Enhanced biodegradation of crude oil by constructed bacterial consortium comprising salt-tolerant petroleum degraders and biosurfactant producers

Weiwei Chen\textsuperscript{a,b,c}, Yachao Kong\textsuperscript{a,b,c}, Junde Li\textsuperscript{a,b,c}, Yanyu Sun\textsuperscript{a,b,c}, Jun Min\textsuperscript{a,c,d}, Xiaoke Hu\textsuperscript{a,c,d,*}

\textsuperscript{a} Key Laboratory of Coastal Biology and Bioresource Utilisation, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai, China
\textsuperscript{b} University of Chinese Academy of Sciences, Beijing, China
\textsuperscript{c} Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China
\textsuperscript{d} Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao, China

\begin{abstract}
Bioremediation is an attractive strategy of utilizing bacteria to remove crude oil contaminants. In this study, two salt-tolerant crude oil-degrading and biosurfactant-producing bacteria, Dietzia sp. CN-3 and Acinetobacter sp. HC8–3S, were functionally combined to construct a bacterial consortium. The consortium achieved 95.8\% degradation efficiency of crude oil in 10 days and various \textit{n}-alkanes, cycloalkanes, branched alkanes and aromatic hydrocarbons were all depleted more effectively than single strains. Functional optimization of the consortium degraded crude oil efficiently in a wide range of pH (4–10) and salinity (0–120 g L\textsuperscript{-1}). Furthermore, two alkane hydroxylase genes, \textit{alkB} in CN-3 and \textit{alkM} in HC8–3S, were cloned and their expression were examined by real-time quantitative polymerase chain reaction, indicating that \textit{alkB} was more prominent in long-chain alkanes (C\textsubscript{26}, C\textsubscript{24} and C\textsubscript{23}) utilization and \textit{alkM} played crucial roles in medium- and long-chain alkanes (C\textsubscript{14}, C\textsubscript{16}, C\textsubscript{20}, C\textsubscript{24} and C\textsubscript{26}) degradation. In soil microcosms artificially contaminated with crude oil and bioaugmented with the consortium, 58.3\% of total petroleum hydrocarbons were depleted after 60 days and the degradation rate (485.8 mg kg\textsuperscript{-1} d\textsuperscript{-1}) was higher than those reported in previous studies. Consequently, the consortium is a promising candidate in crude oil bioremediation.
\end{abstract}

\section{Introduction}
Recently, crude oil spills, due to natural or anthropogenic factors, have been bringing about noticeable environmental pollution and sustainability problems (Head et al., 2006; Gu and Wang, 2015). Diverse physical and chemical remediation technologies are available for crude oil cleanup (Wang et al., 2013; Chen et al., 2017; Mapelli et al., 2017), such as different types of booms and skimmers, thermal treatments, natural or synthetic sorbent materials, etc. Furthermore, bioremediation, using microorganisms to degrade crude oil, is considered to be the most thorough way to mineralize crude oil ultimately, which is also more economical, versatile and environmentally friendly than physical and chemical remediation (McGenity, 2014; Wang and Shao, 2014; Cui et al., 2020). In terms of physiology, ecology and biotechnology, bacteria are recognized as the most important microbes due to their universality and prevalence (Rojo, 2009; Xia et al., 2017, 2019). As bioremediation develops, some typical and valuable crude oil-degrading bacteria are drawing people’s attention. For example, \textit{Alcanivorax} and \textit{Acinetobacter} are good at alkanes degradation (Wang and Shao, 2012a, 2014; Lin et al., 2014); \textit{Cycloclasticus} degrades aromatic hydrocarbons with high efficiencies (Teira et al., 2007); \textit{Dietzia} is capable of degrading \textit{n}-alkanes, branched alkanes, as well as aromatic hydrocarbons (naphthalene, phenanthrene, pyrene and fluoranthene) (Wang et al., 2011, 2014; Chen et al., 2017). Up to now, isolating and preserving bacteria which have outstanding hydrocarbon-degrading abilities to adapt to various environments need ongoing efforts.

After oil spills occurred, petroleum hydrocarbons could be depleted through abiotic factors (natural volatilization, oxidation etc.), as well as natural attenuation relying on indigenous microorganisms. However, the removal process is always slow and long (Head et al., 2006; Varjani and Upasami, 2019). The effectiveness of crude oil depletion could be enhanced by bioaugmentation, by introducing enriched single strain or...
and Funhoff, 2005, 2007; Feng et al., 2007; Throne-Holst et al., 2007; Liu et al., 2011; Wang et al., 2014). Notably, based on the universality of Acinetobacter species in crude oil removal (Thompson et al., 2005; Cui et al., 2020). Because of their wider substrate spectra, higher robustness, more complementary metabolic abilities, and better adaptation to complex environments, bacterial consortia are usually more advantageous than single species in crude oil removal (Thompson et al., 2005; Varjani and Upasani, 2019). Both laboratory and field studies had shown that bioaugmentation contributed to improving crude oil degradation. For instance, in a microcosm experiment, five bacterial strains were combined as a consortium to remediate different crude oil-contaminated soil samples, degrading 25.4%–48.1% of petroleum hydrocarbons after 40 d (Bidja Abena et al., 2019a). In a field study, the supplements of microbes enhanced the crude oil removal by 76% compared with 3.6% in control groups (Mukherjee and Bordoloi, 2011).

The petroleum hydrocarbons degradation always relies on some preconditions, among which hydrocarbon molecules entering the bacterial metabolic systems available through cellular uptake or adsorption is the key (Wang and Shao, 2014). Low solubility and weak bioavailability of hydrocarbons always limit their biodegradation by microbes. As surface activity compounds, biosurfactants are composed of hydrophobic and hydrophilic components (Ron and Rosenberg, 2002; Wang et al., 2014). Generally, bacteria produce two kinds of biosurfactants with different chemical compositions and functional features: low-molecular biosurfactants reduce surface and interfacial tension, and higher-molecular bioemulsifiers bind substrates tightly (Ron and Rosenberg, 2002; Xia et al., 2019). Particularly, thanks to low toxicity, high biodegradation efficiency and biocompatibility, biosurfactants are applicable for crude oil pollution bioremediation (Wang et al., 2013, 2014).

Despite the diversities of crude oil origins, alkanes constitute a large fraction in crude oil components. Terminal oxidation pathway of alkane aerobic degradation is known as the most widespread way, which needs different kinds of alkane hydroxylases (Head et al., 2006; Rojo, 2009). Alkane hydroxylases convert alkane to corresponding alkanols, which is considered to be the initial and crucial step in alkane terminal oxidation (van Beilen and Funhoff, 2007). Depending on different action ranges of alkanes, several types of alkane hydroxylases were reported in bacteria, including methane monoxygenase, integral-membrane alkane monooxygenase AlkB-related hydroxylases, soluble cytochrome P450 enzymes, AlnA and LadA, which had been reported in Pseudomonas, Acinetobacter, Alcanivorax, Dietzia and some other bacteria (van Beilen and Funhoff, 2005, 2007; Feng et al., 2007; Throne-Holst et al., 2007; Liu et al., 2011; Wang et al., 2014). Notably, based on the universality of AlkB-related hydroxylases in oil-degrading bacteria, they were often recognized as functional markers to monitor the crude oil degradation capacities (Paise et al., 2011). For example, alkane hydroxylase gene (alkB) was strongly induced by C_{14} C_{16} and pristane by real-time quantitative polymerase chain reaction (RT-qPCR) detection in Dietzia maris As-13-3 (Wang et al., 2014). Additionally, alkB was successfully amplified by PCR from a bacterial consortium, revealing the presence of alkane-degrading enzymes (Xia et al., 2019).

Herein, taking advantages of two salt-tolerant crude oil-degrading bacteria Dietzia sp. CN-3 and Acinetobacter sp. HC8–3S previously isolated from crude oil-contaminated sediments of Bohai Bay, we constructed a bacterial consortium. The aim of our research was to investigate the effects of combining petroleum degraders and biosurfactant producers in crude oil contaminants removal. The specific targets were: (I) to explore the biosurfactants production of CN-3 and HC8–3S and test their effects on the effective bacterial consortium and optimize environmental tolerances (pH and salinity); (II) to assess the petroleum hydrocarbons degradation characteristics by individual strain and consortium; (IV) to detect the alkane hydroxylase genes (alkB and alkM) involved in alkane degradation in single species; (V) to carry out crude oil-contaminant soil microcosms upon abiotic factors, natural attenuation and bioaugmentation. First-order kinetic model was applied to predict the petroleum hydrocarbons degradation in soil microcosms.

