Isolation, screening, and crude oil degradation characteristics of hydrocarbons-degrading bacteria for treatment of oily wastewater

Xiumei Tian, Xiaoli Wang, Shitao Peng, Zhi Wang, Ran Zhou and He Tian

ABSTRACT

The aim of this study was to isolate hydrocarbons-degrading bacteria for treatment of oily wastewater from long-standing petroleum-polluted sediments in Bohai Bay, China. Six hydrocarbons-degrading bacteria were screened and identified as *Pseudomonas* sp. and *Bacillus* sp. A new approach using a combination of various bacterial species in petroleum biodegradation was proposed and evaluated for its degradation characteristics. Gas chromatography-flame ionization detection (GC-FID) analysis showed that mixed bacterial agents (YJ01) degraded 80.64% of crude oil and 76.30% of crude oil alkanes, exhibiting good biodegradation effect. Besides, after 14 days of culture, the biodegradation assessment markers, pristane and phytane, showed significant degradation rates of 46.75% and 78.23%, respectively. Kinetic analysis indicated that the degradation trends followed a single first-order kinetics model and the degradation half-life (t_{1/2}) of 15 g/L crude oil was significantly shorter (5.48 days). These results indicated that YJ01 could degrade a wider range of hydrocarbons as well as some recalcitrant hydrocarbon components, and can be applied for bioremediation and treatment of oil-contaminated environment.

Key words | hydrocarbons-degrading bacteria, isolation, oily wastewater, treatment

INTRODUCTION

Petroleum hydrocarbons pollution (PHP) is becoming increasingly severe with the continuously growing demands for oil and other oil-related products. This form of pollution has attracted increasing attention owing to its serious harmful effects on both marine and terrestrial ecosystems (Meng et al. 2016). Numerous activities lead to PHP, including use of cooling and lubricant agents, compressor condensates, metal processing, as well as oil exploitation and transportation (Bayat et al. 2016). Exploitation and processing of oil produce massive amounts of residues and industrial effluents, and oil transportation is usually associated with frequent spillages of crude oil (Deng et al. 2014). Oil-polluted effluents generally come from a wide range of sources and contain numerous toxins, such as polyaromatic hydrocarbons (PAHs), petroleum hydrocarbons, and phenols, which are highly lethal and difficult to degrade (Jin et al. 2017). They inhibit the growth of plants and animals, lead to genetic mutation, and pose health risks to humans. Besides, oil-polluted effluents are rich in oil contents and color and exhibit high chemical oxygen demand (Yakimov et al. 2007; Ghanavati et al. 2008), all of which can significantly damage terrestrial and marine ecosystems.

Recently, oily wastewater has been treated using various methods employing different biological, chemical, and physical technologies. The physical remediation methods include adsorption, air flotation, membrane filtration, etc., whereas chemical methods comprise electrocoagulation, wet oxidation, electrolytic oxidation, ozone catalytic oxidation, etc. (Kumari et al. 2012). Although both physical and chemical methods can be used as a quick solution to treat oily effluents, they are very expensive and can easily produce secondary contamination. Many studies have shown that bioremediation is an effective biological method to clean up oil residues and remediate hydrocarbons in a variety of environments owing to its advantages such as self-stimulation, environment-friendliness, and cost-effectiveness. Moreover, application of appropriate microorganisms to petroleum degradation can result in easy mineralization of
oil-rich toxins to nontoxic materials at a very low cost (Ghoreishi et al. 2017).

