell immobilization (Hsu et al., 2004). The formation of multi-cell biofilm has been observed in the previous study by Yang et al. (1998), which is a popular technique used to achieve better stability and maintain biological activity (Castorena et al., 2008).

HC8-3S was chosen to investigate the biodegradation of crude oil by immobilized and planktonic bacteria. After 5 days of incubation, compared with the control flask, the crude oil adsorbed on cotton fibers in the flask filled with the biocarriers, which indicated cotton fibers not only act as a bacterial carrier, but could also absorb crude oil (Ibrahim et al., 2007).

The effect of pH on the biodegradation of saturated hydrocarbons fraction of crude oil by planktonic and immobilized bacteria was investigated at 30 °C in a rotatory shaker (180 rpm). The immobilized bacteria showed a higher degradation rate than that of planktonic bacteria (Fig. 2). In the pH range of 5.6–8.6, the degradation rate of both planktonic and immobilized bacteria showed no obvious change, which maintained above 60%. The highest degradation rate was detected at pH 7.6. When pH was reduced below 5.0, planktonic bacteria showed degradation efficiency lower than 10%, this indicated that the degrading activity of bacteria was inhibited. In contrast, the immobilized bacteria remained a degradation rate about 30%. Overall, pH could affect the biodegradation of the crude oil. The immobilized bacteria have better tolerance ability to acid conditions than planktonic bacteria (Wang et al., 2007).

The biodegradation of crude oil at different values of salinity were investigated in medium with addition of sodium chloride (30–120 g/L). As illustrated in Fig. 3, when the concentration of NaCl was in the range of 30–70 g/L, immobilized bacteria exhibited a higher degradation rate of saturated hydrocarbons fraction, and the degradation rate by both planktonic and immobilized bacteria decreased with increasing of the concentration of NaCl. When the NaCl concentration reached 120 g/L, the planktonic and immobilized bacteria nearly lost all degradation activity (degradation rate lower than 6%), which demonstrated that high salty could inhibit the degrading activity of bacteria. It is interesting to note that the biodegradation rate of saturated hydrocarbons by immobilized bacteria declined sharply when the concentration of NaCl higher than 90 g/L, while planktonic bacteria still maintained a degradation efficiency over 70%. To our knowledge, cotton fibers carry a negative charge, which attribute to the presence of hydroxyl and some carboxylic acid groups (Stan-Kleinschek and Ribbitsch, 1998; Rattanaphani et al., 2007). Upon increasing the concentration of NaCl, the negative charge on the surface of cotton fibers is neutralized through electrostatic interactions by the Na⁺ which distribute between the external solution and internal solution. Due to the adsorbed Na⁺ on the cotton fibers, the concentration of NaCl in internal solution could be higher than that of external solution (Jain et al., 2003), this could be the reason why the degradation efficiency of immobilized bacteria obviously decreased when the NaCl concentration at 90 g/L.

Gas chromatography was used to determine the bacterial biodegradation of the saturated hydrocarbons. The gas chromatograms of the saturated hydrocarbons biodegradation by immobilized bacteria compared with that of planktonic bacteria were shown in Fig. 4. The results indicate that a significant decrease of saturated hydrocarbons occurred during the biodegradation process, and a notable decrease of the n-alkane fractions was observed.

Fig. 1. SEM showing the typical adhesion of the bacterial isolates HC8-3B (a) and HC8-3S (b) to the surface of cotton fibers after 24 h incubation.
after 2 days of biodegradation. With increased biodegradation time, the GC results showed that immobilized bacteria were capable of degrading more of saturated hydrocarbons than the suspended planktonic bacteria. The oleophilic and hydrophilic properties of cotton fibers allowed the contact surface between the crude oil and the immobilized bacterial cells to expand greatly (Mehdi and Giti, 2008), which resulted in more exposure of the absorbed crude oil to the immobilized bacteria. Through the expanded contact areas between oil and bacteria, the rate of biodegradation could be increased. Adherence of hydrocarbons to the bacteria coated bio-carriers is a simple way to boost the substrate uptake speed (Lawnickz et al., 2011).