2. Materials and methods

2.1. Bacteria, crude oil, media and chemicals

The bacteria studied in this research were Dietzia sp. CN-3 (GenBank accession number K7779094) and Acinetobacter sp. HC8–3S (GenBank accession number MN216306), which were both isolated from the crude oil-contaminated marine sediments of Bohai Bay, China. Detailed information about these two strains in crude oil utilization could also be found in our previous study (Lin et al., 2014; Liu et al., 2016; Chen et al., 2017).

The crude oil used in this study was from Shengli Oilfield in China. The API (American Petroleum Institute) gravity of the crude oil was 25.6 and the viscosity was 4896 mPa s (Chen et al., 2017). The compositions of mineral salt medium (MSM) contain (per liter): 1 g (NH_{4})_{2}SO_{4}, 0.8 g Na_{2}HPO_{4}, 0.2 g KH_{2}PO_{4}, 0.2 g MgSO_{4}-7H_{2}O, 0.05 g CaCl_{2}-2H_{2}O, 0.005 g FeCl_{3}-2H_{2}O, 0.001 g (NH_{4})_{2}MoO_{4}·4H_{2}O and 25 g NaCl, pH 7.2. The medium was sterilized by autoclaving for 20 min at 121 °C. Additionally, lysogenic broth (LB) medium was used for strains enrichment. Analytical-grade chemical reagents were bought from Aladdin Chemistry Co., Ltd (Shanghai, China). The n-tetradecane (C_{14}), n-hexadecane (C_{16}), n-eicosane (C_{20}), n-tetracosane (C_{24}) and n-hexacosane (C_{26}) were purchased from Sigma-Aldrich (USA).

2.2. Biosurfactants production detection of bacteria

2.2.1. Oil spreading assay

Biosurfactants activities could be measured through oil spreading assay (Xia et al., 2019). Specifically, C_{16} (0.1%, v/v) or C_{24} (0.1%, w/v) was selected as sole carbon source to cultivate CN-3 or HC8–3S strain in MSM under 30 °C, 180 rpm for 10 d, respectively. The distilled water (25 mL) was added into the petri dishes and crude oil (10 μL) was supplied to water to form a uniform oil film. The cell-free supernatant (10 μL) was acquired by centrifuging (10,000 rpm, 10 min) and then added onto the center of the oil film layer. Exactly, if there was biosurfactant in the supernatant, the oil film would disperse and formed concentric circles, and the diameters would be positively correlated to the biosurfactants activities. The positive and negative controls were Triton X-100 and distilled water, severally.

2.2.2. Emulsification activity

The CN-3 or HC8–3S was incubated in MSM medium with C_{16} (0.1%, v/v) or C_{24} (0.1%, w/v) under 30 °C, 180 rpm for 10 d, respectively. The MSM media in the same substrates without bacteria were as the negative controls. The bacteria-free supernatant was gained by centrifugation (10,000 rpm, 10 min). To measure the emulsification activity, equal volume of C_{16} and bacteria-free supernatant were put into graduated tubes. After homogenizing at top speed for 2 min with vortex, the mixtures were left for 24 h (Xia et al., 2019). According to the formula, emulsification index (E_{24}) was calculated to represent the emulsification activity:

\[ E_{24} = \frac{\text{Height of emulsion layer (mm)}}{\text{Total height of liquid layer (mm)}} \times 100.\]

Additionally, E_{24} was measured every 2 d to detect the influences of culture time.

2.2.3. Cell surface hydrophobicity

Cell surface hydrophobicity of CN-3 or HC8–3S was measured by bacterial adherence to hydrocarbons test, which was modified from Rosenberg (1984). The single strain was incubated in MSM medium supplemented with C_{16} (0.1%, v/v) or C_{24} (0.1%, w/v) under 30 °C, 180 rpm for 10 d, severally. CN-3 or HC8–3S grown in LB medium was as negative control. The cell pellets were obtained by centrifugation (10,000 rpm, 10 min), washed using PUM buffer (22.2 g K_{2}HPO_{4}, 7.26 g KH_{2}PO_{4}, 1.8 g urea and 0.2 g MgSO_{4} in 1 L distilled H_{2}O, pH 7.2) for two

W. Chen et al.
International Biodeterioration & Biodegradation 154 (2020) 105047

consortia into environments (Thompson et al., 2005; Cui et al., 2020).
times and resuspended in the PUM buffer. The optical density 600 nm (OD_{600}) was adjusted to about 0.6 and recorded as A1. After standardization, 1200 µL of cell resuspension and 200 µL of C_{16} were added to tubes and vortexed at maximum speed for 2 min. The mixture was left for 1 h and OD_{600} of aqueous phase was measured immediately, which was recorded as A2. The cell surface hydrophobicity was quantified using the following equation and bacterial adherence (%) was measured every 2 d:

\[
\text{Bacterial adherence (\%)} = \left(1 - \frac{A2}{A1}\right) \times 100
\]

2.3. Bacterial consortium construction

The CN-3 and HC8–3S strains were both isolated from the petroleum-contaminated marine sediments of Bohai Bay. For enhancement of crude oil biodegradation, CN-3 and HC8–3S, which had different oil-degrading characteristics and biosurfactants production capacities, were selected to construct bacteria consortium. In addition, the tolerances to environmental factors (pH and salinity) were also tested in this section. The pH levels were set up as follows: 4, 5, 6, 7, 8, 9 and 10. The salinities were carried out by a series of NaCl concentrations (0, 20, 40, 60, 80, 100 and 120 g L^{−1}). CN-3 and HC8–3S strains were cultivated individually in LB medium with above-mentioned pH and salinities for 3 d and OD_{600} was tested every 3 h to draw corresponding growth curves. To construct the bacterial consortium, CN-3 or HC8–3S was incubated severally in LB medium for 24 h in a shaker and then harvested by centrifugation and washed with MSM medium for three times. The OD_{600} was adjusted to about 1.0, respectively, and the consortium was mixed with the same volume of individual strain. The colony numbers were evaluated by plate counting method in LB agar plates, which was 2.83 × 10^{10} CFU mL^{−1}. As stated, the growth curves of bacterial consortium were also measured in different pH values and salinities.

2.4. Crude oil biodegradation by single strain and constructed bacterial consortium

The single strain (CN-3 or HC8–3S) and constructed bacterial consortium were cultivated in LB medium, harvested by centrifugation and washed with MSM medium for three times, and subsequently they were cultivated in MSM supplemented with 1% (w/v) crude oil for 10 d, respectively. The MSM media with crude oil excluding bacteria were as the controls. The remaining crude oil was disposed as described previously with minor modification (Chen et al., 2017). The n-hexane was used to extract the total petroleum hydrocarbons (TPH), including alkanes and aromatic hydrocarbons in crude oil. To assess the degradation efficiency, the remaining TPH was analyzed and calculated by GC-MS using the following equation and bacterial adherence (%) was measured.

\[
\text{Bacterial adherence (\%)} = \left(1 - \frac{A2}{A1}\right) \times 100
\]

2.5. Functional optimization of constructed bacterial consortium

Optimization of degradation conditions resulted from different environmental factors, such as pH and salinity, affected the bacteria activity distinctly and had significant effects on crude oil biodegradation. The mixed bacterial consortium was constructed and obtained as section 2.3 described. The effects of pH on crude oil biodegradation were tested in the ranges of pH (4, 5, 6, 7, 8, 9 and 10). Similarly, a series of NaCl concentrations (0, 20, 40, 60, 80, 100 and 120 g L^{−1}) were investigated to assess the salinity influences on crude oil biodegradation. After 10 d of cultivation, residual crude oil was extracted and TPH degradation was analyzed by GC-MS.

