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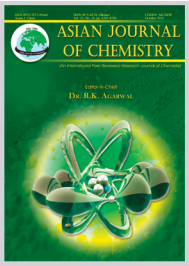

Biodegradation of Heavy Oil with High Asphaltene Content by *Kocuria sp*.

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Heavy oil biodegradation capacity of *Kocuria sp*. isolated from the oil contaminated soil of Tuha oil field in west China was tested at an orthogonal experiment, which showed that the best biodegradation conditions were as following: pollution intensity (PI) 3 %, initial pH 7.0, C:N ratio 100:3 and C:P ratio 100:0.8. Pollution intensity played a main role on the biodegradation. Under the best conditions, the heavy oil from Tuha oil field (viscosity 30 Pa·s at 50 ºC and density 0.9962 g/cm^3 at 90 ºC) and asphaltene from it was degraded 43.26 % and 55.84 % respectively, within 7 days in laboratory. Furthermore, through analyzing changes of crude oil components by a gas chromatography and mass spectrum, the strain could cause significant changes of organic fractions in the oil.

Key Words: Biodegradation, Heavy oil, Contaminated soil, Asphaltene, *Kocuria sp*.

INTRODUCTION

With the rapid development of petroleum industry, the environment has been seriously polluted during the production, transportation and application of petroleum. Bioremediation of oil contaminated soil has been studied a lot for its low cost, improving the environment, application over large areas.

Crude oil contains many kinds of organic compounds, dominated by aliphatic and aromatic hydrocarbons. The difficulty of biodegradation of these hydrocarbons by microorganism varies with the chemical components, generally as short chain hydrocarbons < long chain hydrocarbons < aromatic hydrocarbons < resin and asphaltene[1, 2]. Heavy oil has a high viscosity and density, containing many resin and asphaltene. According to that, biodegradation of heavy oil, especially with high asphaltene content, is considered more recalcitrant to biodegradation than normal hydrocarbons. At present, studies on biodegradation of light crude oil and light aromatic hydrocarbons, show that most of the pollutants are degraded in a relatively short period of time[3-5]. However biodegradation of heavy oil, especially, heavy components like asphaltene in the heavy oil, costs longer time and exhibits less effeciency[6,7]. In laboratory, heavy oil (density 0.9803 g/cm^3 and viscosity 6.031 Pa·s at 50 ºC) was used to study bioremediation in halophilic circumstance, but biodegradation of resin and asphaltene was not obvious[7]. The Prestige fuel oil (density 0.993 g/cm^3 and viscosity 30 Pa.s at 15 ºC, 650 mPa·s at 50 ºC)[8] spilled from oil tanker affected hundreds of Spanish shoreline. Although many methods were conducted to biodegrade hydrocarbons, the bioavailability of heavy fractions was still extremely low[2,9,10].

The genera *Kocuria* has indicated a certain degree to degrade petroleum hydrocarbons[5,11], but it has not been used to treat heavy oil yet. Moreover the oil in our work was very heavy and viscous and it was never used in previous studies. Bioaugmentation, which is defined as adding foreign microbial strains, supplemental nutrients and/or a terminal electron acceptor, is usually performed in order to optimize the microbial activity. Therefore, the aim of this study was to examine the heavy oil biodegradation capacity of *Kocuria sp*. An orthogonal experiment with factors of pH, PI, C:N ratio and C:P ratio was used to optimize the highest heavy oil degradation yield by *Kocuria sp*.

EXPERIMENTAL

Organism: The microorganism, *Kocuria sp*. (accession number of Genbank data was HM 629336), used in our experiment was isolated from the oil contaminated soil of Tuha oil field in Xinjiang, China

Medium and growth conditions: The mineral medium (MM) used in degradation was as follows (g/L): NaCl 1.0; $(NH_4)_2SO_4$; $CaCl_2$ 0.02; $MgSO_4$ 0.5; NaH_2PO_4; $FeCl_3$ traces. The strain inclined to use ammonium nitrogen in previous work. Therefore, the nitrogen source and phosphorus source in the mineral medium were respectively $(NH_4)_2SO_4$ and NaH_2PO_4. The mass of them changed according to the need of orthogonal experiment. The pH was adjusted to 7.0-7.2 with NaOH.The nutrient medium was comprised of the following

compounds (g/L): beef extract 3; peptone 10; NaCl 5. The pH was adjusted to 7.0-7.2 with NaOH (2 mol/L).

