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Enhanced biodegradation of crude oil in contaminated soil by inoculation of hydrocarbon-degraders

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ABSTRACT

Inoculation of hydrocarbon-degraders will be a favorable feature for bioremediation of oil-contaminated soil. In this work, enhanced biodegradation of crude oil was investigated using the process, wherein inoculation of seven active hydrocarbon-degraders were isolated from the contaminated site. Preliminary results showed that isolated seven active bacterial strains were *Acinetobacter* SY21, *Neisseria* SY22, *Plesiomonas* SY23, *Xanthomonas* SY24, *Azotobacter* SY42, *Flavobacterium* SY43, and *Pseudomonas* SY44, respectively. Seven active degraders belong to gram-negative bacteria. They were capable of degrading crude oil and six other hydrocarbons (normally octane, paraffin wax, benzene, methylbenzene, phenol, and naphthalene) as carbon source and energy. Their biodegradation efficiencies of oil were higher than 80% after 8 d. The highest degradation rate of 2.34×10^{-3} mg cell^{-1} d^{-1} was obtained for bacterial strain SY43 (*Etwinia* sp.), followed by a degradation rate of 1.50×10^{-3} mg cell^{-1} d^{-1} for bacterial strain SY23 (*Plesiomonas* sp.). In addition, it was observed that a higher removal efficiency of TPH depends on large number of strains by more inoculums of isolates. Finally, it has been found that the adaptation of isolates was well because there was no decrease in the CFU even when the concentration of crude oil was up to 34250 mg/kg. Moreover, the removal efficiency of total petroleum hydrocarbon (TPH) was up to 88.4% after active oil degrader was inoculated while the corresponding removal efficiency of TPH was 1.4% without inoculums. These results indicated that both the SY43 and SY23 strains are easy to grow and establish a local ecology in the oil-contaminated soil. That is, biodegradation of crude oil in contaminated soil was enhanced by inoculating isolates.

Keywords: Isolating; Hydrocarbon-degraders; Biodegradation of crude oil; Inoculum; Bioremediation

1. Introduction

Areas of contaminated soil have increased rapidly in recent years due to the continuous growth and development of the oil industry in China. There have been several very large spills, chronic small scale spills, and many petroleum hydrocarbon contaminated sites. Meanwhile, the level of contamination becomes severe as time elapses. Contaminated soils include many complex compounds such as alkanes, benzene,

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methylbenzene, and benzene, among others. These contaminants are toxic and usually categorized as carcinogenic substances. They cannot be easily eliminated and will eventually leach into the groundwater systems [1,2]. Consequently, oil contamination is a serious environmental problem to our living ecosystem.

In the last decades, relevant bioremediation techniques have been widely studied because there is a pressing need for bioremediation of oil-contaminated soil with low cost and without leading to secondary pollution [3]. Most of these studies focused on enhancing bioremediation efficiency by increasing the activities of the native micro-organisms by adding nutrients such as nitrogen (N) and phosphorus (P) [4,5]. However, these studies have shown that native microbes need a long time to domesticate due to slow growth rates [6,7]. In addition, low metabolic activities of these native microbes make a rapid decontamination difficult. Therefore, the application of bioremediation using indigenous microbes is restricted. Fortunately, petroleum-degrading active bacteria could be the solution to this problem. For instance, the Baltic General Investment Corporation in America used mixed microbes to improve bioremediation [8]. The research of Wilson and Jones [9] presented bacteria strains which had shown high degradation rates of polycyclic aromatic hydrocarbons (PAHs). Bacteria of the genera *Sphingomonas, Burkholderia*, *Pseudomonas*, *Acinetobacter*, *Rhodococcus*, and *Mycobacterium* are well-known PAH-degraders. They successfully mineralized both low molecular weight PAHs and high molecular weight PAHs [10,11]. Thus, inoculation of hydrocarbon-degraders will be a favorable feature for hydrocarbon pollution bioremediation in contaminated environments. However, bioremediation of petroleum-contaminated soil is a complicated process, in which the pollutants characteristics, the ecological structure of microbes, and the environmental conditions must be considered. The characteristics of hydrocarbon-degrader can influence bioremediation [12,13].