2.6. Cloning of alkane hydroxylase genes and sequence analysis

Alkane hydroxylases take effects on the first step of alkane degradation, converting alkanes to corresponding alkanols. The genomic DNA of CN-3 or HC8–3S was extracted by Microbial DNA Isolation kit (USA) and stored at −20 °C freezer. According to the whole genome sequencing annotation information of CN-3 and HC8–3S, we designed primers for alkane hydroxylase genes amplification (Table S1, Supplementary Information). The PCR procedure was conducted with the following settings: 95 °C for 5 min; 95 °C for 30 s, 58 °C for 30 s and 72 °C for 2 min, 30 cycles; 72 °C for 10 min and stored at 4 °C. The TransStart FastPfu DNA Polymerase (China) was used to amplify the products. Subsequently, PCR products were analyzed by agarose gel electrophoresis and sequenced to determine the validity. The corresponding DNA and amino acid sequences were analyzed and compared in NCBI. Sequence alignments were generated through Bioedit software. Phylogenetic analysis was conducted by neighbor-joining algorithm with 1000 bootstrap trials in MEGA 5.1 software.

2.7. Real-time quantitative PCR

To detect the induction effects of alkB and alkM by different alkanes, CN-3 or HC8–3S was cultivated in MSM supplemented with glucose (10 g L^{−1}), 0.1% (v/v) liquid alkanes (C_{14} or C_{16}) and 0.1% (v/v) solid alkanes (C_{20} or C_{26}), individually. Total RNA of HC8–3S was extracted by Easy Pure RNA kit (China) and total RNA of CN-3 was extracted with the hot phenol method (Min et al., 2016). After detections of RNA quality and concentration, the reverse transcription was performed to transform RNA to cDNA by the Transcript One-Step gDNA Removal and cDNA Synthesis Super Mix kit (China). The cDNA was quantified by RT-qPCR using qPCR Super Mix kit (China), and experimental details were shown in our previous work (Chen et al., 2017). The specific primers were used to amplify the partial fragments of alkB, alkM, 16S rRNA genes in CN-3 or HC8–3S, respectively (Table S1, Supplementary Information). Particularly, the respective 16S rRNA genes were as internal normalization references for the transcription of alkB or alkM. RT-qPCR procedure was performed by the ABI Prism 7500 Fast Sequence Detection System (USA) as follows: 95 °C for 15 min; 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, maintaining 40 cycles. The specificity of RT-qPCR product was detected by melting curve. The 2^{−ΔΔCT} method was adopted to evaluate fold changes of above-mentioned genes (Livak and Schmittgen, 2001).

2.8. Crude oil-contaminated soil microcosm

To evaluate the crude oil bioremediation potential, the constructed bacterial consortium was also applied in the soil microcosm experiments. The soil was collected from a non-contaminated farmland (37°28′18″ N; 121°26′25″ E) in Yantai. The top layer (0–15 cm) of soil was collected and sieved by 2-mm mesh to remove large sands. The microcosm experiments were set up in 250 mL of glass bottles with prior sterilization at 121 °C for 20 min. Soil samples in the sterile groups were sterilized for three times to remove microorganisms thoroughly. Briefly, four different treatments in the microcosm were set up in triplicate: (1) T1: Abiotic control, sterile-soil with crude oil, to study the abiotic loss of crude oil; (2) T2: Natural attenuation, non-sterile soil with crude oil, to study the crude oil depletion by indigenous microorganisms; (3) T3: Bioaugmentation, sterile-soil with crude oil and constructed bacterial consortium.
consortium, to explore the effects of exogenous consortium on crude oil depletion; (4) T4: Natural attenuation + Bioaugmentation, non-sterile soil with crude oil and constructed bacterial consortium, to detect the combination of exogenous consortium and indigenous microorganisms on crude oil removal.

Each microcosm contained 5% (w/w) of crude oil (100 g of soil and 5 g of crude oil). The consortium inoculum (10 mL) was added to T3 and T4 to obtain ~5.65 × 10^8 CFU g⁻¹ and the soil was mixed thoroughly to make sure a uniform strain distribution. Meanwhile, the equivalent volume of MSM was added to T1 and T2 to standardize the moisture content (20%) in the microcosms. All the microcosms were stirred periodically to mix thoroughly and kept at 30 °C during the experimental periods. At each sampling time (0, 20, 40 and 60 d), 10 g of soil was taken from each microcosm. Subsequently, 1 g of dried soil was mixed with 8 mL of carbon tetrachloride for three times and transferred them into centrifuge tubes to extract the TPH thoroughly. The organic phase extracts were collected and dried by anhydrous sodium sulfate. 5 g of magnesium silicate was added into the dissolved solution, mixed vigorously for 1 min and placed on a shaker at 180 rpm for 30 min. The extracts were collected after centrifuging at 4000 rpm for 10 min and metered by 25 mL of volumetric flasks. The TPH concentration was estimated by an infrared spectrometric oil detector (OIL 460, China) (Chen et al., 2017; Bidja Abena et al., 2019a). Furthermore, the individual hydrocarbon components were analyzed by GC-MS and numbers of degraders were analyzed via plate counting method.

2.9. TPH degradation kinetics in soil microcosm

The degradation kinetics were performed to evaluate the TPH degradation of constructed bacterial consortium in microcosm experiments. The kinetics analysis assumed that majority of factors were kept constant and regarded as unlimited factors, such as microbes, nutrients, oxygen and so on (Bidja Abena et al., 2019a). Herein, the TPH degradation was determined by first-order kinetic model (Kachieng’a and Momba, 2017). The formula was as follows:

\[ \ln C_t = \ln C_0 - kt \]

Specifically, \( t \) is the degradation time (d), \( C_0 \) is the TPH concentration at different time (t), \( C_t \) is the original TPH concentration, \( k \) is the degradation rate constant (\( d^{-1} \)).

In addition, the half-life of TPH degradation, which means the time needed for the original concentration to be reduced by one-half, is calculated by the following equation (Kachieng’a and Momba, 2017):

\[ t_{1/2} = \frac{\ln 2}{k} \]

In detail, \( t_{1/2} \) represents the half-life period (d).

2.10. Statistical analysis

Data were expressed as mean ± standard deviation of three triplicates. Origin 8 was used for results analyses and IBM SPSS statistics 22 was conducted for statistical analysis. One-way ANOVA was applied to compare the differences of petroleum hydrocarbons degradation under diverse treatments. The t-test was used to determine transcript levels of alkB and alkM induced by different carbon sources. \( P < 0.05 \) was considered as the significance.

3. Results

3.1. Biosurfactants production by Dietzia sp. CN-3 and Acinetobacter sp. HC8–3S

According to the phenomena of crude oil dispersed and emulsified into small droplets in the presence of CN-3 or HC8–3S, investigations of their abilities to produce biosurfactants were carried out. The emulsification activity, oil spreading assay and cell surface hydrophobicity were effective indicators to evaluate the biosurfactants activities. Hereon, medium-chain alkane C16 and long-chain alkane C24 as the typical hydrocarbons substrates.

3.1.1. Emulsification activity and oil spreading assay

Emulsification activities for CN-3 and HC8–3S were tested in MSM with C16 or C24 as sole carbon and energy source for 10 d, respectively (Fig. 1A). As to CN-3 grown on C16, the emulsification activity increased to maximum (62.5%) on 8 d. Nevertheless, there was little emulsification activity (4.69%) for CN-3 when utilized C24. The variation trend of CN-3 grown on C16 demonstrated the biosurfactants accumulated gradually in the first 8 d. As C16 decreased, the strain might prefer to utilize the biosurfactants as carbon source in the following days, resulting in the diminution of emulsification activity. As to HC8–3S, the maximum E50 was 37.5% on 6 d when grown on C16 and E50 increased sequentially to 43.75% on 10 d when utilized C24. Both the negative controls of CN-3 and HC8–3S showed little emulsification activity to C16 and C24. However, in the oil spreading assay, it seemed that neither CN-3 nor HC8–3S produced obvious oil ripples (only 2–5 mm), indicating negligible ability to produce such kinds of biosurfactants to reduce surface tension of crude oil. Hence, it could be speculated that the biosurfactants synthesized by CN-3 or HC8–3S were extracellular bioemulsifiers.

