MICROBIAL DEGRADATION OF CRUDE OIL: IMPLICATIONS FOR BIOCHEMICAL TREATMENT OF CONTAMINATED WASTEWATER

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Some microbes are able to emulsify and degrade crude oil and can be used for biochemical treatment of oil-contaminated wastewater from oilfields, microbial restoration of oil-contaminated soils, and the extraction of crude oil from contaminated soils. In this study, 6 highly efficient crude oil-degrading bacteria (Microbacterium sp., Ochrobactrum intermedium, Alcaligenesfaecalis, Brevibacilluslaterosporus, Stenotrophomonas, and P. aeruginosa) were isolated, cultivated, and purified from soils exposed to long-term crude oil contamination. For P. aeruginosa, the most efficient conditions for oil degradation were found to be pH ~7–8, inoculums size of ~2%–4%, oil content of ~1%–4%, salt content of ~0.8%–1.0%, shake speed of ~180–200 r/min, a constant temperature of 37°C, and a degradation time of 7 days. A sudden decline in crude oil biodegradation degree was associated with calcium sulfate deposition on the surface of pseudomonas bacteria, which cut off energy and nutrient pathways between the bacteria and its environment. The results of this study provide new insights in the stability of microbial methods for treating highly scaled wastewater from oilfields.

Keywords: *P. aeruginosa*, Crude Oil, Bacterial Growth Conditions, Bacterial Surface Scaling, Calcium Sulfate Scales

1. Introduction

Certain microbes are able to emulsify and degrade crude oil, and the practical use of such microbes in oilfields is increasingly being considered [1]. Firstly, microbes can be used for biochemical treatment of oil-contaminated wastewater through a process where organic materials (e.g., emulsified oils [2]) are removed through degradation into carbon dioxide and water via petroleum-hydrocarbon oxidases within microbes [3]. Secondly, oil-degrading bacteria can be used to restore contaminated soils by degrading and removing crude oil [4-6]. Finally, microbes can be used for microbially enhanced oil recovery (MEOR), during which microbes are injected into strata and the acids and surface-activating agents produced during microbial metabolism reduce interfacial tension between oil and water[7,8].This improves the effectiveness of oil extraction by decreasing oil viscosity, dissolving carbonates within the reservoir, physically displacing oil,

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and plugging highly permeable areas of the reservoir[9], which increases the sweep efficiency of water flooding[10]. Pseudomonas microbes are efficient type of oil-degrading bacteria, whose oil-degradation mechanisms include the production of acids and surface-activating agents, and degradation of long hydrocarbon chains [11-13].

In this work, 6 highly efficient crude oil-degrading bacterial strains were isolated from crude oil-contaminated soils and oily sludges (*D1 Microbacterium sp.*, *D2 Ochrobactrum intermedium*, *D3 Alcaligenesfaecalis*, *D4 Brevibacilluslaterosporus*, *D5 Stenotrophomonas* and *D6 P. aeruginosa*), significantly increasing the known number of bacterial strains capable of crude oil degradation. To provide for the application of this particular strain of pseudomonas, we observed the growth conditions of *P. aeruginosa* and conducted the first analysis of how scaling on bacterial surfaces affects oil-degradation performance. The results of this study provide new insights in the stability of microbial methods for treating highly scaled wastewater.

2. Materials and methods

2.1 Chemicals and Equipments

The experiments in this study utilized agricultural soils extracted from~5–10 cm depth. Soils had been subjected to five years crude oil contamination from the Wuqi oil production plant of the Yanchang oil field, Shaanxi, China. In addition, experiments were conducted using oily sludges previously left standing for two years.

All chemical reagents and medium components were of analytical grade (purity≥99 %), and were purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. Infrared Oil Measuring Instrument (DL≤0.04mg/L, BOEN, Fairborn Industrial development (ShangHai) Co., Ltd., Shanghai, China); Transmission Electron Microscope (Tecnai, FEI, USA); Gas Chromatograph—Mass Spectrometry (Agilent 6890, Agilent Technologies Co. Ltd., USA).

