Biodegradation of hydrocarbons by microbial strains in the presence of Ni and Pb

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Abstract

Microbial strains capable of degrading petroleum hydrocarbons were isolated from the Yellow River Delta and screened for bio-surfactant production. The bio-surfactant-producing characteristics of the isolates were evaluated, and all the isolates which could produce bio-surfactant were identified by 16S rRNA gene sequencing. The results showed that the isolates belong to *Bacillus* sp. (72%), *Ochrobactrum* sp. (0.16%), *Brevundimonas* sp. (0.06%) and *Brevibacterium* sp. (0.06%). The biodegradability of crude oil, gasoline, diesel oil and other hydrocarbons by microbial strains were studied, among which the biodegrading ability of strain P1 and strain P19 is higher than other strains. Both strains P1 and P19 can degrade *n*-hexane and *n*-hexadecane effectively and have wide substrate extensiveness. In addition, Ni promoted the biodegradability of toluene by both strain P1 and strain P19, while Pb inhibited the growth of strain P19 and decreased its ability to biodegrade toluene. The studies revealed that microbes including strain P1 and strain P19 can be utilized in bioremediation of co-contaminated water with petroleum and heavy metals including Ni and Pb.

Keywords Bioremediation · Bio-surfactants · Co-contamination · Heavy metals · Petroleum hydrocarbons

Introduction

Contamination of water, gas or soil by petroleum and its products has become a serious problem in the environment around the world (Jamal and Pugazhendhi 2018; Maddela et al. 2017; Pacwa-Plociniczak et al. 2014; Xi et al. 2014). Bio-augmentation has been shown to be efficient to clean up the environment polluted with petroleum, heavy metals or other organic compounds by introducing microbes into polluted soil or water for the remediation of contaminated sites (Federici et al. 2012; Mishra et al. 2017; Yousafzai et al. 2017).

During the bioremediation of petroleum hydrocarbon-polluted water, hydrocarbon-degrading bacteria are used for the removal of pollutants (Bai et al. 2015; Iqbal et al. 2019; Luz et al. 2004; Mouton et al. 2009; Zhang et al. 2019). One

of the difficulties in the removal of petroleum hydrocarbons is the high hydrophobicity and low water solubility of petroleum hydrocarbons and their derivatives. Bio-surfactants are surface-active compounds produced by different microorganisms, including bacteria, yeast and fungi, which can partition at the water–air and water–oil interfaces to reduce interfacial tension and surface tension in the hydrocarbon and aqueous mixtures (Georgiou et al. 1992; Qi et al. 2017; Sachdev and Cameotra 2013; Youssef et al. 2004). In addition, bio-surfactants have many advantageous features, including low toxicity, high biodegradability and ecological acceptability (Amani et al. 2013; Desai and Banat 1997; Nitschke and Pastore 2006; Santos et al. 2016; Yoshikawa et al. 2017). These features make bio-surfactants good alternatives for microbial-enhanced oil recovery (MEOR) (Lotfabad et al. 2009; Okolo et al. 2016; Santos et al. 2016). Bio-surfactants are also widely used as cleansers of petroleum hydrocarbon-contaminated groundwater (Irorere et al. 2017; Sharma and Pant 2000). A large area of land was polluted by petroleum in the Yellow River Delta, Shandong Province, China. It is necessary to isolate petroleum-degrading microbes and study their bio-surfactant-producing characteristics.

The potential to excrete bio-surfactants by each isolate was evaluated by different methods in this study.

