JCHR (2018) 8(1), 65-74

Journal of Chemical Health Risks

www.jchr.org

ORIGINAL ARTICLE

Biodegradation of the Most Heavier Fraction of Crude Oil, Asphaltene, by *Bacillus toyonensis* BCT-7112

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(Received: 18 May 2017 Accepted: 25 July 2017)

KEYWORDS

Biodegradation;

Asphaltenes;

Crude oil

ABSTRACT: There have been few records on microorganisms with the ability to survive and utilizehigh concentrations of heavy fractions of crude oil like asphaltene. These organisms are applicable in different aspects of petroleum industry from extraction to refining and environmental pollution treatment. To isolate such indigenous bacteria, a highly viscouscrude oil was selected and its asphaltene extracted. Isolation, enrichment, and purification of the bacterium were done in ISO 9439 medium at room temperature and 45°C as well. Studying morphological characteristics, biochemical and molecular tests were performed to identify isolated bacteria. The 16S rRNA gene sequence was subjected. To study the biodegradation of asphaltene, isolated bacteria were cultured in ISO 9439 medium for 2, 20 and 50 d at 25°C and 45°C.The efficiency of asphaltene degradationwas evaluated by FT-IR spectroscopy analysis. The bacterial species, which could use asphaltene as the sole carbon and energy source, were selected. Among all, *Bacillus toyonensis* BCT-7112 had the most degrading ability on asphaltene. The percentage of asphaltene degradation after 50 d of incubation at 25°C was 64.8%, and it was 60% at 45°C. Based on the FT-IR analysis, the isolate had the most biodegrading effect on Aldehyde compounds in comparison with other asphaltene ingredients. This amount of degradation is the most among the present records in literature.

INTRODUCTION

Asphaltene is the most polar and the heaviest fraction of crude oil. It consists of associated systems of polyaromatic sheets bearing alkyl side chains with a high content of O, N, and S heteroatom as well as metals (V, Ni, and Fe) which make it a nutritious environment that can support microbial growth [1–3]. However, its highly complex molecular structure makes it inaccessible for biological growth. These complex structure precipitates in petroleum facilities according to the precipitator factor and reservoir [4, 5] and may

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initiate obstacles and problems in the petroleum processing, for example, they may block extraction facilities, transport pipes and pollute the ecosystems [6–10]. Due to rapid depletion of light oil resources, trends to extract and process heavy crude oils are dramatically raising which contain high concentration ofproblematic components like asphaltenes. These components interfere cracking, refining, and upgrading operations (Figure 1) [11–15]. Nowadays, to remove or prevent heavy hydrocarbon precipitation mechanical, chemical, thermal and electromagnetic techniques or combination of them are applied which are expensive and troublesome. The costs and environmental concerns have raised scientific interests in exploiting biological techniques to overcome the problems of unconventional crude oil consumption [16, 17]. Only a few systematic studies have been done in biologically removal of asphaltene precipitates and the best-recorded result in biological degradation of asphaltene is 51.5% [18-22].

In this study, we have screened Iranian crude oil for an active bacterium that can effectively degrade complex structure of asphaltene. This bacterium could be a promising biological resolution for the environmental concerns of asphaltenic contents of petroleum. It could actively be used as a biological refinery to upgrade unconventional heavy crudes and bio-upgrade them.