2.2 Culture medium and cultivation conditions

2.2.1 The crude oil culture medium was used for residual hydrocarbon analysis, P. aeruginosa growth conditions studies and calcium sulfate scales assessing impacts, comprised 2% crude oil added to the basic inorganic salt culture medium. Crude oil was taken from the crude oil storage tank of the Shibaiwan joint station at Wuqi oil production plant. The basic inorganic salt culture medium comprised 4.8 g of $K_2HPO_4 \cdot 3H_2O$, 1.5 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.5 g of trisodium citrate, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of yeast powder, 0.002 g of $CaCl_2 \cdot 2H_2O$, and 1000 mL of water with a pH of~7.2 – 7.4.

2.2.3 The lysogeny broth (LB) culture medium was used for screening bacterial strains and bacterial identification, comprised 10 g of peptone, 5 g of

yeast extract, 10 g of NaCl, and 1000 mL of distilled water with a pH of 7.0. Cultivation was performed under constant temperature in an incubator set to 37℃, and at a shake speed of 180 r/min.

2.2.4 The glucose fermentation broth was used for bacterial enrichment, 16SrDNA sequence analysis and inoculum supply, comprised 50 g of glucose, 6 g of peptone, 3.5 g of Na_2HPO_4, 2 g of KH_2PO_4, 0.5 g of $MgSO_4$, 0.005 g of $CaCl_2$, and 1000 mL of distilled water with a pH of 7.0.

2.3 Experimental methods

2.3.1 Screening bacterial strains. Sample of contaminated soil and oily sludge (10 g) were dissolved in a normal saline solution. The solution was shaken using an automated-shaker for 2 hours and then bacterial supernatant was drawn and pipetted into 100 mL of the crude oil culture medium. Two bacterial transfers were performed after 7 days of cultivation. The cultivated bacterial fluids were diluted into 10^{-4}, 10^{-5} and 10^{-6} dilutions, and a coating bar was used to evenly paint diluted solutions onto an LB plate. After a further~2 – 4 days of cultivation, bacterial colonies that showed good levels of growth and well developed morphologies were selected for numbering, streaking isolation, purification, and storage.

2.3.2 Morphology, physiology, and biochemistry analyses. According to Bergey's Manual of Determinative Bacteriology, species strains are identified using relative biochemistry experiments and a dyeing experiment as described by Yumoto et al.[14]. Morphology, physiology, and biochemistry analyses of oil-degrading bacteria of six indigenous microorganisms are presented in Table 1.

2.3.3 Bacterial identification. Preliminary identification of the bacterial strains was performed through a comparison of morphological characteristics, physiological and biochemical tests, and comparison with data from previous studies[15]. In addition, deoxyribonucleic acid (DNA) sequencing via 16SrDNA sequence analysis was also performed [16]. Reaction parameters included pre-denaturation for 5 min and denaturation for 1 min at 94℃, annealing for 1 min at 54℃, chain extension for 2 min at 72℃, and a total of 30 cycles. After the final chain extension, the mixture was cooled to 4℃ and PCR (Polymerase Chain Reaction) products were sent to Sangon Biotech for sequencing. Sequences were compared with those in the Genebank [17].

2.3.4 Residual hydrocarbon analysis. After 7 days of degradation, residual oils were extracted using CH_2Cl_2 in a 1:1 ratio, with the processes repeated 3 times. Residual waters were removed by passing the extract through anhydrous sodium sulfate. GC-MS (Gas chromatograph—mass spectrometry[18]) analysis was performed using a DB-5MS chromatographic capillary column (30 m × 0.25 mm × 0.25 μm). The temperature of the column was raised to 80℃ and held for 5 min using an automated temperature program. The temperature was

then increased to 165°C at a rate of 3°C/min, where it was held for 2 min. The temperature was then raised to 260°C at a rate of 5°C/min, where it was held for 10 min. A split ratio of 10:1 and an inlet temperature of 250°C were used. Helium with a flow rate of 1 mL/min was used as the carrier gas.

2.3.5 Analysis of P. aeruginosa growth conditions. Optimal growth conditions for *P. aeruginosa* were determined by varying degradation time (~1 – 14 days), pH (~4 – 11), shake speed (~40 – 200 r/min), inoculum percentage (~0.5 % – 10 %), crude oil content (~1 % – 10 %), and salt content (~0.2 % – 1.6 %).