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Biodegradability of different petroleum hydrocarbons from the petroleum-polluted water by the isolates was also studied. Monoaromatic petroleum hydrocarbons, toluene, benzene, and mixture of xylenes are of more and more concern because of the high water solubility (Kaczorek and Olszanowski 2011; Qi et al. 2017; Yadav et al. 2012); therefore, the dynamic biodegradability of the hydrocarbons was studied. The main pollutants in oil-contaminated soil are petroleum hydrocarbons and heavy metals, such as Pb (Zhang et al. 2019). Environmental pollution by heavy metals has become a serious threat to living organisms in an ecosystem (Okolo et al. 2016). Some of these metals are highly toxic and can cause cancer, abnormality, and are thus serious environmental threats. Petroleum hydrocarbons and heavy metals produce synergistic effect in soils that exacerbate the toxicity and damage to the environment, making treatment more difficult (Bai et al. 2015; Mouton et al. 2009) (Goswami et al. 2017; Klimek et al. 2016). So it is very necessary to develop strains facing to co-polluting of crude oil and heavy metals. The biodegradation of petroleum hydrocarbons by microbial strains in the presence of Ni and Pb was first studied in this study. The tolerance of heavy metals including Ni and Pb by microbial strains was studied. Furthermore, the biodegradation of toluene by the isolates in the presence of Ni and Pb was studied to determine the effect of heavy metals on biodegradability of petroleum hydrocarbons. The research would be helpful to lay the theoretical foundation to bioremediate the co-contamination of petroleum hydrocarbons and heavy metals.

Materials and methods

Chemical reagents

Chemicals, including KH_2PO_4 , NaNO_3 , $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, NaCl , $\text{NH}_4(\text{CH}_3\text{COO})_3\text{Fe}$, $\text{Ni}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$, $\text{Pb}(\text{NO}_3)_2$ and glycerol, were purchased from Shanghai Bio Engineering Co., Ltd. Tryptone and yeast extract were bought from US Biological Co. Blood agar plates were purchased from Shanghai Yihua Science and Technology Co., Ltd. Crude oil was donated by Jinan Refinery, Shandong Province. Both gasoline and diesel oil were bought from Petro China. Other petroleum hydrocarbons including *n*-hexadecane, *n*-hexane and toluene were bought from Sigma Co.

Sample collection

Samples were collected from petroleum-contaminated soil or sewage near Shengli Oilfield in the Yellow River Delta, China. The sites were located in petroleum-polluted area of different counties and have been contaminated with

petroleum for more than 10 years. All samples were stored in aseptic container at 4 °C before analysis.

Isolation of the petroleum hydrocarbon-degrading microbes from the petroleum-polluted area

Petroleum hydrocarbon-degrading microbes were isolated by continual enrichment method with minimal salt media (MSM) containing (g L^{-1}) KH_2PO_4 6.8; NaNO_3 0.085; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.05; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 7.8 and $\text{NH}_4(\text{CH}_3\text{COO})_3\text{Fe}$, 0.01 including trace element solution and 1 ml L^{-1} crude oil as the sole organic carbon source. The trace element solution was prepared according to the formulation by Pfennig and Lippert with minor modifications (Swati et al. 2014). All the microbes were purified and identified by 16S rRNA gene sequencing.

Phylogenetic identification of bacterial isolates

The bio-surfactant-producing bacterial isolates were phylogenetically identified by 16S rRNA gene sequencing. DNA extraction was performed using Genomic DNA isolation Kit (Qiagen Inc., USA). The 16S rRNA gene was amplified using the universal primer sets, the forward was 5'-GAG AGT TTG ATC CTG GCT-3' and the reverse was 5'-CTA CGG CTA CCT TGT TAC-3' (Viramontes-Ramos et al. 2010). The PCR amplicons were purified by the Gel Extraction Kit (QIAGEN) and sequenced by Sangon Biotech. The sequences were compared with the 16S rRNA sequences by the BLAST server at NCBI. All sequences were aligned by CLUSTAL software and analyzed by neighbor-joining method by MEGA software. The sequences of all strains were submitted and deposited in GenBank.

Characteristics of the isolates from the petroleum-polluted area

Bio-surfactant-producing characteristics

Hemolytic activity of all the isolates was screened as described before (Swati et al. 2014). Each isolate was streaked onto blood agar plates and incubated at 28 °C for 48 h. Opaque circles around the colonies were checked and considered as indicative of bio-surfactant production (Pacwa-Plociniczak et al. 2014).