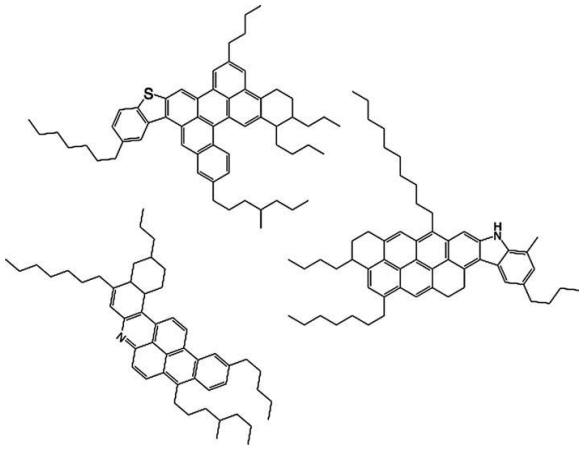

Figure1. Hypothetical structure of asphaltene molecules

MATERIALS AND METHODS

Asphaltenes extraction

Asphaltenes are soluble in aromatic solvents such as benzene and are insoluble in paraffinic solvents such as n-heptan. The transformation rates for these large molecules are limited by its low solubility and mass transfer rates in aqueous phase [23]. Asphaltene was precipitated from the oil sampleby addition with n-heptane (Sigma ACS) in a proportionof 50:1 (n-heptane/sample). The sample was reserved in magnetic bar agitation for 18 h. Then, the mixture was filtrated by using Whatman No. 42 filter. The extracted asphaltenes were dried at 40 °C for 24 h. Finally, they were stored at room temperature for later use [20].

Isolation and screening proper bacteria

To isolate bacteria with ability of asphaltene degradation , 2 mL crude oil sample was added to 50mL of the sterile mineral medium, ISO 9439 [20]: (g L^{-1}) KH_2PO_4, 0.085; K_2HPO_4, 0.21; $Na_2HPO_4.2H_2O$, 0.33; NH_4Cl, 0.005; $MgSO_4.7H_2O$, 0.0225; $CaCl_2.6H_2O$, 0.0275; $FeCl_3.6H_2O$, 0.025; pH 7.2 supplemented with

the extracted asphaltene powder (1 g L^{-1}). Samples were incubated at 45 °C and 200 rpm for 48 h. The samples were transferred to a fresh culture with the same situation, and 2 ml of growth sample was inoculated to 50 ml of ISO 9439 culture with 1 g L^{-1} asphaltene. This action was repeated for five times. Then strick plate was done in ISO 9439 agar supplemented with 1 g L^{-1} asphaltene and 0.5 g L^{-1} yeast extract. The plates were then incubated at 45 °C and 25 °C for 48 h to select the bacteria with the largest temperature endurance range.

Identification of microorganisms

To identify isolated microorganisms morphological, biochemical and molecular tests were carried out. Pure colonies were gram and spore stained [24]. The selected bacteria were tested biochemically including Catalase to recognize Catalase enzyme, Oxidase to recognize cytochrome C oxidase, Voges Proskauer to detect acetoin in a bacterial broth culture, Methyl red to test for the ability to perform mixed-acid fermentation, motility for determining motility, hydrolysis of casein to identify Casease enzyme, hydrolysis of starch to identify Amylase enzyme, utilization of citrate as the sole source of carbon, Nitrate reduction to identify the nitrate (NO_3^-) reduction anaerobically, tryptophan metabolism into indole, sulfur reduction, triple sugar iron (TSI) agar to test microorganism's ability to ferment different sugars and sensitivity to βlactam antibiotics using penicillin. All the media were by Merck.

To identify the selected strain molecularly, the total cell DNA was extracted by General Genomic Extraction Kit. To extract DNA, first Proteinase K was added to the bacterial pellet with Lysis buffer and was incubated at 65 °C for 3 h. Then the sample was centrifuged at 12000 rpm for 10 min at 4 °C. After that Binding buffer, Precipitation buffer was added and was centrifuged at 12000 rpm for 10 min at 4 °C sequentially. The Wash buffer was added and was centrifuged at 14000 rpm for 10 min at 4 °C. The pellet of DNA was dried at 65 °C for 10 min. DNA was dissolved in Solvent buffer and was stored -20 °C. After DNA extraction, PCR carried out. The primers were: fD1and rD1 (Table 1). Table 2 shows the thermal plan that was used for PCR[25]. Finally, the 16S rRNA full gene sequence was subjected to a BLAST search against the NCBI database. The neighbor joining was used to investigate the similarity of our isolate with record gene bank [26-28].

