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Combination of a crude oil-degrading bacterial consortium under the guidance of strain tolerance and a pilot-scale degradation test☆

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ABSTRACT

Under the guidance of strain tolerance, a new combination method for crude oil-degrading bacterial consortium was studied. Firstly, more than 50 efficient crude oil-degrading and biosurfactant producing bacteria were isolated from petroleum-contaminated soil and water in Tianjin Binhai New Area Oilfield, China. Twenty-four of them were selected for further study. These strains were identified as belonging *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Brevibacillus brevis*, *Achromobacter* sp., *Acinetobacter venetianus*, *Lysinibacillus macroides*, *Klebsiella oxytoca*, *Stenotrophomonas rhizophila*, *Rhodococcus* sp. and *Bacillus thuringiensis*. A shake-flask degradation test revealed that 12 of these strains could degrade over 50% of 1% crude oil concentration in 7 d. Of these, 8 strains were able to produce biosurfactants. Furthermore, environmental tolerance experiments indicated that the majority of the strains had the ability to adapt to extreme environments including high temperatures, alkaline environments and high salinity environments. A mixed bacterial agent comprising the strains WB2, W2, W3 and HA was developed based on the environmental tolerance tests and subjected to the pilot-scale degradation test indicating that this bacterial agent could degrade 85.2% of 0.8% crude oil concentration in 60 d. Our results suggest that the application of this mixed agent could remediate crude oil polluted soils in saline and alkaline environments.

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1. Introduction

Oil can pollute the environment when leaked during the processes of production, transport, storage and refinement [1]. Accidental oil spills relating to pipeline ruptures and tank leakages in combination with improper handling are the primary causes of oil spills [2]. Crude oil pollution can cause serious and long lasting damage to the environment and public health [3–5]. Hydrocarbon are the main components of crude oil, they lack the corresponding functional groups and possess low water solubility. Most of them persist in the environment and eventually become recalcitrant pollutants [6,7]. The remediation of crude oil pollution remains a challenge for researchers.

Presently, more attention is being paid to bioremediation. The engineered measures that support the biodegradation of thousands of xenobiotic contaminants constitute bioremediation. The use of natural

populations of microorganisms, such as crude oil-degrading bacteria, is one of the most basic and the most reliable mechanisms for eliminating crude oil pollution from the environment [8–11]. More importantly, microbial bioremediation is better than other remediation technologies such as chemical treatment and physical separation [12]. The advantages of microbial bioremediation include relatively low costs, high efficiency and low adverse impacts on the surrounding environment [13,14].

Currently, many microbial species including bacteria and fungi have been reported to degrade hydrocarbons efficiently. Petroleum-degradation bacteria include *Pseudomonas* spp. [15,16], *Bacillus* spp. [17], *Acinetobacter* spp. [18], and *Microbacterium* spp. [19], while petroleum-degradation fungi include *Aureobasidium* spp., *Rhodotorula* spp. [20], *Candida* spp. [21], *Aspergillus* spp. and *Penicillium* spp., among others. Microorganisms existing in nature are able to degrade environmental pollutants including petroleum hydrocarbons, however the efficiency is generally quite low. Strengthening microorganism remediation efficiency in the natural environment, and increasing remediation efficiency, is currently being researched for the remediation of environmental oil pollution.

Wu *et al.* isolated a novel bacterial strain, *Dietzia* sp. DQ12-45-1b, from the production water of a deep subterranean oil reservoir. They found that this strain could degrade hydrocarbons, polycyclic aromatic hydrocarbons, and crude oil in the C6 to C10 range. The strain had a wider range of degradation than most other strains that are only able

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to degrade a relatively small range of hydrocarbons [22]. S Mnif isolated a *Pseudomonas* strain from Tunisian oilfields named C450R that was able to almost completely degrade the aliphatic hydrocarbons in crude oil. Additionally, the study also revealed the presence of diverse aerobic bacteria in Tunisian oilfields with interesting aliphatic hydrocarbon degradation potentiality, mainly for biosurfactant-produced strains [23]. Wang *et al.* screened an oil degrading and biosurfactant producing strain, the deep-sea bacterium *Dietzia maris* As-13-3, which was able to degrade *n*-hexadecane efficiently, and found that the degradation rate of *n*-hexadecane could reach in excess of 80% [24].

Microbial degradation of petroleum pollution is influenced by many environmental factors, including temperature, oil composition [25], soil composition [26], the proportion of carbon, nitrogen and phosphorus sources [27], surfactants [28], microbial species, pH and salt concentration [29–31].

Biosurfactants are amphipathic compounds that exhibit surface activity [32,33]. Some common biosurfactants include lipopeptides, glycolipids and phospholipids, as well as polymeric and particulate surfactants [34]. A large variety of microbes, for instance, *Acinetobacter*, *Arthrobacter*, *Achromobacter*, *Brevibacterium*, *Bacillus*, *Candida*, *Corynebacterium*, *Pseudomonas* and *Rhodotorula* have already been identified as the producers of biosurfactants [35]. Temperature affects the growth of microorganisms and also influences enzyme activity and oil physical form. The viscosity of oil increases at low temperatures, which leads to a great drop in the dispersion rate of oil. Additionally, microtherm reduces the contact between bacteria and oil, and the decrease in volatility and solubility of oil directly affects the biological toxicity of some oil hydrocarbons [36].

Microbial tolerance to the environment is essential for the remediation of oil-contaminated soil. The combination of a crude oil-degrading bacterial consortium under the guidance of strain tolerance could provide a new approach for the remediation of crude oil polluted soil in saline and alkaline environments. In this study, we aimed to (1) screen the highly efficient crude oil-degrading and surfactant-producing bacteria in local petroleum-contaminated soil and water, (2) characterize their ability to degrade crude oil, (3) explore the tolerance of crude oil-degrading bacteria to the environment, including temperature, pH and salt concentration, (4) improve the degradation efficiency of petroleum pollutants by combining degrading bacteria under the guidance of strain environmental tolerance tests, and (5) validate the performance of our method by means of a pilot-scale experiment.