Substrate spectrum of the isolates

All bacterial strains were inoculated into MSM medium to confirm substrate specificity with 50 mg L^{-1} of different petroleum hydrocarbons as the sole carbon and energy source, including toluene, *n*-hexane, gasoline and diesel oil. Liquid petroleum hydrocarbons were filtered and added

separately to sterile MSM medium with a final concentration of 0.1% (v/v). Incubation was continued for 10 days (Chen et al. 2017; Swati et al. 2014). Degradation and turbidity were monitored continuously during incubation.

Drop-collapse test

The drop-collapse method was always used to detect the bio-surfactant production (Viramontes-Ramos et al. 2010). Cells were collected by centrifugation (12,000g, 5 min) and then re-suspended in 1 ml of M9 broth. 1.8 µl gasoline or diesel oil was added to each well of 96-well microplates and then the plates were equilibrated at room temperature for 24 h. 5 µl of the suspension was added on the surface of the oil. After 1 min, the shape of the drop was inspected. The result was scored as negative (−) if the drop remained beaded; on the other hand, if the drop collapsed, the result was scored as positive (+) (Viramontes-Ramos et al. 2010). All the tests were executed in triplicate and non-inoculated Erlenmeyer flasks were included as controls.

Assessment of surface tension

Each bacterial isolate with positive result in the drop-collapse test was also evaluated for surface tension of its fermentation liquid. Strains were inoculated into M9 broth amended with sucrose (1% w/v) for 5 days. 5 ml supernatant of fermentation liquid of each isolate was transferred into a test tube which was submerged in water bath at 28 °C. Surface tension was determined by measuring the height reached by the liquid when ascending through a capillary tube (Munguia and Smith 2001). Non-inoculation broth was used as the control.

The surface tension was calculated by the following formula:

$$\gamma = \frac{rh\delta}{2}g,$$

where γ surface tension (mN/m), δ density (g/mL), g gravity (980 cm/s²), r capillary radius (0.05 cm), and h height of the liquid column (cm).

Determination of emulsification index

2 ml of cell suspension and 3 ml of gasoline were mixed in a glass tube and vortexed for 2 min to determine the emulsification index. The glass tubes were kept at 25 °C and the height of emulsion layer was detected to calculate the emulsification index based on the following formula (Cooper and Goldenberg 1987):

$$E = \frac{\text{height of emulsion layer}}{\text{height of total solution}} \times 100\%.$$

Degradation of petroleum hydrocarbons by microbial strains

Degradation of crude oil by microbial strains

The degradation of crude oil by microbial strains was carried out in BH liquid medium supplemented with crude oil, diesel or gasoline, respectively. The residual petroleum hydrocarbons were determined gravimetrically by extracting the residual crude oil using diethyl ether after incubation at 28 °C for 7 days (Margesin et al. 2003). For each sample, 2.5 ml diethyl ether was added into the fermentation liquid followed by vigorous shaking. The liquid was then separated by laboratory funnel and evaporated at room temperature to remove the residual solvent. At last, the weight of the crude oil residues was determined using a standard curve. The degradation rates of the petroleum hydrocarbons by different strains were calculated as reported previously (Batista et al. 2006).

Degradation of gasoline and diesel oil

The degradation rates of gasoline and diesel oil by microbial strains were detected in BH liquid medium supplemented with gasoline or diesel oil as single carbon and energy source, respectively. Each treatment was executed in three replicates. After incubation at 28 °C for 7 days, the dry weights of residual gasoline and diesel oil were determined after extraction with *n*-hexane (Borah and Yadav 2014). Then the percentage degradation was calculated based on the weight loss between residual petroleum hydrocarbons before and after inoculation.

Degradation of *n*-hexadecane, *n*-hexane and toluene by isolated microbial strains

Strains with high degradation ability were selected to study the biodegradability of different hydrocarbons. The isolates were grown on BH mineral liquid medium with *n*-hexadecane, *n*-hexane or toluene as single carbon and energy source. The concentration increased from 1000 mg L^{−1}, 2000 mg L^{−1} to 3000 mg L^{−1} gradually. 12 triangular flasks were prepared for each concentration of hydrocarbons, and every 3 flasks were taken to detect the concentration of hydrocarbons every 7 days.