In this research, several potential petroleum degraders were isolated from four oil-contaminated soils in the northern region of the Shaanxi province and identified by different morphological, physiological, and biochemical assays. Furthermore, the capability of biodegrading petroleum and growth of the bacterial strains were assessed according to the increase of microbial count and the changes in hydrocarbon concentration in soil monitored as isolates mineralized different concentrations of petroleum hydrocarbon in soil. The objectives of this study were to investigate enhanced biodegradation of crude oil using inoculation of seven active hydrocarbon-degraders isolated from contaminated site in oil field, and to find out the most effective degraders of crude oil for bioremediation.

2. Methods

2.1. Sampling

Samples of crude oil and oil polluted soil used in this experiment were collected from oil wells (14#, 28#, 4#, and 6#) located in the northern region of the Shaanxi province. Soil samples were collected in sterilized seal pack polythene bags. Later, they were ground and sieved through a 2 mm pore size sieve and stored at 4℃ for further physico-chemical and microbial assay to isolate oil degrading micro-organisms. In addition, 1 g of fresh soil was diluted and 0.1 mL of 10^7 dilutions were plated on nutrient agar and on mineral salts agar containing 0.5 mL sterile crude oil, and incubated for 30℃, respectively. Colonies were directly counted and expressed as CFU/g dry matter.

2.2. Isolation and characterization

2.2.1. Mineral medium

The composition of mineral medium used in this work was as follows: 2 g of NH_4NO_3, 1.5 g of K_2HPO_4, 3 g of KH_2PO_4, 0.1 g of $MgSO_4{\cdot}7\ H_2O$, 0.01 g of anhydrous $CaCl_2$, 0.01 g of $Na_2EDTA{\cdot}2H_2O$, and 1,000 mL of distilled water, with a pH ranging from 7.2 to 7.4.

2.2.2. Methodology of isolation

Enrichment cultures were prepared in screw-capped 250 mL Erlenmeyer flasks by addition of 10 g of fresh contaminated soils mixed with four soil samples into 100 mL sterilized mineral salts medium. The cultures were incubated at 30℃ in a THZ-82 shaker, manufactured by Changzhou Guohua Electronic Appliance Ltd, China, at a speed of 180 rpm for 7 d. Then, 50 mL of the enriched culture was transferred into 250 mL flask with 100 mL of fresh sterile mineral broth containing 1 mL of the sterile crude oil, and then were shaken again at a speed of 180 rpm for 7 d at 30℃ for the second enrichment. After such four successive weekly transfers, the culture was inoculated on the mineral salts agar containing crude oil to obtain the enriched consortium. Oil degrading micro-organisms were separated with a clearing zone around the inoculated region. The isolation and purification of the bacterial consortium were carried out on nutrient agar plates by conventional spread plate techniques [5]. Plates were incubated at 30℃ for 48 h after which isolated colonies were selected for further

identification. All isolates were stored at below zero that is at −20˚C.

2.3. Identification of isolated hydrocarbon-degrading active bacteria

The microbial isolates were first identified based on the morphological, cultural characteristics of individual colonies, then by traditional biochemical tests. Individual isolated colony was restreaked on mineral agar plates for identification. The isolated colony was gram-stained and different standard morphological, physiological, and biochemical tests were performed using BiobioA kit (96 Elisa plate, Shanghai, China).

2.4. Utilization of carbon substrates by isolated hydrocarbon-degrading active bacteria

In order to investigate the growth of isolates, the experiment was carried out using six common hydrocarbon compounds which included normal octane, paraffin wax, benzene, methylbenzene, phenol, and naphthalene as carbon source. The inocula containing 5% of the total volume were sampled in 100 mL flask containing 50 mL of mineral medium broth including hydrocarbon compounds mentioned above. Then, the mixture was incubated for 7 d with orbital shaking (180 rpm) under 30˚C. For utilization of phenol and naphthalene the inocula were added after all the acetone was evaporated from the mixture with phenol and naphthalene mixed with acetone. Finally, the optical density of the culture solution under 600 nm (OD_{600}) was measured.

2.5. Utilization of crude oil by isolated hydrocarbon-degrading active bacteria

About 10^3 CFU/mL of isolates were inoculated in mineral medium broth with 1,000 mg/L crude oil and incubated at 30˚C in an orbital shaker (180 rpm) for 5 d (120 h) to study their growth. Then, dry weight of bacterial strain was recorded. Same series were set in order to measure crude oil removal.