2. Materials and Methods

2.1. Chemicals

Peptone, glucose and yeast extract were purchased from Tianjin Guangfu Institute of Fine Chemicals (China). NaCl, K_2HPO_4 and KH_2PO_4 (all with purity $\geq 99\%$) were purchased from Tianjin Yuanli Chemical Co., Ltd. (China). Crude oil was provided by China Offshore Environmental Service Ltd.

2.2. Sampling

In order to isolate and screen crude oil-degrading bacteria, a sample of petroleum-contaminated soil/water was collected from an oil production well (NO.Xi-51-5-1: 38°70' N; 117°49' E) in Tianjin Binhai New Area Oilfield, near the Bohai Sea, southeast of Tianjin in northeastern China.

The oil production well we sampled belongs to Dagang Oilfield, which produces about 5 million tons of oil annually. However, the soil in the vicinity of the oil well was polluted and we were unable to sample from an uncontaminated source. Six sample points are there including 4 soil samples and 2 water samples.

2.3. Culture media

Luria-Bertani medium (LB) was used and was composed of peptone 10, yeast extract 5 and NaCl 5 in $g \cdot L^{-1}$ de-ionized water. For the preparation of nutrient agar plates or slants, 15.0 $g \cdot L^{-1}$ agar was added.

Trypticase Soy Broth medium (TSB) was composed of peptone 20, glucose 2.5, NaCl 5 and K_2HPO_4 2.5 in $g \cdot L^{-1}$ de-ionized water. For the preparation of petroleum-degradation experiment, 1.0 $g \cdot L^{-1}$ crude oil (1%) was added.

Bushnell-Haas medium (BH) was composed of NH_4NO_3 1, NaCl 1, K_2HPO_4 1.5, KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.2, CaCl 0.02, $FeSO_4 \cdot 7H_2O$ 0.05, to which 0.05 g yeast extract was added in order to offer growth factor. For the preparation of petroleum-degradation bacterial domestication, 15.0 $g \cdot L^{-1}$ agar was added. To the BH crude oil plates, another 20 μl crude oil was surface coated on the agar plates.

All the media were adjusted to pH 7.3 and autoclaved at 121 °C for 20 min.

2.4. Isolation and screening of biosurfactant-producing and crude oil-degrading strains

About 10 g of either soil sample or water sample were added aseptically to 100 ml BH plus 1% crude oil in a 250 ml flask, and incubated in a rotary shaker at 30 °C and 220 $r \cdot min^{-1}$. After 7 d, 1 L was transferred to another fresh BH plus 1% crude oil media and then incubated under the same conditions for another 7 d. This operation was repeated 3 times. After repeated cultivation, 100 μl liquid was spread for broth culture precipitates and then added to agar plates, and then cultured for about 3 d at 30 °C for the first screening.

Different colonial morphologies were selected and inoculated in 100 ml TSB media in a 250 ml flask and incubated on a rotary shaker at 30 °C and 220 $r \cdot min^{-1}$ for 10 h. They were then spread on the BH crude oil agar plates and cultured for 3 d at 30 °C for the second screening. The aim of re-screening was to select the strain that could grow with crude oil as the only organic carbon source. Eventually, we screened colonies with different colonial morphologies and incubated each of them on LB agar plates, cultured at 30 °C for 24 h and stored at 4 °C until the next evaluation.

The fermentation liquor was centrifuged in order to remove the bacteria. Then 15 ml distilled water and 8 ml liquid paraffin were blended and added to the solid plate. After 30 min, 50 μl concentrated fermentation liquor was added and dropped onto the layer of oil on the paraffin. Following this, each exclusive circle of methods of the different fermentation liquors were recorded and used to test the existence of biosurfactants.

2.5. Identification

2.5.1. 16S rRNA gene sequence analysis

Total DNA of crude oil-degrading bacteria was extracted using the SDS method for 16S rRNA gene sequence analysis. The 16S rRNA gene was amplified by PCR using the universal forward primer 27 F (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer 1492 R (5'-GGTACCTTGTACGACTT-3'), and then sequenced by GENEWIZ (Suzhou) Biological Technology Co., Ltd., and compared with the reference sequences on the NCBI database (<http://www.ncbi.nlm.nih.gov>).

2.5.2. Phylogenetic tree reconstruction

A phylogeny of the 24 crude oil-degrading strains in Tianjin Binhai New Area oilfield was reconstructed and analyzed.

The phylogenetic tree was constructed with MEGA V. 5.1 using the neighbor-joining method and the tree topology was evaluated by bootstrap analysis based on 1000 resampling replicates. The phylogenetic tree included the 16S rRNA gene of the representative strains of the 35 groups. The bootstrap values (%) are indicated at the nodes. The scale bar represents 0.02 substitutions per site. The strains with labels in bold were isolated in our present study.

2.6. Crude oil biodegradation

One milliliter of strains was inoculated in 100 ml BH plus 1% crude oil in a 250 ml flask and incubated in a rotary shaker at 30 °C and 220 r·min⁻¹ for 7 d. Each strain was tested with 1 blank control trial and 3 parallel experiments. The crude oil remnants were extracted by *n*-hexane, and the treated residues obtained were dissolved in methylene dichloride and analyzed by Gas Chromatography–Mass Spectrometry (GC/MS).

The GC–MS system consisted of a gas chromatograph (Agilent 6890N, USA) equipped with a 30-m HP-5MS (0.25-mm-by 0.25-μm film, USA), an Agilent 5975C MSD (Agilent Technologies, USA) and an Agilent 7683B operating at 70 eV. Helium was employed as the carrier gas and the average flow rate was 35.0 ml·min⁻¹. Separation on the column was achieved by using a temperature program from 50 (temperature remains at 50 °C for 3 min) to 310 °C at the speed of 8 °C·min⁻¹. The sample size was 1 μl with a diversion ratio 1:1.

For the crude oil biodegradation rate, we computed the integral for the peak area as the total oil content. In each sample, including the control and experimental groups, the peak area of the internal standard naphthalene and the alkane residues were isometric. The crude oil degradation rate was computed using the integral for the peak area of naphthalene and the alkane residues. After computing the integral for the peak area of the alkane residues, the corrective area of the different samples was calculated using naphthalene as the internal standard.