The inoculation volume was 200 µl in each triangular flask with the same density. Precultures were inoculated into the BH mineral liquid medium supplemented with *n*-hexadecane, *n*-hexane or toluene as single carbon and

energy source. Blank medium was used as control. To dislodge abiotic contamination, volatilization procedures were performed to remove *n*-hexadecane. OD₆₀₀ was determined to evaluate total biomass by spectrophotometric method. Before inoculation, cell suspensions were centrifuged and re-suspended to remove nutrients and other impurities. All treatments were executed in triplicate. After shaking on the orbital shakers for 7 days, the residual *n*-hexadecane in the fermentation liquid was determined by gas chromatography (GC) after extraction with *n*-octane (Noordman and Janssen 2002). *N*-Hexane was also quantified in all samples by the method provided by Valenzuela-Reyes et al. (Valenzuela-Reyes et al. 2014), while the concentration of toluene was detected by high-performance liquid chromatography (HPLC) (Kim et al. 2013).

Tolerance of the isolates to heavy metals

Ni (100 mmol/L) and Pb (100 mmol/L) stock solutions were prepared in Milli-Q water with Ni (CH₃COO)₂·4H₂O and Pb(NO₃)₂ (Sigma-Aldrich), respectively. The metal solutions were added into the MSM medium after being autoclaved and the final concentration of the heavy metals was 100 μM, 10 mM, 100 mM, respectively. Each treatment was carried out in three replicates. The isolates were incubated in LB medium overnight, harvested by centrifugation at 5000 rpm for 10 min, and washed three times with sterilized water. The supernatant of the isolates was inoculated in the MSM medium with heavy metals and incubated at 37 °C for 24 h. Then all the inoculants were counted by colony counting method to study the tolerance of the isolates to the heavy metals.

Biodegradation of toluene by the isolates with the presence of heavy metals

When studying the biodegradation of toluene by strains P1 and P19 in the presence of heavy metals, 100 μM Ni or Pb and toluene were added to the BH mineral liquid medium, respectively. After shaking in the orbital shakers for 7 days, the residual toluene in the fermentation liquid was determined by HPLC (Kim et al. 2013).

Results and discussion

Isolation and screening of bio-surfactant-producing bacteria

Isolates with different phenotypes were obtained from the samples. All isolates were used to screen the bio-surfactant-producing bacteria by hemolytic experiment. Bio-surfactants can reduce surface tension so that the red blood cells rupture

and release hemoglobin to produce opaque circles. After inoculation into the blood plates for 48 h, the appearance of opaque cycle showed that the bacteria can produce bio-surfactants. The diameter of the opaque cycle is proportional to the bio-surfactant-producing ability of bacteria. The results showed that nine isolates could produce opaque circle on the blood agar plates. Data are shown in Fig. 1. Opaque cycles produced by strains P2, P3, P7, P8, P11, P19 and P25 were much larger than other strains, which showed that they can produce more bio-surfactant. Opaque cycles were indicative of bio-surfactant production (Pacwa-Plociniczak et al. 2014); therefore, strains P2, P3, P7, P8, P11 and P25 can produce more bio-surfactants than other strains at the detection time.

Phylogenetic identification of bacterial isolates

The isolation and screening strategy of this study resulted in the isolation of different phenotypes of microbial strains in petroleum-polluted area in the Yellow River Delta. Generally, the native strains are efficient degraders of the petroleum hydrocarbons than exogenous pure strains. All the isolates from the petroleum-polluted area in the Yellow River Delta which could produce opaque cycles were identified by 16S rRNA gene sequencing, and the results showed that the isolates belong to *Bacillus* sp. (72%), *Ochrobactrum* sp. (0.16%), *Brevendimonas* sp. (0.06%) and *Brevibacterium* sp. (0.06%). Data are shown in Table 1.

