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Biodegradation of heavy oils by halophilic bacterium

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Abstract

A halophilic bacterial strain TM-1 was isolated from the reservoir of the Shengli oil field in East China. Strain TM-1, which was found to be able to degrade crude oils, is a gram-positive non-motile bacterium with a coccus shape that can grow at temperatures of up to 58 °C and in 18% NaCl solution. Depending on the culture conditions, the organism may occur in tetrads. In addition, strain TM-1 produced acid from glucose without gas formation and was catalase-negative. Furthermore, strain TM-1 was found to be a facultative aerobe capable of growth under anaerobic conditions. Moreover, it produced butylated hydroxytoluene, 1,2-benzenedicarboxylic acid-bis ester and dibutyl phthalate and could use different organic substrates. Laboratory studies indicated that strain TM-1 affected different heavy oils by degrading various components and by changing the chemical properties of the oils. In addition, growth of the bacterium in heavy oils resulted in the loss of aromatic hydrocarbons, resins and asphaltenes, and enrichment with light hydrocarbons and an overall redistribution of these hydrocarbons.

Keywords: Halophilic bacterium; Metabolite; Heavy oil; Degradation

1. Introduction

Halophiles are usually found in salted products, salt fields, salt lakes, brines, saline soils, cold saline habitats, alkaline saline habitats and unusual habitats (desert plants and desert animals) [1]. Halophiles exhibit optimum growth at a salinity of approximately 15–20% but are often able to grow in saturated brine with salinities as high as 32%. The extreme halophiles that have been isolated to date belong to the genus *Halobacterium* [2].

Heavy oil has a high viscosity and density and contains many resins and asphaltenes. Currently, physical methods are used to recover heavy oil; however, such methods are expensive and result in pollution of the ambient environment. As a result, microbial systems have been used in heavy oil reservoirs to overcome such problems, thereby improving oil recovery. Conversely, the level of environmental pollution becomes more severe as the quantity of heavy oil produced increases. Therefore, it is important to develop methods that enable the remediation of heavy oil-contaminated environments by microbes to address this critical problem.

Bacterial metabolites (especially polysaccharides) are of great value as enhancers of oil recovery due to their surfactant activity and bioemulsifying properties [3]. Because the conditions in oil deposits are often saline, the use of salt-resistant metabolites may be advantageous to the recovery of oil. Furthermore, hypersaline water and soil are often contaminated with crude oils, heavy metals or other toxic compounds from anthropogenic sources. However, conventional microbiological treatment processes do not function at high salt concentrations; therefore, the use of moderately halophilic bacteria should be considered.

Most of the oil reservoirs at the Shengli oil field in East China that are subject to enhanced oil recovery processes have extreme physical conditions. The depths of the oil wells are between 1000 and 2000 m, they have *in situ*

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temperatures that range from 60 to 90 °C, and the average *in situ* pressure of the wells is 10 MPa [4]. Additionally, the water and soil around the oil field are saline and contain halophilic organisms and plants.

Many studies have been conducted to evaluate halophilic bacteria; however, halophilic bacteria isolated from oilfields have been relatively less studied, and the interaction between halophilic bacteria and heavy oils has not been evaluated until now. Therefore, this study was conducted to describe the basic characteristics of halophilic strain TM-1, which was isolated from the Shengli oil field in East China. In addition, in this study, we evaluated the effect of strain TM-1 on various types of heavy oil.

2. Materials and methods

2.1. Organism

The halophilic bacterium, TM-1, was used for this study. TM-1 was originally isolated from the Shengli oil field, which contains halophilic organisms and plants. TM-1 can grow at temperatures of up to 58 °C and in solution that contained 18% NaCl.

2.2. Medium and growth conditions

The experimental medium (EM) used for culture, stock maintenance and experimental studies contained the following compounds (per liter): $NaNO_3$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.01 g; NaCl, 180.0 g; K_2HPO_4, 2.7 g; NH_4Cl, 0.1 g; yeast extract, 0.5 g; and glucose, 3.0 g.

In addition, medium Y was used to evaluate the capacity of the strain to use organic substrates. Medium Y contained the following compounds (per liter): $NaNO_3$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.01 g; NaCl, 180.0 g; K_2HPO_4, 2.7 g; NH_4Cl, 0.1 g; and yeast extract, 0.2 g.

