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Characterization of crude oil degrading microbial cultures isolated in Qingdao China

Yongrui Pi [a,b], Mutai Bao[a,b*], Yiming Li[a,b], Guangmei Li[c], Jinren Lu[b], Peiyan Sun[c]

[a] Key Laboratory of Marine Chemistry Theory and Technology, Ministry of Education, Ocean University of China, Qingdao 266100, China

[b] College of Chemistry & Chemical Engineering, Ocean University of China, Qingdao 266100, China

[c] Key Laboratory of Marine Spill Oil Identification and Damage Assessment Technology, North China Sea Environmental Monitoring Center, State Oceanic Administration, Qingdao 266033, China

Abstract

9 hydrocarbon-degrading strains were isolated based on their ability to grow with crude oil as the sole carbon source from the water and sediments samples of Qingdao offshore. The isolated microbes, pure and mixed cultures, were demonstrated to degrade petroleum, and petroleum samples that contain higher concentrations of lower molecular hydrocarbons experience greater biodegradation. These cultures are phylogenetically related to previously characterized hydrocarbon degrading microbial cultures, dominated by members of the *Pseudomonas* cluster; *Brevundimonas* cluster;

* Corresponding author: Mutai Bao, *E-mail address*: mtbao@ouc.edu.cn (M. Bao); *Full postal address*: 238 Songling Road, Qingdao 266100, Shandong, PR China; *Tel:/Fax*: +86-532-66782509.

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Bacillus cluster; *Peptoclostridium* cluster. The mixed culture and some individual cultures can form stable emulsions of oil in water and that the surfactant activity possessed by these cultures is predominantly associated with the bacterial cells and extracellular polymeric substances.

Keywords: enzyme activity, extracellular polymeric substances, hydrocarbon-degrading bacteria, spill oil

Introduction

With the increasing demand for oil energy of human society, oil production, transportation and other activities increase frequently. During these activities, there are a large amount of the oil released into the marine environment, such as the "Deepwater Horizon" oil spill of the Gulf of the Mexico[1]. The principal methods to manage oil spill are physical methods, chemical methods and bioremediation. Physical and, on rare occasions, chemical methods are capable of rapidly removing the majority of beached oil, but they are rarely completely successful[2].

Microbial degradation is an environmental-friendly strategy gaining increasing prominence for its potential to clean up oil contaminated water or sediments. The ability to fully degrade all of the compounds found in oil is thought to be beyond the capability of any single species, so mixed microbial cultures are preferred for the bioremediation of petroleum-contaminated water and soil, and even then biodegradation is limited to

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the lower molecular weight hydrocarbons. More than 79 known genera of marine hydrocarbon-degrading bacteria distributed over several (sub) phyla (*α*-, *β*- and *γ-proteobacteria*; *Gram positives*; *Flexibacter-Cytophaga-Bacteroides*) have been described so far.[3-10]

Enzyme activity of the hydrocarbon-degrading bacteria was investigated and confirmed different enzyme activity has important influence on the degradation of oil spill or polycyclic aromatic hydrocarbons (PAHs).[11-14] Recently, the bacteria was employed to stabilize oil in the water,[15] and some microorganisms can emulsify hydrocarbons even in the absence of cell growth or uptake of hydrocarbons.[16] The latter suggested that emulsification may be associated with the surface properties of the cells, as a result of attachment to the oil-water interface by general hydrophobic interactions rather than specific recognition of the substrate. What's more, bacterial cells may behave as fine solid particles at interfaces.

Extracellular polymeric substances (EPS) which are secreted by microorganisms during growth, consisting of various organic substances such as polysaccharides, proteins, nucleic acids and lipids, may express on the cell surface or release into the surrounding seawater. EPS production by microbial cells is commonplace, and serves a number of different functions, principally stabilization and protection of a biofilm structure, because an EPS covering on a cell surface alters the physicochemical characteristics of the surface such as charge, hydrophobicity and the polymeric property. EPS has an ability to interface with hydrophobic organic chemicals, such as

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hydrocarbons[17-19]. The potential significance of marine bacterial EPS to influencing the fate and ultimate degradation of hydrocarbon pollutants in the ocean, particularly during oil spills, remains an important issue needed for further study.

The overall goal of our research is to isolate efficient petroleum hydrocarbon degrading bacteria under in *situ* conditions in the marine environments impacted by the spilled oil in the ocean. The degradation effect on crude oil was investigated and the activity of dehydrogenase and peroxidase which were produced during the biodegradation process was studied. The emulsification activity of the bacteria and extracellular polymeric substances on the crude oil was investigate to explore the contribution of the emulsification activity on the biodegradation of the crude oil.

Materials and methods

Chemicals and samples

All chemicals used in this paper were analytic grade and obtained from various commercial sources. The three kinds of crude oil used in this study were all from the Shengli oilfield. The percentages of saturates, aromatics, resins, and asphaltenes for Haierzhan crude oil are 62.3%, 22.7%, 9.5% and 0.9%. The percentages of saturates, aromatics, resins, and asphaltenes for thick oil are 23.5%, 20.6%, 42.8% and 3.0%. The percentages of saturates, aromatics, resins, and asphaltenes for residual oil are 27.7%, 18.6%, 28.4% and 20.1%. Ten samples including 6 sediments and 4 water samples were collected from offshore of Qingdao (Loushan River, 36°12’N, 120°20’E), which was polluted by petroleum products.

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Enrichment experiments and isolation

Enrichment medium contained beef extract 3.0 g/L, peptone 10 g/L, and NaCl 5.0 g/L. The basic medium used for screening was mineral salt medium (MSM) with crude oil as the sole carbon source. The MSM contained K_2HPO_4 0.5 g/L, $NaSO_4$ 2.0 g/L, NH_4Cl 1.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.02 g/L, $CaCl_2$ 0.07g/L [20] and 1.0 mL of trace salt solution per liter. The trace salt solution was defined as 30 mg/L $FeCl_3$, 0.5 mg/L $CuSO_4$, 0.5 mg/L $MnSO_4 \cdot H_2O$, and 10 mg/L $ZnSO_4 \cdot 7H_2O$.The pH was adjusted to 7.0-7.2 with 1.0 M NaOH and 1.0 M HCl before sterilization.

Hydrocarbon-degrading bacteria present in the soil and water samples were isolated in two ways: (a) by direct cultivating of dilutions of the samples on mineral salts agar containing crude oil as the sole carbon and energy sources; and (b) by plating of enrichment cultures of the samples prepared in mineral salts broth, also containing crude oil as the sole carbon and energy sources.