Crude oil consists of asphaltenes, resins, aromatics, and alkanes, along with numerous other organic chemicals containing oxygen, nitrogen, and sulfur (Das & Chandran 2011). It is a mixture of various complex and simple hydrocarbons that can be degraded using a number of indigenous microorganisms, each of which could break down particular molecules (Zanaroli et al. 2010). For example, Acinetobacter, Rhodococcus, Bacillus, Pseudomonas, and Sphingomonas have been reported to utilize saturated hydrocarbons (n-alkanes) during petroleum oil degradation (Liu & Liu 2011; Wang et al. 2011; Hassanshahian et al. 2012; Hassanshahian et al. 2014a). However, the efficiency of petroleum hydrocarbons biodegradation has been noted to significantly differ with different bacterial genera, presenting low biodegradation efficiency (Varjani 2017). One of the reasons for this difference and low degradation efficiency is the complex composition of petroleum hydrocarbons (Khan et al. 2018), indicating that petroleum biodegradation by simple bacterial genera may not be suitable for practical applications. Recently, a new approach using a combination of various bacterial species in petroleum biodegradation was proposed. This technology can generally present higher degradation efficiency in petroleum hydrocarbons remediation owing to broader enzymes activities (Hassanshahian et al. 2014b), and has been successfully applied in remediation of petroleum hydrocarbon contaminated soil (Wu et al. 2017). However, only limited relevant information is available on the remediation of petroleum hydrocarbon contaminated water in bay areas.

Bohai Bay is a semi-enclosed bay located in the western Bohai Sea in northern China. This bay is the only recipient of approximately one billion tons of wastewater from Beijing, Tianjin, and Hebei Province in China (Sun et al. 2018). Owing to the presence of high concentration of petroleum hydrocarbons in the effluents, such discharge may increase the petroleum hydrocarbons content in the seawater of Bohai Bay. Petroleum hydrocarbons have been the main pollutants in Bohai Bay, causing severe risk to the marine ecosystem (Li et al. 2010; Peng 2015). To decrease the concentration of petroleum hydrocarbons in Bohai Bay waters, it is necessary to control the discharge of wastewater containing petroleum hydrocarbons. One of the most efficient ways to decrease petroleum hydrocarbons pollution in Bohai Bay is microbial degradation of petroleum hydrocarbons wastewater before being discharged into the bay.

The present study was conducted to isolate and screen petroleum-degrading bacteria from oil-contaminated sediments of Bohai Bay, and use them to treat the oily wastewater. These petroleum-degrading bacteria can utilize oil as the only energy and carbon source. The aims of this study was to (1) reveal the biodegradability of oil through the preparation of mixed bacterial agent involved in oil degradation and (2) analyze the petroleum hydrocarbons degradation characteristics of the mixed bacterial agent on a laboratory scale. The results obtained could be useful to understanding the abilities of petroleum-degrading indigenous strains in remediating sediments and oil-polluted water of Bohai Bay.

**MATERIALS AND METHODS**

**Samples, media, and culture**

Samples of oil-polluted sediment were collected from the Tianjin coast of the Bohai Bay, China (39°85′N, 117°49′E). The sampling site is located near the Tianjin Port and comprises some wastewater discharge outlets. The surface sediment was collected by using a sterile knife, transported to the laboratory, and stored at 4 °C. The Iraqi Basra crude oil, which is dark brown and thick, was employed in this study.

Mineral salt medium (MSM) contained the following: distilled water (1 L), trace element mixture (5 mL), NH4NO3 (1.0 g), CaCl2 (0.02 g), MgSO4 (0.05 g), K2HPO4 (1.0 g), and KH2PO4 (1.0 g). The mixture of trace elements (pH 7.0) comprised the following: CaCl2 (2 mg/L), FeCl3·6H2O (0.5 mg/L), MnCl2·4H2O (0.5 mg/L), ZnSO4·7H2O (10 mg/L), and CuSO4 (0.5 mg/L). For solid medium, bacterial agar (20 g/L) was added to the MSM. All the media were autoclaved at 121 °C for 30 min. A rotary shaker was used to incubate the liquid culture at 170 rpm, and both solid and liquid cultures were incubated at 30 °C.