2.3.6 Assessing impact of calcium sulfate scales. To investigate the effects of calcium sulfate scales on the oil-degradation performance of *P. aeruginosa*, ion solutions of calcium, sulfate, and sodium were prepared using 0.1 mol/L of calcium chloride, sodium sulfate, and sodium chloride, respectively. EDTA (Ethylene Diamine Tetraacetic Acid) titration was used to determine the concentration of calcium cations and to calculate the rate of calcium loss[19].

2.3.7 Assessing crude oil biodegradation degree. After each experiment, the biodegradation degree of crude oil was measured using an infrared oil measuring device and Equation (1)[20]:

$$\text{Biodegradation degree}, \% = (A - B)100/(A) \quad (1)$$

where A is the total hydrocarbon content in the crude oil control sample (mg/L) and B is the total hydrocarbon content in the inoculated crude oil sample (mg/L).

3. Results and Discussion

3.1 Isolation and identification of bacterial strains

Following enrichment cultivation, 6 bacterial strains highly effective at degrading crude oil were isolated, (*D1, Microbacterium sp.*; *D2, Ochrobactrum intermedium*; *D3, Alcaligenesfaecalis*; *D4, Brevibacilluslaterosporus*; *D5, Stenotrophomonas*; and *D6, P. aeruginosa*; Table 1). Each bacterial strain was inoculated within the crude oil degradation culture medium with an inoculum size of 2% from the glucose fermentation broth. All 6 strains achieved biodegradation degrees above 80%, with *D6 P. aeruginosa* achieving a crude oil biodegradation degree of 93.16% (Fig. 1). Crude oil contains a complex hydrocarbon compound with range mainly from C_{11} to C_{36}[21]. GC-MS analyses confirmed that *D6 P. aeruginosa* is highly effective at degrading straight-chained alkanes, including hexadecane (C_{16}), octadecane (C_{18}), heneicosane (C_{21}), tetracosane (C_{24}), hexatricontane(C_{36}), and other mid-to-long-chained alkanes (Fig. 2). The MEGA 5.1 software package with the Neighbor-Joining command was used to construct a phylogenetic tree for *P. aeruginosa* (Fig. 3).

Table 1.

Morphology, physiology, and biochemistry of oil-degrading bacteria[a]

a. + = Positive reaction; − = Negative reaction

Strain Number	D1	D2	D3	D4	D5	D6
Morphology	regular	regular	Irregular	Irregular	regular	regular
Colony color	Yellow	Cream	White	Off-white	Cream	White
Bacterial shape	Rod-shaped	Rod-Shaped	Club-shaped	Rod-shaped	Rod-shaped	Rod-shaped
Transparency	Translucent	Opaque	Transparent	Opaque	Opaque	Opaque
Gram staining	+	−	−	+	−	−
Spore production	−	−	−	+	−	−
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Glucose oxidation and fermentation	Oxidized into acid	Oxidized into acid	Oxidized into acid	Oxidized into acid	Oxidized into acid	Oxidized into acid
M.R[b]	−	−	−	−	−	−
V.P[c]	−	−	−	−	−	−
Citrate usage	+	+	−	−	+	+
Starch hydrolysis	−	+	+	+	−	−
Gelatin hydrolysis	−	−	−	+	−	+
Nitrate Reduction	+	+	−	+	−	+
Registration Nº.	KX665599	KX665600	KX665601	KX665602	KX665603	KX665604

b. M.R = methyl red
c. V.P = acetyl methyl alcohol

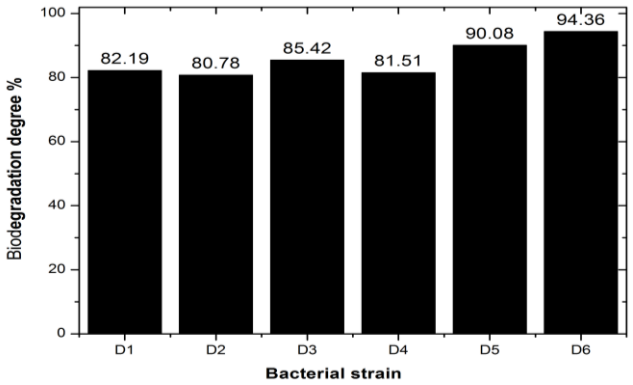

Fig. 1. Experimental biodegradation degrees (%) of six bacterial strains: D1 Microbacterium sp.; D2 Ochrobactrum intermedium; D3 Alcaligenesfaecalis; D4 Brevibacilluslaterosporus; D5 Stenotrophomonas; D6 P.aeruginosa