2.6. Bioremediation experiment

2.6.1. The degradation ability of isolated hydrocarbon-degrading active bacteria

Bench-scale experiments were performed to evaluate the ability of degradation of crude oil by seven hydrocarbon-degrading active bacteria (SY21, SY22, SY23, SY24, SY42, SY43, and SY44). The treatment was conducted in batch on 10 g of fresh soil sample with 50 mL of medium in 100 mL serum vials. At first, isolates were enriched in sterile mineral broth containing 1,000 mg/L of crude oil for 24 h at 30˚C. One milliliter of each inoculum having ~1 OD_{600} nm was centrifuged at 3,000 rpm for 15 min to get pellets of intact bacterial cells. After resuspension of the intact cells in buffer solution, the inocula containing 5% of the total volume were added to serum vials above mentioned in a THZ-82 shaker at 30˚C for 8 d. Residual concentrations of oil were measured in the soil samples during degrading period. Two blank samples were needed here, one of which was sterilized and the other was non-sterilized. The sterilized blank sample was prepared by adding 0.2 wt.% of mercury chloride into one of the oil-contaminated soil samples. A control test was conducted, which consisted of a contaminated soil sample in the absence of active bacteria but containing the indigenous or native micro-organisms' samples. All experiments were conducted in triplicate, and data shown are the means of the results. The relative standard deviation was always smaller than 5%.

2.6.2. Bioremediation of oil-contaminated soil by isolated hydrocarbon-degrading active bacteria

The bioremediation experiments were carried out in basin ports with diameters of 15 cm and depths of 15 cm. A mass of 1,000 g of fresh soil was used in each group. Each experimental group was conducted in triplicate. Experiments were carried out at room temperature, which varied in the range of 16–20˚C. Samples were taken for analysis at time intervals to measure pH and total petroleum hydrocarbon (TPH) concentration. About 1 g of soil was gradient diluted with deionized water, and 0.1 mL of 10^7 dilutions were plated on mineral salts agar containing sterile crude oil and incubated at 24–30˚C in dark. Colonies were directly counted and expressed as CFU/g [14].

2.6.3. The adaptability of the environment of isolated hydrocarbon-degrading active bacteria

Four soil samples 1–4 taken from different oil wells mentioned above were used to do this experiment. A potential hydrocarbon-degrading strain SY23 was selected as the inoculum to investigate the application of isolates. At first, strain SY23 was enriched in broth containing 1,000 mg/L of crude oil for 24 h at 30˚C. One milliliter of each inoculum having ~1 OD_{600} nm was centrifuged at 3,000 rpm for 15 min to get pellets of intact bacterial cells. In each sample the volume of inoculum was also varied as follows: 2, 5, and 10%.

The treatments were conducted (in triplicates) separately in Erlenmeyer flask (250 mL) containing 10 g of fresh each soil with 50 mL of mineral medium. All the flasks were placed in a THZ-82 shaker (set at 180 rpm) at 30℃ for 18 d incubation period.

2.7. Extraction and analysis

Soil samples and carbon tetrachloride were mixed using a solid/liquid ratio of 1:4 g mL^{-1} in a flask. The obtained slurry was sonicated for 15 min and then shaked at room temperature for 1 h (175 rpm, Gerhardt Laboshaker). Subsequently, carbon tetrachloride (20 mL) was added into the slurry and the slurry was shaken for 1 h again (175 rpm, Gerhardt Laboshaker). Finally, an extraction was set at a 50 mL of constant volume. The concentration of TPH was determined by an OCMA-350 non-dispersive infrared oil analyzer [15].

Detailed analysis of TPH (1 μl extracts) was performed on a GC system (model Trace 2000) and a Mass spectrometer model Voyager 5975B, manufactured by Agilent USA, which equipped with a capillary column (30 m × 320 μm × 0.25 μm) (DB-5). Nitrogen was used as carrier gas (3.5 mL/min). The injector temperature was 320℃. The initial oven temperature of 40℃ was maintained for 5 min, after which the oven was heated at 10℃/min, up to 300℃. This temperature was maintained for 10 min. The GC was equipped with an FID detector. The GC was started and ended with injection of pure carbon tetrachloride and boiling point calibration sample (5,632 mg/L) purchased from Agilent Technology. The measurable range of relative molecular weights was 30–450.

