Pet.Sci.(2008)5:367-374
DOI 10.1007/s12182-008-0061-x

Study of a plugging microbial consortium using crude oil as sole carbon source

Wang Jing *, Yan Guiwen, An Mingquan, Liu Jieli, Zhang Houming and Chen Yun

School of Chemical Science & Engineering, China University of Petroleum, Beijing 102249, China

Abstract: A microbial consortium named Y4 capable of producing biopolymers was isolated from petroleum-contaminated soil in the Dagang Oilfield, China. It includes four bacterial strains: Y4-1 (Paenibacillus sp.), Y4-2 (Actinomadura sp.), Y4-3 (Uncultured bacterium clone) and Y4-4 (Brevibacillus sp.). The optimal conditions for the growth of the consortium Y4 were as follows: temperature about 46 °C, pH about 7.0 and salinity about 20.0 g/L. The major metabolites were analyzed with gas chromatography-mass spectrometry (GC-MS). A comparison was made between individual strains and the microbial consortium for biopolymer production in different treatment processes. The experimental results showed that the microbial consortium Y4 could produce more biopolymers than individual strains, and the reason might be attributed to the synergetic action of strains. The biopolymers were observed with optical and electron microscopes and analyzed by paper chromatography. It was found that the biopolymers produced by the microbial consortium Y4 were insoluble in water and were of reticular structure, and it was concluded that the biopolymers were cellulose. Through a series of simulation experiments with sand cores, it was found that the microbial consortium Y4 could reduce the permeability of reservoir beds, and improve the efficiency of water flooding by growing biomass and producing biopolymers. The oil recovery was enhanced by 3.5% on average. The results indicated that the consortium Y4 could be used in microbial enhanced oil recovery and play an important role in bioremediation of oil polluted environments.

Key words: Microbial enhanced oil recovery, plugging microbial consortium, biopolymers

1 Introduction

Microbial plugging technology is used for enhanced oil recovery by using microbes and their metabolites, which could adjust the profile of absorbing water so as to improve the oil recovery efficiency of the mature oilfield. With this technique microbes were injected into reservoirs, and their growth was stimulated with nutrients. The growth of bacteria in the oil-bearing formation and the production of biopolymers could change the pathway of injection water and force oil into new channels to become part of the produced fluid, and improve the sweep efficiency of the water flooding operation. This technique has been proven to be effective in residual oil recovery from mature fields, in which the conventional production and recovery mechanisms are ineffective. The conventional water flooding processes employ polymers or surfactants to recovery residual oil (Cui et al, 2004), but these processes leave a lot of oil in productive strata, and can pollute strata and the ground surface of the oilfield at the same time. However, as a technique of microbial enhanced oil recovery (MEOR), microbial plugging has many advantages (Soudmand-asli et al, 2007). It is a simple process with low investment, quick effect, more flexibility, and no pollution to the environment. Since reservoir conditions of anaerobiosis, temperature, pressure and salinity can not be manipulated, it is necessary to find appropriate bacteria which can produce the desired metabolites under formation conditions. Bacteria that can produce lots of biopolymers under harsh reservoir condition are little known. Some bacillus species that could produce biopolymers are not affected by increasing pressure, but decrease greatly with increasing NaCl (Yakimov et al, 1997). currently, most researchers focus on the effect of a single bacterium on MEOR. However, effective microorganisms (EM) (Jin et al, 2005) may show good effect on production of biopolymers and their adaptability under reservoir condition due to the synergetic action of strains. A microbial consortium Y4 was isolated from petroleum-contaminated soil from the Dagang Oilfield, North China in our laboratory, and it could produce biopolymers using crude oil as sole carbon and energy source, making them potentially useful for MEOR. This work investigated experimentally the characteristics and growth of the bacterial strains and their adaptability to different environments and their influence on enhancing oil recovery (core flooding) by producing biopolymers.

*Corresponding author. email: swhgwj898@cup.edu.cn
Received January 5, 2008

2 Materials and methods

2.1 Materials

Crude oil was taken from the Daqing Oilfield in Heilongjiang province, China. All the other reagents were analytical-reagent grade. All the experimental materials and crude oil were sterilized.