Bacterial identification

The degrader was identified by morphology, physiobiochemical characteristics including catalase reaction, methyl red, V-P test, amylohylysis, nitrate reduction, nitrite reduction, and denitrification performed using standard procedures and genetic analysis based on 16S rDNA gene sequence as well as API identification systems. Colony morphology was observed on enrichment medium incubated at 25°C. Genomic DNA was extracted directly with the DNA extraction kit (Cwbio, China) according to manufacturer's instructions. The 16S rDNA gene was PCR amplified with universal

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primers as described previously[21]. PCR products were purified with GeneJET Gel Extraction Kit (Thermo Scientific) and sequenced by NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA). The resulting 16S rDNA gene sequences were compared with the sequences in the GenBank nucleotide library using BLAST program. Multiple sequence alignment was carried out using Clustal X 1.8.1 and phylogeny was analyzed using MEGA 6.0. Phylogenetic tree was constructed using the neighbor-joining method.

Measurement of crude oil degradation.

After the identification, 9 microbial cultures were isolated and their biodegradation abilities on the crude oil was tested, which were carried out in the 250mL-flasks with 0.3g oil in the 150mL MSM inoculate 7.5 mL bacteria suspension(OD_{600}=1.0). After the biodegradation, the remaining oil in the flasks was extracted twice from the culture fluid with 50 mL petroleum ether and the petroleum ether phase was then collected 20min after extraction. The organic phase was subsequently analyzed by UV spectrophotometer, which was used to determine the percentage degradation of the oil samples. Also, the biodegradation factors such as temperature, N and P-sources and their concentration was analyzed in this method. All treatments except the sterile control were performed in triplicate.

The Haierzhan crude oil samples after biodegradation process under the optimized conditions were analyzed by GC-FID and GC-MS. GC-FID analysis of the *n*-alkane distributions was performed on a Shimadzu (Kyoto, Japan) GC-2010 equipped with a

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flame ionization detector. The details for the experimental conditions and quality control can be found in Wang [22] and Sun[23].

Measurement of surface tension of the MSM

The surface tension of the aqueous phase of the MSM after the remaining oil in the flasks was extracted was measured by surface tension meter (BZY-2, from Shanghai equity instrument). Then the aqueous phase of the MSM was centrifuged for 5 minutes (11,293×g). The surface tension of the supernatant was analyzed again.

Measurement of the dehydrogenase and peroxidase activity

The precipitation of above was cleaned with 0.9% physiological saline more than 3 times. And then it was suspended in the 0.9% physiological saline until its OD_{600}=2.0. The measurement of dehydrogenase activity was performed by a modified spectrophotometric method according to the literature[24]. Peroxidase activity is measured as the rate of substrate oxidation in the presence of added H_2O_2 [25].

Emulsification assays

For emulsification assays, cell-free supernatants (11,293×g; 5 min) and cells which were washed twice with 0.9% physiological saline were employed in the emulsification assays. Emulsification assays were performed by mixing samples (1 ml) with an equal volume of *n*-tetradecane or *n*-hexadecane or *n*-tetradecane + crude oil or *n*-hexadecane + crude oil in screw-cap glass tubes (4mL). The tubes were manually shaken (30 s) and vortexed (30 s) to homogeneity, left to stand for 10 min, and shaken as before, and the height of the emulsion layer, denoted as emulsification index (EI_{24}), was measured after

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allowing the mixture to stand for 24 h at room temperature.

Results

Enrichment of isolated strains with crude oil and isolation

All the bacteria from the samples of Loushan River were cultivated in the MSM with Haierzhan crude oil for 7 days and transferred into the fresh MSM. At the end of the fourth week, the components changed during the biodegradation process of the Haierzhan crude oil were analyzed by GC-FID and GC-MS (Fig.1). From Fig. 1a, the cultivated microorganism could metabolize *n*-C_9 to C_{38} effectively. The *n*-C_9-C_{22} were almost completely degraded at 25 ^oC, while *n*-C_{23}-C_{33} were partly degraded. However, *n*-C_{34}-C_{38} were recalcitrant to the biodegradation. Fig. 1b shows the distribution changes of five targeted alkylated PAHs. The alkyl homologues of naphthalene were easier than the other four PAHs to utilize by the bacteria. As shown in Fig. 1c, other low-molecular aromatics, such as acenaphthalene and acenaphthene, were also oxidized to a certain extent. The *n*-C_{17}/Pr and *n*-C_{18}/Ph among all the diagnostic ratios were great larger compared with others'.

After cultivated in the MSM with Haierzhan crude oil for 4 weeks, the bacteria was isolated on the MSM agar plates with Haierzhan crude oil. 9 strains with good biodegradation ability were selected to be adjudged as hydrocarbon-degraders since they were able to grow on mineral salts medium in the absence of any other substrate except Haierzhan crude oil. By providing crude oil, any other contaminant substrate present in crude oil may be available to the organisms for growth [20].

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Identification of the hydrocarbon-degrading bacteria

After the enrichment, the solid BP medium was employed to isolate the hydrocarbon-degrading bacteria. Then they were tested in MSM with crude oil as sole carbon source. 9 strains which was names LSH-series bacteria with good biodegradation ability were selected to investigate the morphological, biochemical and physiological characteristics. The characteristics of the select sequences are shown in Table 1. Analysis of bacterial 16S rDNA gene sequences were strongly dominated by members of the *Pseudomonas*, *Brevundimonas*, *Bacillus* and *Peptoclostridium* cluster (Fig. 2).

Degradation of crude oil

For the purpose of investigating the bioremediation agents in contaminated seawater, the LSH-series bacteria were applied in our experiments. Except Haierzhan crude oil, another two kinds of crude oil, thick oil and residual oil, were employed to test the biodegradation ability of LSH-series bacteria. Also, another two strains, N2 and LZ-3, isolated formerly in our lab were used to compare the biodegradation ability with LSH-series bacteria. The remaining organic phase after 7 day biodegradation was extracted with petroleum ether, and then subsequently analyzed by UV spectrophotometer, which was used to determine the percentage degradation of the oil samples.

The degradation removal of the LSH strains were in Fig. 3. All the strains could degrade the Haierzhan crude oil more completely in a week compared to the thick oil and residual oil. This may be contributed to the compositions of the three kind of oil.