Finally, medium YG, which was used for the metabolites assay, was comprised of the following components (per liter): $NaNO_3$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.01 g; NaCl, 180.0 g; K_2HPO_4, 2.7 g; NH_4Cl, 0.1 g; yeast extract, 0.5 g, and glucose, 5.0 g.

Strain TM-1 was cultured in 100 ml of medium EM at 37 °C and atmospheric pressure for 24 h, after which it was grown on agar EM plates at 37 °C for 24 h.

2.3. Growth characteristics

Strain TM-1 was characterized using biochemical tests including the hydrolyzation of arginine, starch and glycerol, the reduction of nitrite, the indole test, the oxidase test and the catalase test. All tests were conducted following the methods described in Ref. [5].

To evaluate the ability of strain TM-1 to use organic substrates, the low concentration complex, medium Y, was supplemented with different substrates (citrate, D-glucose, D-mannitol, D-xylose, fructose, glycerol, L-arabinose, lactose, L-rhamnose, maltose, starch and sucrose), each at a concentration of 2 g/l.

2.4. Analysis of metabolic products

After 72 h of growth in 100 ml of medium YG at 37 °C, cells were removed by centrifugation (8000*g*, 60 min, 4 °C). The supernatant was then extracted using mixed chloroform–methanol (2:1), after which it was distilled. Next, the final metabolic products were collected in sterile bottles and analyzed using a gas chromatograph mass spectrometer (Shimadzu QP5050A). The samples were chromatographed by passing them through a GDX_{103} column (0.25 μm × 0.53 mm × 30 m) using the following gradient temperature program: an initial temperature of 80 °C for 2 min, followed by an increase in temperature to 250 °C at 10 °C/min, where it was held for 10 min. The injection temperature was 280 °C and the transport line was maintained at 260 °C. The helium flow was 0.5 ml/min, and the injection volumes were 2 μl.

2.5. Bacterial influence on crude oil

All tests were conducted under aerobic conditions, and all heavy oils evaluated in this study were collected from the Shengli oil reservoir. The selected oils were divided into three types according to viscosity: heavy oil A, which had a density of 0.9803 g/cm^3 and a viscosity of 6.031 Pa s, heavy oil B, which had a density of 0.9538 g/cm^3 and a viscosity of 1.283 Pa s, and heavy oil C, which had a density of 0.9668 g/cm^3 and a viscosity of 1.657 Pa s. The viscosities of the various heavy oils were measured using a DVIII Brook Viscometer at 50 rpm and 50 °C. To acclimatize strain TM-1 to the different crude oils, it was precultured in 100 ml of EM with 5 ml of the three different oils individually at 37 °C for 24 h prior to use in the experiment. All oils used for the experiment were autoclaved. The acclimated cultures and 65 ml of the three different sterilized oils were then added to 500 ml conical flasks that contained 200 ml of EM. In addition, 300 ml of EM and 70 ml of each of the three various sterilized oils were also added to 500 ml conical flasks as controls for comparison. The flasks were then sealed with foam stoppers and incubated in a shaker at 150 rpm and 37 °C for 5 days, at which time the crude oils were sampled and analyzed.

The oil components were analyzed according to the industrial analytical standards of the China National Petroleum Corporation (CNPC). The group components (saturated hydrocarbons, aromatic hydrocarbons, resins, and asphaltenes) were extracted by chloroform (standard code: SY/T 5118-1995) and then measured using an IATROSCAN MK-5 thin layer chromatography/flame ionization detector (TCL/FID, standard code: SY/T 6338-1997), which has an analytical error of 0.1%. The components of the saturated and aromatic hydrocarbons were analyzed using a gas chromatograph mass spectrometer (HP5890II GC/5970B MSD), which has an analytical error of 0.1%. The samples were chromatographed using a

gradient temperature program, which was as follows: an initial temperature of 110 °C, which was held for 1 min and then increased to 320 °C at 4 °C/min, where it was held for 22 min for saturated hydrocarbons, or to 335 °C at 3 °C/min, where it was held for 11.68 min for aromatic hydrocarbons. The column used to analyze the saturated hydrocarbons was a DB5-MS (60 m × 0.25 mm; 0.25 μm film), and a BPX5 (30 m × 0.22 mm; 0.25 μm film) was used to analyze the aromatic hydrocarbons. The injection temperature was 320 °C, and the transport line was maintained at 320 °C. The helium flow rate was 25 cm/s, and the injection volumes were 2 μl. The contents of carbon, hydrogen, nitrogen and sulfur were analyzed using an Elemental Analyzer (Germany Elementar vario EL), which has an analytical error of 0.1% for carbon and hydrogen, and of 0.01% for nitrogen and sulfur.