In general, saturated hydrocarbons of crude oil are more easily to be degraded, while the branched alkanes, pristane (Pr) and phytane (Ph) are more resistant to biodegradation than saturated hydrocarbons, which attribute to the branched alkanes are less vulnerable to microbial attack (Röling et al., 2003). The ratios of $n$-C$_{17}$/Pr (n-heptadecane/pristane) and $n$-C$_{18}$/Ph (n-octadecane/phytane) have been known as reliable indicators of biodegradation, with the decreasing values indicating enhanced biodegradation (Asif et al., 2009). For evaluating the degree of biodegradation, the ratios of $n$-C$_{17}$/Pr, $n$-C$_{18}$/Ph and Pr/Ph were determined. The ratios in Table 1 showed a trend consistent with GC analysis. Comparing the biodegradation of the immobilized bacteria and planktonic bacteria, the ratio of $n$-C$_{17}$/Pr and $n$-C$_{18}$/Ph showed significant differences. Initially, planktonic bacteria showed a higher-efficiency of degradation. The ratios changed from 1.19 to 0.625 for $n$-C$_{17}$/Pr and from 0.547 to 0.285 for $n$-C$_{18}$/Ph. In comparison, the ratios of $n$-C$_{17}$/Pr and $n$-C$_{18}$/Ph changed to 0.925 and 0.426 by degradation of the immobilized bacteria, respectively. Bacteria grew on the surface of cotton fibers first, and the amount of suspended bacteria in the flask was lower than that of the planktonic bacteria flask, this might cause the low degradation efficiency of immobilized cells. With increased incubation time, the results demonstrated a significant enhancement in the biodegradation of saturated hydrocarbons with the immobilized bacteria. This might attribute to the absorption of crude oil by cotton fibers, and higher contact areas of oil and bacteria could enhance the biodegradation. After 5 day incubation, the ratios of $n$-C$_{17}$/Pr and $n$-C$_{18}$/Ph decreased to 0.046 and 0.019 by degradation of the immobilized bacteria, this revealed most of saturated hydrocarbons fraction of crude oil degraded. The degradation efficiency of planktonic bacteria (0.212 and 0.089 for $n$-C$_{17}$/Pr $n$-C$_{18}$/Ph, respectively) was lower than that of immobilized bacteria. With the oil adsorbing property of cotton fibers, the immobilized bacteria on cotton fibers could be employed to improve the biodegradation of floating oil from oil spills.

4. Conclusion

In our study, two bacterial strains identified as Acinetobacter sp. HC8-3S and Vibrio sp. HC8-3B, were isolated from oil contaminated sediment collected from Bohai bay. Bacterial cells were successfully

![Fig. 2. Effect of pH on biodegradation of planktonic and immobilization bacteria. Bacteria were grown at 30 °C for 5 days with shaking at 180 rpm in liquid medium (concentration of NaCl was 30 g/L).](image1)

![Fig. 3. Effect of NaCl concentration on biodegradation of planktonic and immobilization bacteria. Bacteria were grown at 30 °C for 5 days with shaking at 180 rpm in liquid medium (pH 7.6).](image2)

![Fig. 4. Biodegradation rate of the saturated hydrocarbons by planktonic and immobilized bacteria on cotton fibers.](image3)

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<th>Table 1</th>
<th>n-alkane and regular isoprenoid ratios of the analyzed crude oil samples by biodegradation for 0, 1, 2, 3, 5 days. F: free bacteria cells, M: immobilized bacterial cells, $n$-C$<em>{14}$: n-heptadecane, $n$-C$</em>{16}$: n-octadecane, Pr: pristane, Ph: phytane.</th>
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<td>Ori</td>
<td>1.19 0.625 0.925 0.488 0.481 0.308 0.240 0.212 0.046</td>
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<tr>
<td>n-C$_{17}$/Pr</td>
<td>0.547 0.285 0.426 0.221 0.212 0.138 0.106 0.089 0.019</td>
</tr>
<tr>
<td>n-C$_{18}$/Ph</td>
<td>0.503 0.498 0.485 0.494 0.447 0.465 0.442 0.461 0.444</td>
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immobilized on the surface of cotton fibers for biodegradation of crude oil. The crude oil biodegradation efficiency of the bacteria strain immobilized onto the cotton fibers was higher than that of the planktonic bacterial cells. The biodegradation rates of both planktonic and immobilized bacteria were above 60% with the pH ranged from 5.6 to 8.6, and the degradation activity was not affected significantly when the NaCl concentration reached 70 g/L. Cotton fibers were proved to be a useful crude oil adsorbent and good bacterial biocontainers. Bacteria were attached onto the surface of cotton fibers probably through extracellular polysaccharides produced by HC8-3S. Further experiments are being conducted to elucidate the role of extracellular polysaccharides during the immobilization process of HC8-3S.

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References