The crude oil degradation rate formula is shown as the following: A_H refers to the peak area of the residues alkane, with A_T refers to the peak area of naphthalene.

$$\text{degrading} = \frac{A_{H \text{ control}} - \frac{A_{H \text{ experimental}} \times A_{T \text{ control}}}{A_{T \text{ experimental}}}}{A_{H \text{ control}}}$$

2.7. Environmental tolerance

The environmental tolerances of the 24 strains including temperature, pH and salt concentration were systematically studied. Under the conditions for optimum growth, the degradation rate experiments on crude oil could then be performed. The growth conditions of the strains were measured by the detecting optical density at 600 nm (OD₆₀₀).

A single factor (5 levels) experiment was used for the gradient experiments. The parameters included temperatures of 20 °C, 25 °C, 30 °C, 35 °C and 40 °C; pH values of pH 5, pH 6, pH 7, pH 8 and pH 9; and salt (NaCl) concentrations of 0.5%, 2%, 4%, 6%, 8%. All gradient experiments used TSB culture medium and each level was subjected to 3 parallel controlled experiments.

2.8. Pilot-scale test

2.8.1. Bioremediation reagent preparation

We selected highly efficient oil-degrading and biosurfactant-producing strains with good environmental tolerances that were likely to produce good results in the pilot test. We prepared several different mixed bacterium agents and tested their degradation effects of 1% crude oil and we chose the agent that exhibited the best degradation effects for the following pilot-scale test. All of the mixed bacterium agents were composed of the strains isolated from the Tianjin Binhai New Area Oilfield.

The strains selected were activated overnight at 30 °C and 250 ml zymotic fluid was concentrated to 40 ml and added to 200 g solid medium. The solid medium was produced from bran, peat and distilled water. They were then cultivated at 30 °C for 2 d and diluted to 10⁷ CFU·g by peat. The same quantity of each strain was mixed to compound the bioremediation reagent.

2.8.2. Pilot test site construction

Three bioremediation pools (4.5 m × 3 m) were selected for the pilot-scale site in an approximately 40 m² area. These 3 bioremediation pools were labeled pool 1, pool 2 and pool 3 and were filled with gravel, sand, silt and clay as the matrix, and the filling ratio of each filler was adjusted based on the contents of the Tianjin native soil. To simulate the crude oil-contaminated soil environment, 7.0 kg BXPT crude oil and 7.0 L diesel were combined thoroughly and sprayed onto the surface of the substrate.

Pool 1 constituted the control group in which only water-soluble fertilizer NH₄NO₃ (2.0 kg) and KH₂PO₄ (0.4 kg) were added. Pool 2 and pool 3 were the experimental groups. In addition to equal amounts of water-soluble fertilizer, 1.0 kg bioremediation reagent (2.8 L) was added to pools 2 and 3, while 2.0 kg organic fertilizer was only added to pool 3.

Temperature sensors were installed in each pool in order to monitor the temperature.

2.8.3. Sample collection and detection

Soil samples were obtained once a week during the bioremediation experiments. In order to collect representative samples, 3 equally spaced samples were selected in each pool and numbered A, B and C. The columnar sampler used was 5 cm thick and 5 cm in diameter. The soil samples were combined uniformly and stored until testing. The testing mainly included temperature, pH, moisture content, bacterial content and TPHs.

For the temperature test, the surface temperature of each experimental pool was recorded twice daily. The recording times were 9 am and 2 pm. For the other tests, samples were taken once a week and 1 sample was divided into 3 parts. The pH of the soil samples was tested using a pH meter, while the water content was tested using the oven dry method and then estimated in the environmental media of bacteria growth. The bacterial content in each soil sample was tested using the dilution-to-extinction method and used to estimate the bacterial density of the environment. For petroleum hydrocarbon residue testing, each soil sample was dried and the petroleum hydrocarbons were extracted by *n*-hexane, then the total petroleum hydrocarbons (TPH) were measured by GC.

3. Results and Discussion

3.1. Isolation, screening and identification of biosurfactant-producing and crude oil-degrading strains

3.1.1. Isolation and screening

We screened different colonial morphologies first and then re-screened using shake-flask fermentation in order to obtain highly efficient crude oil-degrading bacteria. In total, more than 50 colonies with different colonial morphologies were selected. Following re-screening, 24 strains which could grow well with crude oil as the only organic carbon source were identified.

Table 1 shows the final selection of 24 highly efficient crude oil-degrading and biosurfactant-producing strains and their performance parameters.

3.1.2. 16S rRNA gene sequence analysis

The 16S rRNA region of these 24 strains was amplified by 16S PCR and sequenced by GENEWIZ (Suzhou) Biological Technology Co., Ltd. Following alignment and comparison with the reference sequences, the bacteria were identified.

The majority of the 24 strains isolated constituted gram-negative bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter venetianus*, *Klebsiella oxytoca* and *Achromobacter* sp., as well as gram-positive bacteria such as *Bacillus* sp., *Bacillus subtilis*, *Rhodococcus* sp. It has previously been reported that some of these bacteria (such as *Achromobacter* sp., *Lysinibacillus macroides* and *Bacillus* spp.) constitute petroleum-

Table 1
Characters of trains

Number	Name	Identification	Optimum temperature	Optimum pH	Salt-tolerance	Degradation rate
1	HA	<i>Klebsiella oxytoca</i>	25 °C	8	2%	46.55%
2	HA0	<i>Stenotrophomonas rhizophila</i>	30 °C	7	0.5%	47.33%
3	HA1	<i>Klebsiella oxytoca</i>	35 °C	7	2%	40.41%
4	HA2	<i>Achromobacter</i> sp.	25 °C	9	6%	45.09%
5	SA1	<i>Pseudomonas aeruginosa</i>	30 °C	7	8%	52.16%
6	SA2	<i>Pseudomonas aeruginosa</i>	30 °C	5	0.5%	47.68%
7	SA3	<i>Pseudomonas aeruginosa</i>	30 °C	5	2%	57.26%
8	W1	<i>Lysinibacillus macroides</i>	40 °C	7	0.5%	53.90%
9	W2	<i>Pseudomonas aeruginosa</i>	35 °C	8	6%	81.54%
10	W3	<i>Acinetobacter venetianus</i>	30 °C	6	6%	86.20%
11	WB1	<i>Acinetobacter venetianus</i>	30 °C	5	6%	69.80%
12	WB2	<i>Pseudomonas aeruginosa</i>	35 °C	7	6%	76.16%
13	T1	<i>Rhodococcus</i> sp.	35 °C	7	6%	56.71%
14	T2	<i>Rhodococcus</i> sp.	40 °C	7	6%	76.50%
15	J1	<i>Bacillus subtilis</i>	35 °C	7	6%	23.16%
16	J2	<i>Bacillus subtilis</i>	35 °C	9	8%	17.85%
17	J3	<i>Bacillus tequilensis</i>	30 °C	6	6%	68.92%
18	J4	<i>Bacterium</i>	35 °C	6	2%	42.03%
19	J5	<i>Brevibacillus brevis</i>	35 °C	8	4%	75.32%
20	J6	<i>Bacillus subtilis</i>	35 °C	6	4%	33.27%
21	C1	<i>Brevibacillus brevis</i>	35 °C	6	4%	23.21%
22	M1	<i>Bacillus thuringiensis</i>	25 °C	9	4%	59.26%
23	S-10-3	<i>Bacillus</i> sp.	25 °C	9	6%	31.02%
24	S-13-1	<i>Bacillus</i> sp.	25 °C	9	4%	46.25%