3. Results and discussion

3.1. Isolation and characterization

3.1.1. Isolation

Four oil-contaminated soils taken from 14#, 28#, 4#, and 6# oil well, which located at Yan chang oil field, Northern of Shaanxi province, China, were used as source of isolating hydrocarbon-degrading active bacteria. The physico–chemical characteristics of contaminated soil are presented in Table 1. The varying concentrations of TPH in four soil samples taken from different oil wells were 209 ± 8 mg/kg dry matter, 148 ± 6 mg/kg dry matter, 28, 100 ± 128 mg/kg dry matter, and 572 ± 13 mg/kg dry matter, respectively (Table 1). In addition, the microbial measurement showed the presence of aerobic bacteria and petroleum-degrading bacteria in the soil. The corresponding numbers are at the range from 10^3 to 10^5 CFU/g dry matters (Table 1). Isolation was operated at 30℃ using mineral medium inoculated with oil-contaminated soils as source of bacteria and crude oil as the sole source of carbon.

A total of 45 isolates were selected to be adjudged as hydrocarbon-degraders because they were able to grow on mineral salts medium in the absence of any other substrate except crude oil. All of 45 isolates which obtained from the primary screening on mineral salts agar also degraded crude oil in liquid culture. However, they varied widely in their ability to degrade crude oil (10–90%) as sole carbon and energy source. Finally, seven degraders (>70% of substrate degradation) were selected after second screening.

3.1.2. Identification

In order to identify the isolated micro-organisms, different physiological and biochemical tests were conducted (Table 2). All seven isolates were gram-negative bacteria. Some were micrococcus, and others were bacillus [16]. The seven identified isolates were *Acinetobacter* SY21, *Neisseria* SY22, *Plesiomonas* SY23, *Xanthomonas* SY24, *Azotobacter* SY42, *Flavobacterium* SY43, and *Pseudomonas* SY44, respectively.

3.1.3. The characteristics of bacterial strains growth

C_{10}–C_{61} of alkanes is a major component of petroleum-contaminated soil of the oil field located in Northern part of China. Hence, two hydrocarbons such as paraffin wax, normal octane, and four monoaromatic hydrocarbons were selected as the model compounds for the microbial degradation in order to determine the range of hydrocarbon compounds that can serve as sole sources of carbon for isolates. The growth of seven isolates is presented in Table 3. All of seven strains grew well in the paraffin wax media (The optical density D_{600} measured range from 0.117 to 0.450 shown in Table 3). It was found that strain SY43 had the highest growth rate in paraffin wax (the degradation efficiency of paraffin wax reached 81.3%), whereas strain SY21 grew slowly with the lowest efficiency of 43.7%. The bacterial growth in paraffin wax broth indicated that all isolates showed a high degradation capability toward middle and long-chain alkane because 90% of paraffin wax consisted of C_{18}–C_{61} normal and isomeric alkanes [17].

In addition, it was observed that a wide spectrum of substrate was utilized by strains SY23, SY24, and SY43. Strains SY23 and SY24 showed high abilities to degrade benzene, methylbenzene, and phenol as the corresponding degradation efficiency reached 80–90%.

Table 1
Characteristics of oil-contaminated soil samples used as source

Soil sample	Total petroleum hydrocarbon (TPH) concentration (Conc.) (mg/kg dry matter)	pH	Moisture content (wt. %)	Number of bacteria (CFU) (CFU/g dry matter)	
				Aerobic bacteria	Petroleum-degrading bacteria
Sample 1 (14#)	209 ± 8	8.89	8.3	9.2×10^3	1.3×10^3
Sample 2 (28#)	148 ± 6	8.66	15.5	1.3×10^5	5.6×10^3
Sample 3 (4#)	28,100 ± 128	8.55	13.9	4.4×10^4	2.2×10^4
Sample 4 (6#)	572 ± 13	8.91	15.1	3.4×10^5	1.0×10^5

Table 2
Physiological and biochemical characteristics of seven isolates

Strain	SY22	SY23	SY24	SY21	SY42	SY43	SY44
Shape	Micrococcus	Bacillus	Bacillus	Bacillus	Micrococcus	Bacillus	Bacillus
Gram stain	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Glycolysis	–	+	+	+	–	++	+
Lipolysis	++	+	++	++	++	+++	+
Catalase production	+	+	+	+	+	+	+
Milk litmus	Peptonize	Peptonize	Peptonize	Peptonize	Peptonize	Peptonize	Peptonize
Starch hydrolysis	–	–	+	–	–	+	+
Indoles production	–	+(after 7 d)	+(after 4 d)	–	+(after 7 d)	+(after 2 d)	+(after 7 d)
Methyl red test	–	–	–	–	–	–	–
Citric acid utilization	–	+	+	+	–	+	+
Identified results of isolated strains	*Neisseria* sp.	*Plesiomonas* sp.	*Xanthomonas* sp.	*Acinetobacter* sp.	*Azotobacter* sp.	*Etwinia* sp.	*Pseudomonas* sp.