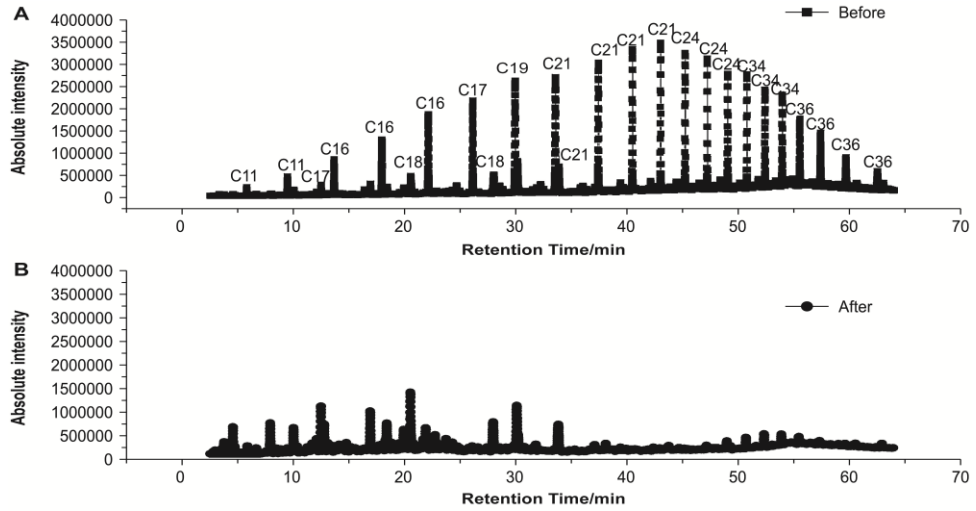

Fig. 2. Gas chromatograph—mass spectrometry (GC-MS) analysis of *P. aeruginosa*
(A) Before degradation. (B) After degradation.

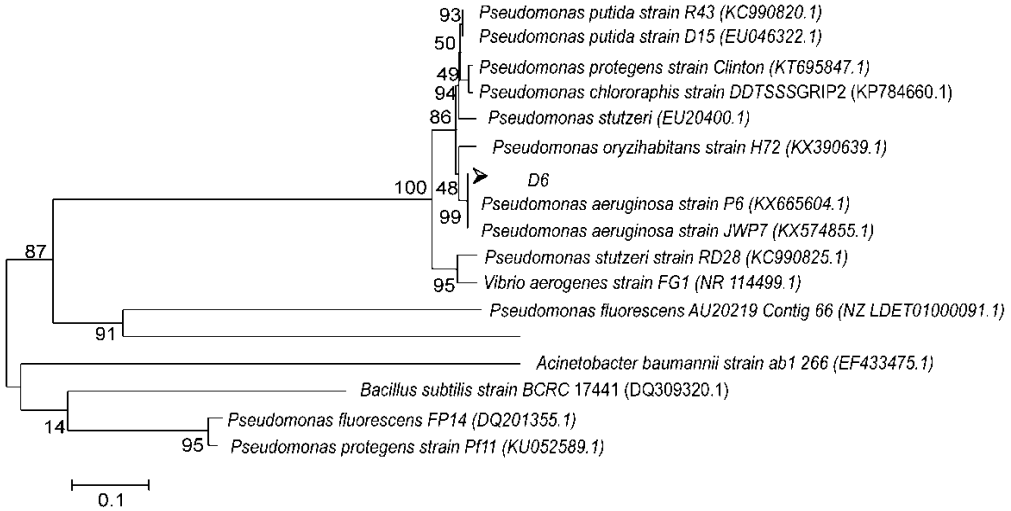

Fig. 3. Phylogenetic trees for *P. aeruginosa*

3.2 *P. aeruginosa* growth factors

Over 13 days of degradation, the crude oil biodegradation degree of *P. aeruginosa* first increased, then plateaued at 82.3% on day 7 (Fig. 4). The highest rates of degradation were associated with pH values of~7–8, while lower rates of degradation saw pH values of ≤ 6 or ≥ 9 (Fig. 5). To an extent, microbe adaptability reflects the environmental adaptability of the microbe [20,21]; for example, pH impacts on the effectiveness of crude oil degradation through changes to cellular charge, the activity of metabolic enzymes, and the ability of the microbe to absorb nutrients through changes to the permeability and stability of the cytoplasmic membrane [22]. As observed in the experiments, both high and

low pH values are detrimental for the degradation of crude oil.