3.1.2. Cell surface hydrophobicity

The cell surface hydrophobicity (CSH) of CN-3 or HC8–3S against C16 and C24 was also tested. In this study, CN-3 showed the highest CSH toward C16 (78.82%) and a relatively high CSH toward C24 (55.82%) on 8 d. In contrast, the maximum CSH of HC8–3S was recorded to be 62.94% for C16 and 19.35% for C24, respectively (Fig. 1B). Compared to the long-chain alkane C24, both CN-3 and HC8–3S strains had higher cell surface hydrophobicity to middle-chain alkane C16, demonstrating higher cell affinity to C16. CSH value of negative controls revealed no hydrophobicity ability towards C16 and C24.

3.2. Construction of a mixed bacterial consortium

In order to obtain superior degradation efficiency, broader substrate spectrums and better environmental tolerances for petroleum bioremediation, we constructed a mixed bacterial consortium consisting of both petroleum degraders and biosurfactant producers (CN-3 and HC8–3S). The substrates preferences and environmental tolerances of the two strains were shown as follows: CN-3 (substrate preferences: relatively wide spectrums, including n-alkanes, branched alkane, cycloalkanes and polycyclic aromatic hydrocarbons; pH tolerance: 4–10; salinity tolerance: 0–120 g L⁻¹ NaCl) and HC8–3S (substrate preferences: relatively wide spectrums, including n-alkanes and aromatic hydrocarbons; pH tolerance: 4–10; salinity tolerance: 0–120 g L⁻¹ NaCl). Two strains could survive harmoniously in the consortium, and the growth curves under different pH and salinity conditions demonstrated the consortium grew well in pH ranging from 4 to 10 and salinity up to 120 g L⁻¹ NaCl.

3.3. Crude oil biodegradation by constructed bacterial consortium

Crude oil was a complex mixture composing of identifiable TPH and a great many unknown chemicals. Based on different carbon numbers, TPH with distinct peaks on the chromatogram were identified as C7–C35. From the point of individual compounds, TPH contained n-alkane, cycloalkane, branched alkane, BTEX (Benzene, Toluene, Ethylbenzene, Xylene) and PAH (Polycyclic Aromatic Hydrocarbon). The individual hydrocarbons with different carbon numbers and various components were summarized and analyzed severally to demonstrate the degradation characteristics of CN-3, HC8–3S and constructed bacterial consortium. The most probable crude oil components, along with the corresponding retention time, formula and degradation ratio under
different treatments are shown in Table S2 (Supplementary Information). Exactly, from decane (C\textsubscript{10}) to pentatriacontane (C\textsubscript{35}) were identified as n-alkanes. 1,3-dimethylcyclohexane and undecylcyclohexane were the dominant cycloalkanes, while among the branched alkanes, pristane, phytane, 2,6,10-trimethyldodecane and 2,6,10-trimethylpentadecane were abundant. Toluene, ethylbenzene and p-xylene were the primary BTEX. Additionally, the dominant PAHs were naphthalene, 1,6-dimethylnaphthalene and 1-methylphenanthrene.

To determine the crude oil biodegradation by the single strain (CN-3 or HC8–3S) and constructed bacterial consortium, cells were inoculated in MSM medium with 1% (w/v) petroleum for 10 d. As shown in Table S2, the consortium obtained the maximum TPH degradation ratio of 95.8%, significantly higher (p < 0.05) than that of single strains (90.8% for CN-3 and 90.3% for HC8–3S), whereas the natural depletion of the control group was only 6.7%. Furthermore, biodegradation efficiencies of individual hydrocarbons with different carbon numbers and diverse components (n-alkane, cycloalkane, branched alkane, BTEX and PAH) are displayed in Fig. 2 A and Fig. 2 B. As seen from Fig. 2 A, biodegradation efficiencies of TPH with different carbon numbers were significantly improved (p < 0.05) by the consortium than by the single species, particularly for those substrates with carbon chain ≤ C\textsubscript{10} and C\textsubscript{26}–C\textsubscript{35}. For example, biodegradation ratio of C\textsubscript{31}–C\textsubscript{35} by the consortium was 78.3%, which was a prominent increase of more than 13% compared with HC8–3S (65.2%). A similar variation trend was discovered in C\textsubscript{26}–C\textsubscript{30} degradation. Nevertheless, the degradation of C\textsubscript{21}–C\textsubscript{25} by the consortium had no significant difference than CN-3 or HC8–3S, which could be explained by the fact that both CN-3 and HC8–3S had excellent utilization to such n-alkanes. Similarly, biodegradation efficiencies of different components (n-alkane, cycloalkane, branched alkane, BTEX and PAH) by the constructed bacterial consortium were all higher than 86% and achieved significant enhancements (p < 0.05) than each single strain (Fig. 2B), especially for branched alkane, BTEX and PAH. Although CN-3 and HC8–3S were both good at n-alkane degradation (> 90%), the consortium had the supreme degradation of 97.4%, illustrating the powerful combination performance of these two strains.

3.4. Functional optimization of constructed bacterial consortium

To obtain optimal degradation capacities of crude oil, strains should have favorable physiological characteristics that are appropriate for specific environments. Based on the growth adaptabilities of CN-3 and HC8–3S, the effects of pH and salinity on crude oil biodegradation by the single species and constructed bacterial consortium were investigated at 30°C for 10 d. As shown in Fig. 3 A, in the pH range (4–10), the TPH degradation efficiencies of consortium were significantly improved than the single strains (p < 0.05), varying from 51.8% to 96.3%.

![Emulsification activity](A) and cell surface hydrophobicity (B) of Dietzia sp. CN-3 and Acinetobacter sp. HC8–3S grown on C\textsubscript{16} and C\textsubscript{24} in 10 d, respectively. Con group is the negative control.

![Degradation efficiency](A) of individual hydrocarbons with different carbon numbers (A) and different components (n-alkane, cycloalkane, branched alkane, BTEX and PAH) (B) by Dietzia sp. CN-3, Acinetobacter sp. HC8–3S and constructed bacterial consortium. Groups sharing different letters (a, b, c) indicate significant differences between two treatments by one-way ANOVA test (P < 0.05).
Dramatically, the degradation ratios were higher than 90% in the pH values 6–9 and the optimal pH was 8. It was worth noting that the degradation efficiency was still maintained at 82.4% at strong alkaline condition (pH = 10), illustrating the bacterial activity was not restrained visibly at this pH. Thanks to the synergistic action of CN-3 and HC8–3S, this constructed bacterial consortium had better ability for petroleum degradation in neutral and alkaline pH situations.

To investigate the salinity tolerances and crude oil degradation activity of this consortium, the experiments were performed at different NaCl concentrations (0–120 g L\(^{-1}\)). Apparently, the salinity had less influence on TPH degradation than pH. As shown in Fig. 3B, TPH degradation ratios of CN-3 or HC8–3S were all higher than 70% in NaCl concentrations of 0–100 g L\(^{-1}\), revealing good salinity tolerances of these two strains. Thanks to the synergistic effects and powerful combination in the consortium, TPH degradation efficiencies were significantly increased compared to the single strains (p < 0.05), even under the high salinity condition (120 g L\(^{-1}\)), where the degradation remained 80.8%. The optimal NaCl concentration was 20 g L\(^{-1}\) (95.2%), with slightly advantages than 0 g L\(^{-1}\) (93.6%) and 40 g L\(^{-1}\) (93.1%).