degrading bacteria [15–18]. Others, including *Acinetobacter venetianus*, *Bacillus subtilis* and *Brevibacillus brevis* have previously exhibited biosurfactant production capacity [35], while *Pseudomonas aeruginosa* possesses both degradation capability and biosurfactant production capacity [37].

One strain (HA1) was identified as *Klebsiella oxytoca* and constitutes an uncommon crude oil-degradation bacterium. It has been increasingly reported that *K. oxytoca* is able to efficiently degrade hydrocarbons. In fact, Chamkha *et al.* isolated and characterized *K. oxytoca* strain that exhibited good crude oil degradation [38]. Furthermore, the sequence alignment of the strain HA2 indicated that it might be a new strain based on the uncultured cloned partial sequence of the Ach-2 16S ribosomal RNA gene. The sequence comparison showed that HA2 was only 98% similar to *Achromobacter* sp. It has been reported that *Achromobacter* sp. could degrade crude oil and plays a large role in the bioremediation of soils heavily contaminated with crude oil [39]. HA2 possibly constitutes a novel high crude oil-degrading bacterium belonging to *Achromobacter*.

3.1.3. Phylogenetic tree reconstruction

The Phylogenetic tree of the 24 strains is indicated in Fig. 1.

The phylogenetic tree showed that the isolated strains from Tianjin Binhai New Area Oilfield, China, by and large, were closely associated and comprised typical crude oil-degrading bacteria of the region. *Pseudomonas aeruginosa* and *Bacillus* sp. were the major constituents of the local bacteria and more than half of all the strains belonged to the class Bacilli, which was strongly supported in the tree.

Recent research has documented that a large number of *Bacillus* spp., including *Brevibacillus brevis*, are able to utilize hydrocarbons as their only carbon source. Guo *et al.* found that a mixed culture of *B. brevis* and *Bacillus cereus* could improve the efficiency of crude oil degradation [40]. Furthermore, Hou *et al.* applied hydrocarbon-degrading bacteria comprising *B. brevis* in the low permeability but high hydrocarbon oilfields of Daqing, and obtained good results [41]. We isolated *Brevibacillus brevis* J5 and other *Bacillus* spp., including *Bacillus subtilis* J1, J3 and J6. These strains could possibly be combined to produce different mixed cultured forms. Effective synergies between these strains might improve the bioremediation of crude oil pollution especially in the local environment.

Pseudomonas aeruginosa has previously been found to degrade crude oil and produce biosurfactants [37]. Additionally, Zhang *et al.* discovered

a method to enhance the dispersion and biodegradation of octadecane by taking advantage of a rhamnolipid biosurfactant produced by *Pseudomonas* [42]. Noordman *et al.* also confirmed that the biosurfactants produced by *P. aeruginosa* as the substrate enhanced hexadecane degradation to a certain extent [43]. Doong *et al.* established the same conclusion in polycyclic aromatic hydrocarbon degradation [44]. The *P. aeruginosa* we isolated included SA1, SA2, SA3, W2 and WB2. As hydrocarbon decomposers, these strains could degrade crude oil to some extent and as biosurfactant producers, these strains possess the ability to enhance the dispersion of oil–water mixtures. This could further enhance the degradation of crude oil.

The strain pool of the isolated strains from Tianjin Binhai New Area Oilfield was representative. All these strains revealed that they were able to grow well in crude oil-polluted environments. The appropriate applicability of these strains could offer an effective means of local bioremediation and their co-cultivation might even offer a more significant role in the bioremediation of severely environmentally polluted areas.

3.2. Crude oil biodegradation

3.2.1. Oil flask culture and degradation rate test

After 7 d of incubation, the oil-flasks in the experimental groups exhibited a noticeable change in comparison to the control groups (Fig. 2). It appears that the concentration of the bacteria increased to a great extent, as the increasing amount of bacteria indicated that the strains inoculated were able to proliferate. Additionally, it also appears that the crude oil content in the liquid medium declined. The reduced crude oil content indicated that the inoculated strains were able to consume crude oil. In other words, the strain was capable of growing in the condition of 1% crude oil, and even capable of growing with crude oil as its sole carbon and energy source.

After several tests, most of the experimental groups exhibited the above phenomena. Furthermore, some of the experimental groups consumed the crude oil more noticeably, and thus possibly comprise highly efficient crude oil-degrading bacteria.

Fig. 3 shows the GC–MS analysis of crude oil biodegradation by the isolated strains.