2.2 Enrichment and isolation of the biopolymer-producing microbial consortium

Mineral salt medium (MSM) was used as the culture medium and Daqing crude oil was used as a single carbon and energy source. MSM has the following composition (in g/L-distilled water): $K_2HPO_4 \cdot 3H_2O$, 1; KH_2PO_4, 0.5; NaCl, 0.5; $(NH_4)_2SO_4$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.025; Daqing crude oil, 20. The medium pH was around 6.9. An oil polluted soil sample (10 g) from the Dagang Oilfield was suspended in 50 ml of sterilized distilled water. Ten mL soil suspension was supplemented with 100 mL MSM as enrichment substrate and incubated with shaking at 120 rpm and 46 °C. After two weeks, turbidity was observed and there were a few round biopolymers suspended in the flask. Then, an aliquot of 4 mL enriched culture was inoculated into another 250 mL conical flask containing 100 mL fresh MSM for the second enrichment.

After four consecutive enrichments, the cultures with a series of concentration gradient were inoculated on the nutrient agar (with a nutrient composition of 0.3 g beef extract, 1 g peptone, 0.5 g NaCl per 1L distilled water) plates, respectively, to obtain enriched consortium Y4 and separated biopolymer-producing microorganisms. The isolation and purification of the bacterial consortium were carried out on nutrient agar plates with conventional spread plate technique.

2.3 Identification of the microbial consortium

The isolated species was first identified by color and morphology of individual colonies, then by traditional biochemical tests (The Group of Bacteriology Classification of the Institute of Microbiology, Chinese Academy of Sciences, 1978). Further, the purified bacterium was identified by the sequence of 16S rDNA in variable regions V6-V8 after amplification of the gene by PCR using the universal set of primers 960f-GC1 [5'-AAC GCG AAG AAC CTT AC-3'] and 1392r (5'-ACG GGC GGT GTG TAC A-3'). The amplification fragments were V6-V8 regions of 16S rDNA (Liu et al, 1996). The PCR reaction mixture (final volume = 25 μl) was prepared in a single tube as follows: 1×PCR buffer, 2 mM $MgCl_2$, 0.1 mM dNTPs, 0.1μM of each primer, about 30 ng genomic DNA from isolates, and 0.625 U Taq DNA polymerase. Thirty-five thermal cycles were performed in a FTGENE5D DNA Engine (Techgene Ltd.) as follows: 94 °C for 1min, 54 °C for 1min and 72 °C for 1min. Purification and DNA sequencing of PCR products (about 432 bp) were conducted by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

The obtained partial DNA sequences (about 450 bp) were initially submitted to Genbank database of National Center of Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and were checked by using the Basic Local Alignment Search Tool (BLAST) algorithm to roughly determine their phylogenetic affiliation. The partial sequences with a difference less than 3% were considered to belong to the same phylotype.

2.4 Growth characteristics of the microbial consortium

The growth of the microbial consortium Y4 was measured with the dilute plate method in a condition of 46 °C, 120 rpm. Based on the growth curve of Y4, we can know its logarithmic phase and stationary phase. Through this method, the effect of temperature, initial pH and salinity on the growth of the consortium was investigated in the stationary phase.

2.5 Extraction and analysis of the major metabolites and residual oil

To extract the major metabolites, 50 mL of cell suspension was harvested after 7 days culture. Extraction was conducted twice, first with equal volumes of petroleum ether, then with half volumes of dichloromethane. Dichloromethane and petroleum ether solvents were evaporated at room temperature. Then both residues were dissolved in dichloromethane again to be mixed together. The mixed extracts were evaporated to less than 1mL for GC-MS analysis (Xie et al, 1999). At the same time, the upper oil of the system and the oil adhered to biopolymers were analyzed by GC-MS, respectively.

GC-MS analysis was performed with Thermo-Finngan Trace DSQ by the Analytical Services Center of State Key Laboratory of Heavy Oil Processing, China University of Petroleum (Beijing).

2.6 Separation and analysis of biopolymers

To extract microbial consortium metabolites, 100 mL of culture medium was harvested after 4, 12, 16, 20 days respectively of culture in four flasks at 46 °C and 120 rpm. Extraction and weighing were conducted in three steps as follows: Firstly, filter paper was oven-dried, and then put into a desiccator and weighed when it reached room temperature. Secondly, the crude oil free culture medium was filtered by using a dried filter paper. Finally, the filter paper containing biopolymers was treated as in the first step. The control flask was treated in the same way. Biopolymers were observed by optical microscopy and scanning electron microscopy.