3. Results

3.1. Growth of bacteria

Cells of strain TM-1 stained as gram-positive and were found to be cocci without spores. The diameter of the cells ranged from 0.5 μm to 1.0 μm. After incubation in liquid EM at 37 °C for 24 h, the cells were distributed in a tetrad formation. Strain TM-1 was grown on EM amended with 15 g/l agar. After incubation at 37 °C for 1 day, round colonies with diameters of approximately 0.1–0.3 mm that were milk-white and smooth had developed.

Next, the ranges of temperature, pH and salinity that strain TM-1 was capable of growth in were determined. The results of the experiments indicated that the temperature growth range for strain TM-1 was 32–58 °C, with the maximal growth rate occurring at 37 °C. In addition, the strain was capable of growth at a pH of between 4.5 and 8.5, with the optimal growth occurring at approximately pH 6.5–7.0. Strain TM-1 required NaCl for growth, and could grow in medium containing up to 18% NaCl.

3.2. Metabolism and metabolic products

Strain TM-1 was a facultative aerobe that was capable of growth under anaerobic conditions. In addition, strain TM-1 was a homofermentative lactic bacterium that fermented glucose and produced lactic acids. The strain could not hydrolyze arginine, starch or glycerol, and could not reduce nitrite or produce indole. Strain TM-1 was found to be oxidase and catalase negative.

Strain TM-1 could utilize various organic substrates, with good growth being observed on D-glucose, fructose, maltose and D-mannitol. No growth occurred on L-arabinose, citrate, D-xylose, L-rhamnose, lactose, sucrose, starch or glycerol.

When grown in YG medium, strain TM-1 produced butylated hydroxytoluene ($C_{15}H_{24}O$); 1,2-benzenedicarboxylic acid-bis ester ($C_{16}H_{22}O_4$); and dibutyl phthalate ($C_{16}H_{22}O_4$). The ester, which is a better organic solvent and also a low-molecular-weight surfactant with lower surface tension, was by far the most abundant metabolite, accounting for approximately 63.3% of the total products.

3.3. Biodegradation of heavy oils

Biodegradation often induces changes in the contents of saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes, as well as a reduction in the sulfur and nitrogen content. The effects of induced biodegradation of various heavy oils on four major fractions are shown in Table 1. When compared with sterile controls (SC), the relative percentages of saturated hydrocarbons, aromatic hydrocarbons, resin and asphaltene vary. However, in oil A and oil C, each of these factors decreased, with the exception of saturated hydrocarbons. In addition, the contents of carbon, hydrogen, nitrogen and sulfur in various oils were lower following biotreatment when compared with the sterile controls (Table 2).

Table 1
Distribution of saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes in various heavy oils before and after biotreatment.

Crude oil	Saturated hydrocarbons (%)		Aromatic hydrocarbons (%)		Resin (%)		Asphaltene (%)	
	SC	BT	SC	BT	SC	BT	SC	BT
A	38.44	41.85	25.08	21.54	28.66	28.62	6.19	4.00
B	38.70	37.72	24.86	20.76	27.40	23.68	5.93	4.76
C	38.00	39.83	23.99	21.75	27.08	26.55	5.46	3.95

SC, sterile control; BT, biotreatment.

Table 2
Variations in the concentrations of carbon, hydrogen, nitrogen and sulfur in various heavy oils before and after biotreatment.

Crude oil	Carbon (%)		Hydrogen (%)		Nitrogen (%)		Sulfur (%)		H/C (atom/atom)	
	SC	BT	SC	BT	SC	BT	SC	BT	SC	BT
A	86.6	80.9	13.1	12.0	0.6	0.5	0.2	0.1	1.82	1.78
B	86.7	86.3	12.9	12.2	0.6	0.5	0.2	0.1	1.79	1.70
C	86.6	84.8	12.5	11.5	0.7	0.5	0.2	0.1	1.73	1.63

SC, sterile control; BT, biotreatment.