However, it was observed that the majority of these strains showed low degrading efficiency toward normal octane, with the exception of strains SY24 and SY43, which showed degradation efficiencies of 54.4 and 56.8%, respectively. There are several reports available on the utilization of hydrocarbon as source of carbon and energy by pure bacterial strains [18]. Faster growth of isolates might have impact on degradation of petroleum hydrocarbon.

These observations indicate that seven isolates belong to hydrocarbon-degrading active bacteria, bacterial strains SY22, SY23, SY24, SY42, and SY43 are capable of degrading more than one hydrocarbon and have a strong resistance to toxic compounds, which makes them potential candidate bacterial strains for the bioremediation of petroleum-contaminated soil.

The relationships between the removal of crude oil and growth of seven isolates were investigated (Table 3). It was shown that dry weight of strain was increased to a great extent (80 ~ 110 mg), and about 10 ~ 18 mg of crude oil was removed after 120 h incubation (Table 3). Among the seven strains, the maximum removal of crude oil was recorded for SY23 (18 mg), which was the same as the trend of increasing growth (the maximum dry weight was 110 mg for SY23), followed by SY43 (16.3 mg), SY21 (13.4 mg), SY42 (11.5 mg), SY22 (11.3 mg), SY44 (10.8 mg), and the minimum was recorded in SY24. The higher cell conversion rates coming from degradation of crude oil (26.7%) were obtained for SY23 and SY43. The results indicated that 16.1 ~ 26.7% of carbon and energy for cell growth comes from crude oil, which agreed with the data in other report (22 ~ 26%) [19]. Higher cell conversion rates of SY23 and SY43 might have enhanced the degradation of crude oil in soil. However, isolates reached a pseudo-linear growth phase when their growth was limited physically by the maximum dissolution of crude oil which was converted in cells. In the pseudo-stationary phase, crude oil consumption of individual cells reached the maintenance level and consequently the growth ceased [10].

3.2. Crude oil removal in soil by hydrocarbon-degrading active isolates

3.2.1. Crude oil removal

Seven bacteria differed widely in their ability to degrade crude oil in slurry sample. As evident from Table 1 the rate of crude oil degradation was initially slow up to 2 d of incubation and then enhanced in all

Table 3
Utilization of carbon substrates and crude oil by isolated hydrocarbon-degrading bacteria

Bacterial strain	Utilization of crude oil				Utilization of carbon substrates[a]					
	Bacterial strain growth (mg)	Crude oil removal (mg)	Assimilation of Carbon (mg)	Cell conversion rate (%)	Normal octane	Paraffin wax	Benzene	Naphthalene	Phenol	Xylene
SY21	80	13.4	48.8	25.4	+[b]	++	–	+	+	–
SY22	90	11.3	54.9	20.6	–[b]	++	–	+	–	+
SY23	110	18	67.1	26.7	+[b]	+	++	+	++	+
SY24	80	10.7	48.8	22.3	++[b]	+	+	+	++	+
SY42	100	11.5	61.0	17.2	–[b]	++	–	+	+	+
SY43	100	16.3	61.0	26.7	++[b]	++	+	+	+	–
SY44	110	10.8	67.1	16.1	–[b]	++	–	+	+	–

[a]Growth was followed by measuring the increase of OD (600 nm) of the culture for 7 d.
[b]++, Good growth: $OD_{600\,nm} > 0.2$; +, growth: $OD_{600\,nm} > 0.1$; –, no growth: $OD_{600\,nm} < 0.02$.

strains gradually. However, in the cases of SY23 and SY43, the rates of crude oil degradation were recorded always higher than the other five strains namely SY21, SY22, SY24, SY42, and SY44. After eight days of incubation, the maximum degradation was recorded in SY23 (86%), followed by SY43 (85%), SY24 (79%), SY42 (78%), SY22 (77%), SY44 (75%), and the least was observed in SY21 (74%).