**Isolation and selection of crude-oil-degrading bacteria**

A total of 10 g of contaminated soil samples were soaked in 250-mL conical flask containing sterile water (100 mL). After adequate shaking and blending, the flask was allowed to stand for 20 min, and the supernatant (10 mL) was extracted, added into MSM (100 mL) containing 0.5% (w/v) crude oil (Iraq Basrah crude oil) as the only energy and carbon source, and incubated for 7 days at 30 °C and 170 rpm in a rotary shaker. Subsequently, the supernatant (10 mL) was again extracted and added into fresh medium containing 1.0% (w/v) crude oil. After a series of three
further subcultures, the crude oil content was increased by a 0.5-g gradient. Then, tenfold serial dilution was performed with 1 mL of the liquid culture to a concentration of $10^{-7}$, and 100 mL of the dilution aliquots were inoculated onto MSM plates and incubated at 30 °C. After 48 h of incubation, colonies with phenotypic differences were isolated by streaking onto fresh MSM plates, and then transferred into fresh oil-containing medium. This procedure was repeated until colonies that showed obvious tendency to grow were obtained. These colonies were transferred into fresh MSM and then stored on Luria–Bertani (LB) agar slant in a fridge at −20 °C (Cappello et al. 2012).

**Identification of petroleum-degrading bacteria**

Optical microscopy was used to examine the morphological properties of the isolated colonies after incubation for 24–48 h on LB agar slant. Subsequently, systematic analysis of the isolated strains was conducted based on physiological and biochemical characteristics in accordance with Bergey’s Manual for Determinative Bacteriology and related studies (Holt et al. 1998; Chettri et al. 2016; Mulet et al. 2018). Taxonomic characterization of the isolated bacterial strains was performed by 16S rDNA sequencing analysis. For molecular identification of the isolated strains, polymerase chain reaction (PCR) amplification of 16S rDNA universal forward primers (5’-CCCCACACATGACCTACC-3’) and 16S universal reverse primer (5’-CTACTCCAGGGCTTGTAGT-3’) was conducted. A detailed description of all the methods employed in this study has been provided elsewhere (Sarkar et al. 2017). The PCR conditions were as follows: initial denaturation for 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min, and final extension at 72 °C for 5 min. The PCR products were purified in 1% agarose gel by PCR purification kit (Takara Biotechnology, Dalian, China) and sent for automated sequencing (Takara Biotechnology). The BLAST tool of NCBI was used to match the DNA sequences with those in the database. A phylogenetic tree was constructed by neighbor-joining method using MEGA 5.0 software (Liu et al. 2016a).

**Preparation of mixed bacterial agents**

We selected highly efficient oil-degrading strains with higher environmental tolerances, which we considered to be promising to produce good results in the test. We then established different mixed bacteria agents and tested their degradation effects on 1.5% (w/v) crude oil. We opted for agents with the best degradation effects for subsequent test. The isolated individual colonies were inoculated into LB liquid medium and cultivated overnight at 180 rpm and 30 °C in a shaker. By using a dilution plate method, the bacterial solution was spread onto LB plate, and the number of colonies was recorded. This procedure was repeated until more than $10^8$ CFU/g was achieved. The same quantity of each strain was mixed to prepare the bioremediation reagent.

**Growth and crude oil removal analysis**

A total of 5% (v/v) of bacterial suspension was inoculated into 1.5% (w/v) crude oil medium and incubated at 30 °C and 180 rpm in a shaker for 14 days. As a control, sterile medium was employed. After incubation, UV-visible spectrophotometry (UV-9600, USA) was used to determine the optical density of the culture at 600 nm. The remaining crude oil was dissolved in dichloromethane (DCM) and the optical density of the oil extracts was ascertained at 230 nm to determine crude oil removal. Oil degradation was calculated using Equation (1), and the experiment was performed in triplicate.

$$C_d = 1 - \frac{C_a}{C_O} \times 100\%$$  (1)

where $C_O$, $C_a$, and $C_d$ represent the concentration of oil hydrocarbons in the control, concentration of oil hydrocarbons in the sample culture, and rate of oil degradation, respectively.