Crude oil biodegradation by each strain for 7 d was computed in the same manner and compared with the blank control. After computation, most of the isolated strains indicated a good degradation effect towards crude oil. Specifically, W3 was able to reduce 86.20% of 1% crude oil in

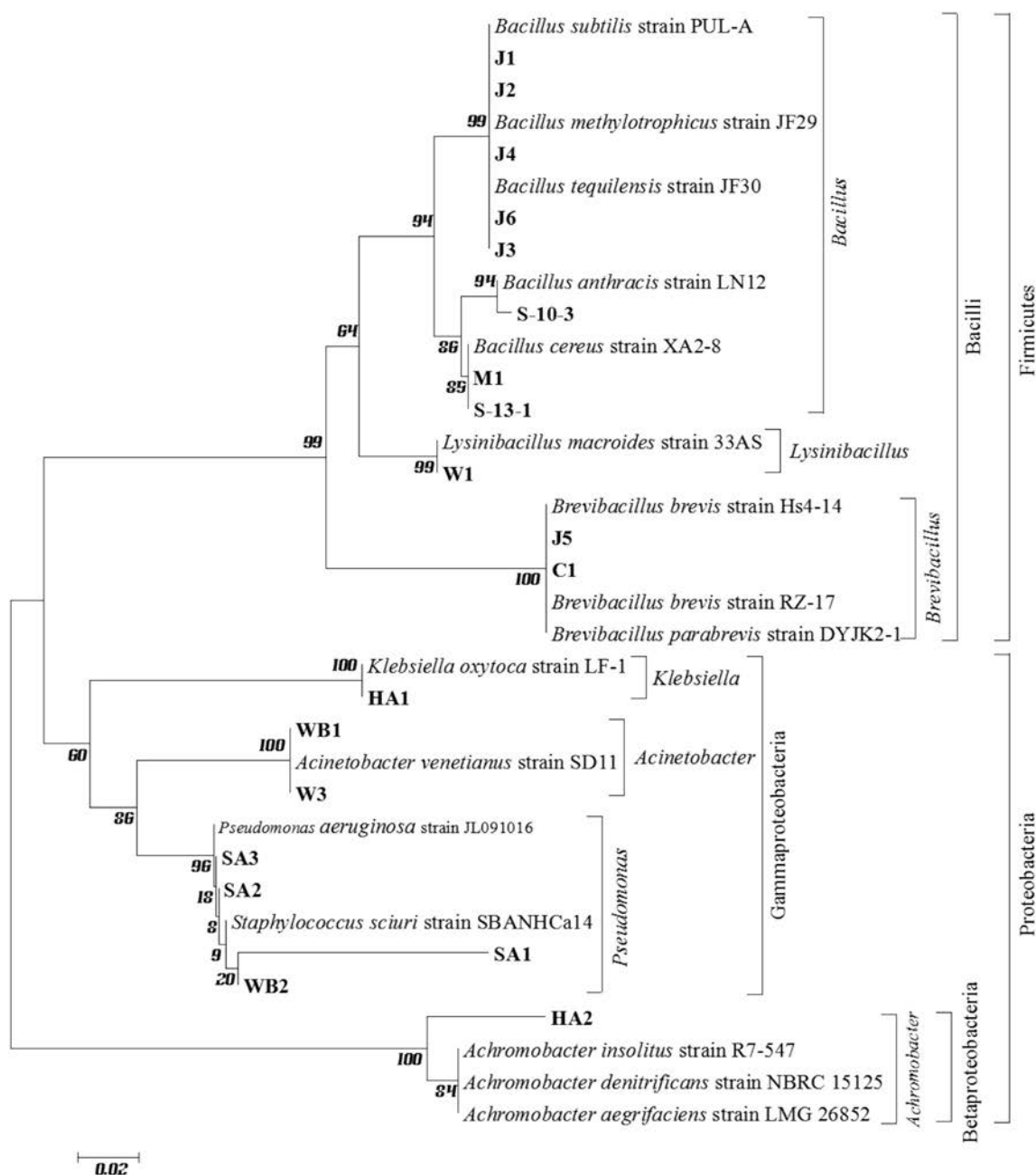


Fig. 1. Phylogenetic tree.

7 d (Fig. 3(a)), and it was concluded that W3 exhibited good biodegradation towards short chain and medium-long chain alkanes. Additionally, W2 degraded 81.54% of the 1% crude oil (Fig. 3(b)), while WB2 degraded 76.16% (Fig. 3(c)). Both W2 and WB2 tended to degrade short-chain alkanes. Ten strains out of the 24 tested were responsible for degrading over 50% of the crude oil, while 7 other strains degraded over 40%.

3.2.2. Identification of biosurfactant producers

An increasing number of biosurfactant producers have recently been identified, including *Acinetobacter* [45], *Arthrobacter* [46], *Achromobacter*, *Brevibacterium*, *Bacillus*, *Candida*, *Corynebacterium*, *Pseudomonas* and *Rhodotorula*. Guerra-Santos *et al.* found that *P. aeruginosa* cultured with glucose could produce biosurfactants [47]. A few years later, Yakimov *et al.* found a new lipopeptide surfactant producer, *Bacillus licheniformis*, growing in subsurfaces as well [48].

Upon observation of the oil-flasks containing these 24 strains, some of them appeared to form obvious emulsifications, as the interface of oil and water was not distinct when they were shaken. Approximately 2 cm to 3 cm of thick white foam formed among the oil–water layers, and the white foam did not dissipate for a long while. This suggested that a surfactant-like matter existed in the oil–water mixture. Among these 24 strains, SA1, SA2, SA3, W1, W2, W3, WB1 and WB2 exhibited the emulsification characteristics described above. It was initially thought that these 8 strains could produce biosurfactants and needed subsequent validation. Therefore, we cultured these strains overnight and subjected the clear zone to testing.

Fig. 4 shows the diameters of the clear zones of these strains. It could be concluded that these strains all had a certain size exclusive circle. However, SA1, SA2, W3 and WB2 possessed larger clear zones and were thus good biosurfactant producers with greater biosurfactant production capacity.