The strain was inoculated in 30 mL of the nutrient medium and incubated at 30 ºC on rotary shaker at 180 rpm until the cell number reached 10^8 cfu/mL.

Microbial consortia suspension was obtained by centrifuging the mineral medium with biomass at 4500 rpm for 20 min twice. The cells were washed twice with sterilized mineral medium. The biomass was re-suspended in mineral medium to obtain an aqueous suspension.

Different carbon sources: The strain was inoculated in 30 mL of the mineral medium ($(NH_4)_2SO_4$ 1.0 g/L1, NaH_2PO_4 1.0 g/L) with different carbon sources at 30 ºC on rotary shaker at 180 rpm for 4 days. The concentration of the carbon sources was 1.5 %, including *n*-hexane, liquid wax, cyclohexane, lubricant, toluene and naphthalene. The inoculation of microbial consortia suspension was 6 %.

Contaminated soil: The contaminated soil with heavy oil was artificially prepared. Natural soil dried at 60 ºC was selected as the substrate and cleaned out the impurities. The viscosity and density of the crude oil, from Tuha oil field in Xinjiang, were 30 Pa·s at 50 ºC and 0.9962 g/cm^3 at 90 ºC. A certain weight of heavy oil was added into the dried soil substrate using chloroform as a solvent and the percentage content of oil in the soil was named pollution intensity. After inoculating 6 % of microbial consortia suspension and adding mineral medium in the ratio of 4:5 to contaminated soil, the contaminated soil was cultivation at 30 ºC on rotary shaker at 180 rpm for 7 days.

Gravimetric determination of hydrocarbon content of remediated soil: The degradation yield of oil was obtained through a gravitational method. After a 7 days test, the contaminated soil was acidified by adding 2 mL HCl (6 mol/L) for 0.5 h to exclude the disturbance of organic alkali in the substrate. Then, the soil was dried, triturated and extracted by chloroform in a soxholet extractor till the solvent appeared clear. The extraction was washed by 6 % (w/v) NaOH and distilled water twice respectively to remove organic acid and other impurities. After filtrating through Na_2SO_4, the solvent was dried and the residua were weighted. The degradation yield of heavy oil was calculated by equation: Degradation % = $[(m_0-m_t)/m_0] \times 100$ %, where m_0 and m_t represented the initial and final mass of the crude oil in the soil.

Biodegradation characteristics: An orthogonal experiment of four factors with four levels was conducted to determine optimum conditions, which resulted in the highest biodegradation yield of crude oil. The biodegradation conditions of the orthogonal experiment were showed in Table-1. And a control without microorganism and mineral medium was set to eliminate absorption of the soil with 0.2 g crude oil adding into 20 g soil.

Analysis of biodegradation products: Asphaltene content was obtained through a gravitational method. The asphaltene were separated and purified using a similar method originally from Cassani and Duyck[12,13]. The oil was separated into asphaltene and maltene by adding 40:1 volume of *n*-hexane with 10 min ultrasonic treatment and then the mixture was kept in the dark for 24 h. The precipitated asphaltene was filtered out and washed by *n*-hexane for 3 times. The degradation yield of asphaltene was calculated by equation:

$$\text{Degradation}\% = [(m_0-m_t)/m_0] \times 100\ \%$$

where m_0 and m_t represented the initial and final mass of the asphaltene in the oil.

TABLE-1
FACTORS AND LEVELS OF ORTHOGONAL EXPERIMENT

Level/factor	A	B	C	D
1	1	6	100:1	100:0.6
2	3	7	100:3	100:08
3	5	8	100:5	100:1.0
4	7	9	100:7	100:1.2

A, B, C and D represented PI, initial pH adjusted by 1 M HCl or 0.6 % NaOH, C:N ratio and C:P ratio respectively. C:N ratio was the ratio between C and N elements and nitrogen source was added once at the beginning. So did the C:P radio