**Crude oil degradation kinetics**

Previous studies have indicated that kinetics models could be applied to measure the degradation kinetics of bacteria (Dai et al. 2011). In the present study, the chemical kinetic model for crude oil degradation was based on Equations (2)–(4):

$$n = 1: \ln \frac{S}{S_0} = -kt$$  (2)

$$n = 2: \frac{1}{S} - \frac{1}{S_0} = kt$$  (3)

$$n = 3: \frac{1}{S^3} - \frac{1}{S_0^3} = 2kt$$  (4)

where $S$ is the mass concentration of crude oil as a function of time, $S_0$ is the initial mass concentration of crude oil, and $k$ is the rate constant of degradation.
Gas chromatography analysis of the residual crude oil

The biodegradation and removal of hydrocarbons were accurately estimated using gas chromatography-flame ionization detection (GC-FID). First, several CH2Cl2 phases of same volume were respectively added into each flask. After that, extraction of the residual crude oil was performed and an adequate amount of Na2SO4 was used for removing the remaining water. Column chromatography was applied to achieve fractionation of the extractive components. Bottom-up was silica gel (3 g), which was activated at 145 °C for more than 8 h, and alumina (2 g), which was activated at 450 °C for more than 4 h. The activated silica gel was used to pack the glass column of 9 cm in length and 0.9 cm in internal diameter until the mark of 9 cm. Polar compound removal was achieved by passing the alumina through the column. HPLC-grade hexane was used for sample elution in triplicate (Mukherji et al. 2004; Liu et al. 2016b). GC-FID was applied to analyze the extractive composition under the following operating conditions: initial temperature was kept at 60 °C for 2 min, increased to 150 °C at a gradient of 10 °C/min and maintained for another 2 min, and again raised to 180 °C at the rate of 5 °C/min and maintained for another 2 min to 15 °C. The rate of min rose to 290 °C with the rate of flow at 1.0 mL per minute. The inlet temperature was kept at 260 °C, manual injection volume was 5.0 μL, and split ratio was 100:1. Quantitative analysis of the mass fraction of n-alkanes was performed by the method of peak area normalization using the following Equation (5):

\[ C_i = \frac{A_i}{\sum A_i f_i} \times 100\% \] (5)

where \( C_i \) is the mass fraction of a hydrocarbon component expressed as percentage, \( A_i \) is the peak area of a hydrocarbon component, and \( f_i \) is the mass correction factor for a hydrocarbon component.

RESULTS

Isolation and identification of oil-degrading bacteria

The growth and isolation of oil-degrading bacteria with morphological differences were performed on MSM containing crude oil as the only energy and carbon source. A total of six pure cultures, which could utilize crude oil as the sole carbon and energy source, were isolated on the LB agar slants under similar conditions. These six strains were respectively labeled as HDB-1–HDB-6 (Table 1). First, classic biochemical tests were applied to identifying these six strains, and the results showed that the strains were significantly different from each other. In addition, both six strains were catalase positive, whereas six strains were indole and methyl red negative.

Table 1 | Typical physiological and biochemical characteristics of the six strains

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HDB-1</th>
<th>HDB-2</th>
<th>HDB-3</th>
<th>HDB-4</th>
<th>HDB-5</th>
<th>HDB-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony form</td>
<td>Irregular</td>
<td>Inerratic</td>
<td>Irregular</td>
<td>Inerratic</td>
<td>Inerratic</td>
<td>Irregular</td>
</tr>
<tr>
<td>Cell shape</td>
<td>Rounded</td>
<td>Bacilliform</td>
<td>Bacilliform</td>
<td>Elliptical</td>
<td>Elliptical</td>
<td>Bacilliform</td>
</tr>
<tr>
<td>Colony color</td>
<td>White</td>
<td>Light Yellow</td>
<td>Light Yellow</td>
<td>Light Yellow</td>
<td>Yellow</td>
<td>White</td>
</tr>
<tr>
<td>Smoothness</td>
<td>Rough</td>
<td>Smooth</td>
<td>Rough</td>
<td>Smooth</td>
<td>Smooth</td>
<td>Smooth</td>
</tr>
<tr>
<td>Gram stain</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Hydrolysis of starch</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Nitrate to nitrite</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Citrate test</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin liquefaction</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Indol test</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Methyl red</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Catalase activity</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Symbols: +——growth positive, ———growth negative.
Degradation of the different mixed bacterial strains