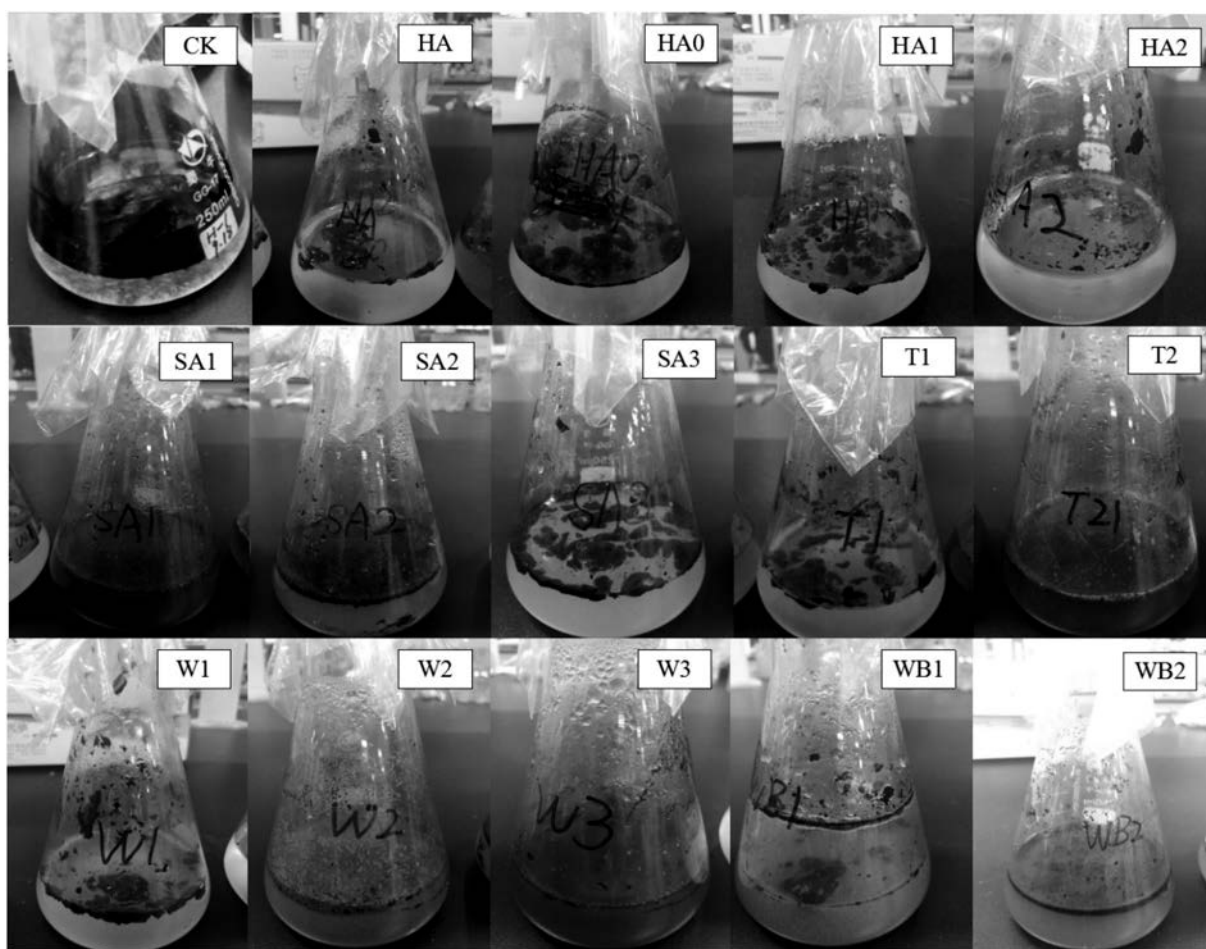


Fig. 2. Experimental and control groups incubated for 7 d.

Biosurfactants are effectively able to reduce the surface tension of petroleum hydrocarbons in the environment, which is of vital significance in practical oil pollution environment restoration. Yadav *et al.* isolated a bacterium producing a lipopeptide biosurfactant that aided the degradation of diesel oil by lowering the surface tension, and was able to degrade 88.6% of diesel oil in soil [49]. For other oil components, biosurfactants may also improve the effect of oil degradation strains degrading petroleum hydrocarbons indirectly. Zhao *et al.* discovered a bioemulsifier that was able to increase the light crude oil degradation rate of a bacterial strain from 37.5% to 58.3% within 56 d [50]. These examples all indicate the strong potential for biosurfactants in the bioremediation of oil polluted environments, and the discovery of the biosurfactant producers, particularly SA1, SA2, W3 and WB2, may considerably improve the bioremediation of crude oil.

3.3. Environmental tolerance

Almost all the 24 strains could grow well at temperatures ranging between 25 °C and 35 °C. While the crude oil-degrading efficiency was highest at 30 °C for the majority of the crude oil-degrading strains, some of the strains exhibited good growth even at 20 °C or 40 °C. The best temperature condition for W1 and T2 was 40 °C, while for other strains such as HA1, W2 and WB2, the best growing condition was 35 °C. These experimental results indicated that most of the strains isolated in the region were able to adapt to high temperatures to a certain degree, and are adaptable to a wide range of temperatures for growth, thus potentially offering practical solutions towards the realistic bioremediation of crude oil pollution.

Although pH 7 was generally optimal for the crude oil-degrading bacteria, some strains including SA2, SA3 and WB1 had the highest degradation efficiency at pH 5, while HA2 degraded crude oil best at pH 9. These all constitute good acid-fast bacteria or alkali-fast bacteria. The majority of the strains therefore exhibited good growth within a certain pH range. While some tended towards acidic conditions in the pH 6 to pH 7 range, others tended towards alkaline conditions in the pH 7 to pH 8 range. As the soil of the region is saline and alkaline bacteria have difficulty growing. This creates difficulties for bioremediation [51]. However, good alkali-fast bacteria could solve this problem. The strains isolated in this area are largely adapted to the saline-alkali soil, which is a great advantage for bioremediation of crude oil pollution. In fact, Dane *et al.* previously observed that these alkali-fast bacteria are able to effectively deal with this issue [52]. Fortunately, 21 of the strains tested in this study exhibited good growth at pH 8, while more than 80% possess a certain growth at pH 9. These strains are likely to produce good crude oil bioremediation results.