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Saturated *n*-alkanes were the main compositions of the Haierzhan oil, while the thick oil contains much more aromatic hydrocarbon. Among the three, residual oil has the highest percentage of resin and asphalt. Also, Figure 3 shows greater hydrocarbon degradation by pure cultures LSH-5, 7, and 9 than for the mixed culture that contains all these cultures. This could be explained that the cell density in the mixed culture was lower than in the pure cultures, the number of effective microorganism who could biodegrade the crude in the mixed culture was less than in the pure cultures. And another reason maybe the antagonism effect between different strains. It also could be explained by variability in the results according the limited data obtained.

The surface tension of the MSM

After extracted remaining oil, the aqueous phase was MSM and bacteria. We analyzed its surface tension. Then the aqueous phase of the MSM was centrifuged to take out the bacteria. The surface tension of the supernatant was analyzed again. And the D-value was calculated as the difference of the two test. All of the hydrocarbon-degrading bacteria in our study could reduce the surface tension of the MSM (Fig.4). Major surface tension reductions were obtained for LSH-7, LSH-5 and LSH-mix. Especially, LSH-7 could reduce the culture medium surface tension below 40 mN m^{-1}. 3 isolates (LSH-2, LSH-5, and LSH-6) were able to form emulsions, which also could reduce surface tension. In general the isolates that produced larger volume emulsions reduced surface tension, but with lower stability than those produced by the isolates that could also reduce surface tension.

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Dehydrogenase and peroxidase activity of the hydrocarbon-degrading bacteria

The activities of dehydrogenase and peroxidase in the biodegradation of the crude oil of the LSH strains were monitored and shown in the following Fig. 5. As shown in Fig. 5a, the presence of Haierzhan crude oil demonstrated great effect on the dehydrogenase activity after 7 days. The dehydrogenase activity in the presence of thick oil was low except bacteria LSH-2 after 7 days, indicated the low stimulation effect of the strains on the thick oil during the biodegradation process. Compared the data in Fig. 3 and Fig. 5, what confused us was that there was no correlation between oil biodegradation efficiency and dehydrogenase and/or peroxidase activity. Maybe we can explore the reason using microbial genomics technology to find out the corresponding genes of the enzymes in the isolates in the future.

Emulsification assays

The evolution of emulsion stability for the LSH strains was evaluated against *n*-tetradecane substrates. Emulsification activity was associated only with the cells suspension in physiological saline. The emulsification index (EI_{24} values) of LSH strains was presented in Fig.6. Some other isolates showed little emulsion-stabilizing capacity, with the emulsions formed breaking up after only a few minutes. The relationship of OD_{600} with the D-value of the surface tension which was calculated as the difference of surface tension with and without bacteria ($\Delta\gamma$) and the EI_{24} values of LSH-2, LSH-5, LSH-6 and LSH-7 was measured (Fig.7). From Fig.7a, with the increase of the OD_{600} of the bacteria LSH-2, D-value of the surface tension and EI_{24}

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values increased, while there was a sudden rising of EI_{24} values as OD_{600}>0.8. As Fig.7b shown, the D-value of the surface tension and EI_{24} values increased similarly with OD_{600} of LSH-5. For LSH-6 (Fig. 7c), when the OD_{600} was rising, the D-value of the surface tension and EI_{24} values increased while there was platform of the D-value of the surface tension (OD_{600} between 1.4 to 2.3). The little pictures in Fig. 7b and 7c shows the different emulsion along with different OD_{600} of LSH-5 and LSH-6. It was interesting that the EI_{24} values showed a little change with the OD_{600}, while the D-value of the surface was an overall rising trend with two decrease (Fig. 7d).

Emulsion stability of the bacteria LSH-5 were measured of the cream layer height with *n*-tetradecane and *n*-tetradecane + crude oil (crude oil) (Fig. 8). However, visual inspection of the overlying *n*-tetradecane and crude oil phases indicated that they became increasingly emulsified with the increase of the crude oil. Microscopic examination of samples taken from the *n*-tetradecane confirmed that the oil was breaking up into smaller droplets. Similarly, microscopic examination of samples taken from the *n*-tetradecane + crude oil showed size of the oil droplets become smaller as the increase of the crude oil, which appeared to be stabilized by the attachment of strain cells onto the surface of the droplets.

The emulsifying activity on *n*-tetradecane or *n*-hexadecane or *n*-tetradecane + crude oil or *n*-hexadecane + crude oil were detected, these substrates were emulsified in the suspension cells in 0.9% physiological saline. Gutierrez[15] confirmed that due to the cell-associated EPS, the stain TG409^T cells can adsorb accumulate PAHs from the

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surrounding seawater and emulsify the hydrocarbons. However, the emulsifying activity was every low. The corresponding light microscopic images of droplets were Fig.8a and Fig.8b. The bacterial cells was attached to the surface of the crude oil droplets in the Fig.8c. From Fig.8d, EPS was surrounding and attached to the emulsified oil droplets. These results indicated that amphiphilic EPS were produced and expressed on the cell surface to mediate this attachment, the emulsification of the bacteria cells probably also resulted from the production of amphiphilic EPS.

Discussion

The overall goal of our research is to isolate efficient petroleum hydrocarbon degrading bacteria under in situ conditions in the marine environments impacted by the spilled oil in the ocean. The oil degradation capacity of microbial populations in marine sediments is likely limited by environmental factors such as temperature or nutrient starvation, as well as ecological interactions such as mutualistic production and exchange of biosurfactants between bacterial populations[26]. Knowledge of bacterial community structure and the response of key microbial players in oil-contaminated environments provide a first glance at metabolic potential and the physiological mechanisms that might drive hydrocarbon degradation.

Degradation of crude oil

Accidental spills of oil during production and/or transport were thousands/year. For example, in 2009, 3492 spills in the US were happened and released about 195189 gallons oil and petroleum based products in the waters (www. census. gov/ compendia/

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statab/ 2012/ tables/ 12s0386.xls). The technique of microbial remediation has played an important role in the treatment of petroleum contaminants.

After 7 days biodegradation, the removal efficiency of *n*-alkanes was determined to be 10%-100% (Fig. 1a), and degradation rate of five targeted alkylated PAHs ranged between 95.4 and 9.6%, which was consistent with those reported in previous work on biodegradation of crude oils and derivatives[34,35]. And also the sequence of degradation of the PAHs depends on their molecular weight and number of substituents: N > P > D > F > C (Fig. 1b). The ratios *n*-C_{17}/Pr and *n*-C_{18}/Ph were used to estimate the degree of crude oil degradation by microbial agents. During the biodegradation experiment, pristane and phytane were not susceptible to biodegradation effect. The ratio of pristane/phytane was steady throughout the whole process in both the control and the inoculated flask. We determined that the ratios of *n*-C_{17}/pristane and *n*-C_{18}/phytane in the inoculated flask clearly decreased from 2.51 and 3.56 to 0.11 and 0.18, respectively.