Halophilic TM-1 was capable of degrading various components of different heavy oils. Consistent with the aforementioned analyses, biodegradation of these oils leads to enrichment with lighter hydrocarbons and an overall redistribution of these hydrocarbons, as shown by the peak clusters at retention times of 20–30, 30–40 and 40–50 min. The analytical results of the gas chromatograph analysis of saturated hydrocarbons in various heavy oils state that the strain degraded different fractions of crude oils (Fig. 1). When added to oil A, strain TM-1 effectively transformed alkanes C_{12}–C_{15}, C_{23}, C_{25}, C_{26} and C_{28} and completely degraded C_{32} (Fig. 1(a)). When strain TM-1 was tested against oil B, it effectively transformed C_{12} and C_{19} and completely degraded alkanes longer than C_{19} (Fig. 1(b)).

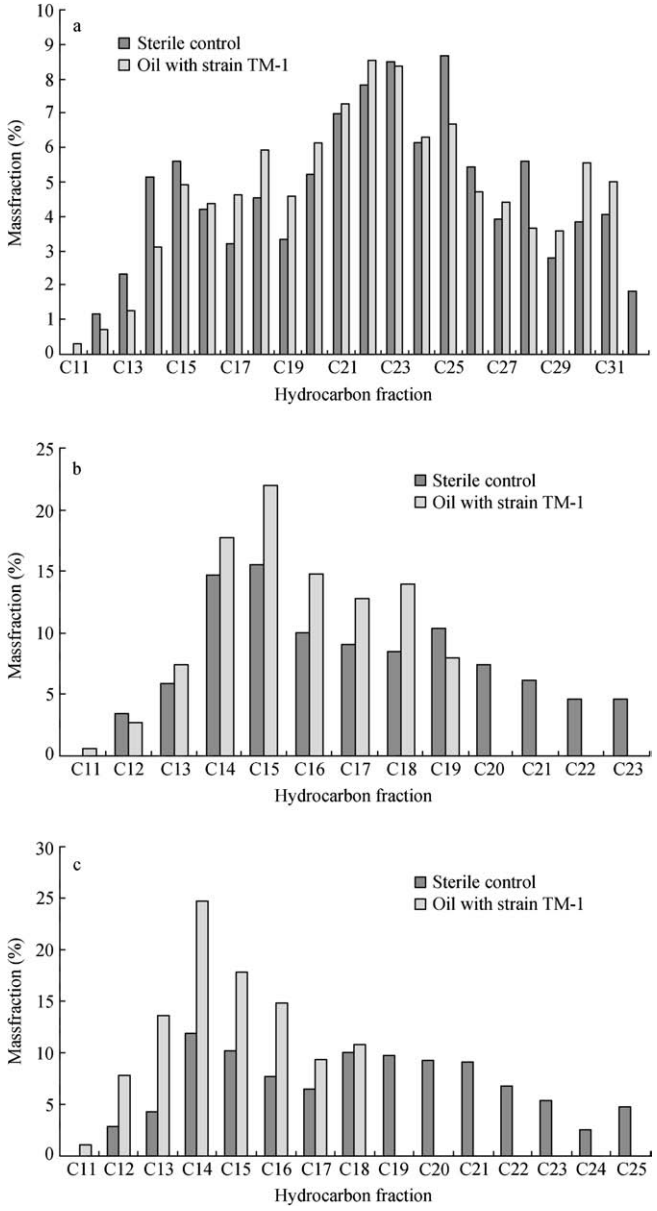

Fig. 1. Distribution of different alkane groups in various heavy oils before and after biotreatment. (a) Type A oil; (b) type B oil; (c) type C oil.

Table 3
Variation in the parameters of different heavy oils before and after biotreatment.

Crude oil	Pr/*n*-C_{17}		Ph/*n*-C_{18}		Pr/Ph		Carbon of main number *i* peak	
	SC	BT	SC	BT	SC	BT	SC	BT
A	2.73	0.80	3.08	1.02	0.63	0.61	25	22
B	2.50	1.92	3.17	2.53	0.85	0.69	15	15
C	1.83	1.28	1.94	1.60	0.61	0.70	14	14

SC, sterile control; BT, biotreatment.

When strain TM-1 was tested against oil C, it completely degraded alkanes longer than C_{18} (Fig. 1(c)). For all oils, the mass fraction of undecane increased. These results indicate that the differences in the degradation of different crude oils by strain TM-1 primarily depend on the physicochemical properties of crude oils and the growth potential of strain TM-1.