The biodegradation products obtained by Soxholet extraction were analyzed through a gas chromatography and mass spectrum (GC/MS-QP2010, Shimadzu Company, Japan). Data analytical system was GC/MS Postrun analysis. Standard mass spectrums were NIST05s. LIB and NIST05. LIB. The samples were chromatographed by passing them through a 50 % phenyl 50 % methyl polysiloxane column, of which the diameter was 0.25 mm and the length was 30 m, using the following gradient temperature program: an initial temperature of 80 ºC, followed by an increase in temperature to 100 ºC at 15 ºC/min, then, raised to 200 ºC for 2 min at 5 ºC/min, finally, raised to 280 ºC for 20 min at 6 ºC/min. The flow of carrier gas, purely helium, was 1.0 mL/min and the injection volume was 2.0 µL. The analytical condition of MS was as follows: EI, electronic pressure 70 eV, ion source temperature 230 ºC, detector temperature 280 ºC.

RESULTS AND DISCUSSION

Growth of the strain in different carbon source medium: As shown in the Table-2, the strain could utilize saturated and aromatic hydrocarbons, which was propitious to biodegrade heavy oil with complex components. The concentration in naphthalene was higher than in any other hydrocarbons, demonstrating aromatic hydrocarbon biodegradation capacity of the strain. Overall, the growth of the strain in different carbon source medium varied as follow: aromatic hydrocarbons > cycloalkanes > straight-chain hydrocarbons, except toluene for its toxicity. The studies on biodegradation mechanisams, enzymes and genes of the stains which could degrade naphthalene had been reported[14,15]. Moreover, the genera *Kocuria* had demonstrated the ability to degrade polycyclic aromatic hydrocarbons, such as naphthalene[16]. Therefore, it was specu-

TABLE-2
GROWTH OF THE STAIN IN DIFFERENT CARBON SOURCES MEDIUM

	N-hexane	Liquid wax	Cyclohexane	Lubricant	Toluene	Naphthalene
Concentration (10^7 cfu mL^{-1})	1.40	1.66	1.91	2.47	1.00	28.1

lated that the stain could biodegrade polycyclic aromatic hydrocarbons.

Biodegradation of heavy oil: 99.5 % of crude oil was abstracted out from the control, showing that soil absorption was negligible after extraction. The orthogonal experiment showed that highest degradation reached 42.00 % in the 6th experiment. Analyzing the data in the Table-3: $K_{2A}/4 > K_{3A}/4 > K_{1A}/4 > K_{4A}/4$, $K_{2B}/4 > K_{3B}/4 > K_{1B}/4 > K_{4B}/4$, $K_{2C}/4 > K_{1C}/4 > K_{3C}/4 > K_{4C}/4$, $K_{2D}/4 > K_{1D}/4 > K_{3D}/4 > K_{4D}/4$, the maximum of the every factor was $K_{2A}/4$, $K_{2B}/4$, $K_{2C}/4$ and $K_{2D}/4$, obtaining the best combination of oil biodegradation conditions that was PI 3 %, initial pH 7.0, C:N ratio 100:3 and C:P ratio 100:0.8. Because the combination didn't appear in the Table-3, an experiment was performed to verify the result of the orthogonal experiment. The degradation yield turned out to be 43.26 %, which was higher than any others.

TABLE-3
DATA OF OIL-BIODEGRADATION ORTHOGONAL EXPERIMENTS

No.	A	B	C	D	Degradation rate (%)
1	1 %	6	100:1	100:0.6	28.78 ± 0.56
2	1 %	7	100:3	100:0.8	41.71 ± 1.87
3	1 %	8	100:5	100:1.0	29.21 ± 1.44
4	1 %	9	100:7	100:1.2	25.4 ± 0.55
5	3 %	6	100:3	100:1.0	40.99 ± 0.88
6	3 %	7	100:1	100:1.2	42.00 ± 0.76
7	3 %	8	100:7	100:0.6	38.89 ± 1.48
8	3 %	9	100:5	100:0.8	34.33 ± 0.95
9	5 %	6	100:5	100:1.2	27.07 ± 0.43
10	5 %	7	100:7	100:1.0	30.15 ± 0.48
11	5 %	8	100:1	100:0.8	35.05 ± 1.32
12	5 %	9	100:3	100:0.6	33.03 ± 1.66
13	7 %	6	100:7	100:0.8	30.71 ± 0.61
14	7 %	7	100:5	100:0.6	36.50 ± 1.18
15	7 %	8	100:3	100:1.2	30.55 ± 0.65
16	7 %	9	100:1	100:1.0	24.66 ± 0.48
$K_i/4$	31.28	31.89	32.62	34.30	
$K_2/4$	39.05	37.59	36.57	35.45	
$K_3/4$	31.33	33.43	31.78	31.25	
$K_4/4$	30.61	29.36	31.29	31.26	
R	8.45	8.24	5.28	4.20	
PSF	A	B	C	D	