Isolation, identification, and characterization of hydrocarbon-degrading bacteria

Microorganisms with the capacity to degrade hydrocarbons are among the best-studied microbial groups in applied and environmental microbiology. Indeed, more than 200 bacterial, algal, and fungal genera, encompassing over 500 species, have been recognized as capable of hydrocarbon degradation[26].

All 9 of our isolates were screened initially in minimal media with oil as the sole carbon and electron source, thereby assessing their potential to degrade oil. The majority of strains showed high 16S rDNA gene sequence (Fig.2) identity to isolates

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previously cultured from marine or saline habitats that were contaminated with oil hydrocarbons[27-31]. All of these organisms were demonstrated to degrade oil hydrocarbons in pure culture in the present study or by others or were detected in oil-contaminated marine environments. Kostka[32] identified and characterized predominant oil-degrading taxa that may be used as model hydrocarbon degraders or as microbial indicators of contamination and characterized the in *situ* response of indigenous bacterial communities to oil contamination in beach ecosystems. The results concluded that oil contamination from the DH spill had a profound impact on the abundance and community composition of indigenous bacteria in Gulf beach sands, and pointed out the *Gammaproteobacteria* (*Alcanivorax*, *Marinobacter*) and *Alphaproteobacteria* (*Rhodobacteraceae*) as key players in oil degradation. Coupled PhyloChip and GeoChip microarray analyses, Beazley demonstrated the microbial community structure and hydrocarbon-degrading microbial populations (*Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*), which increased in hydrocarbon-contaminated sediments and then decreased once hydrocarbons were below detection[33].

A further understanding of the ecophysiology of hydrocarbon degraders will be crucial to uncovering the *in situ* controls of oil degradation and to the development of improved mitigation strategies for oil spills. Through the isolation of model organisms, physiological testing of isolates, and genome sequencing, the activity, physiological potential and environmental distribution of hydrocarbon degraders can be confirmed and understood.

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The emulsification of the bacteria and its stability of oil-drops

When oil drops or slices are formed and dispersed in water, the oil droplets tend to coalesce to minimize the system energy. However, Pickering confirmed that small colloidal particles situated at the oil-water interface which were referred as Pickering emulsion were able to prevent the coalescence of oil droplets, such as to stabilize the oil-water emulsion. Francy[36] isolated some hydrocarbon-degrading microorganism and examined their abilities to emulsify petroleum hydrocarbon, which confirmed that some microorganism had emulsifying ability due, at least in part, to the whole cells themselves and retained emulsifying ability after removal of cells.

Gutierriez[15] demonstrated that cells of strain TG409^T were found to be preferentially attached to oil droplets during enrichment on hydrocarbons, indicating an ability by the strain to express cell surface amphiphilic substances (biosurfactants or bioemulsifiers) as a possible strategy to increase the bioavailability of hydrocarbons. Dorobantu[37] reported the ability of certain intact bacterial cells to stabilize oil-in-water and water-in-oil emulsions without changing the interfacial tension, by inhibition of droplet coalescence as observed in emulsion stabilization by solid particles like silica. Except to strong adhesion of the hydrophobic bacteria to the oil-water interface, they possess an affinity for each other, leading to the self-assembly of bacteria at the oil-water interface, which resists coalescence and deformation. Wongkongkatep[38] studied an oil-in-water Pickering emulsion stabilized by biobased material based on a bacteria-chitosan network, which was obtained through the electrostatic interactions

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between polycationic chitosan and the negative charge of the bacterial cell surface, proven to stabilize the *n*-tetradecane/water interface, promoting formation of highly stable oil-in-water emulsion.

On the contrary, Mohebali[39] isolated an efficient de-emulsifying bacterium *Ochrobactrum anthropi* strain RIPI5-1 examined using a model multiple water-crude oil (w/o/w) emulsion. The initial rate of breaking of the multiple water-crude oil emulsion by whole culture and whole cells was calculated as 11% and 54%, respectively. However, overall de-emulsification for whole culture and whole cells was calculated as 63% and 72%, respectively. De-emulsification proceeds via two steps: (i) flocculation or aggregation of droplets, and (ii) coalescence of droplets to form a continuous second phase. Depending on the cell surface hydrophobicity, cells may aggregate at the water-oil interface, promoting flocculation and coalescence of oil droplets.

Extracellular polymeric substances

The microbes may promote mineralization by changing the local solution chemistry through metabolic activity, or the microbes may provide a nucleation surface by binding calcium ions, which react with carbonate ions resulting from degradation of low molecular weight organic acids or labile forms of EPS[40,41]. A key function of extracellular protein is as enzymes, which can trap, bind, and concentrate organic materials in close proximity to the cells. Extracellular enzymes, which are also localized close to the cells, can hydrolyze the adsorbed organic matter. This proximity of extracellular hydrolysis to the cells facilitates efficient uptake of low-formula-weight

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hydrolysis products by reducing diffusion loss of products to the surrounding water[42]. Mikutta[43] studied the interaction of EPS derived from *Bacillus subtilis* with ferrihydrite and bentonite and the subsequent effects on heavy metal sorption (Pb^{2+}, Cu^{2+}, Zn^{2+}) to the respective EPS-mineral associations, and confirmed the association of EPS with mineral surfaces can have opposite consequences for the retention of heavy metals depending on the type of mineral present. Tsuneda[44] investigated the influence of EPS on bacterial cell adhesion onto solid surfaces, and suggested that electrostatic interaction and polymeric interaction due to the EPS covering on the cell surface promoting cell adhesion. Wang[45] detailed chemical compositions of the biomolecules in EPS from both pure cultures of bacteria and mixed species biofilm, indicated that proteins in EPS have a greater influence on disinfection byproduct (DBP) formation, especially on the formation of nitrogenous DBPs (N-DBPs). Fahs[46] characterized several EPS of a *P. fluorescens* biofilm using a combination of vibrational spectroscopies and the single molecule force technique, and provided complementary information about the structural and conformational properties of the EPS of the bacterial biofilm.