Deduction of 9% of crude oil under the abiotic condition was observed in the control test containing mercury chloride, while 37% of crude oil removal was observed in the control test with native microbes. This indicates that the indigenous microbes contributed to 25% of crude oil removal. Therefore, the actual degradation attributed to isolates was found to be 49% by SY23, 48% by SY43, 42% by SY24, 41% by SY42, 40% by SY22, 38% by SY44, and a minimum of 37% by SY21.

Degradation of crude oil was initially slow due to its hydrophobic nature which restricted its availability to microbes. However, after initial degradation, the polarity was probably introduced into the crude oil which enhanced its availability to strains for degradation by extracellular enzymes. Karsa and Porta [20] have reported involvement of monoxygenases and dioxygenases synthesized by bacteria in crude oil degradation. Most of PAH degradation reports have been made by gram-negative bacteria such as *Pseudomonas* sp. and *Mycobacterium* sp. [21,22]. Also, in our investigation, all of seven strains belong to gram-negative bacteria that were found to be efficient to remove crude oil.

3.2.2. Number of bacterial strain

Number of strain in slurry was monitored during the experiment. It was found to be increasing along the incubation time within a period of 8 d experiment and remained constant as the incubation progressed towards the end of the experiment (Table 4). In SY23, SY24, SY22, SY42, and SY44, the maximum CFU value was recorded after 2 d of incubation, while in SY43, and SY21, the highest growth was recorded after 8 d of incubation (Table 4). The CFU of seven isolates increased to 10^5 per gram soil after 8 d of incubation. This showed that isolated strain multiplied 100 times during the same incubation period, while native bacterial strain multiplied by 10 times only as compared to initial inoculum. The increase in cell count of the isolates during degradation was an indication that crude oil supported microbial biomass product even as a sole source of carbon and energy. Researchers have shown that

population of bacteria increase in slurry was a reflection of degradation process and proliferation of cell mass. This indicates that the indigenous microbes were inefficient in degrading crude oil and that the inoculation of hydrocarbon-degrading active isolates shows potential in removing hydrocarbons contaminants.

Bacterial strains evaluated showed different oil degradation capacities and different patterns. Table 4 presents the degradation rates of crude oil by seven bacterial strains. It was observed that the biodegradation rates of TPH by strains SY21, SY22, SY23, and SY44 were higher in the second day, while the biodegradation rates of SY42 and SY43 were higher in day 4, while in day 6, the highest degradation rate was shown by strain SY24. The average degradation rates of crude oil were within 0.01~0.1 g kg^{-1} d^{-1}, which were higher than degradation data previously reported [23–25], consequently these isolated strains could degrade crude oil more rapidly.

Previous work [26] using marine filamentous bacteria reported biodegradation rates of cell at the range from 7.92×10^{-11} to 4.8×10^{-10} mg $cell^{-1}$ d^{-1}. Similarly, other work [27] using *Pseudomonas* showed that the highest oil degradation rate was from 1.44×10^{-10} to 3.77×10^{-9} mg $cell^{-1}$ d^{-1}. Table 4 presents the average degradation rate per cell of seven isolated strains used in this work. It shows that the highest degradation rate of 2.34×10^{-3} mg $cell^{-1}$ d^{-1} was obtained for bacterial strain SY43 (*Etwinia* sp.), followed by bacterial strain SY23 (*Plesiomonas* sp.) with a degradation rate of 1.50×10^{-3} mg $cell^{-1}$ d^{-1}. The degradation rate of the remaining strains ranged from 1.15×10^{-3} to 4.57×10^{-4} mg $cell^{-1}$ d^{-1}. These results demonstrate that the rate of degradation shown by these strains was tens of thousands times higher than the degradation rate reported in a previous work [28]. Thus, hydrocarbon-degrading active isolates show great potential for the bioremediation of oil-contaminated soils.

3.2.3. Bioremediation of oil-contaminated soil by hydrocarbon-degrading active isolates

The oil-contaminated soil taken from oil wells was selected because it was expected to strain SY23 and SY43 adapted and survived in contaminated site and also has potential to degrade crude oil. Table 5 shows that significant removal of crude oil took place in the soil samples inoculated with hydrocarbon-degrading active bacteria (SY23 and SY43) compared with the removal of crude oil in the control sample without inoculum. After 6 d of bioremediation, the percentage of crude oil removal in the samples inoculated with strain SY43 and SY23 were 17.2 and 19.2%, respectively, while the percentage of crude oil removal in the control sample was only 1.4%. On day 9, the removal efficiency of strain SY43 was 30.5%, which was higher than the removal efficiency observed for the strain SY23 (24.9%).