In the oil-flask experiments, the degradation performances of the different mixed bacterial strains were tested. The combination of six strains including HDB-1, HDB-2, HDB-3, HDB-4, HDB-5 and HDB-6 exhibited the best degradation performance (Figure 3(a)), all constituting highly efficient crude oil degraders. Combining the antagonism and synergy between the strains, we chose several different combinations for degradation: HDB-1, HDB-2, and HDB-5 belong to *Bacillus* sp., while HDB-3, HDB-4 and HDB-6 belong to *Pseudomonas* sp. They are the highly efficient degrading bacteria that are finally screened out. The mixed bacteria agent (YJ01) could grow under normal conditions and the bacteria density showed the highest OD600 (1.133) after 3 days (Figure 5(b)). In particular, YJ01 showed the highest oil degradation rate (80.64%).

Petroleum degradation kinetics of YJ01

The time course of petroleum degradation at different initial concentrations and microbial growth was evaluated. As illustrated in Figure 4(a), YJ01 exhibited high biodegradation ability for degrading 15, 10, and 5 g/L crude oil, and removed 81.84%, 74.70%, and 73.01% hydrocarbons after 14 days, respectively. The biodegradation effect of YJ01 on different initial concentrations of crude oil was similar (Figure 4(b)). Meanwhile, the time for YJ01 to adapt to the new environment was only 48 h. After this adaptation period, YJ01 exhibited sufficient carbon as well as energy metabolism, thereby rapidly increasing the degradation rate.

The kinetic parameters of oil removal by YJ01 were well-regressed. As shown in Table 2, degradation of crude oil at different initial concentrations by YJ01 followed a single first-order kinetics model. The oil degradation half-life ($t_{1/2}$) of 15 g/L crude oil was found to be 5.48 days, whereas that of 10 and 5 g/L crude oil was 6.63 and 7.65 days, respectively. These findings illustrated that the concentration of YJ01 was higher during growth and metabolism, and the required carbon source content was higher. However, low concentration of crude oil could not support growth of microorganisms, and the degradation rate was significantly slow, whereas the degradation half-life was long. These results established that YJ01 could efficiently degrade a high concentration of oily wastewater.

Alkane biodegradation capacities of YJ01

The ability of YJ01 to degrade n-alkanes (C9–C39) was determined by incubating YJ01 with each n-alkane as the only

Growth rate and crude oil removal efficiency of the isolated strains

Each isolated strain was incubated in a shaker containing 1.5% (w/v) crude oil for 14 days. The growth and crude oil degradation performance of the isolated strains were monitored by UV spectrometry. Figure 2(a) illustrates the growth and crude oil removal efficiency of the isolated strains. The OD600 of all the six strains on the first day was not obvious, which may be owing to adaption of the strains to the new environment. However, after 2 days, the OD600 of all the strains rapidly increased, suggesting that the six strains could degrade crude oil. The crude oil utilization capability of the bacterial isolates was constantly observed. After inoculation of the isolates, the culture started to become turbid and black, and an extensive dispersion of crude oil was noted in the flasks. It appears that the concentration of the bacteria increased to a great extent, as the increasing in the number of bacterial colonies indicated that the six strains inoculated were able to proliferate.

Furthermore, the crude oil was better dissolved in the medium and its content was decreased, resulting in more effective contact with the microorganisms, which accelerated degradation. The UV spectrophotometry was employed to determine the petroleum degradation rate, and the oil degradation rates for HDB-1, HDB-2, HDB-3, HDB-4, HDB-5, and HDB-6 were 45.76%, 40.52%, 57.88%, 43.29%, 54.01% and 49.48%, respectively (Figure 2(b)).