The capacity of LSH-5 cells to emulsify *n*-tetradecane or crude oil appears to be related to production of cell-associated EPS. The emulsifying activity were investigated in the cells suspension with model compounds and model compounds + crude oil, and the cells were attached to the emulsified oil droplets (Fig.8c). These results indicate that amphiphilic EPS were produced and expressed on the cell surface to mediate this attachment (Fig.8d). The direct physical attachment of the cells to oil droplets can be

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inferred to be a mechanism to access this poorly soluble substrate. From the light microscopic images of crude oil droplets, its emulsification and degradation probably also resulted from the production of amphiphilic EPS.

Zhang[47] confirmed the EPS contents in biofilms displayed significant correlations with the biodegradation efficiencies of phenanthrene and pyrene, indicating that the bacterial-produced EPS was a key factor to mediate bacterial attachment to other surfaces and develop biofilms, thereby increasing the bioavailability of poorly soluble PAH for enhanced biodegradation. More[48] reviewed many environmental applications of EPS such as water treatment, wastewater flocculation and settling, removal of toxic organic compounds, soil remediation and so on. However, exploring the potential of field applications of EPS are required to investigate.

Conclusions

We isolated 9 strains of organisms from the offshore of Qingdao, which showed high 16S rDNA gene sequence identity to isolates previously cultured from marine or saline habitats that were contaminated with oil hydrocarbons. They were strongly dominated by members of the *Pseudomonas*, *Brevundimonas*, *Bacillus* and *Peptoclostridium* cluster. Microbes showed a satisfied crude oil removal efficiency when the artificial seawater was contaminated with high concentration of petroleum containing high percentage of low molecular hydrocarbons. From the results of the emulsifying activity and light microscopic images of droplets, we can confirmed that the EPS or the microorganisms as little particles (or may be both) play a crucial role on contacting with

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the organic pollutants. However, further study is needed to verify the mechanism of the EPS on the efficient removal of crude oil.

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Table 1 Morphological, physiological and biochemical characteristics of the LSH strains

Test indicators	LSH-1	LSH-2	LSH-3	LSH-4	LSH-5	LSH-6	LSH-7	LSH-8	LSH-9
Strain color	Light yellow	yellow	white	Light yellow	milk white	Light yellow	milk white	Light yellow	milk white
Strain surface	Smooth, moist	Smooth, moist	Smooth, moist	Not smooth and moist	Smooth, moist	Not smooth and moist	Smooth, moist	Not smooth and moist	Not smooth and moist
Strain morphology transparent	Inerratic, no halo ring semitransparent	Inerratic, no halo ring opaque	Inerratic, halo ring opaque	Anomalous, no halo ring opaque	Inerratic, halo ring semitransparent	Inerratic, halo ring opaque	Anomalous, halo ring opaque	Anomalous, no halo ring transparent	Anomalous, halo ring transparent
Catalase reaction	+	+	+	+	+	+	+	+	+
Methyl red test	+	+	+	+	+	_	_	_	_
V-P test	_	_	_	_	_	_	_	_	_
Amylohylysis	_	_	_	_	_	+	+	+	_
Nitrate reduction	+	+	+	+	+	+	+	+	+
Nitrite reduction	+	+	_	_	_	+	+	+	+
Denitrification	_	_	_	+	_	+	+	+	+

Figure legends

Fig.1 Degradation of crude oil by LSH-series bacterial consortium at 25 ℃ for 7 days. (a) Distribution changes of n-alkane hydrocarbons. (b)Distribution changes of alkylated PAHs. (c)Distribution changes of other target aromatic compounds. (d)Distribution changes of the diagnostic ratio.

Fig.2 Phylogenetic trees of the nine isolates. The tree was constructed by the neighbor-joining method using the software MEGA 6.06.

Fig.3 Degradation effect on different oil of the hydrocarbon-degrading bacteria isolated from different soil and water samples collected from the offshore in Qingdao.

Fig.4 The change of surface tension of MSM before and after centrifugal process.

Fig.5 Activities of dehydrogenase (4a) and peroxidase (4b) during the oil biodegradation

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process produced by the LSH strains.

Fig.6 The emulsification index (EI_{24} values) of LSH strains.

Fig.7 The relationship of OD_{600} with the D-value of the surface tension and the emulsification index (EI_{24} values) of (a)LSH-2, (b)LSH-5, (c)LSH-6 and (d)LSH-7.

Fig.8 Microscopic images of oil emulsion formation by strain LSH-5 during incubation in a MSM medium with crude oil or *n*-tetradecane. Light microscopic image of *n*-hexadecane emulsified by LSH-5(a). Light microscopic image of droplets from LSH-5 with *n*-hexadecane + crude oil (b, c). Light microscopic images of extracellular polymeric substances from LSH-5(d).

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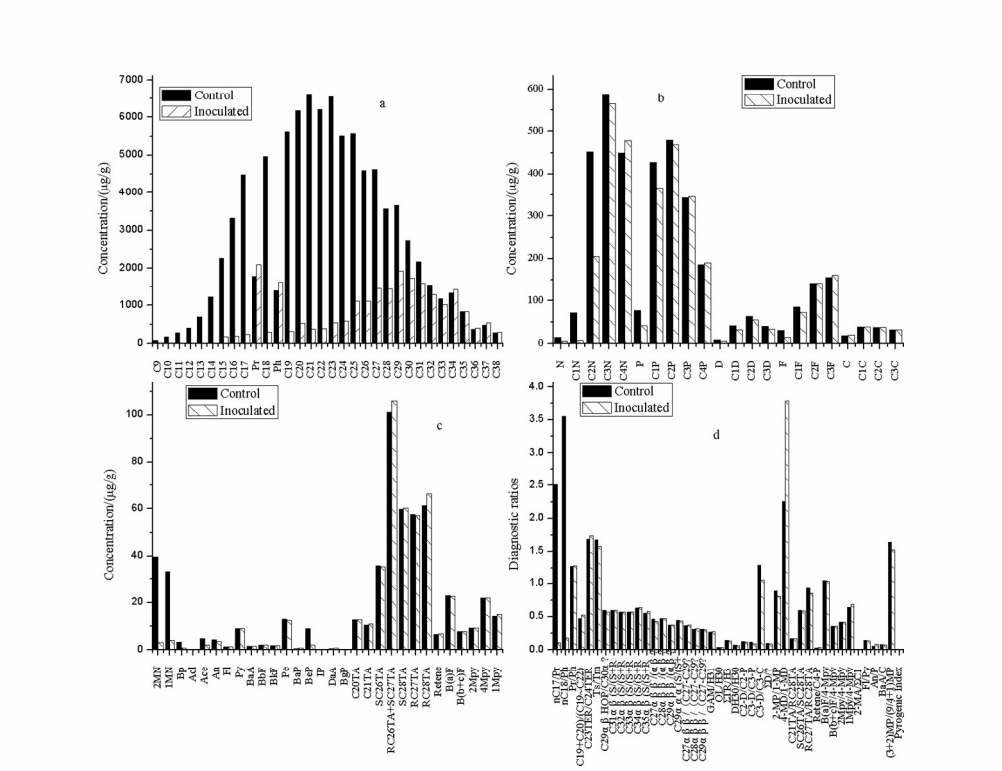