### 3.5 Cloning and sequence analysis of alkane hydroxylase genes (alkB and alkM)

Cloning and analysis of functional genes, which encode key enzymes related to hydrocarbon degradation, are very crucial for understanding the mechanisms of specific biochemical processes. In our study, two putative alkane hydroxylase genes, alkB in CN-3 and alkM in HC8–3S were cloned by PCR, generating complete alkB gene (GenBank accession number MT418879) and alkM gene (GenBank accession number MF573947), respectively. The analysis of multiple sequence alignments indicated alkB gene encoded a 495-amino-acid protein with 89.6% sequence identity to the alkane hydroxylase of *Dietzia* sp. E1, followed by 52.78%–85.63% identity to the corresponding protein in *Nocardioides* sp. CF8, *Rhodococcus* sp. Q15 and *Dietzia* sp. DQ12-4S-1h. Similarly, alkM gene encoded a 415-amino-acid protein, which had 95.17% identity to the AlkMa protein of *Acinetobacter* sp. M-1 and 39.68%–60.71% identity to the alkane hydroxylase in *Pseudomonas aeruginosa* PA01, *Alcanivorax borkumensis* SK2, *Pseudomonas fluorescens* CHAO, and *Acinetobacter* sp. M-1. The amino acid sequences (AlkB and AlkM) were aligned with some typical alkane hydroxylase sequences. We found that there were three His boxes containing eight histidines and a HYG motif in AlkB and AlkM (Fig. 4A), which were highly conserved and always as the common features in AlkB-related alkane hydroxylases. Furthermore, the phylogenetic analysis of alkane hydroxylases (Fig. 4B) demonstrated that AlkB in CN-3 formed a single cluster, which was closed to alkane hydroxylases in *Dietzia* strains, but distant from Gram-negative bacteria. Oppositely, AlkM in HC8–3S was clustered with AlkMa in *Acinetobacter* sp. M-1, close to other alkane hydroxylases from Gram-negative bacteria, such as *Alcanivorax*, but distant from those in Gram-positive bacteria. To sum up, AlkB and AlkM could be identified as alkane hydroxylase and their specific functions need further verification and characterization.

#### 3.6 Induction detection of alkB and alkM by different alkanes

The transcript levels of alkB and alkM grown on different alkanes were quantified to verify their specific roles in alkane degradation. Total RNA was extracted from CN-3 or HC8–3S grown on medium-chain alkanes (C\(_{14}\), C\(_{16}\)) and long-chain alkanes (C\(_{20}\), C\(_{24}\) and C\(_{26}\)), respectively. Their own 16S rRNA gene was the internal reference and transcriptions of alkB and alkM were detected by RT-qPCR (Fig. 4C). The alkB was dramatically induced by C\(_{20}\) (79.9-fold) and C\(_{24}\) (93.5-fold), while moderately induced by C\(_{26}\) (6.4-fold) and weakly induced by C\(_{14}\) and C\(_{16}\). However, except for C\(_{26}\), transcription of alkM was significantly different (p < 0.05) from alkB. Specifically, alkM was induced by C\(_{14}\), C\(_{16}\), C\(_{20}\) and C\(_{24}\) with high expression (>15-fold), while slightly low expression induced by C\(_{26}\) (6.3-fold). From another perspective, the transcript level of alkB improved with the increasing chain length for the alkanes (C\(_{14}\), C\(_{16}\), C\(_{20}\) and C\(_{26}\)), but it was downtrend of alkM induced by alkanes (C\(_{16}\), C\(_{20}\), C\(_{24}\) and C\(_{26}\)). In addition, alkB played crucial roles in medium- and long-chain alkanes (C\(_{14}\), C\(_{16}\), C\(_{20}\), C\(_{24}\) and C\(_{26}\)) degradation.

#### 3.7 Crude oil degradation in soil microcosms upon abiotic factors, natural attenuation and bioaugmentation

The ability of constructed bacterial consortium to degrade crude oil was also carried out in soil microcosms after 20, 40 and 60 d of incubation, by setting up four different treatments: T1, abiotic control; T2, natural attenuation; T3, bioaugmentation; T4, natural attenuation + bioaugmentation. Regardless of sample points, crude oil depletion efficiencies of T3 and T4 were significantly higher than T1 and T2 (p < 0.05) (Fig. 5A), indicating the positive influences of bioaugmentation. In T1 microcosm, TPH concentration was reduced from 49,950 to 41,805 mg kg\(^{-1}\) with a total depletion of 16.31%. In T2 microcosm, TPH
The concentration of crude oil was reduced from 49,975 to 39,751 mg kg\(^{-1}\), obtaining a total depletion of 20.46%. According to the abundance of crude oil components on GC-MS chromatograms, some volatile compounds, such as BTEX, short- and medium-chain alkane (C\(_{10}\)–C\(_{16}\)), decreased in T1, indicating the natural volatilization and weathering of crude oil (Fig. S1-AB, Supplementary Information). While in T2, C\(_{10}\)–C\(_{18}\), C\(_{21}\), C\(_{22}\), as well as some branched alkanes (pristane and phytane) were reduced, which could be attributed to the indigenous microbes in the soil (Fig. S1-ABC, Supplementary Information). Through bioaugmentation in T3 and T4, crude oil degradation increased rapidly with incubation time, and different classes of TPH (n-alkane, cycloalkane, branched alkane, BTEX, and PAH) were almost reduced after 60 d (Fig. S1-DE, Supplementary Information). Signally, the maximum TPH degradation ratio of bioaugmentation in T3 was 58.3% with a high degradation rate of 485.8 mg kg\(^{-1}\) d\(^{-1}\). Moreover, the degradation ratio of T4 was slightly higher than T3 without significant difference (p > 0.05), indicating a weak cooperation of exogenous consortium and indigenous microorganisms on crude oil removal herein.

Colony forming units were determined in each of T2, T3 and T4 microcosms on 0, 20, 40 and 60 d by the plate counting method (Fig. 5B). Total bacterial counts were significantly higher in the bioaugmented microcosms (T3 and T4) than in natural attention microcosm (T2) (p < 0.05), which increased from 5.62 to 9.86 × 10\(^9\) CFU g\(^{-1}\) in T3, and from 5.71 to 9.47 × 10\(^9\) CFU g\(^{-1}\) in T4 after 60 d of incubation. A small increase of CFU was also achieved in T2, from 0.56 to 1.35 × 10\(^9\) CFU g\(^{-1}\) on 60 d.

3.8. TPH degradation kinetics in soil microcosms

First-order kinetic model was applied to predict the TPH degradation in soil microcosms. The results of the kinetic equations, degradation rate constant (k), correlation coefficients (R\(^2\)) and half-life (t\(_{1/2}\)) in 60 d were summarized in Table 1. Correlation coefficients (R\(^2\)) were generated by linear regression analysis and they were in the range of 0.8176–0.9813.
of which T3 had the maximum to simulate the TPH degradation kinetic more accurately. In consideration of degradation rate constant (k), it was calculated to be comparative level in T3 and T4, which was 0.0144 d\(^{-1}\) and 0.0158 d\(^{-1}\), severally. Correspondingly, the half-life in T3 (48.14 d) was slightly longer than T4 (43.87 d). However, both the minimum degradation rate constant (0.0028 d\(^{-1}\)) and maximum half-life time (247.55 d) were found in T1, and T2 had a slightly higher degradation rate constant (0.0037 d\(^{-1}\)). To sum up, all the samples revealed a higher degradation rate and a shorter half-life in bioaugmentation microcosms than natural attention and abiotic control microcosms.

4. Discussion

Bioremediation of crude oil pollution, by isolating, identifying and combining functional bacteria, has been deemed to be more effective, economical and environmentally sustainable compared to physical and chemical remediation (McGenity, 2014; Cui et al., 2020). In this study, two bacterial strains, with capacities of withstanding broad pH and salinity conditions, producing biosurfactants and degrading petroleum hydrocarbons efficiently, were employed to construct the bacterial consortium. Furthermore, we investigated crude oil degradation by the consortium in liquid media and contaminated soil microcosms, as well as the functional genes (alkB and alkM) involved in alkane metabolic mechanism, providing extremely valuable resources for bioremediation and bioremediation of crude oil contaminants.