After 12 d of bioremediation, the crude oil concentration of the sample inoculated strain SY43 declined to 213.3 mg kg^{-1} and the maximum removal efficiency of crude oil was 81.1%, while crude oil removal efficiency of the sample containing the strain SY23 and in the control sample were 60.3 and 25.6%, respectively. After 18 d of inoculation, the removal efficiency in the three samples climbed up to 88.4, 73.4, and 37%, respectively. These observations indicate that the contaminated samples inoculated with hydrocarbon-degrading active

Table 4
Removal of crude oil in slurry by seven isolates

Bacterial strain	Crude oil removal (%)	Number of bacterial strain (log CFU g^{-1})		Maximum degradation rate of TPH (mg kg^{-1} d^{-1})		Average degradation rate of cell ($\times 10^{-3}$ mg $cell^{-1}$ d^{-1})
		0 d	8 d			
Blank (sterilization)	9	–	–	–	–	–
Blank (native microbes)	37	2.00	2.88	After 6 d	0.05	1.24
SY21	74	3.30	4.00	After 2 d	0.25	2.34
SY22	77	3.20	4.40	After 2 d	0.24	0.82
SY23	86	3.28	4.60	After 2 d	0.23	0.48
SY24	79	3.37	4.60	After 2 d	0.18	0.54
SY42	78	3.40	4.80	After 4 d	0.17	0.62
SY43	85	3.20	4.20	After 4 d	0.19	2.52
SY44	75	3.40	4.60	After 2 d	0.22	0.46

Table 5
Removal of crude oil in soil by inoculation of isolates

	Volume of inoculums								
	TPH concentration ($g\,kg^{-1}$)			Removal of TPH (%)			Number of bacterial strain (log CFU g^{-1})		
Time (d)	Blank	SY23	SY43	Blank	SY23	SY43	Blank	SY23	SY43
0	1,067	982	1,067	–	–	–	6.40	7.40	7.60
6	995	794	899	6.7	19.2	15.8	6.30	7.18	7.40
9	881	739	741	12.0	24.9	30.6	6.48	7.15	7.45
12	793	391	202	25.6	60.3	81.1	6.65	7.15	7.48
15	680	280	191	36.3	71.5	82.1	6.78	7.30	7.78
18	670	262	124	37.0	73.4	88.4	6.98	7.65	8.04

isolates had higher oil removal efficiency than in the control sample. The average degradation rates of TPH by SY43 and SY23 was $0.044\,g\,kg^{-1}\,d^{-1}$, $0.034\,g\,kg^{-1}\,d^{-1}$, respectively, while the corresponding average degradation rate of the cells was $2.14\times10^{-6}\,mg\,cell^{-1}\,d^{-1}$ and $1.64\times10^{-6}\,mg\,cell^{-1}\,d^{-1}$, respectively. Thus, the ability of crude oil removal by SY43 was higher than that by SY23.

The number of bacteria in the inoculated soil samples was 100 times higher than that in the control sample (Table 5). In addition, it was observed that the number of bacteria in the soil sample inoculated with SY43 was higher than that in the soil sample inoculated with SY23. It's just explained why the highest removal of crude oil was obtained in soil sample inoculated bacterial strain SY43. Thus, the number of hydrocarbon-degrading active bacteria in the contaminated soil sample seems to be the main factor improving the bioremediation of the soil. The results show that both SY43 and SY23 could adapt rapidly to environment and degrade crude oil in the contaminated soil. It was found that bacterial strains SY43 and SY23 were the most effective degraders of crude oil in screening, which is evident in the TIC profiles (Fig. 1) of crude oil substrate following 18 d of incubation in the presence (test strain) and the absence (control sample) of bacterial inocula (Table 5).