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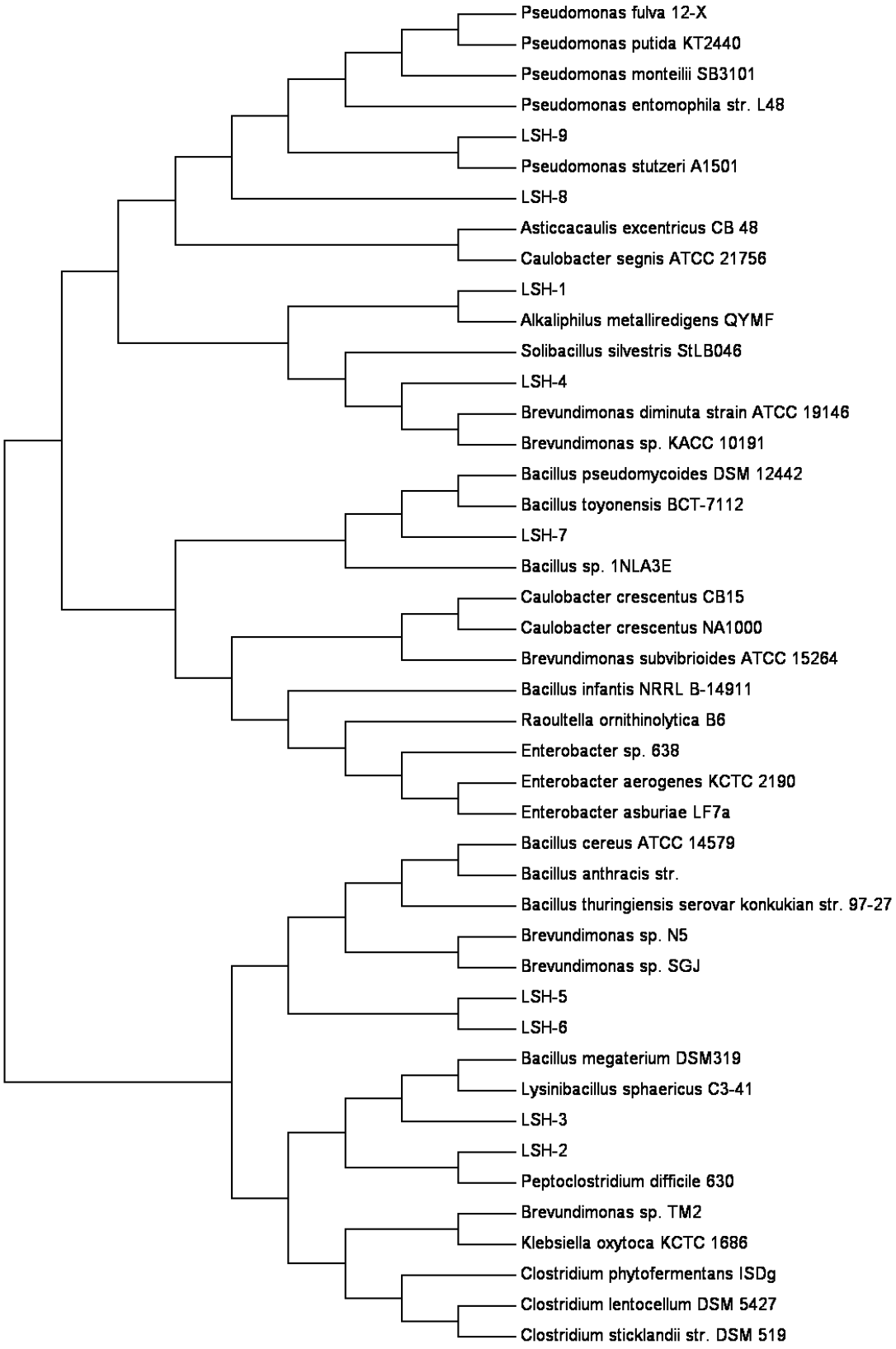

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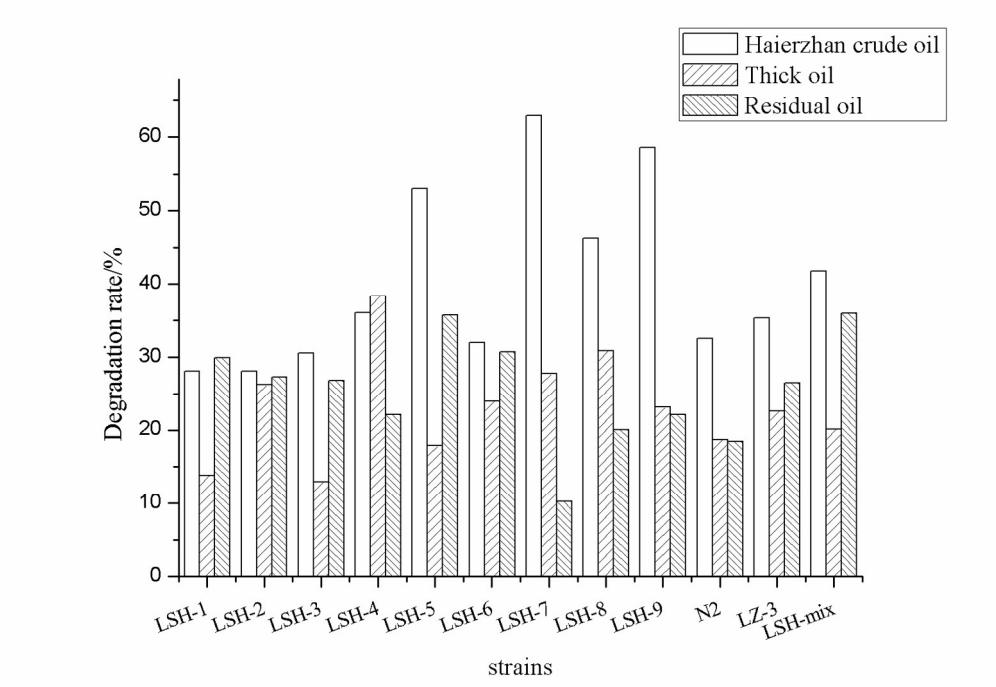