Biopolymers were analyzed in the following steps by using paper chromatography (Cao, 1986): After being rinsed by sterile water several times, biopolymers were put in an ampoule containing 2 mol/L H_2SO_4 and hydrolyzed at 100 °C for 10 hours, then excessive $BaCO_3$ was added and the suspension centrifuged. The upper solution, the product of hydrolysis, was taken and analyzed by using paper chromatography and compared with control solution (glucose, xylose, mannose and lactose).

2.7 Core flooding experiments

Before the microbial consortium was used in the oilfield,

simulation experiments with sand cores were conducted to investigate the enhanced oil recovery by the microbial consortium. The detailed processes are as follows.

In order to simulate inhomogeneous formations, 80-100 mesh quartz sand was wet-process filled into a stainless steel core tube which was 30 cm in length and 2.5 cm in diameter. The porosity of the core was measured by weighing the sands. A specific concentration of brine was prepared, and injected into the core tube to saturate the core of quartz sands until the pressure was stable. The velocity of the peristaltic pump was adjusted to 4 mL/min at 46 °C in the whole simulation process. Oil was injected into the core until oil flowed out, when the pressure was stable, and the original oil saturation of the core was calculated. The core model was left overnight to saturate with oil before the first water flooding was conducted. Brine was injected into the core for the first water flooding until the water content of effluent was above 98%. The recovered volume of crude oil by the first water flooding was used to calculate the oil recovery. Then 2 pore-volumes (pv) culture of the microbial consortium was injected to the core and the core model was placed in oven at 46 °C for a specific period of time. Finally, brine was injected into the core for the second water flooding until the water content of effluent was above 98%. In the whole process, the pressure of the model core was monitored with computer to analyze the effect of water flooding on oil recovery.

3 Results and discussion

3.1 Isolation of the biopolymer-producing consortium

A microbial consortium named Y4 was isolated after 4 weeks of enrichment and selected by repeated section of subcultures. It consisted of 4 bacterial strains: Y4-1, Y4-2, Y4-3 and Y4-4(Fig.1), which use crude oil as the sole carbon and energy source. After 7 days growth of the microorganisms, their biopolymers product appeared in the liquid medium.

3.2 Identification of microbial consortium Y4

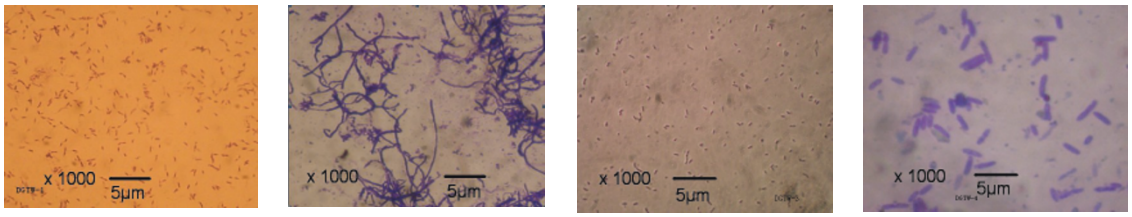
Y4-1, Y4-2, Y4-3 and Y4-4 were identified as *Paenibacillus sp., Actinomadura sp., Uncultured bacterium clone and Brevibacillus sp.*, respectively, on the basis of 16S rDNA sequence in combination with morphological, transmission electron micrographs, and physiological and biochemical tests (Tables 1 and 2).

Flagella were found on the cell surface of Y4-1 and Y4-4, and it might be crucial for growth of these strains in oil. At the same time, cilia were found on the cell surface of Y4-3. These flagella and cilia were postulated to enable these strains to adhere to the surface of the mixture of hydrophobic and biopolymers, for using energy and resisting harsh conditions.

Table 1 Physiological & biochemical characteristics of microbial consortium Y4

Experiments for strains	Y4-1	Y4-2	Y4-3	Y4-4
Gram stain	-	-	-	-
Acid-fast stains	-	-	-	-
Glucose oxidation	+	+	+	+
Ethanol oxidation	-	+	-	-
Catalase	+	+	+	-
Citrate growth	+	-	-	+
Methyl-red	-	-	-	-
Oxidase	+	-	-	-
Levan	+	-	-	-
Urea enzyme	+	+	-	+
Denitrification	-	+	+	+
Starch hydrolysis	-	++++	+++	++
Nitrate deoxidize	+	+	-	-
Nitrite deoxidize	+	+	+	+
Glucose oxidation Zymolysis	Zymolysis type	oxidation type	Zymolysis type	oxidation type

Notes: The experiments is marked by plus sign "+" when the change in growth was observed; and marked by minus sign "–" when no change in growth was observed. The number of the plus signs indicates reaction intensity.