The constructed bacterial consortium was composed of Dietzia sp. CN-3 and Acinetobacter sp. HCB–3S, which were isolated from crude oil-contaminated marine sediments of Bohai Bay, China. Generally, Dietzia genus had been confirmed as alkane degraders, whereas some strains could also utilize polyacrylamic compounds, such as naphthalene, phenanthrene, pyrene, chrysene and fluoranthene (Wang et al., 2011, 2014; Chen et al., 2017). Thanks to preeminent growth and degradation characteristics, Acinetobacter genus was always as active member in crude oil degradation and consortium construction (Lin et al., 2014; Bidja Abena et al., 2019a). In this study, one type of heavy crude oil was utilized by CN-3 and HCB–3S as the sole carbon and energy source. Noteworthily, crude oil-degrading abilities of CN-3 (90.8%) and HCB–3S (90.3%) were much stronger than plenty of reported bacteria (Wang et al., 2014; Xia et al., 2017; Bidja Abena et al., 2019b), suggesting both the two strains were potential crude oil degraders. Through powerful combination, the consortium was constructed and degraded 95.8% of crude oil after 10 d, exhibiting faster degradation speed, higher degradation efficiency and stronger degradation ability than the individual strains. As Xia et al. (2019) reported, the consortium acquired highest crude oil degradation efficiency (85.26%) in 15 d, whereas the single strains had relatively low efficiencies: Serratia proteamaculans S1BD1 (68.0%), Alcaligenes sp. OPKDS2 (63.7%), and Rhodococcus erythropolis OSDS1 (54.9%). In another recent study, a consortium consisting of five strains degraded 94.4% of crude oil without yeast extract, while 95.1% was degraded with yeast extract after 10 d (Bidja Abena et al., 2019b). Briefly, this consortium had higher biodegradation efficiency than multiple similar studies, displaying the superiority of this consortium in crude oil removal.

In particular, both CN-3 and HCB–3S had capacities of producing biosurfactants to accelerate petroleum hydrocarbons degradation, through typical emulsification activity, oil spreading assay and cell surface hydrophobicity detections. Secreting extracellular biosurfactants to improve hydrocarbons accessibility, and altering cell surface hydrophobicity to enhance the interaction between hydrocarbons and cells, were two crucial strategies to degrade hydrophobic hydrocarbons, which occurred in bacterial strains simultaneously or separately (Dastgheib et al., 2011; Wang et al., 2013). Considering the E24 and CSH results, these two indicators displayed a nearly constant variation tendency to C16, but quite different to C24, speculating utilization of C16 or C24 by two strains may rely on different strategies. Hydrocarbons degradation by bacteria was related to cell affinity. Hydrocarbons with high affinity to cells, such as C16, could always be utilized more effectively than those with low affinity (Rosenberg, 1984; Ron and Rosenberg, 2002). Additionally, according to the emulsification activity and oil spreading assay, CN-3 and HCB–3S produced biosurfactants for certain but no obvious oil rings on C16 or C24, which could be deduced that the biosurfactants synthesized by two strains were kinds of bioemulsifiers, breaking up the hydrocarbon droplets into smaller units without surface or interfacial tension reduced obviously (Ron and Rosenberg, 2002; Xia et al., 2019). Numerous studies had shown that

![Fig. 5. Crude oil degradation by T1, T2, T3 and T4 treatments in soil microcosm after 20, 40 and 60 d. (A) Crude oil depletion efficiency. (B) Numbers of colonies. Groups sharing different letters (a, b, c, d) indicate significant differences between two treatments by one-way ANOVA test (P < 0.05).](image-url)
Acinetobacter was outstanding bacterium to produce different biosurfactants, especially high molecular weight polymeric bioemulsifier. For instance, Acinetobacter calcoaceticus RAG-1 produced emulsan as extra cellular bioemulsifier and it had been put into production in the markets (Nerurkar et al., 2009). Likewise, a few Dietzia strains had ability to produce biosurfactants as well. Wang et al. (2014) reported Dietzia maris As-13-3 generated di-rhamnolipid as biosurfactant, with high E<sub>42</sub> (55.88%) and CSH (61.2%) to C<sub>T16</sub>, but a weak CSH to pristane (38.8%).

Although multiple studies had reported that bacterial consortia degraded crude oil more effectively than single strains, few had analyzed the biodegradation efficiencies of individual crude oil components. herein, biodegradation of individual petroleum hydrocarbons with different carbon numbers (C<sub>7</sub>–C<sub>36</sub>) and diverse components (n-alkane, cycloalkane, branched alkane, BTEX and PAH) were analyzed by single strains and bacterial consortium. According to the substrate preferences of CN-3 and HC8–3S, the consortium achieved remarkably complementary advantages, especially to those substrates with carbon chain < C<sub>20</sub> and C<sub>28</sub>–C<sub>36</sub>, as well as branched alkane, BTEX and PAH, whose degradation ratios were all significantly improved (p < 0.05) by this consortium than single species. Metabolic complementation of two strains could be applied as actual strategies for effective crude oil degradation. Universally, the susceptibility of petroleum hydrocarbons to bacterial attack differed from each other (Rojo, 2009; McGinity, 2014). The overall pattern of petroleum hydrocarbons degradation in our study was: n-alkane > branched alkane > cycloalkane > BTEX > PAH. Due to simple structures and low hydrophobicity, the low-molecular weight alkanes could be directly absorbed by cells, in turn causing easy biodegradation. Nevertheless, high-molecular weight alkanes, BTEX and PAH, were degraded with difficulty because of the complex structures and low solubility, which always required biosurfactants to assist in uptaking and assimilating (Hennessey and Li, 2016). Hence, the enhanced degradation efficacy by consortium could be attributed to the metabolic complementation of two strains on crude oil utilization, as well as the presence of biosurfactant producers, which took effects on dispersion, emulsification of crude oil and bioavailability improvements (Abalos et al., 2004).

In addition, the functional optimization of consortium under a range of pH and salinity conditions contributed to complementing the advantages and addressing the deficiencies of CN-3 and HC8–3S. Especially to the acidic pH 4, alkaline pH 10 and high NaCl concentration (120 g L<sup>-1</sup>) conditions, the TPH degradation ratios were all significantly increased by consortium than individual strain (p < 0.05). Crude oil pollution was often accompanied by some high-salinity environments, which will obviously disturb the function of non-salinity-tolerant strains (Vasudevan and Rajaram, 2001; Cui et al., 2020). Generally, marine sediments represent a significant sink for petroleum hydrocarbons after oil spills (Mapelli et al., 2017). Thanks to natural selection and adaption to marine sediment characteristics, both CN-3 and HC8–3S strains displayed outstanding tolerance to broad pH ranges (4–10) and high levels of salinity (0–120 g L<sup>-1</sup>), thus leading to better bacterial vitality and competitiveness in crude oil degradation, which would have great values in practical application of crude oil bioremediation.

To date, although multiple reports had shown that consortium degraded crude oil better than single strains, little is known about the mechanism, such as the key functional genes responsible for individual hydrocarbon compounds. As the largest components of crude oil in this study, mechanisms could be deployed. CN-3 and HC8–3S, which contained high efficiencies (98%). Two alkane hydroxylase genes (alkB and alkM) were cloned and detected to investigate their specific roles in alkane degradation mechanism. Due to RT-qPCR results, alkB and alkM may have different effects on alkane hydroxylation. The alkB gene was more prominent in long-chain alkanes (C<sub>20</sub>, C<sub>24</sub> and C<sub>28</sub>) and alkM gene played crucial roles in medium- and long-chain alkanes (C<sub>16</sub>, C<sub>18</sub>, C<sub>20</sub>, C<sub>24</sub> and C<sub>26</sub>) whereas the specific functions need further characterization and identification. The alkane hydroxylases responsible for short- and medium-chain alkanes were studied well, but the enzyme systems oxidizing long-chain alkanes (> C<sub>16</sub>) are still from clear (Nie et al., 2011; Wang and Shao, 2013; Varjani, 2017). LadA, identified in Geo-bacillus thermodenitrificans NG80-2, was the first experimentally confirmed long-chain alkane hydroxylase to oxidize C<sub>15</sub>–C<sub>36</sub> but not involved in C<sub><14</sub> alkanes. LadA was considered to be distinct from AlkB-related alkane hydroxylases (Feng et al., 2007). AlmA from Acinetobacter sp. DSM 17874, was responsible for the degradation of > C<sub>32</sub> alkanes via verification of almA gene disruption mutants (Throne-Holst et al., 2007). Nevertheless, almA was also reported in Alcanivorax, Marinobacter and Parvibaculum (Wang and Shao, 2012a, 2012b, 2013). However, LadA and almA were not found in CN-3 and HC8–3S. Among the AlkB-related alkane hydroxylases, Wang and Shao (2012a) reported alkB was induced apparently by C<sub>12</sub>–C<sub>16</sub>, but no effects on C<sub>26</sub> in Alcanivorax hongkongensis strain A-11-3. Two alkane hydroxylase genes (alkW1 and alkW2) were cloned and detected grown on C<sub>8</sub>–C<sub>26</sub> in Dietzia sp. DQ12-45-1b, demonstrating alkW1 was induced notably by C<sub>9</sub>–C<sub>12</sub>, while alkW2 had negligible effects (Nie et al., 2011). Thanks to the potential synergistic effects of alkB and alkM in different alkanes, the consortium had more outstanding performance on alkane metabolism. Furthermore, diverse genes responsible for BTEX, PAH etc., such as xylE gene encoding catechol dioxygenase, and nahAc gene encoding naphthalene dioxygenase (Varjani, 2017; Xia et al., 2019), should also be taken into consideration in the future. With the development of genomics, metabolomics and molecular biology, studies on hydrocarbons degradation and metabolic mechanisms are supposed to understand the interactions between bacteria and hydrocarbons, providing more reliable theoretical supports for crude oil bioremediation.