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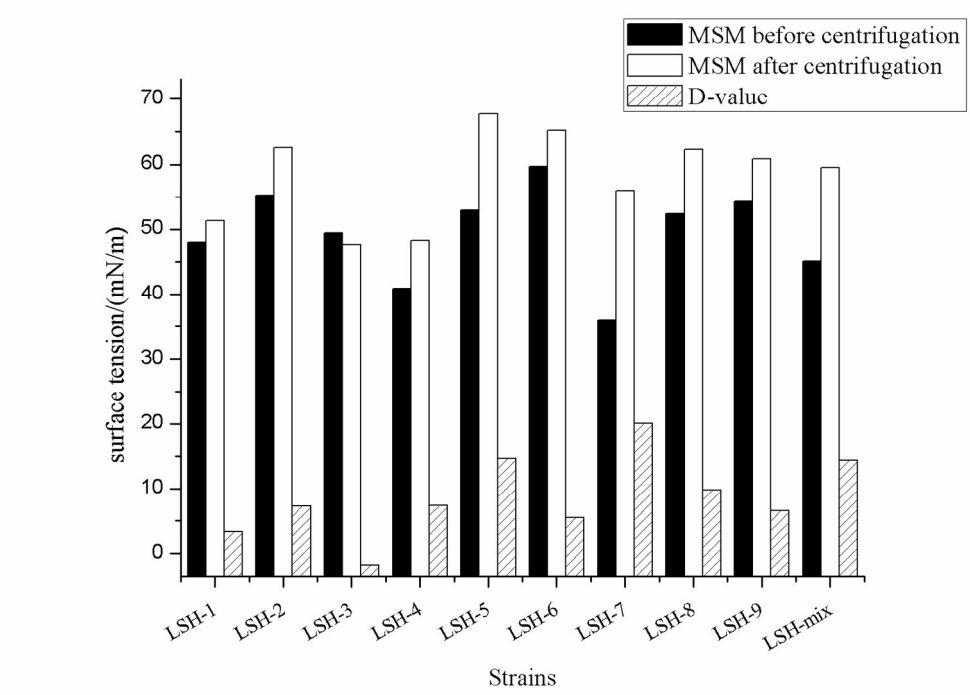

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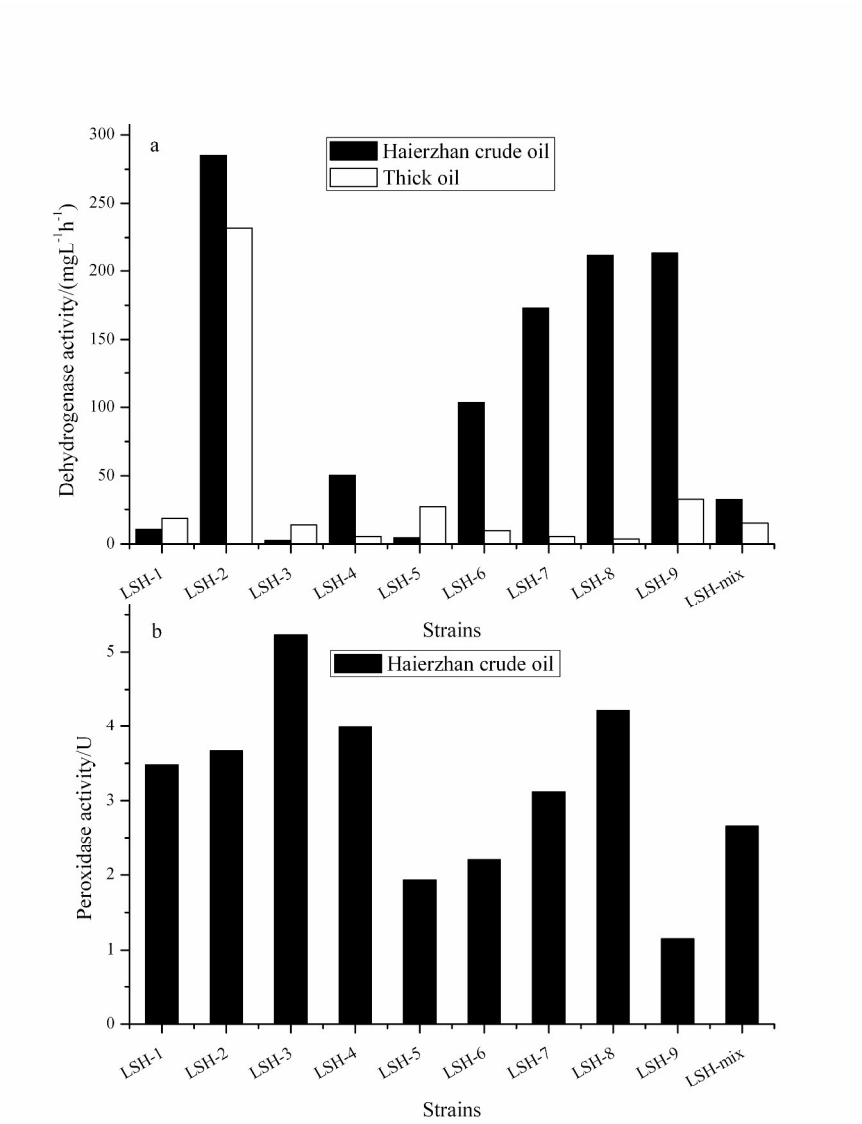