Characteristics of the isolates from the petroleum-polluted area

Substrate spectrum of bio-surfactant-producing bacteria

Microbes can grow in medium with different hydrocarbons as single carbon and energy sources. The substrate extensiveness of different strains was also determined and the data are shown in Table 2, while the bacteria without signs of growth on the medium are not shown. Toluene, *n*-hexane, gasoline and diesel oil were important contaminants. Some isolates can grow in MSM with the above hydrocarbons as single carbon and energy source. The results showed that the isolates have a broad substrate spectrum.

Drop-collapse test

A drop-collapse method has been used as both quantitative and qualitative assay to screen bio-surfactant-producing bacteria (Das et al. 2014). The drop-collapse test was carried out in 96-microwell plates. The efficacy of the qualitative drop-collapse method has once been analyzed to determine the production of rhamnolipid (George and Jayachandran 2013). The phenomena of negative and positive results are

Fig. 1 Strains with opaque cycles on the blood plates

Table 1 Identification of strains isolated from the petroleum-polluted area in the Yellow River Delta

Isolate	Identification	Similarity (%)	Accession no.
P1	<i>Ochrobactrum</i> sp.	99	KF987808
P2	<i>Bacillus</i> sp.	99	KR703632
P3	<i>Bacillus</i> sp.	99	KR703646
P7	<i>Bacillus</i> sp.	99	KR703633
P8	<i>Bacillus</i> sp.	99	KR703634
P9	<i>Bacillus</i> sp.	98	KR703635
P11	<i>Bacillus</i> sp.	99	KR703636
P15	<i>Bacillus</i> sp.	98	KR703637
P19	<i>Bacillus</i> sp.	99	KF990489
P20	<i>Brevundimonas</i> sp.	99	KR703639
P25	<i>Ochrobactrum</i> sp.	99	KR703641

shown in Fig. 2. When the drop expanded on the oil surface of the 96-microwell plate, the positive isolates showed collapsed liquid drops in 1 min, suggesting that these strains could excrete bio-surfactant or that the bio-surfactant may

remain intracellular (Bodour and Miller-Maier 1998). The results of drop-collapse test are listed in Table 3.

Surface tension of each isolate's fermentation liquid

Surface tension is a force between molecules that develop on the interface between the two immiscible fluids and lies on the interface. Generally, surface tension is expressed in force per unit length, as milliNewtons/meter or dynes/centimeter. Research on surface tension of solutions has been one of the interesting subjects in practical and scientific points of view, which is an index to certify the biodegradation of petroleum hydrocarbons (Abou-Shanab et al. 2016). However, when microbes excrete bio-surfactants, particles are introduced into the liquid and the surface tension would be influenced. The results showed that the excretion of surfactant would change the surface tension of liquid. The surface tensions of liquids are listed in Table 4. Results showed that the surface tension of fermentation liquid of strains P2, P3, P8 and P11 was smaller than that of strains P1, P19, P20 and P25,

Table 2 Substrate extensiveness and comparative growth of different isolates

Substrate	Strain	Qualitative growth
Toluene	P1	+++
	P3	+++
	P11	++
	P15	+++
	P19	+++
N-Hexane	P1	+++
	P11	++
	P19	+++
	P25	++
Gasoline	P1	+++
	P7	+++
	P8	++
	P19	+++
	P1	+++
Diesel oil	P2	++
	P19	+++
	P20	+++

+ Poor growth, ++ good growth, +++ luxuriant growth

which indicated that the former strains could produce more bio-surfactants at the detection time.

Emulsification index of fermentation liquid of the isolates (%)

Surfactants can reduce the interfacial tension and increase oil–water emulsification so as to enhance biodegradation of contaminants (Hazen et al. 2010). Some strains isolated from the petroleum-polluted area in the Yellow River Delta could excrete bio-surfactants and enhance the biodegradation of petroleum. The emulsification indexes of fermentation liquid of the isolates were detected as previously reported (Hazen et al. 2010). Data are shown in Table 5. The results showed that emulsification indexes of different fermentation liquid were different. In addition, when the petroleum hydrocarbons changed, the emulsification index changed. The emulsification indexes of both P1 and P19 were higher than other strains, and emulsification of different petroleum hydrocarbons by each isolate was different.