The salt concentration gradient assay indicated that the crude oil-degrading efficiency was highest at 0.5% towards most of the crude oil degrading strains. However, most of the isolated strains from the study area had the capacity to grow at higher salt concentrations. In particular, the strain WB2 exhibited optimal growth at a salt concentration of 2%, and is therefore salt-tolerant. As previously mentioned, the soil of the region is saline and alkaline. Wang *et al.* previously studied the bioremediation of alkane pollutants in high salinity environments [53]. Our approach was to screen the salt-tolerant bacteria to obtain a more effective response for local crude oil pollution bioremediation. We made some progress in this regard, as all of the crude oil-degrading bacteria isolated possessed degradation at 2% salt concentration. In fact, 17 of the

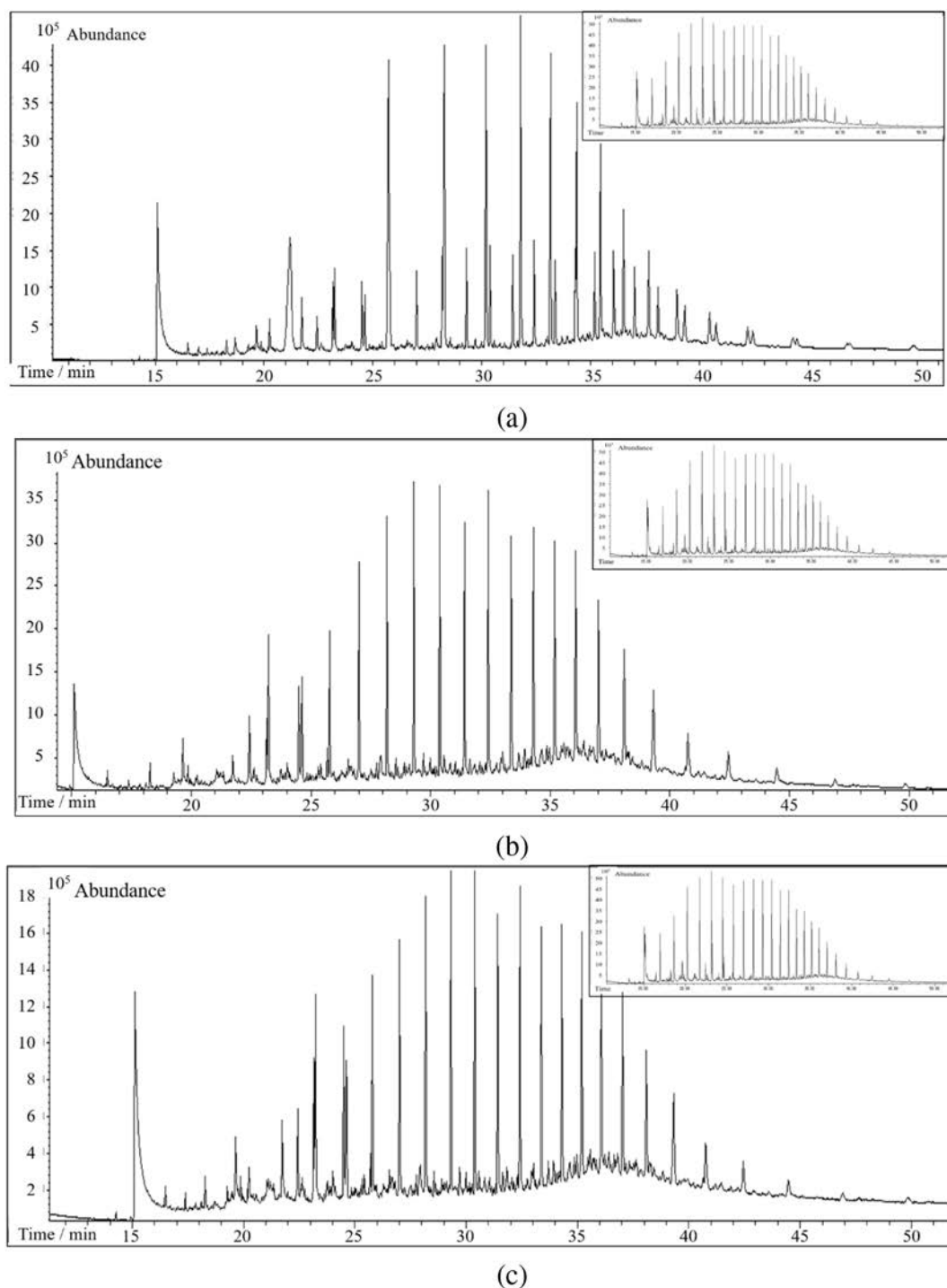


Fig. 3. GC-MS analysis of crude oil biodegradation.

strains were even able to grow well at 4% salt concentration. This indicates that the strains are adaptable to a wide range of salt concentrations for growth and may provide a practical means for the realistic bioremediation of crude oil pollution.

The data and figures regarding the environmental tolerances relating to temperature, pH and salt concentration are all provided as Supplementary materials.

3.4. Pilot-scale test

In the early stage of the oil-flask experiments, the degradation performances of the different mixed bacterial strains were tested.

The combination of 4 strains including HA, W2, W3 and WB2 exhibited the best degradation performance (Fig. 5), all constituting highly efficient crude oil degraders. Combined with the environmental tolerance test, all 4 of these strains possessed good growth at temperatures ranging from 25 to 35 °C. In addition, W3 is good acid-fast bacteria, while HA and W2 are both good alkali-fast bacteria. Furthermore, they are all salt-tolerant strains, in fact, W2, W3 and WB2 were even able to tolerate salt concentrations of 6%. Moreover, WB2 was found to be an excellent biosurfactant producer. Finally, HA, W2, W3 and WB2 were selected for the bioremediation reagent. The pilot test was developed based on the environmental aspects tested, including temperature, pH, moisture content and bacterial

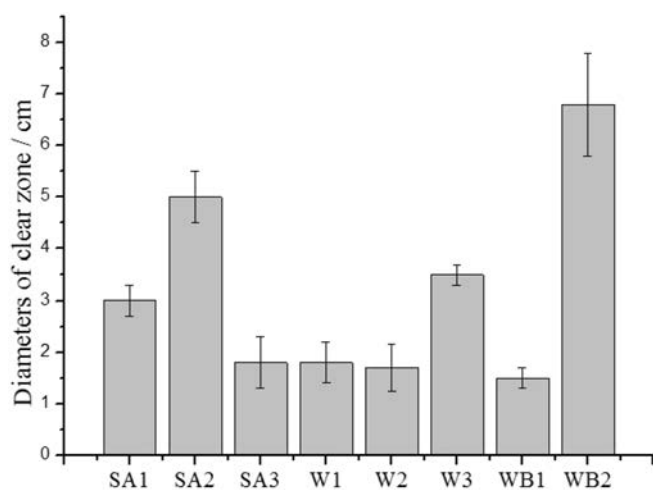


Fig. 4. Clear zone diameters.