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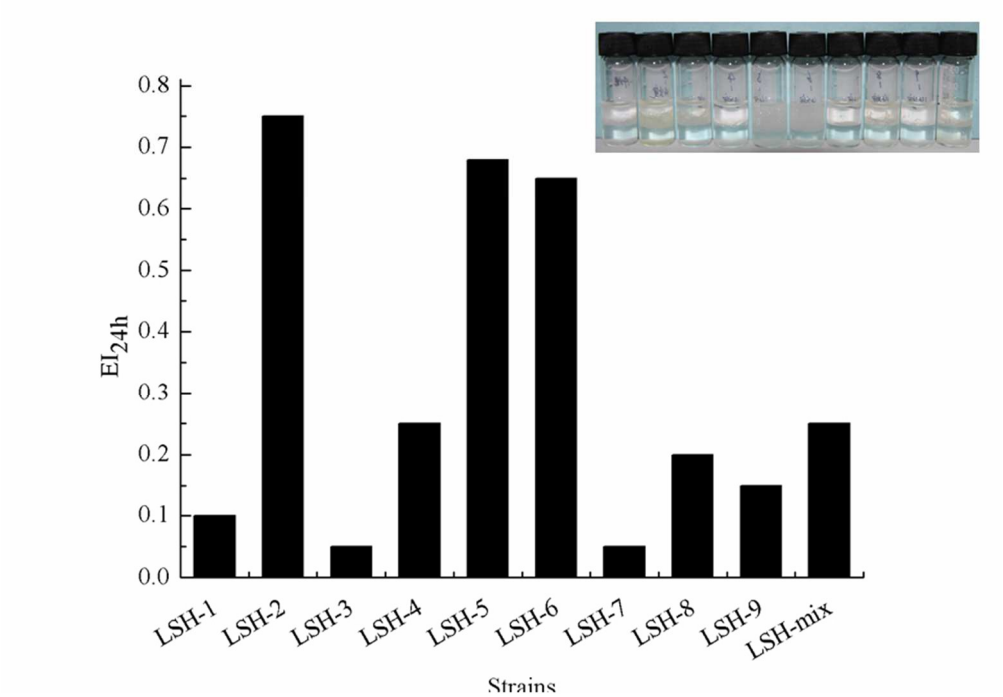

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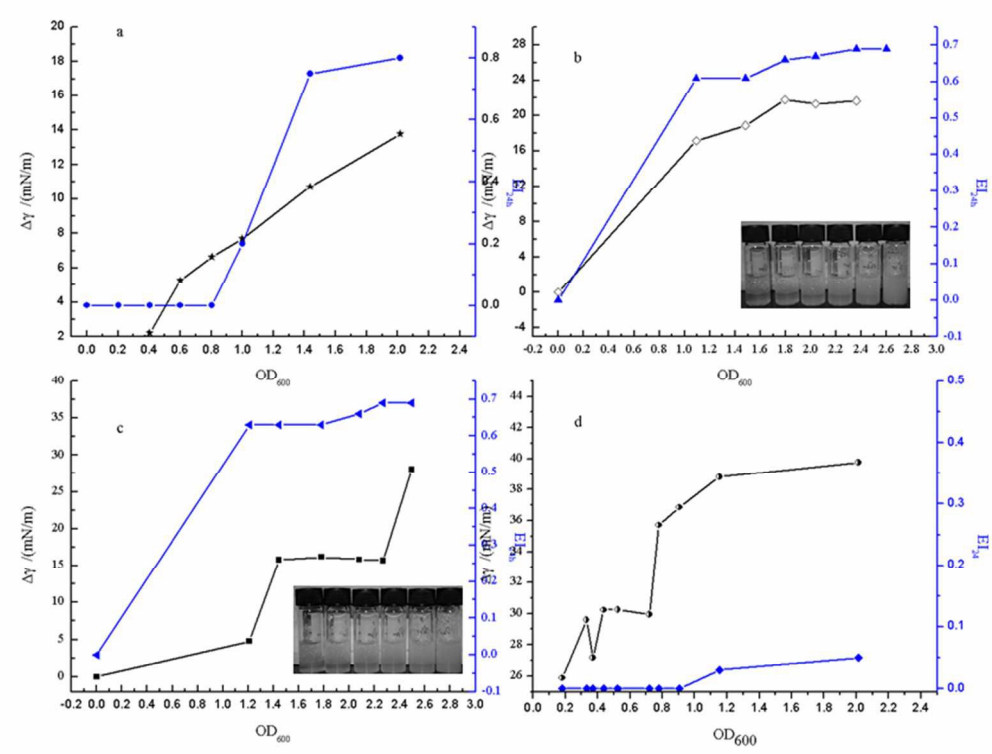

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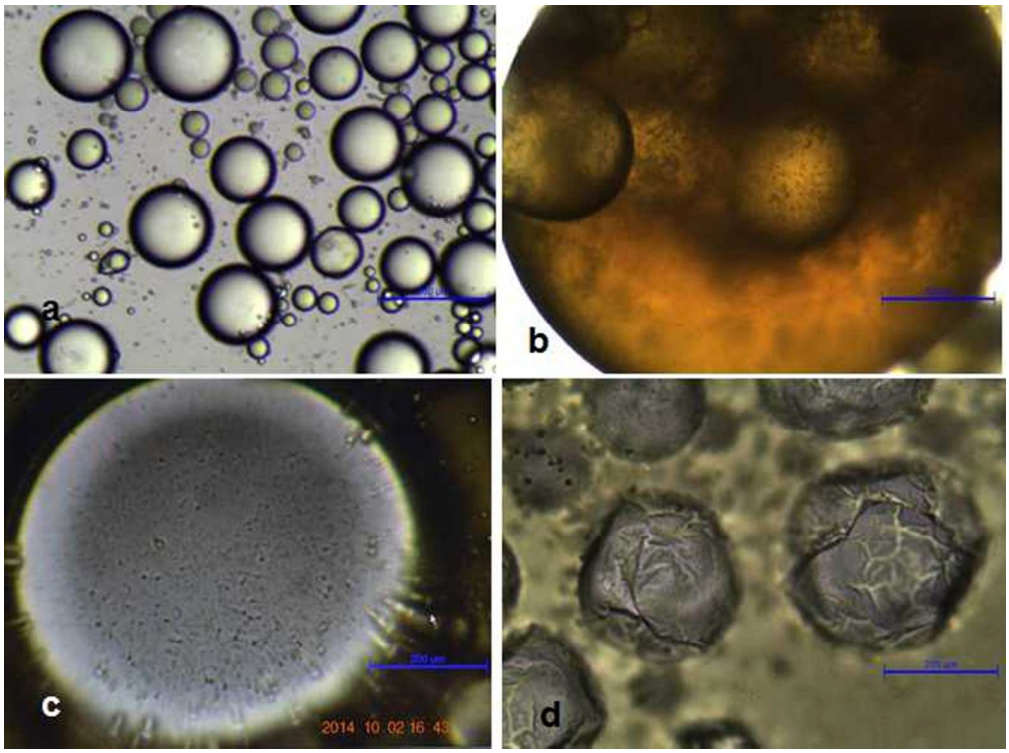

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