Bio-surfactants are a kind of surface-active chemical compounds produced by a wide variety of microbes. The exploitation of new strains for the production of novel bioactive substances is very important (Plociniczak et al. 2013; Sriram et al. 2011). Generally, surface-active substances produced by microbes can reduce surface tension at the water–air interface (bio-surfactants) and decrease the interfacial tension between immiscible liquids or at the liquid–solid interface (bio-emulsifiers) (Batista et al. 2006; Silkina et al. 2017). Bio-surfactants often exhibit emulsifying capacity but bio-emulsifiers do not necessarily decrease surface tension (Desai and Banat 1997; Qi et al. 2017).

Degradation of crude oil, diesel oil and gasoline by microbial isolates

All the isolates could grow on BH medium plate with crude oil as the single carbon and energy source. But the biodegrading ability of the isolates was different. The results showed that both strain P1 and strain P19 could degrade crude oil, diesel oil and gasoline obviously. Among all the detected strains, strain P1 could degrade crude oil mostly, with the highest biodegradability of 54.37%, while the biodegradability of diesel oil by strain P1 was 66.23%. Strain P19 could degrade 55.20% of gasoline in the triangular flask

Fig. 2 Negative and positive results of drop-collapse test**Table 3** Drop-collapse results of the isolates in gasoline, diesel oil and mineral ether

Strain	P1	P2	P3	P7	P8	P9	P11	P15	P19	P20	P25
Gasoline	+	–	–	+	–	–	–	+	+	+	+
Diesel oil	+	–	–	+	–	–	–	+	+	+	+
Mineral ether	+	–	–	+	–	–	–	+	+	+	+

+ Positive, – negative

Table 4 Surface tension of different isolates in fermentation liquid with crude oil as carbon resources (mN/m)

Strain	P1	P2	P3	P7	P8	P9	P11	P15	P19	P20	P25
Surface tension	57.2 ± 2.5	28.7 ± 3.5	26.7 ± 2.9	43.3 ± 18	28.3 ± 5.8	31.7 ± 5.0	27.6 ± 2.9	48.6 ± 2.6	58.9 ± 2.8	58.3 ± 2.9	58.9 ± 2.0

The results are shown as average value ± standard deviation

Table 5 Emulsification indexes in fermentation liquid of each isolate (%)

Strain	Gasoline	Diesel oil	Hexadecane
P1	42.6 ± 3.1	47.9 ± 2.9	37.9 ± 2.3
P2	19.2 ± 1.0	0.0	0.0
P3	26.2 ± 3.1	6.3 ± 2.4	15.2 ± 4.1
P7	32.4 ± 4.4	42.7 ± 5.8	18.2 ± 2.2
P8	32.5 ± 4.7	58.2 ± 4.7	0.0
P9	17.5 ± 4.6	46.2 ± 3.4	5.6 ± 1.8
P11	13.4 ± 6.5	5.4 ± 2.1	25.4 ± 1.4
P15	14.6 ± 1.5	15.4 ± 2.9	0.0
P19	36.9 ± 2.6	46.7 ± 2.8	39.2 ± 3.1
P20	16.5 ± 1.4	6.5 ± 0.7	9.2 ± 1.0
P25	15.7 ± 0.7	12.0 ± 3.8	5.6 ± 0.0

The results are shown as average value ± standard deviation

Fig. 3 Biodegradability of crude oil, diesel oil and gasoline by the isolates

(Fig. 3). Crude oil is a kind of complex mixture of different, predominantly pure hydrocarbons, or other compounds with atoms of nitrogen, sulfur and oxygen. Strains P1 and P19 may degrade not only one of those compounds, but also some other constituents of the crude oil.

Data in Fig. 3 show that the degradation rates of crude oil, diesel oil and gasoline by both strain P1 and strain P19 were higher than other isolates, which indicated that strain P1 and strain P19 could degrade constituents of crude oil.

Therefore, further study about whether strains P1 and P19 could degrade linear or aromatic hydrocarbons need to be conducted.