4. Discussion

Microbial degradation of crude oil in soil is limited by the low number of bacteria. Thus, inoculation of hydrocarbon-degrading active micro-organisms will be a favorable feature for hydrocarbon pollution bioremediation in any environment. However, subsurface bioremediation contamination, mainly initiated by microbes, is a complex process, and increasing the population density of the isolates in the contaminated site is very important for bioremediation. Thus, in the bioremediation experiment, initial concentrations 1,250, 560, 34,250, and 2,090 mg/kg of TPH were taken for samples 1–4, and the volume of inoculums (SY23) was also varied as follows: 2, 5, and 10% in order to investigate the adaptation of isolates. The results are shown in Table 6.

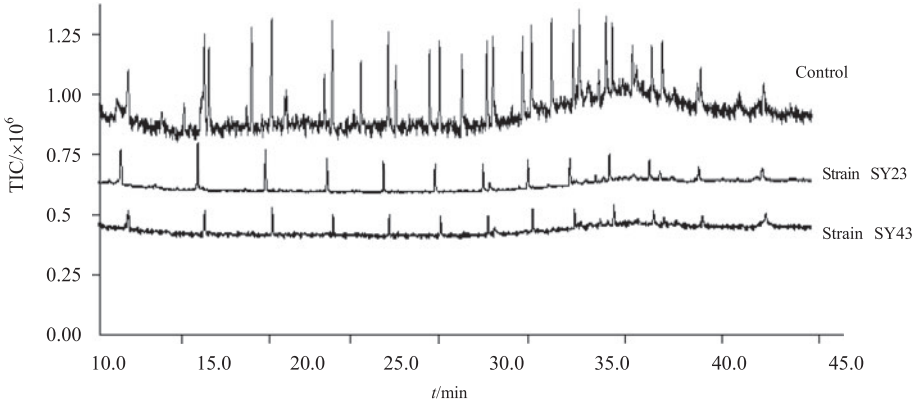

Fig. 1. GC mass spectrum of remaining crude oil in soil.

Table 6
The effect of the volume of inoculums on removal of crude oil

	Volume of inoculums					
	Removal of TPH (%)			Number of bacterial strain (log CFU g^{-1})		
Sample	2%	5%	10%	2%	5%	10%
Sample 1 (1,250 mg kg^{-1})	30.9	35.1	69.7	3.00	4.36	4.60
Sample 2 (560 mg kg^{-1})	20.3	43.1	46.5	2.48	3.00	3.11
Sample 3 (34,250 mg kg^{-1})	32.8	39.3	41.3	3.28	5.46	5.64
Sample 4 (2,090 mg kg^{-1})	48.1	82.0	85.0	2.30	2.80	4.90

The experimental findings indicated that in cases of 1,250 and 2,090 mg/kg crude oil, degradation started within 7 d, i.e. lag period observed at this concentration was very short. This crude oil concentration about 80% of TPH was degraded within 17 d. Biodegradation of lower and higher concentration of crude oil like 560 and 34,250 mg/kg were found relatively slow for consortium as very little decrease in concentration of about 40% was observed in 17 d span. However, there was no decrease in the CFU during the degradation even at higher concentration of crude oil. These results indicated that the bacterial strain SY23 supported growth even at higher concentration. The number of bacteria increased as the volume of inoculums increased in all treatments (Table 6). Hence, population density of isolates was enhanced by increasing the volume of inoculums. The experimental results indicate that the higher the inoculum volume the higher the removal efficiency of crude oil. The number of bacteria and removal efficiency of TPH for the soil samples were low when the inoculum volume was 2%. However, when the inoculum volume was increased from 2 to 5%, the removal efficiency of TPH increased. The removal efficiency of TPH was similar when the inoculum volumes were 5 and 10%. This performance might indicate that a high inoculum volume enhanced the competition ability of petroleum-degrading bacteria in relation to native micro-organism. It could also aid the quick bacterial adaptation to the environment, which also increased the TPH degradation efficiency. It was found that naturally selected microbial populations are enriched with micro-organisms carrying catabolic gene that degrades pollutants, and such adapted population have the advantage of being adapted to polluted sites.

5. Conclusions

Seven active degraders were isolated and identified successfully. The results show that they belong to gram-negative bacteria and grew up well as crude oil and seven other hydrocarbons (normal octane, paraffin wax, benzene, methylbenzene, phenol, and naphthalene) served as carbon and energy sources. These growth conditions are beneficial to the adaptation for bioremediation of oil-contaminated site as increasing the amount of inoculum enhances the competition ability of hydrocarbon-degrader against native bacteria. Further optimization showed that SY23 and SY43 were the most effective strains for bioremediation of oil-contaminated soil.

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