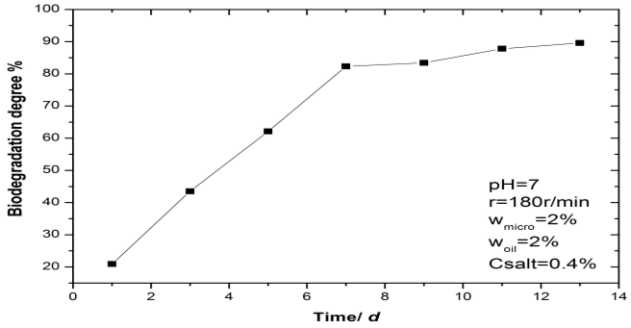

Fig. 4. Crude oil biodegradation degrees as a function of time for *P. aeruginosa*

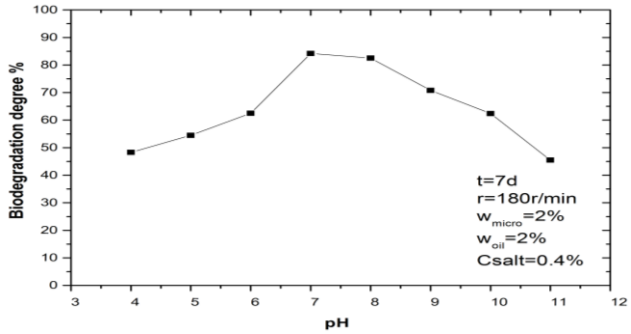

Fig. 5. Crude oil biodegradation degrees as a function of pH for *P. aeruginosa*

Biodegradation degree was positively correlated with shake speed, with the maximum biodegradation degree achieved at 180 r/min (Fig. 6).

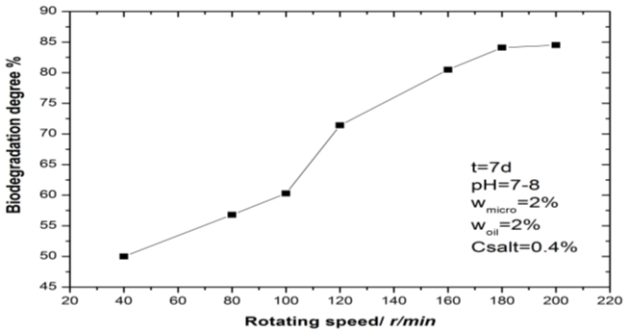

Fig. 6. Crude oil biodegradation degrees as a function of shake speed for *P. aeruginosa*.

These results reflect the fact that low shake speeds result in low dissolved oxygen within the culture medium, which inhibits the degradation of crude oil by *P. aeruginosa*. The highest crude oil biodegradation degree for *P. aeruginosa* was associated with moderate salinity [23], with the optimal salt content found to be 0.8 % (Fig. 7). These results confirm that a certain level of salinity is needed to promote enzyme reactions, maintain membrane homeostasis, regulate osmotic

pressure, and control cellular redox potential during microbial growth processes; however, very high levels of salinity have a toxic effect on microbial growth, decrease microbial respiration rates, and induce a decline in enzyme activity [24].

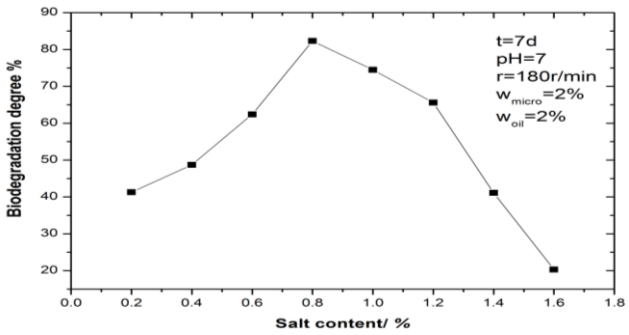

Fig. 7. Crude oil biodegradation degrees as a function of salt content for *P. aeruginosa*