Biodegradability of *n*-hexadecane, *n*-hexane and toluene by strain P1 and strain P19

Concentration of *n*-hexadecane decreased at different levels during incubation. After inoculation, the concentration of *n*-hexadecane in fermentation liquid of strains P1 and P19 decreased from 0.10% to 0.0104% and 0.00323%, which indicated that strains P1 and P19 could degrade 81.6% and 88.8% of *n*-hexadecane, respectively, while the concentration of *n*-hexadecane did not decrease obviously in fermentation liquid with inoculation of other strains. When concentration of *n*-hexadecane increased in the medium, biomass of all strains was stressed, for the fermentation liquid was very well distributed when 0.1% *n*-hexadecane was added to the medium as the sole carbon and energy source, while a ball was converged in the fermentation liquid when the concentration of *n*-hexadecane increased to 0.2%, and the converged ball grew gradually when the content of *n*-hexadecane increased to 0.3%. So high concentration of *n*-hexadecane had stress effect on the bacteria.

The results showed that both strains P1 and P19 could degrade the three kinds of hydrocarbons obviously, especially *n*-hexane. Strain P1 could degrade 89.3% of all the *n*-hexane in the fermentation liquid, while strain P19 could degrade 92.24%. As a kind of aromatic hydrocarbon, 53.7% of toluene in the fermentation liquid could be degraded by strain P1 in 7 days. The biodegradability increased with time. Data are shown in Fig. 4. The biodegradability of both strains P1 and P19 increased slowly, while there was a small increase of the degradation rate of toluene, especially during the first 14 days. The degradation of *n*-hexadecane, *n*-hexane and toluene could be induced if there was no other carbon source in the medium. Strain P1 could degrade more toluene than strain P19 when detected. After 28 days, degradation rate of *n*-hexane by strains P1 and P19 was found to be 92.3% and 93.4%, respectively, while degradation rate of *n*-hexadecane by strains P1 and P19 was found to be 85.4% and 92.6%, respectively. Some factors inhibiting the degradation rate of hydrocarbons need to be changed to enhance the degradation of hydrocarbons.

Qiu et al. isolated a bacterial strain of the genus *Ochrobactrum* (Qiu et al. 2006). Later Zhong et al. reported the

Fig. 4 Biodegradability of different hydrocarbons by strains P1 and P19 during incubation

degradation of aromatic compounds and *p*-nitrophenol by *Ochrobactrum* sp. B2 (Zhong et al. 2007). In addition, degradation of *n*-alkanes, polyaromatic hydrocarbons (PAH) benzo[a]pyrene and chlorinated hydrocarbon by *Bacillus* sp. has been studied (Bieszkiewicz et al. 2002; Wu et al. 2007; Xia et al. 2014). Invariably, microbes play important roles in the bioremediation of petroleum-polluted sites. The action mechanism of different microbes to degrade hydrocarbons and also the interaction between microbes and plants need to be studied for application to ecological remediation of petroleum-polluted area.

Tolerance of strains P1 and P19 to heavy metals

Besides contamination of hydrocarbons, heavy metal contamination in soils and waters has created global concern (Ayangbenro and Babalola 2017). Sometimes both petroleum hydrocarbons and heavy metals co-pollute the environment (Goswami et al. 2017; Wang et al. 2015). So it is very important to study the biodegradation of hydrocarbons by strain P1 and strain P19 in the presence of heavy metals. After incubation overnight in MSM medium with Ni and Pb,

strains P1 and P19 were counted by colony counting method. Data are shown in Table 6.

The results showed that the growth of strain P1 could be inhibited by Ni and Pb. With the increasing concentration of the heavy metals, the inhibition of strain P1 was enhanced. Considering that the growth of strain P1 was inhibited by 0.1 mM of Ni, while the degradability of P1 increased (Fig. 5), we supposed that Ni may activate some enzymes produced by strain P1 which could help degrade toluene during the growth. The growth of strain P19 was inhibited by Pb, while low concentration of Ni could promote the growth of strain P19. Heavy metal may be a key substance of some enzymes and proteins, and sometimes trace heavy metals are essential for the growth of bacteria (Liu et al. 2012; Yousafzai et al. 2017), which might be the reason that strain P19 grew better in MSM with 100 μ M Ni.