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Alkane biodegradation capacities of YJ01

The ability of YJ01 to degrade n-alkanes (C9–C39) was determined by incubating YJ01 with each n-alkane as the only
carbon source for 14 days under constant shaking (Table 3). The degradation of YJ01 was obvious and rapid when C11, C21, C23, C27, and C29 alkanes were used, whereas the degradation slightly decreased when C12, C14, C22, C30, and C34 alkanes were employed. Nevertheless, the majority of the alkanes were well degraded (23.59–91.35%) by YJ01 (Table 3 and Figure 5). The average crude oil alkanes degradation rate was 26.77% after 7 days. In particular, the
The average degradation rate of short-chain alkanes (C9–C20) was 19.98%, medium-chain paraffins (C21–C26) was 28.41%, and long-chain alkanes (C27–C39) was 31.94%, indicating that the degradation rate of long-chain alkanes was relatively high. However, the degradation rate of alkanes significantly improved with incubation time. When compared with the degradation rate of alkanes after 7 days, the average degradation rate of alkanes after 14 days was as high as 76.30%, exhibiting an increase of 49.53%.

The GC–FID chromatograms for these strains compared to the blank are shown in Figure 6. As shown in this figure, the many peaks of crude oil in the blank were decreased dramatically by strains, furthermore, many of the peaks in the control were not even detected in the experimental group. Figure 6 illustrates the degradation of each alkane by YJ01. It can be inferred from the figure that the alkanes corresponding to the wave peaks that disappeared were degraded by the strains, indicating that YJ01 can effectively degrade crude oil paraffins after 14 days at 30 °C and initial pH of 7.0.

**DISCUSSION**

Petroleum hydrocarbons are the most hazardous environmental pollutants, and will remain as the most important energy and chemical source as well as the most challenging organic pollutants in future (Peterson et al. 2003; Macaulay & Rees 2014; Khan et al. 2018). At present, a green alternative approach based on the principle of petroleum hydrocarbons degradation by indigenous or exogenous microorganisms to bioremediate pollutants and treat them safely is gradually emerging, which is very feasible and necessary for the current technological and social conditions (Frutos et al. 2015). Different kinds of potential strains have been isolated from the specific contaminated environment (Aurepatipan et al. 2012). For instance, eight potent degradative bacterial strains were enriched in the petroleum reservoir wastewater of Tehran and Kerman Provinces of Iran to separate alkane-degrading bacteria, with petroleum hydrocarbons as the sole source of carbon and energy (Hassanshahian et al. 2013). In the present study, we isolated six bacterial strains from oil-polluted sediment of Bohai Bay (Figure 1). These strains were found to degrade 37.88–54.01% of crude oil (Figure 2(b)), indicating their significant potential in degrading petroleum hydrocarbons in water. Some of the previous studies have suggested that bacterial strains can be used to degrade petroleum hydrocarbons. For instance, different
hydrocarbons degradation rates for *Bacillus subtilis* were observed in liquid medium and soil after 14 days (38% and 30%, respectively) (Al-Dhabaan 2012). Nevertheless, the hydrocarbons degradation rate of a single bacterial strain is low, which may be owing to the fact that certain bacteria can only remove specific petroleum hydrocarbons. For example, *thermophilic bacillus*, NG80-2, has been found to degrade only C15–C36 alkanes, and not C8–C14 alkanes and alkanes with a chain length of more than C40 (Sorkhoh et al. 1996). Besides, out of the 28 n-alkanes analyzed (nC8–nC35), four were significantly degraded (nC12–nC15) by *Alteromonas* sp. strain TK-46(2), and another two (nC14, nC15) were degraded by *Cycloclasticus* sp. strain TK-8; the rest of the n-alkanes were either not biodegraded or underwent abiotic degradation (Gutierrez et al. 2018). These results indicated that it was difficult to use a single bacterial strain for bioremediation.