Y4-3 (100x) Y4-2(100x) Y4-1(100x) Y4-4(100x)

Fig. 1 Morphological characteristics of microbial consortium Y4 in optical microscope images

Table 2 Lists of sequenced microorganisms and corresponding species in Genbank

Microorganism	Length of 16S rDNA V6-V8 fragments, bp	Source of the most similar genbank sequence	Alignment, % similarity
Y4-1	441	*Paenibacillus sp. SAFN*-016	99%
Y4-2	444	*Actinomadura echinospora*	98%
Y4-3	445	*Uncultured bacterium clone BFS*-7	98%
Y4-4	439	*Brevibacillus sp.* 682-2	99%

3.3 Effect of temperature, pH, and salinity on growth of consortium Y4

A typical growth curve of the consortium Y4 in batch cultivation is shown in Fig. 2. Y4 could adapt to the environment well. There was a short lag phase of 2 hours and the growth reached a steady phase about 12 hours later. Twenty-four hours growth of bacteria was adopted in experiments to investigate the effect of temperature, initial pH, and salinity on the growth of the consortium Y4.

The main factors influencing bacterium growth and metabolism are temperature, initial pH, and salinity. Our work was aimed at the optimal parameters for the consortium Y4. The optimal temperature for the consortium was around 46 °C (Fig. 3). The relationship between the growth of the consortium Y4 and initial pH is shown in Fig. 4. The optimal pH was about 7.0, and the growth of Y4 decreased sharply when pH was less than 7.0. The influence of different salinities of culture medium ranging from 5.0 to 25.0 g/L was investigated and the results are presented in Fig. 5. With the increase of the salinity from 15.0 to 20.0 g/L, the growth of Y4 increased greatly. The optimal salinity was found to be about 20.0 g/L, indicating that the consortium Y4 can adapt to high salinity.

The pH of the system decreased continuously with cultivation time. After a week, pH was stable at about 6.0 (Fig. 6). It could be concluded that biological oxidation reactions took place and organic acids were produced during metabolism of oil by the consortium Y4. However, another experiment (not yet published) showed that initial pH 9 was optimum for the consortium Y4, possibly, because the microbial consortium Y4 could secrete organic acids, which reduced the pH.