The analytical results of GC of the saturated hydrocarbons in various heavy oils demonstrate that the values of parameters of heavy oils changed following biotreatment (Table 3). The ratios of Pr/*n*-C_{17} and Ph/*n*-C_{18} of all oils decreased. There are two possible reasons for this decrease in the ratios. One possibility is that strain TM-1 may exert a greater effect on pristane and phytane than on C_{17} and C_{18}. However, it is also possible that most of the heavy fractions were converted to light fractions, which resulted in increased concentrations of C_{17} and C_{18}. As shown in Fig. 1, the fraction of light saturated hydrocarbons in the heavy oils increased following biotreatment. In addition, with the exception of oil C, the ratios of Pr/Ph were reduced. Taken together, these findings indicate that strain TM-1 degraded pristane before phytane.

As shown in Table 4, the paraffin contents of the heavy oils were 4.06–55.05% lower in the treated oils than in the sterile controls.

4. Discussion

The interaction between bacteria and crude oils is a complex biochemical process. In general, bacteria can have direct and indirect effects on the properties of crude oils that include (1) inducing changes in the physical and chemical features of oils and (2) biodegradation of crude oils.

Table 4
Changes in the paraffin content of various heavy oils before and after biotreatment.

Crude oil	Content of paraffin (%)		
	SC	BT	Percentage decrease (%)
A	2.71	2.60	4.06
B	6.16	4.43	28.08
C	6.34	2.85	55.05

The precision of the method used to determine the paraffin content was 0.1%. SC, sterile control; BT, biotreatment.

These functions occur as a result of microbial growth, bacterial enzymes, and metabolites. Microbial degradation occurs in the entire petroleum range from gas to residuum [6].

The halophilic coccus TM-1 was able to grow in high-salinity environments. In addition, strain TM-1 was capable of metabolizing several low-molecular-weight organic substrates which other halophilic bacteria cannot. As a result, treatment with TM-1 led to transformation of the components of the different crude oils evaluated in this study. Additionally, strain TM-1 was capable of producing metabolic products that act as a bridge between the bacteria and crude oils by enabling them to contact each other. Indeed, it has been reported that bacterial adherence to hydrocarbons facilitates the reproduction of microbes in crude oils during the biodegradation of petroleum [7–10].

Pfiffner et al. [11] isolated halotolerant bacterial strains capable of producing extracellular polysaccharides from oil wells and oil well-associated environments. Additionally, moderate halophiles isolated from the Great Salt Lake [12] and Antarctic saline lakes [13] have been shown to degrade hydrocarbons. Furthermore, Rosenberg [14] isolated *Pseudomonas halodurans* capable of degrading benzoate and other aromatic compounds. Finally, moderate halophiles belonging to the family *Halomonadaceae*, which were isolated from highly saline sites, were found to utilize chloroaromatic compounds as sources of carbon and energy [15].

Strain TM-1 differs from other halophiles in that it primarily affects crude oils. Specifically, strain TM-1 and its metabolites changed the chemical properties of crude oils by decreasing the concentration of high-molecular alkanes in the oil, which resulted in an increase in the light fractions. In addition, the contents of aromatic hydrocarbons, resins and asphaltenes in the various oils evaluated in this study were reduced by strain TM-1. As a result, the paraffin contents of heavy oils A, B and C were reduced by 4.06%, 28.08% and 55.05%, respectively.

The interactions between microorganisms and different crude oils occur through complex biochemical and chemical reactions. These reactions depend on multiple variables within and at the interface of a multicomponent system comprised of organic and inorganic components. The biochemical action on bacteria and crude oil occurred rapidly within the first 7 days, after which time it gradually slowed down [16]. In this study, the interactions between strain TM-1 and different heavy oils lasted for 5 days; however, if sufficient nutrients were available, especially nitrogen and phosphorus, the biochemical action could be sustained for as long as 28 days. Such an extended length of biochemical action would lead to the components of the heavy oils being further degraded.

In this study, the concentrations of each group of compounds in heavy oils A, B and C were changed (Table 1). Based on the data described in Fig. 1, strain TM-1 biodegraded heavy hydrocarbons into light compounds, which implies that the hydrogen contents (and H/C ratio) of those oils increased as the carbon number decreased. However, the H and C contents, as well as the H/C ratios of the oils, were reduced (Table 2) due to the partial degradation of organic compounds into inorganic compounds.

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