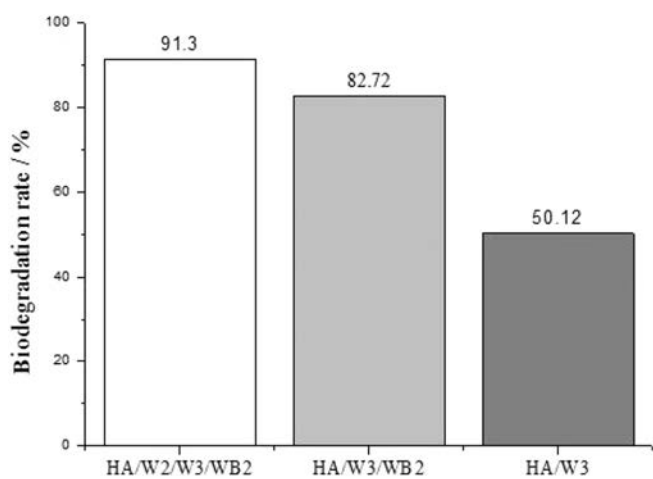


Fig. 5. Biodegradation of the mixed agents.

content, and the degradation efficiency at 0.8% initial oil was measured.

For the 60 d pilot-scale test, the 3 pools were all maintained under the same environmental conditions. The surface temperature of each experimental pool varied between 25 and 35 °C, changing with the change in environmental temperature. The pH ranged from 6.70 to 7.35, and slowly declined as a result of the production of organic acids. The moisture content of each experimental pool varied between 15% and 30%. And the mixed bacteria agent could grow under condition of normal in pilot plant that the bacteria density rose from about $1 \times 10^2 \text{ A} \cdot \text{g}^{-1}$ to $5 \times 10^4 \text{ A} \cdot \text{g}^{-1}$. The changes in temperature, pH, water content and bacterial content of the pilot-scale test are all provided as Supplementary materials.

Fig. 6 indicates the changes in TPH content. Due to the growth of the crude oil-degrading bacteria, the TPH in each pool evidently declined during the pilot test. The TPH of pool 1, in the absence of microbe inoculation, decreased from 0.8% to 0.6% as a result of the aboriginal microbes. As exogenous crude oil-degrading bacteria were added to pools 2 and 3, the TPH declined more evidently than pool 1. Following the 60 d pilot test, the petroleum hydrocarbon degradation rates reached 63.9% and 85.2% in pools 2 and 3, respectively. This indicated that majority of the hydrocarbon component of the oil had been degraded as the experiment progressed. The addition of exogenous bacterial agents (pools 2 and 3) resulted in a higher degradation rate than the

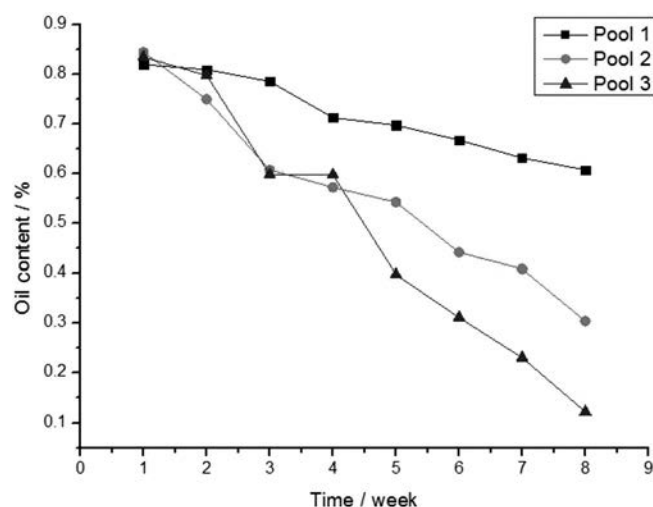


Fig. 6. Total petroleum hydrocarbon changes in the pilot-scale test.

control (pool 1). Better oil degradation was observed in the environment in which organic fertilizer additional had been added (pool 3).

Using an optimized tropical plant, Sanusi achieved 85.5% biodegradation of 3% diesel after 72 d [54], while Priya used co-culture strains and degraded 96% of the total crude oil in 4000 L natural seawater after 28 d [55]. Murygin *et al.* performed a pilot test and obtained a TPH concentration decrease by 23.0% in the 0–10 cm layer and 72.6% in the 10–25 cm layer [56]. However, we achieved a relatively higher biodegradation rate in crude oil-polluted soil in our pilot-scale test.

After adjusting the measures to local conditions and screening the local high efficiency crude oil-degrading bacteria and biosurfactant-producing bacteria, we prepared a mixed bacterial agent based on the results of our environmental tolerance experiments. Indigenous microorganisms possessed higher diversity and were well-adapted to the local oil-polluted environment [57]. The results of our 60 d pilot test confirmed that through domestication and mixed culture, these microorganisms might greatly aid the development of *in-situ* bioremediation of oil-polluted environments.

4. Conclusions

The 24 strains that we isolated were all able to grow using crude oil as a carbon source. They included *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Acinetobacter venetianus*, *Klebsiella oxytoca*, to mention a few. Some of the strains constituted highly efficient degrading bacteria, while others comprised efficient biosurfactant producers. Ten strains could degrade over 50% of the 1% crude oil concentration in 7 d. The majority of the strains had the ability to adapt to extreme environments, including high temperatures, alkaline environments and high salinity environments. Constituting acid-fast and alkali-fast bacteria, as well as salt-tolerant bacteria, they could be effectively applied to saline and alkaline environments. These properties suggest that these strains could realistically aid the remediation of crude oil pollution in Tianjin Binhai New Area Oilfield, China.

Using our environmental tolerance test results, we produced a mixed bacterial agent that could grow well in both high alkalinity and high salinity polluted environments. This mixed strain culture possessed a higher degradation efficiency to crude oil. Our 60 d pilot test suggested that the degradation rate of the mixed bacterial agent degrading 0.8% crude oil could reach 85.2%, realizing an excellent degradation. These results suggest that this method could provide guidance for the remediation of oil-polluted environments.

Supplementary Material

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cjche.2017.02.001>.

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