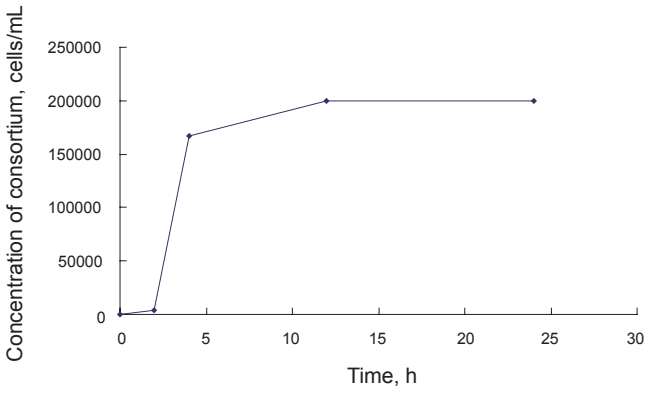


Fig. 2 Growth curves of microbial consortium

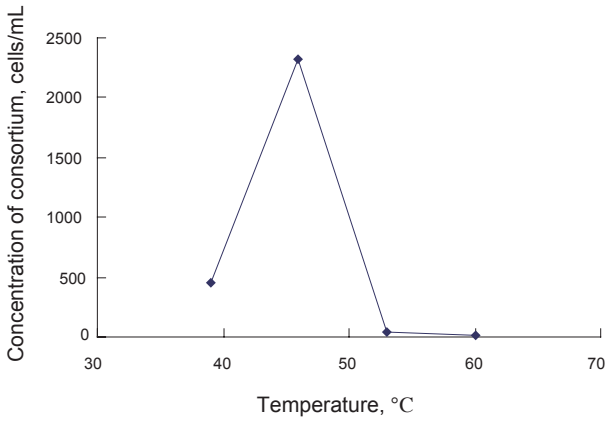


Fig. 3 Effect of temperature on growth of consortium

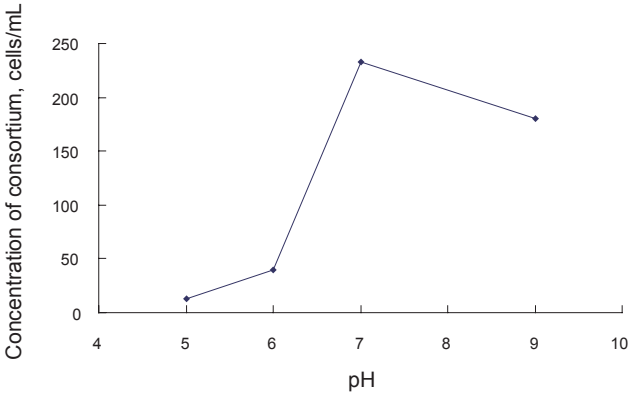


Fig. 4 Effect of pH on growth of consortium

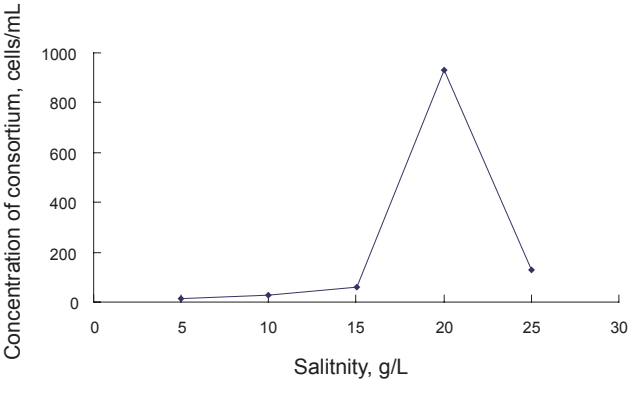


Fig. 5 Effect of salinity on growth of consortium

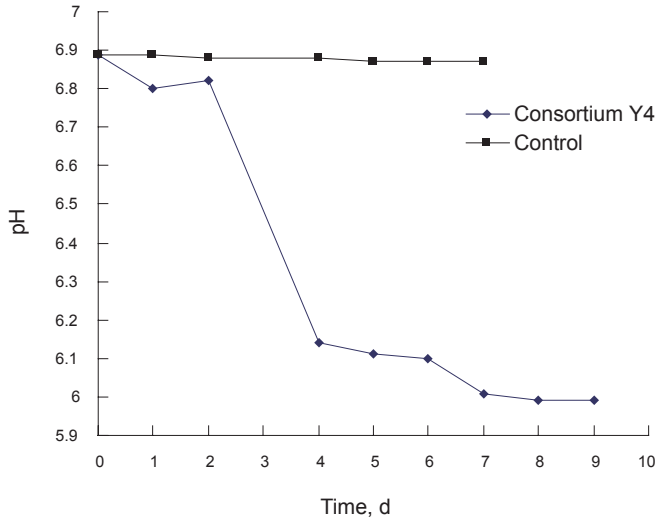


Fig. 6 pH value of the system with change of cultivation time

3.4 Analysis of major metabolites of microbial consortium Y4

When the extracts were subjected to GC-MS analysis (Fig. 7; Table 3), the major metabolites were identified as phthalates, with retention time being 18.37 min, 19.32 min and 24.49 min, respectively. It indicated that Y4 might degrade polycyclic aromatic hydrocarbons (PAHs) via o-phthalic acid to CO_2 and H_2O (Wang et al, 2006). At the same time, acids, aldehydes and esters were also major metabolites with retention time being 10.81, 8.69, 23.05, 5.88, 14.99, 16.23 and 19.74 min, respectively. It indicated that Y4 was capable of utilizing oil as both carbon and energy sources. After analyzing major metabolites, it could be concluded that aliphatic and aromatic hydrocarbon might be used as carbon and energy source at the same time.