Biodegradability of toluene by strain P1 and strain P19 in the presence of Ni and Pb

The accumulation of contaminants including heavy metals and hydrocarbons often existed in soil due to wastewater used for irrigation, which raises concern about public exposure to contaminants in the environment (Zhang et al. 2017). It is essential to study the biodegradability of

Fig. 5 Biodegradability of toluene by strains P1 and P19 during incubation with Ni and Pb

Table 6 CFU of strain P1 and strain P19 in MSM containing Ni or Pb ($\times 10^7$)

Strain no.	Concentration of Ni or Pb						
	0	100 μ M		1 mM		10 mM	
		Ni	Pb	Ni	Pb	Ni	Pb
P1	7.81 $\pm$ 0.32	5.19 $\pm$ 0.23	2.64 $\pm$ 0.31	2.60 $\pm$ 0.11	0.65 $\pm$ 0.09	2.10 $\pm$ 0.13	0.02 $\pm$ 0.005
P19	2.80 $\pm$ 0.16	6.87 $\pm$ 0.45	2.59 $\pm$ 0.10	2.43 $\pm$ 0.09	0.56 $\pm$ 0.06	1.85 $\pm$ 0.13	0.85 $\pm$ 0.16

Results are shown as average value $\pm$ standard deviation

hydrocarbons by strain P1 and strain P19 in the presence of heavy metals. Ni was added to BH liquid medium to study the biodegradability of toluene by strain P1, and the results showed that Ni could promote the biodegradability of toluene, while Pb could inhibit the biodegradability of toluene. The data are shown in Fig. 5. Hausinger P.R. reported that nickel ion was an essential micronutrient for many microorganisms in which it is incorporated into at least four microbial enzymes, and the enzymes can participate in the important metabolic reactions including acetogenesis, ureolysis, methane biogenesis and hydrogen metabolism (Hausinger 1987). On the other hand, Gao reported that biosurfactant can remove heavy metals including Pb and Ni from the polluted sludge (Gao et al. 2012), and we determined that both strain P1 and strain P19 could produce biosurfactant, which can decrease the toxicity of the heavy metals. When the biosurfactant was used to remove the toxicity of Ni and Pb, more biosurfactant would be produced to help degrade toluene. The results showed that the biodegradability of toluene by strain P1 and strain P19 could be promoted by 100 μM Ni after incubation for 28 days, while Pb had little effect on the biodegradability of toluene by strain P1 and strain P19. The action mechanism of strain P1 and strain P19 regarding the combined resistance to heavy metals and petroleum hydrocarbons needs to be studied deeply in later research.

Conclusions

Microbes that produced bio-surfactant were isolated in petroleum hydrocarbon-polluted area near Shengli oil field in the Yellow River Delta. All the strains have potential utilization in the bioremediation of petroleum-polluted sites. Strains P1 and P19 could produce bio-surfactants to help the degradation. Biodegradation abilities of both strains P1 and P19 are high. Strain P1 could degrade 89.3% of all the *n*-hexane in the medium, while strain P19 could degrade 92.24%. In 7 days, 53.7% of toluene in the fermentation liquid could be degraded by strain P1. After 28 days, degradation rates of *n*-hexane by strains P1 and P19 reached 92.3% and 93.4%, respectively, while degradation rates of *n*-hexadecane by strains P1 and P19 reached 85.4% and 92.6%, respectively.

Both strains P1 and P19 could degrade different hydrocarbons because they can produce bio-surfactants which are helpful for the degradation of petroleum hydrocarbons. Both strains P1 and P19 have wide substrate extensiveness. Results showed that 100 μM Pb could promote the biodegradation ability of strain P19. The combined resistance of heavy metals and petroleum hydrocarbons, and the action mechanism by the microbes need to be studied deeply.

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Compliance with ethical standards

Conflict of interest The authors of this work declare that they have no conflict of interest.

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