It is known that inoculum size impacts on the effectiveness of crude oil degradation, primarily because it impacts upon the lag phase of microbes [25]. At very small inoculum size, the lag phase becomes extended, delaying the initiation of crude oil degradation; conversely, very large inoculum size lead to competition for nutrients, causing individual microbes to receive an insufficient quantity of nutrients, which is also detrimental for crude oil degradation. In our results, biodegradation degree increased as *P. aeruginosa* inoculum size increased from 0.5% to 2%; however, as inoculum size increased from 2% to 10%, only small increases in biodegradation degree were observed (Fig. 8). Excessively high crude oil contents are also toxic for crude oil-degrading bacteria and inhibit normal growth and reproduction [26]. This is consistent with the results of this study, which showed that biodegradation degree declines rapidly beyond a crude oil content of 4% (Fig. 9).

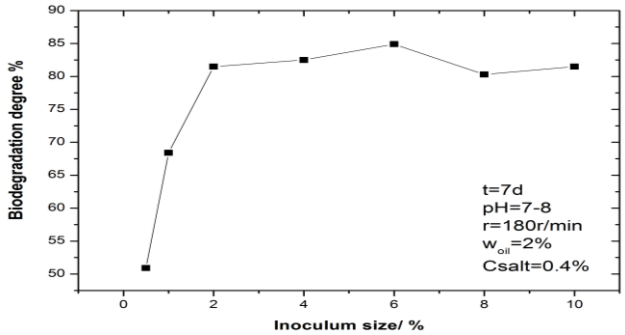

Fig. 8. Crude oil biodegradation degrees as a function of inoculum size for *P. aeruginosa*.

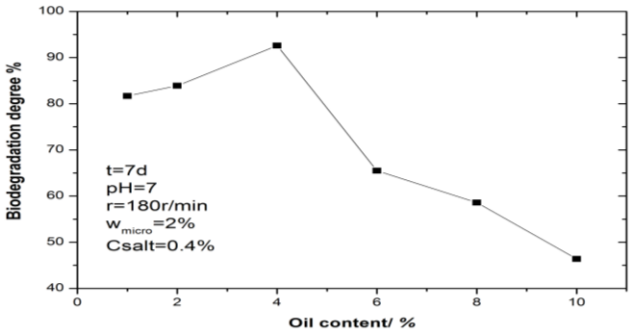

Fig. 9. Crude oil biodegradation degrees as a function of crude oil content for *P. aeruginosa*.

3.3 Calcium sulfate scales and degradation capacity

To investigate the impact of calcium sulfate scales on crude oil degradation by *P. aeruginosa*, different concentrations of calcium cations and sulfate anions (0.01–0.3 mol/L of each) were added to the crude oil culture medium for experiments conducted with a crude oil content of 4%, inoculum size of 2%, pH of 7.5, temperature of 37°C, shake speed of 200 r/min, and an experimental duration of 7 days (Fig. 10). The results showed that when 0.01 mol/L of calcium and 0.01 mol/L sulfates were added, calcium sulfate scale formation was relatively low, the rate of calcium loss was 48.62%, and the biodegradation degree was 57.04%. When 0.05 mol/L of each were added, the rate of calcium loss reached 70.68%, and the biodegradation degree decreased to 21.72%. The biodegradation degree remained between 20% and 30% as the rate of calcium loss increased, while biodegradation degree remained between 42.19% and 77.47% for the sodium sulfate control group. These results show that once a certain level of scaling has been established, calcium sulfate scales have a significant effect on the crude oil degradation performance of *P. aeruginosa*.

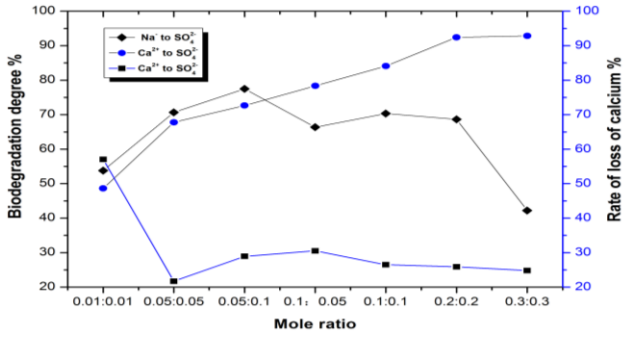

Fig. 10. Effects of calcium sulfate scales on the crude oil degradation performance of D6 *P. aeruginosa*