Most substrates promoting microbial growth need to undergo cellular uptake or attachment to become accessible by the catabolic machinery of the cells. Here, cell contact with hydrophobic substrates is crucial, because the initial step in utilizing aliphatic and aromatic hydrocarbons is often mediated by oxidation reactions catalyzed on cell-surface-associated oxygenases (Wentzel, et al, 2007). Two mechanisms for accessing these substrates are generally considered for bacteria: 1) Interfacial adsorption by direct contact of the cell with the hydrocarbon and 2) Biosurfactant-mediated accessing by cell contact with emulsified hydrocarbons (Wentzel et al, 2007). Considering that the microbial consortium Y4 could use hydrocarbon as sole carbon source, it may have a relation with producing biosurfactants including fatty acid, esters and so on (Table 3) , which could emulsify oil.

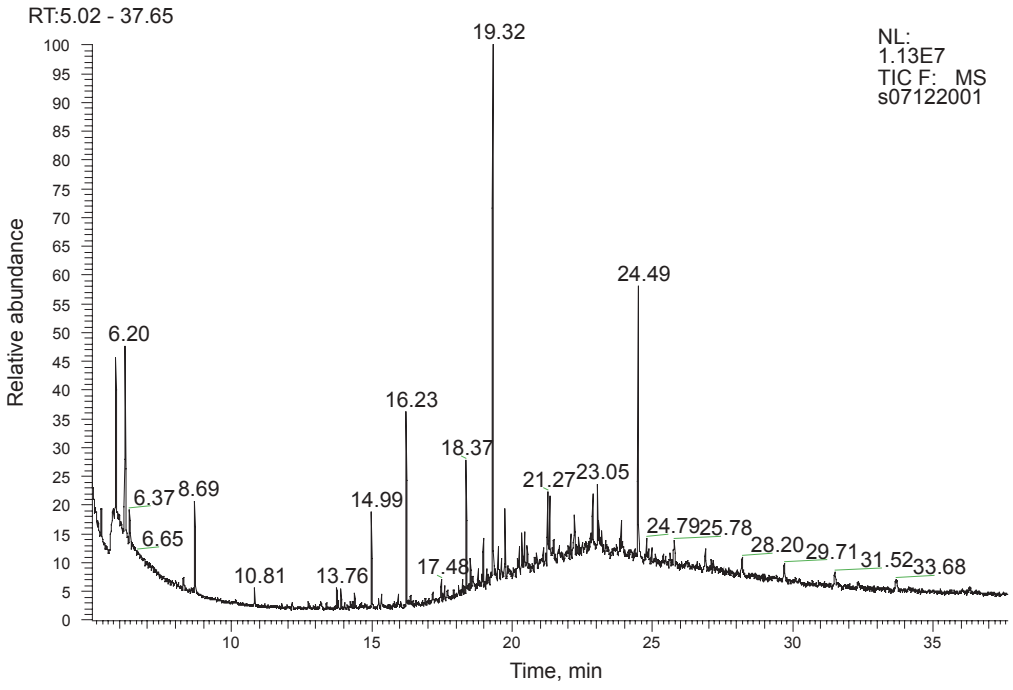
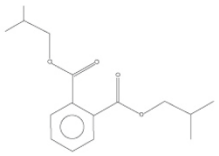
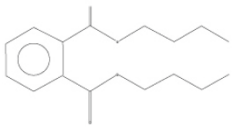
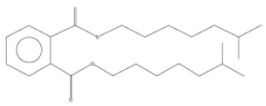
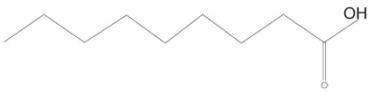
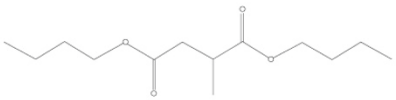
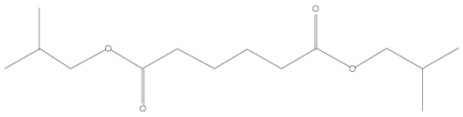
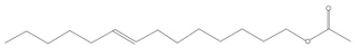


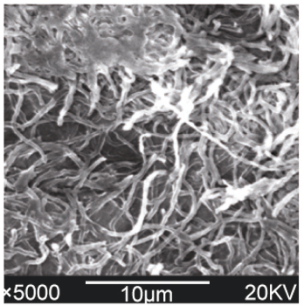
Fig. 7 GC-MS analysis of extra cellular intermediates of Y4