PSF: primary and secondary factor; K_{ii} was sum of degradation yield of factor j and level i (j = A, B, C and D; i = 1, 2, 3 and 4); R_i was difference between the maximum and minimum of $K_{ij}/4$

C:N ratio 100:3 and C:P ratio 100:0.8 indicated that nitrogen and phosphorus demands of the strain were similar with studies reported, in which the C:N:P ratio values were assumed to be at the level of 100:9:2, 100:10:1 or 250:10:3[17,18]. Carbon, nitrogen and phosphorus source were macronutrient. In this study, carbon source was the contamination itself. Nitrogen and phosphorus were added resulting in enhancing biodegradation evidently. However, excessive adding could inhibit biodegradation.

In Del'Arco's study, degradation yield of light Arabian oil decreased with pollution intensity increasing from 1.4 to 2.8 %[19]. However, comparing $K_{1A}/4$, $K_{2A}/4$, $K_{3A}/4$ and $K_{4A}/4$, it indicated that: when pollution intensity < 3 %, degradation yield increased with pollution intensity increasing and degradation yield decreased with pollution intensity increasing when pollution intensity > 3 %. Bioavailability of oil components was an important regulator of biodegradation rates[20]. Hence, deficiency of bioavailable hydrocarbons in the heavy oil limited growth of the strain with pollution intensity < 3 %, because quickly utilized light hydrocarbons were poverty. When pollution intensity > 3 %, high content oil hindered transmission of oxygen and contact of microbial cells with hydrocarbons resulting in decrease of degradation yield. Efficient biodegradation of oil pollution demanded a suitable pollution intensity range. In previous studies of *ex situ* bioremediation, the concentration of oil was usually relatively low[19,21,22], given the cytotoxicity and recalcitrant contents in heavy oil affecting biodegradation efficiency. In this article, the degradation yield was highest at 3 % pollution intensity and also reached 36.50 % at 7 % pollution intensity, indicating that the strain could tolerate high concentrations of heavy oil.

Comparing the value of R_j, the orders of affecting biodegradation yield were ranked from high to low as pollution intensity > initial pH > C:N ratio > C:P ratio. On account of the difference between R_A and R_B was not distinct, the variance analysis in the Table-4 indicated that pollution intensity had a bigger effect on the degradation yield than other factors.

TABLE-4
DATA OF THE VARIANCE ANALYSIS

Variance origin	Square of deviance	Freedom	F value	F critical value
Pollution intensity	192.53	3	6.94	$F_{(0.90)} = 5.39$
Initial pH	143.02	3	5.16	
C:N	69.19	3	2.50	
C:P	55.10	3	1.99	
Error	27.73	3	1.00	
Sum	487.57	15		

Analysis of biodegradation products: The asphaltene content of the heavy oil was 26.26 %. After 7 days' treatment, 58.44 % of the asphaltene was degraded. However, the biodegradation rate of asphaltene was still smaller than heavy oil, for its lighter absolute mass. In laboratory, the degradation yield of the strain reached a higher level at a short time of 7 days than before studies[5,11]. Especially, 58.44 % of the asphaltene was degraded, which was higher than similar studies[7,22,23]. On the one hand, in the laboratory condition, the compositions of oils were well dispersed in the soil, including asphaltene, resulting in contact probability of microorganisms and asphaltene increasing; the other hand, a sort of hydrocarbons were co-metabolism substrates of asphaltene, which was biodegraded into maltene (dissolved in *n*-hexane). In previous study, asphaltene was biodegraded after adding saturated hydrocarbons[24,25].