In general, mixed bacterial strains have higher petroleum hydrocarbons degradation rates than single bacterial strains (Van Hamme & Ward 2001; Li et al. 2015; Ghorbannezhad et al. 2018). In the present study, to improve the removal rate of petroleum hydrocarbons in water, we employed mixed bacterial strains isolated from oil-polluted sediment of Bohai Bay. In total, six petroleum-degrading hydrocarbons degradation rates for *Bacillus subtilis* were observed in liquid medium and soil after 14 days (38% and 30%, respectively) (Al-Dhabaan 2012). Nevertheless, the hydrocarbons degradation rate of a single bacterial strain is low, which may be owing to the fact that certain bacteria can only remove specific petroleum hydrocarbons. For example, *thermophilic bacillus*, NG80-2, has been found to degrade only C15–C36 alkanes, and not C8–C14 alkanes and alkanes with a chain length of more than C40 (Sorkhoh et al. 1996). Besides, out of the 28 n-alkanes analyzed (nC8–nC35), four were significantly degraded (nC12–nC15) by *Alteromonas* sp. strain TK-46(2), and another two (nC14, nC15) were degraded by *Cycloclasticus* sp. strain TK-8; the rest of the n-alkanes were either not biodegraded or underwent abiotic degradation (Gutierrez et al. 2018). These results indicated that it was difficult to use a single bacterial strain for bioremediation.

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bacterial strains were screened from the oil-polluted sediment and mixed to prepare a bacterial consortium YJ01, which exhibited a degradation rate of as high as 80.64% (Figure 3(a)). This high crude oil degradation rate may be owing to the complementary effects of the six strains on the metabolic pathways, leading to better degradation of petroleum hydrocarbons and stronger ability to adapt to complex petroleum hydrocarbons wastewater, when compared with individual strains. Effective degradation of pollutants was achieved through symbiosis and synergy among different strains, generating complex microbial flora and significantly improving the available range and utilization efficiency of petroleum-based matrix for the purpose of degradation (Owsianiak et al. 2013). Besides, a combination of bacterial consortia containing various enzymes with strong abilities to degrade complex hydrocarbons is necessary for effective remediation of oil-polluted water. In a study conducted by Ghorbannezhad et al. (Ghorbannezhad et al. 2018), the total petroleum hydrocarbons degradation rates of strains S1, S2, and S3 were 14.28%, 10.68%, and 15.67%, respectively; however, the synergistic effect of the three bacterial strains produced higher total petroleum hydrocarbon degradation (19.59%), which significantly improved crude oil biodegradation, when compared with the use of single strains.

In the present study, the mixed bacterial agent YJ01 also presented significant degradation effect on alkanes. The GC-FID chromatograms (Figure 6(a)–6(c)) exhibited different intensities resulting from time variations in the experimental group, when compared with that in the control. In contrast

<table>
<thead>
<tr>
<th>Alkanes</th>
<th>Carbon number</th>
<th>7 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonane</td>
<td>C9</td>
<td>5.43</td>
<td>23.59</td>
</tr>
<tr>
<td>Undecane</td>
<td>C11</td>
<td>13.53</td>
<td>91.35</td>
</tr>
<tr>
<td>Dodecane</td>
<td>C12</td>
<td>12.21</td>
<td>65.09</td>
</tr>
<tr>
<td>Tetradecane</td>
<td>C14</td>
<td>29.53</td>
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![Figure 5](http://iwaponline.com/wst/article-pdf/78/12/2626/525455/wst078122626.pdf)
to the previous reports suggesting that the effect of n-alkanes biodegradation is quite constrained owing to the unresolved problem of highly complex hydrocarbons mixture (Speight & Arjoon 2012), YJ01 presented high alkanes (C9–C39) degradation efficiency of 23.59–91.35%. It must be noted that the biodegradation effect of YJ01 on alkanes was better than that on other hydrocarbons because of the simplicity of the alkanes structure (Fedorak & Westlake 1981), YJ01 had better biodegradation effect (average of >80%) on medium-chain paraffins (C21–C26), when compared with other long- or short-chain alkanes, because the long-chain alkanes have poor solubility and short-chain alkanes are generally toxic to the degrading microorganisms. It has been reported that the degradation of simple-structured alkanes in crude oil occurs before that of complex-structured alkanes, resulting in higher degradation rates (Nkem et al. 2016). While a low C9 degradation rate could be due to the degradation of complex components (C27–C39), generating poisonous intermediates, Suganthi et al. (Suganthi et al. 2018) used a combination of Shewanalla chilikensis, Bacillus firmus, and Halomonas hamiltonii for the biodegradation of oil sludge, and their results suggested that microbial consortia were capable of degrading hydrocarbons ranging from C14 and C34. This may be related to the degradation pathway of alkanes. Furthermore, to complete the degradation of hydrocarbons, the microbiota often need to oxidize the methyl groups at the ends of the hydrocarbons to form fatty alcohols that are ultimately converted into different types of fatty acids that can be used for degradation by their own transformation or change. These fatty acids can then be utilized by the β-oxidation pathway to degrade the corresponding hydrocarbons, ultimately producing carbon dioxide and water. The two isoparaffins, Pr (2,6,10,14-tetramethylpentadecane) and