3.5 Separation and analysis of biopolymers

The characteristics of biopolymers including their physical structure were determined. Biopolymers were composed of microbial cells which in the latter stages of the growth cycle were embedded in an exopolymer matrix. It was speculated that there were several steps in the formation of biopolymers: initial adhesion, substrate attachment, and microcolony formation, leading to mature biopolymers consisting of cells, oil droplets and exopolymer matrix. They were observed by using scanning electronic microscopy and were found to be reticular structures (Fig. 8). The Y4 system showed a remarkable ability to survive and grow in spite of most growth-limiting factors, including high salinity, high temperature, and nutrient deficiency. Exopolymers have

Table 3 GC-MS of major metabolites formed in the degradation of Daqing oil by Y4

Category	Compound	Retention time min	Major characteristic peaks in the mass spectrum, m/z (%)
Phthalates		18.37	M+149.05, 104.06, 57.08, 41.09
		19.32	M+149.07, 150.11
		24.49	M+149.07, 167.07, 57.10, 71.16
Fatty acid		10.81	M+60.10, 73.21, 57.18, 43.09
Aldehydes		8.69	M+57.09, 41.09, 98.16, 70.18
		23.05	M+57.10, 59.09, 83.17, 43.11
Esters		5.88	M+43.03, 61.07, 87.07, 84.00
		14.99	M+115.05, 171.16, 57.10, 87.10
		16.23	M+129.12, 185.16, 57.10, 55.03
		19.74	M+211.25, 95.17, 55.06, 109.11



(5000X)

Fig. 8 Micrographs of biopolymers byscanning electron microscope (SEM)

been shown to enhance nutrient capture and resistance to environmental stress (McLandsborough et al, 2006).

In the paper chromatography (Fig. 9, Table 4), hydrolysis product (Rf: 0.30-0.35) displayed the same retention factor as glucose and other carbohydrates with a large molecular weight (Rf: 0.31-0.37). Biopolymers were analyzed and identified to be mostly glucose, and combining with the result of structural analysis in Fig. 8, we suggested that they were cellulose.

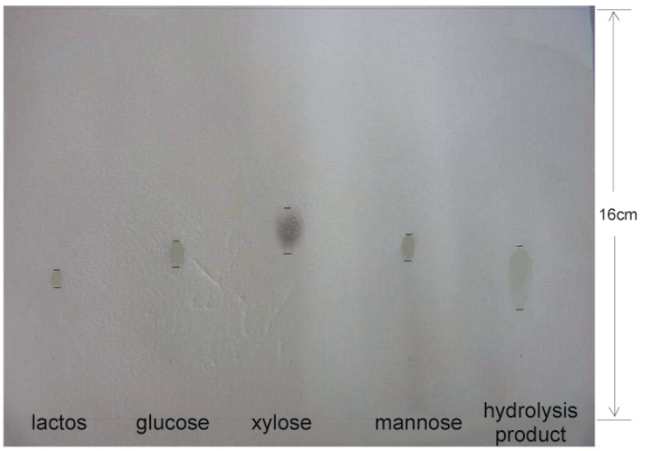


Fig. 9 Paper chromatogram of the hydrolyzed sample solution

Table 4 Rf values of hydrolysis product by ascending paper chromatography, using filter paper Xinhua No. 1 at room temperature

Substance	Rf
Hydrolysis product	0.30-0.35
Glucose	0.31-0.37
Xylose	0.37-0.45
Mannose	0.34-0.41
Lactose	0.28-0.32

3.6 Simulation experiments with sand cores

The objective of this core flood experiments was to assess the result of MEOR after inoculation with the consortium Y4 in the steady phase. No biopolymers were found in the core effluents, indicating that biopolymers produced by the Y4 were completely adsorbed within the cores. The MEOR efficiency for cores inoculated with the Y4 was about 3.5%. After 4 days of culture of incubating core at 46 °C, the pressure of the core was enhanced. These results indicated that enhanced MEOR efficiency was obtained mainly due to plugging by the consortium Y4, which was attributed to the growth of microorganisms and their metabolized biopolymers. The results showed that the consortium Y4 can adapt well to high temperature, high salinity and the sand core environment, which was similar to oilfield formation. If the concentration of microorganisms was increased, better result would be obtained according to other research (Liu et al, 2008).