The curves in the Figs. 1 and 2 were the results of initial oil and biodegraded oil treated by GC/MS-QP2010 GC/MS, and percentage contents of hydrocarbons from C9 to C34 showed in the Fig. 3. Comparison of observed GC peaks in the fingerprints demonstrated that, though components of oil were quite complex, *Kocuria sp*. was able to degrade various types of hydrocarbon molecules. The datum in the Fig. 3 demonstrated that: C9-C13, C28, C29 and C34 were completely degraded; percentage content of C15, BC16 and C18 increased after degrading, which was contrary to the C17, C19-C21, C24, C25 and C27; C22 and C26 were newly formed.

New fractions came out because microorganisms not only decreased or eliminated the amount of organic pollutants, but also metabolized the substrates to another forms. The hydrocarbons, of which the carbon atom number was less than 13 were completely degrade into CO_2 and H_2O or organic acid. The interaction between bacteria and crude oils was a complex biochemical process. From the the results of the GC/MS, it could be concluded that the strain had obvious effect on the heavy oil, besides both on the saturated and aromatic hydrocarbons in the heavy oil, which was consistent with the requirements of the strain on the carbon source. In previous studies on remediation of oils, light saturated hydrocarbons were firstly and quickly biodegraded and polycyclic aromatic hydrocarbons were considered recalcitrant[7,9,26]. However, the strain could biodegrade saturated and aromatic hydrocarbons, and especially had obvious effect on asphaltene.

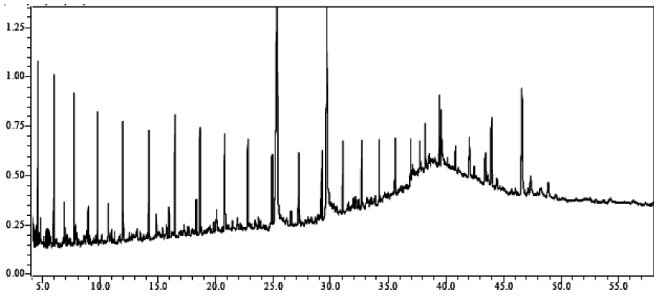

Fig. 1. GC fingerprints of origin oil

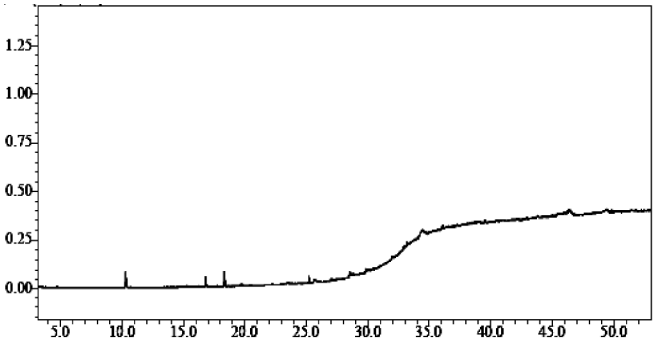

Fig. 2. GC fingerprints of residual oils with YC-X 4

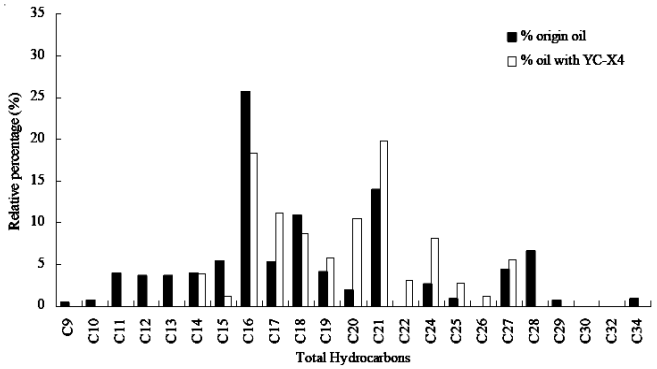

Fig. 3. Relative percentage changes of samples

Conclusion

In control flask (contaminated soil without inoculation) there was no reaction between heavy oil and soil. This result proved the heavy oil (viscosity 30 Pa·s at 50 ºC and density 0.9962 g/cm^3 at 90 ºC) was degraded only by the stain tested in this study. The strain had the highest heavy oil degradation yield (43.26 %), which was found in pollution intensity 3 %, initial pH 7.0, C:N ratio 100:3 and C:P ratio 100:0.8. The strain tolerated high concentrations of heavy oil. These data indicated that has the potential to be used to bioremediation of soil contaminated by heavy oil with high asphaltene content.

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