Table 1. Sequences of fD1 and rD1 primers

fD1	5'CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG 3'
rD1	5' CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC 3'

Table 2. Thermal program for PCR

1 cycle	5 min	95 °C
30 cycles	1 min	95 °C
	1 min	55 °C
	1/30 min	72 °C
1 cycle	10 min	72 °C

Figure 2. The colonies of bacteria in nutrient agar

Table 3. The results of biochemical testbacteria in nutrient agar

Biochemical test	Results
Catalase	+
Oxidase	-
Formation of indole	-
Production of hydrogen sulfide	-
Motility	+
Methyl red	+
Voges Proskauer	-
Utilization of citrate	-
Nitrate reduction	+
Triple Suger Iron	Alk/A
Hydrolysis of starch	+
Hydrolysis of casein	-
Sensitivity to penicillin	Sensitive

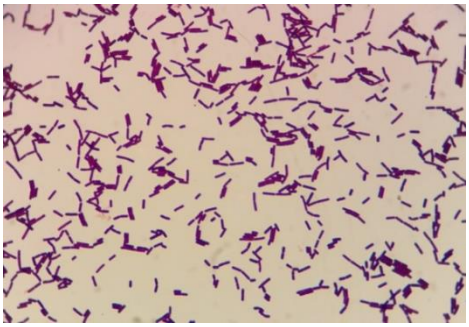

Figure 3. The microscopic morphology of *Bacillus toyonensis*(gram srain)

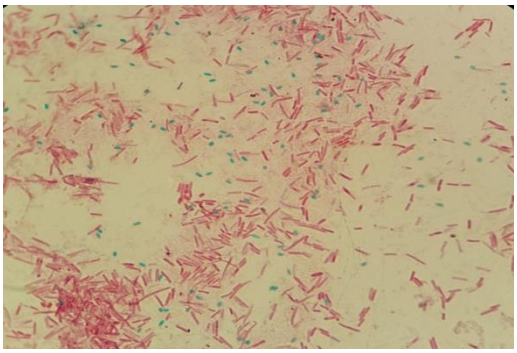

Figure 4. The microscopic morphologyof *Bacillus toyonensis*(malachite green srain)

Effect of the salinity

Salinity endurance of indigenous crude oil bacteria was studied. Our selected bacterium tolerates 0.5% to 5% salinity [29].

Evaluation of asphaltene biodegradation

Bacterial asphaltene degradation observed in 2, 20 and 50 d at 25°C and 45 °C. The increasing percentage of biodegradation in time is shown in Table 4. Since the initial amount of asphaltene was 0.05 gr. After 2 d' incubation at 25 °C and 45°C, it decreased to 0.0296 and 0.025 gr respectively. After 20 d of incubation, the amount of asphaltene was reduced to 0.0195gr at 25 °C and 0.024 gr at 45 °C. Eventually after 50 d of incubation at 25°C the amount of asphaltene was 0.0176 gr and at 45 °C, it was 0.020 gr.

Table 5 represents the percentage of asphaltene biodegradation. Biodegradation after 2 d of incubation at 25 °C and 45 °C were 40.8 and 50%, respectively. Totally, 61% asphaltene broken down After 20 d of incubation at 25 °C, and 52% at 45 °C. After 50 d of incubation at 25 °C asphaltene biodegradation percent was increased to 64.8% and at 45 ° C to 60%.