The result of microbial plugging using crude oil as sole carbon and energy source depends on a variety of factors, including physical conditions and the bioavailability of the substrate, so more research is needed.

Table 5 Laboratory microbial plugging experiment with microbial consortium Y4

Parameters	Results
Sample of oil	Daqing oil
Viscosity, mPa·s	93.49
Temperature, °C	46
Saturated crude oil, mL	43
Porosity, %	54.82
Rate of injection, mL/min	0.4
Permeability, mD	599
Concentration of microorganism, cells/mL	13300
Quantity of injection, mL	24
Recovery ratio with water flooding, %	48.8
Incubated time, d	4
Recovery ratio with microbial plugging, %	3.5
Final recovery ratio, %	52.3

4 Conclusions

1) The consortium Y4 isolated from Dagang oilfield soil by using crude oil as a single carbon and energy source had outstanding microbial plugging potential. The optimal conditions for growth of the consortium Y4 were as follows: temperature about 46 °C, pH about 7.0 and the salinity about 20.0 g/L. Under these conditions, it was able to effectively produce biopolymers.

2) The consortium Y4 can produce more insoluble biopolymers than the single strain Y4-2. It might be attributed to the synergetic action of the four strains. The filamentous biopolymers wrapped bacteria and oil droplets to form a ternary complex. Biopolymers were determined as cellulose by using paper chromatography.

3) The consortium Y4 had good microbial plugging capability for enhanced oil recovery in the simulated experiment. Domestication in the laboratory could lead to the consortium Y4 having still better flexibility and producing more biopolymers for tertiary oil recovery.

4) The consortium Y4 can utilize aliphatic and aromatic hydrocarbon as carbon and energy source, indicating that they might play an important role in bioremediation of oil polluted environment.

Acknowledgements

This paper is supported by the following Grants:

(1) National High Technology Research and Development Program of China (863 Programs) (Grant No: 2007AA021306);

(2) Department of Scientific and Technical Development of CNPC(Grant No: 2008A-1403)

References

Cao Z Q. Chromatography color reagent manual. Beijing: China Commercial Press. 1986 (in Chinese)

Cui J, Zhang Z Z, Song S F, et al. Research on the Jilin Oilfield Field trials of microbial enhanced oil recovery. Petroleum Science Special Issue of Petroleum Engineering. 2004. 4 (1): 30-35 (in Chinese)

Jin M, Wang X W, Gong T S, et al. A novel membrane bioreactor enhanced by effective microorganisms for the treatment of domestic wastewater. Appl. Microbiol. Biotechnol. 2005. 69 (2): 229-235

Liu W T and Ritalahti K. Denaturing gradient gel electrophoresis (DGGE) protocol. ROME Lab DGGE Workshop. 1996. 5-6

Liu J, Wang J, Zhang H W, et al. Studies on cultivation and core simulated test of bacillus TP-1 for microbial profile modification. Microbiology. 2008. 35(4): 491-495 (in Chinese)

McLandsborough L, Rodriguez A, Pe' rez-Conesa D et al. Biofilms: At the interface between biophysics and microbiology. 2006. FOBI. DOI 10.1007/s11483-005-9004-x

Soudmand-asli A, Ayatollahi S S, Mohabatkar H, et al. The in situ microbial enhanced oil recovery in fractured porous media. Journal of Petroleum Science and Engineering. 2007. 58(1-2): 161-172

The Group of Bacteriology Classification of the Institute of Microbiology Chinese Academy of Science. Common Methods of Determinative Bacteriology. 1978. Beijing: Science Press (in Chinese)

Wang J, Xu H K, Liu Y Q, et al. Advance in mechanism of microbial degradation of phenanthrene. Microbiology. 2006. 33(5): 138-144 (in Chinese)

Wentzel A, Ellingsen T E, Kotlar H K, et al. Bacterial metabolism of long-chain n-alkanes. Appl. Microbiol. Biotechnol. 2007. 76: 1209-1221

Xie M J, Xie Z F and Cao W W. Study on surface-active agent production from paraffin degradation by pseudomonas aeruginosa strain BS01. 1999. 19 (1): 12-15

Yakimov M M, Amro M M, Bock M, et al. The potential of bacillus licheniformis strains for in situ enhanced oil recovery. Journal of Petroleum Science and Engineering. 1997. 18(1-2): 147-160

(Edited by Zhu Xiuqin)