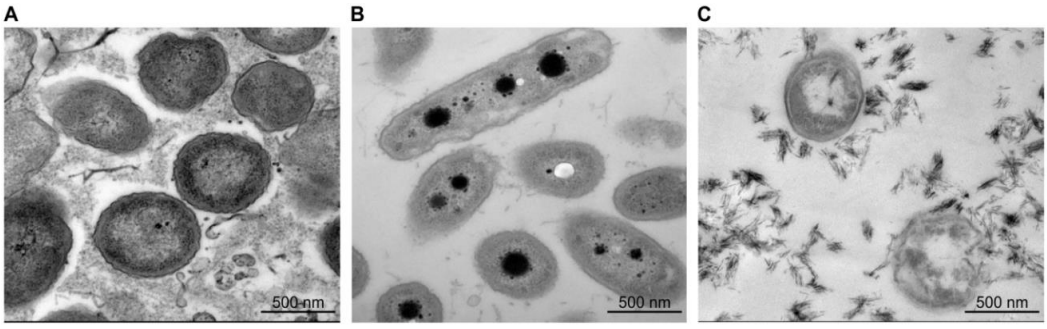

Fig. 11. Transmission electron microscopy morphologies of D6 P. aeruginosa in different culture mediums: (A) Inorganic salt culture medium (B) Crude oil culture medium (C) High-scaling crude oil culture medium

Under transmission electron microscope (TEM), differences in the bacterial fluid from the crude oil culture medium and from the highly scaled culture medium (0.1 mol/L calcium and 0.1 mol/L sulfate) showed that *P. aeruginosa* performs degradation of crude oil by ingesting crude oil into its cells (Fig. 11); however, the formation of calcium sulfate scales hinders entry of crude oil into pseudomonas cells inhibiting exchanges of energy and nutrients with the environment. As the introduction of crude oil to the cells decreases, there is a sudden decline in crude oil biodegradation degrees.

3.4 Implications for microbial technology at oilfields

After the development of an oilfield reaches the middle-to-late stages, the water content of extracted fluids continuously increases with time [26]. The re-injection of dewatered and treated fluids is an important mode of operation in the waterflooding of oilfields [27]. The water extracted from different reservoir layers may have differences in ion content and type; for example, the water extracted from the 6th layer of the Yanchang and Changqing oil fields contain large quantities of calcium (~20000 mg/L), such that this water is essentially a calcium chloride solution. In contrast, the water extracted from the 2nd *layer* contains large amounts of sulfate (~8600 mg/L), such that it is essentially a sodium sulfate solution. The mixing of these waters during mixed mining, transport, and treatment processes generates large quantities of calcium sulfate scales. The results of this study show that if microbial methods are used to treat this highly scaled water, calcium sulfate scales will aggregate on the surface of microbes, inhibiting their metabolic processes and crude oil degradation capacity. Furthermore, the stability of the water quality resulting from microbial treatment will fall and, in severe cases, the treatment process could fail. Before microbes can be effectively employed to treat highly scaled oil wastewaters, pre-treatment methods to remove scales must be developed.

4. Conclusions

In this study, 6 highly efficient crude oil-degrading bacteria were isolated, cultivated, and purified from soils exposed to long-term crude oil contamination, and the factors impacting on the efficiency of crude oil degradation by *P. aeruginosa* were investigated. The results provide new insights into the deposition of calcium sulfate scales on microbial surfaces, and on the use of microbes for the treatment of oil wastewaters. In particular, it was found that: (1) six highly effective crude oil-degrading bacterial strains occur in contaminated soils from the Yanchang oilfield; (2) degradation time, pH value, shake speed, salt content, oil content, and inoculums size all have a significant effect on the oil biodegradation degree of *P. aeruginosa*, with suitable ranges for each parameter identified; (3) the deposition of calcium sulfate scales on the surface of pseudomonas bacteria hinders the processes through which *P. aeruginosa* exchanges nutrients and energy with the environment. In turn, this significantly inhibits the crude oil biodegradation degree; and (4) further research into the deposition of scales on the surfaces of crude oil-degrading bacteria is needed to improve the technical stability of microbial treatments for highly scaled oil wastewaters.

Acknowledgments

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