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**Figure 6** | Gas chromatograms indicating the variations in the components of crude oil before and after degradation by YJ01 in MSM medium at 30 °C for 14 days. (a) The oil control; (b) the n-alkanes degradation of YJ01 after 7 days; (c) the n-alkanes degradation of YJ01 after 14 days.
Ph (2,6,10,14-tetramethylhexadecane), are commonly used as biodegradation assessment markers (Asif et al. 2009). In the present study, the Pr and Ph degradation rates were significantly higher, reaching 46.75% and 78.23%, respectively. These results indicated that YJ01 could degrade a wider range of hydrocarbons and some recalcitrant hydrocarbon components.

Kinetic modeling plays a critical role in the process of biodegradation. In the present study, the synergistic effect among the six bacterial strains on the bioremediation process was analyzed by kinetic analysis. It must be noted that assessment of kinetic parameters is necessary to understand the biodegradation process in nature. The kinetic equation showed that with the increasing rate of biodegradation, the process of biodegradation was faster and, consequently, the degradation half-life was reduced (Sarkar et al. 2017). In the present study, oil degradation by YJ01 at different initial crude oil concentrations followed a single first-order degradation kinetics model. Furthermore, kinetic analysis of the biodegradation process of YJ01 showed that the degradation half-life (5.48 days) was significantly decreased at high concentrations of crude oil (15 g/L), indicating that the active hydrocarbons-degrading strains had an interactive effect on the existing microbial populations in mitigating oil-polluted wastewater.

Petroleum hydrocarbon pollution in Bohai Bay has severely affected the ecological and environmental security of that region (Chen et al. 2017). Because of the immediate and long-term environmental damages, marine petroleum pollution has been regarded as an increasingly serious international concern. Microbial bioremediation is a useful tool for long-term remediation of petroleum hydrocarbons pollution in Bohai Bay (Li et al. 2016). The findings of this study showed the higher capabilities of different microbial mixed cultures in the degradation of petroleum hydrocarbons, suggesting that the developed bacterial consortium YJ01 has significant potential in petroleum pollution remediation.

CONCLUSION

Six strains presenting with highly efficient hydrocarbons degradation were isolated from heavy oil contaminated soil in Bohai Bay, China, and were identified as Bacillus sp. and Pseudomonas sp. The mixed bacterial agents (YJ01) comprising these strains showed good survival and petroleum-degrading activity (80.64%), indicating synergy among the isolates. Furthermore, YJ01 could degrade high concentration of crude oil (15 g/L) with a short degradation half-life (5.48 days). The strains not only degraded short-chain alkanes, but also more complex long-chain alkanes. Besides, the strains also exhibited higher paraffin (Pr and Ph) degradation activity. Thus, the isolated strains have potential applications in the remediation of crude oil contaminated soil as well as treatment of oily sewage.

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