Successful bioremediation depends not only on the catabolic abilities of introduced microorganisms against the contaminants, but also on strong survival of inoculum in actual environments (Zhao et al., 2009; Varjani and Upasani, 2019). We have proved that the consortium played crucial roles in removing hydrocarbons in liquid media. Herein, soil microcosms with four different treatments were also carried out to investigate their own effects. In the whole experimental periods, crude oil depletion ratios of bioaugmentation T3 and natural attenuation + bioaugmentation T4 were significantly higher than that of abiotic control T1 and natural attenuation T2 (p < 0.05), indicating positive influences of bioaugmentation. T1 showed the natural evaporation and oxidation of crude oil with TPH depletion of 16.31%, while T2 obtained TPH depletion of 20.46% due to degrading elements to utilize xenobiotic contaminants by native microorganisms. In soil microcosms, one of the main factors limiting hydrocarbon degradation is the low solubility. Relying on the producing biosurfactants, hydrocarbons bioavailability might be increased in the consortium. Bioaugmentation of the consortium achieved TPH degradation efficiency of 58.3% on 60 d with a rate of 485.8 mg kg<sup>-1</sup> d<sup>-1</sup>, which was significantly higher than many previous studies (Table 2). For instance, Varjani and Upasani (2019) reported a consortium of five strains degraded 82.56% of crude oil (30, 000 mg kg<sup>-1</sup>) in 60 d at a rate of 412.8 mg kg<sup>-1</sup> d<sup>-1</sup>. Similarly, the consortium of seven strains removed 45.3%–61.9% of different concentrations of TPH with the highest rate of 377.5 mg kg<sup>-1</sup> d<sup>-1</sup> (Zhao et al., 2011). However, there were still some counter-examples to show bacterial consortium was less effective than single strains in crude oil degradation. Magdalena et al. (2019) reported the TPH removal efficiency of consortium (Rhodococcus erythropolis CD 130 and CD 167) (29.72%) was worse than single strain CD 167 (38.40%), which might be explained due to the two strains and similar metabolisms competed intensely to release antagonists (toxins or antibiotics) that create inhospitable space finally. Besides, the first-order kinetic model successfully predicted the TPH degradation in T1, T3 and T4 microcosms (R<sup>2</sup> > 0.9), displaying a strong linear correlation between incubation time and TPH degradation. The degradation rate constant (k) of T3 was approximately 3.9-fold faster than T2, while 48.14 days were required for T3 to degrade 50% of TPH, against 187.34 days for T2. Additionally, TPH depletion ratio of T4 was significantly increased (p < 0.05) than T3
on 20 d, but no significant increase on 40 d or 60 d, which could be explained that indigenous microorganisms resisted against and/or competed with consortium in the soil microcosms (Magdalena et al., 2019), consequently the bacteria numbers of T4 were less than T3 at that time.

In a summary, the constructed bacterial consortium comprising petroleum degraders and biosurfactant producers, had capabilities of withstanding broad pH (4–10) and salinity (0–120 g L$^{-1}$), and degraded petroleum hydrocarbons efficiently both in liquid media and soil microcosms, indicating this consortium had great potential in crude oil bioremediation. The analysis of alkane hydroxylase genes (alkB and alkM) involved in alkane degradation contributed to explaining synergistic interactions of two strains at molecular level. However, although a number of crude oil-degrading bacteria had been investigated previously, their survivability, sustainability and degradation activity in actual environments were still severe challenges in crude oil bioremediation. Due to the complexity of crude oil, it is hard to identify specific intermediate metabolites and elucidate complete mechanisms in the degradation process. Therefore, more intensive researches are still needed to understand the microbial physiology and metabolic mechanisms in order to develop superior microbial biotechnological applications for crude oil bioremediation.

5. Conclusion

A functional bacterial consortium comprising salt-tolerant petroleum degraders and biosurfactant producers of Dietzia sp. CN-3 and Acinetobacter sp. HC8–3S, was constructed and achieved 95.8% degradation efficiency of crude oil in 10 d, with abilities to withstand broad pH (4–10) and salinity (0–120 g L$^{-1}$). The alkane hydroxylase genes (alkB and alkM) played different roles in medium- and long-chain alkanes degradation. Bioaugmentation of this consortium significantly enhanced TPH depletion than abiotic factors and natural attenuation in soil microcosms, which could be a promising candidate in crude oil bioremediation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The funding source(s) and country names in our article are listed as follows: Ministry of Science and Technology of the People’s Republic of China (2016YFC1402300); Youth Innovation Promotion Association of the Chinese Academy of Sciences, China (2020218); Yantai Science and Technology Bureau, China (2017ZH092); Chinese Academy of Sciences, China (QYZDB-SW-DQC041); Chinese Academy of Sciences, China (133337KYSB20180015).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2020.105047.

References

Abalos, A., Viñas, M., Sahate, J., Manresa, M.A., Solanas, A.M., 2004. Enhanced biodegradation of Casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by Pseudomonas aeruginosa AT10. Biodegradation 15, 249–266.

Bidja Abena, M.T., Li, T., Shah, M.N., Zhong, W.H., 2019a. Biodegradation of total petroleum hydrocarbons (TPH) in highly contaminated soils by natural attenuation and bioaugmentation. Chemosphere 234, 864–874.

Bidja Abena, M.T., Sodibaatar, N., Li, T.T., Dadineureuen, N., Choidash, B., Zhong, W.H., 2019b. Crude oil biodegradation by newly isolated bacterial strains and their consortium under soil microcosm experiment. Appl. Biochem. Biotechnol. 189, 1223–1244.

Chen, W.W., Li, J.D., Sun, X.N., Min, J., Hu, X.K., 2017. High efficiency degradation of alkanes and crude oil by a salt-tolerant bacterium Dietzia species CN-3. Int. Biodeterior. Biodegrad. 118, 110–118.

Cui, J., Huang, L., Wang, W.W., Xu, P., Zanaroli, G., Tang, H.Z., 2020. Maximization of the petroleum biodegradation using a synthetic bacterial consortium based on minimal value algorithm. Int. Biodeterior. Biodegrad. 150, 104964.

Das, K., Mukherjee, A.K., 2007. Crude petroleum oil-biodegradation efficiency of Bacillus subtilis and Pseudomonas aeruginosa strains isolated from a petroleum-oil contaminated soil from North-East India. Bioresour. Technol. 98, 1339–1345.

Dastgheib, S.M.M., Amoozegar, M.A., Khajeh, K., Ventosa, A., 2011. A halotolerant bacterium Dietzia species CN-3S, was constructed and achieved 95.8% degradation efficiency of crude oil in 10 d, with abilities to withstand broad pH (4–10) and salinity (0–120 g L$^{-1}$). The alkane hydroxylase genes (alkB and alkM) played different roles in medium- and long-chain alkanes degradation. Bioaugmentation of this consortium significantly enhanced TPH depletion than abiotic factors and natural attenuation in soil microcosms, which could be a promising candidate in crude oil bioremediation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25, 402–408.

Mukherjee, A.K., Bordoloi, N.K., 2011. Bioremediation and reclamation of soil contaminated with petroleum oil hydrocarbons by exogenously seeded bacterial consortium: a pilot-scale study. Environ. Sci. Pollut. Res. Int. 18, 471–478.

Nie, Y., Liang, J.L., Fang, H., Tang, Y.Q., Wu, X.L., 2011. Two novel alkane hydroxylase systems in a marine alkane degrader, Alcanivorax dieselolei B-5. Environ. Microbiol. 13, 1168–1178.

Min, J., Zhang, J.J., Zhou, N.Y., 2016. A two-component para-nitrophenol monooxygenase initiates a novel 2-chloro-4-nitrophenol catalysis pathway in Rhodococcus planticola. Front. Microbiol. 7:122.

Paisse, S., Duran, R., Coulon, F., Goni-Urriza, M., 2011. Are alkane hydroxylase genes (alkB) relevant to assess petroleum bioremediation processes in chronically polluted sediments? Environ. Microbiol. 13, 249–252.

Rojo, F., 2009. Degradation of alcanes by bacteria. Environ. Microbiol. 11, 2477–2490.

Ron, E.Z., Rosenberg, E., 2002. Biosurfactants and oil bioremediation. Curr. Opin. Biotechnol. 13, 249–252.

Rosenberg, M., 1984. Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. FEMS Microbiol. Lett. 22, 289–295.

Thompson, I.P., Van Der Gast, C.J., Ciric, L., Singer, A.C., 2005. Bioaugmentation for bioremediation: the challenge of strain selection. Environ. Microbiol. 7, 909–915.

Van Beilen, J.B., Funhoff, E.G., 2005. Expanding the alkane oxygenase toolbox: new enzymes and applications. Curr. Opin. Biotechnol. 16, 308–314.

Varjani, S.J., 2017. Microbial degradation of petroleum hydrocarbons. Bioresour. Technol. 223, 277–286.

Wang, W.P., Cai, B.B., Shao, Z.Z., 2014. Oil degradation and biosurfactant production by the deep sea bacterium Dietzia maris AS-13-3. Front. Microbiol. 5, 711.

Nie, Y., Hu, X.K., Liu, H., 2016. Industrial-scale culturing of the crude oil-degrading marine Acinetobacter sp. strain HCB-3S. Int. Biodeterior. Biodegrad. 107, 56–61.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2ΔΔCT method. Methods 25, 402–408.

Magdalena, P.P., Joanna, C., Tomasz, P., Zofia, P.S., 2019. The effect of bioaugmentation of petroleum-contaminated soil with Rhodococcus erythropolis strains on removal of petroleum from soil. Environ. Sci. 169, 615–622.

A. Niger, A. Suthar, H. G., 2009. Bioemulsifiers from marine microorganisms. Trends Biotechnol. 27, 46–54.

Min, J., Zhang, J.J., Zhou, N.Y., 2016. A two-component para-nitrophenol monooxygenase initiates a novel 2-chloro-4-nitrophenol catalysis pathway in Rhodococcus planticola. Front. Microbiol. 7:122.

Paisse, S., Duran, R., Coulon, F., Goni-Urriza, M., 2011. Are alkane hydroxylase genes (alkB) relevant to assess petroleum bioremediation processes in chronically polluted sediments? Environ. Microbiol. 13, 249–252.

Rojo, F., 2009. Degradation of alcanes by bacteria. Environ. Microbiol. 11, 2477–2490.

Ron, E.Z., Rosenberg, E., 2002. Biosurfactants and oil bioremediation. Curr. Opin. Biotechnol. 13, 249–252.

Rosenberg, M., 1984. Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. FEMS Microbiol. Lett. 22, 289–295.

Suja, F., Rahim, F., Taha, M.R., Hambley, N., Razali, M.R., Khalid, A., Hamzah, A., 2014. Effects of local microbial bioaugmentation and biostimulation on the bioremediation of total petroleum hydrocarbons (TPH) in crude oil contaminated soil based on laboratory and field observations. Int. Biodeterior. Biodegrad. 90, 115–122.

Teira, E., Lekunberri, I., Gasol, J.M., Nieto-Cid, M., Alvarez-Salgado, X.A., Figueras, F. G., 2007. Dynamics of the hydrocarbon-degrading Cycloclasticus bacteria during mesocosm-simulated oil spills. Environ. Microbiol. 9, 2551–2562.

Thompson, I.P., Van Der Gast, C.J., Ciric, L., Singer, A.C., 2005. Bioaugmentation for bioremediation: the challenge of strain selection. Environ. Microbiol. 7, 909–915.

Throne-Holst, M., Wentzel, A., Ellingsen, T.E., Kotlar, H.K., Zichev, S.B., 2007. Identification of novel genes involved in long-chain n-alkane degradation by Acinetobacter sp. strain DSM 17874. Appl. Environ. Microbiol. 73, 3327–3332.

van Beilen, J.B., Funhoff, E.G., 2005. Expanding the alkane oxygenase toolbox: new enzymes and applications. Curr. Opin. Biotechnol. 16, 308–314.

van Beilen, J.B., Funhoff, E.G., 2007. Alkane hydroxylases involved in microbial alkane degradation. Appl. Microbiol. Biotechnol. 74, 13–21.

Varjani, S.J., 2017. Microbial degradation of petroleum hydrocarbons. Bioresour. Technol. 223, 277–286.

Varjani, S.,Upasani,V.N., 2019. Influence of abiotic factors, natural attenuation, bioaugmentation and nutrient supplementation on bioremediation of petroleum crude contaminated agricultural soil. J. Environ. Manag. 245, 358–366.

Vausdevan, N., Rajaram, P., 2001. Bioremediation of oil sludge-contaminated soil. Environ. Int. 26, 409–411.

Wang, W.P., Cai, B.B., Shao, Z.Z., 2014. Oil degradation and biosurfactant production by the deep sea bacterium Dietzia maris AS-13-3. Front. Microbiol. 5, 711.

Wang, W.P., Shao, Z.Z., 2012a. Genes involved in alkane degradation in the Alcanivorax hongdengensis strain A-11-3. Appl. Microbiol. Biotechnol. 94, 437–448.

Wang, W.P., Shao, Z.Z., 2012b. Diversity of flavin-binding monooxygenase genes (almA) in marine bacteria capable of degradation long-chain alcanes. FEMS Microbiol. Ecol. 80, 523–533.

Wang, W.P., Shao, Z.Z., 2013. Enzymes and genes involved in aerobic alkane degradation. Front. Microbiol. 4, 116.

Wang, W.P., Shao, Z.Z., 2014. The long-chain alkane metabolism network of Alcanivorax dieselolei. Nat. Commun. 5, 5755.

Wang, X.B., Ni-Urriza, M., Nic, Y., Tang, Y.Q., Tan, Y., Wu, G., Wu, X.L., 2011. Degradation of petroleum hydrocarbons (Cn-C25) and crude oil by a novel Dietzia strain. Bioresour. Technol. 102, 7755–7761.

Wang, X.B., Nie, Y., Tang, Y.Q., Wu, G., Wu, X.L., 2013. N-alkane chain length alters Dietzia sp. strain DQ12-45-1b biosurfactant production and cell surface activity. Appl. Environ. Microbiol. 79, 400–402.

Wu, M., Li, W., Dick, W.A., Ye, X., Chen, K., Kost, D., Chen, L., 2017. Bioremediation of hydrocarbon degradation in a petroleum-contaminated soil and microbial population and activity determination. Chemosphere 169, 124–130.

Xia, M., Liu, Y., Taylor, A.A., Fu, D., Khan, A.R., Terry, N., 2017. Crude oil depletion by bacterial strains isolated from a petroleum hydrocarbon impacted solid waste management site in California. Int. Biodeterior. Biodegrad. 123, 70–77.

Xia, M.Q., Fu, D.F., Chakraborty, R., Singh, R.P., Terry, N., 2019. Enhanced crude oil depletion by constructed bacterial consortium comprising biosurfactant producer and petroleum hydrocarbon degraders. Bioresour. Technol. 282, 456–463.

Zhao, D., Liu, C., Liu, L., Zhang, Y., Liu, Q., Wu, W.M., 2011. Selection of functional consortium for crude oil-contaminated soil remediation. Int. Biodeterior. Biodegrad. 65, 1244–1248.

Zhao, S., Ramette, A., Niu, G.L., Liu, H., Zhou, N.Y., 2009. Effects of nitrobenzene contamination and of bioaugmentation on nitrification and ammonia-oxidizing bacteria in soil. FEMS Microbiol. Ecol. 70, 159–167.