The best record of biological degradation of asphaltene reported in literature shows 51.5% by now. The degradation of asphaltene was studied from Maya crude oil by a microbial mixed culture included strains of *Bacillus*, *Brevibacillus*, *Staphylococcus*, and *Coryne bacterium*. The isolated strains degraded and utilized 8% asphaltene as their only carbon and energy source for 13 d at 25 °C [20]. About 46% biodegradation of asphaltenes were reported after two months at 28 °C, with the initial concentration of 5 g/L. The capability of the microorganisms, identified as *Pseudomonas* sp, *B.licheniformis*, *B.lentus*, *B.cereus* and *B.firmus*[29]. The capability of microbial biodegradation of asphaltene was studied in oil contaminated sludge and soil. They investigated at 4 groups including strains of *Pseudomonas*, *Citrobacter*, *Enterobacter*, *Staphylococcus*, and *Lysini bacillus*. The biodegradation was tested for two months in both static and shaking condition at 40 °C. They reported 11%-51.5% of biodegradation of asphaltene [21]. *Garciaella petrolearia* TERIG02 were performed 42% viscosity reduction, of crude oil [22]. This bacterial mixed culture to decontaminate asphaltene-polluted enviroment [20].

In our study, we recorded 64.8% biodegradation of asphaltene at 25 °C in 50 d and 60% at 45 °C by *Bacillus toyonensis*. This result is remarkably higher than previous records in literature. Thus, it indicates the potential use of this bacterium for environmental pollution treatment and in some other petroleum biotechnology aspects like heavy oil bioupgrading, MEOR (microbially enhanced oil recovery) and pipeline clogging resolve.

Table 4. The amount of asphaltene after 2, 20 and 50 d incubation at 25 and 45 °C

The amount of asphaltene after 50 d incubation (g)	The amount of asphaltene after 20 d incubation (g)	The amount of asphaltene after 2 d incubation (g)	The amount of asphaltene at the first (g)	Temperature °C
0.0176	0.0195	0.0296	0.05	25
0.020	0.024	0.025	0.05	45

Table 5. Percentage biodegradation of asphaltene after 2, 20 and 50 d incubation at 25 and 45 °C

% biodegradation after 50 d incubation	% biodegradation after 20 d incubation	% biodegradation after 2 d incubation	Temperature °C
64.8	61	40.8	25
60	52	50	45

Gravimetry test

Table 6 shows the results of biomass quantification that has done [21, 29].

Table 6. The amount of biomass after 2, 20 and 50 d incubation at 25 and 45 °C

The amount of biomass after 50 days incubation	The amount of biomass after 20 days incubation	The amount of biomass after 2 d incubation	Temperature °C
0.002	0.004	0.0212	25
0.001	0.0012	0.0013	45

Bacterial growth

At the beginning, optical density (OD) was 0.106, after 1 d it was increased to 0.729 after 2 d was 0.756. Then after the third day, a decrease in the turbidity was observed (Figure 5).

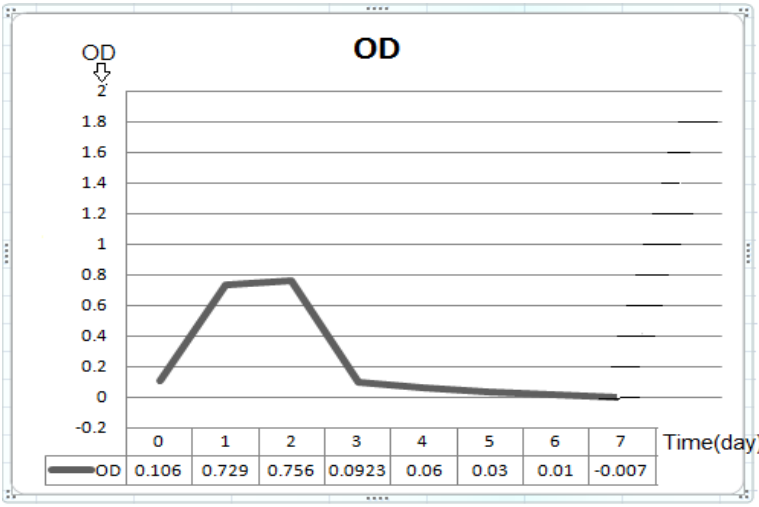

Figure 5. Bacterial growth graph

FT-IR analysis

Initially, asphaltene was analysis with FT-IR before bacterial treatment (Figure 6). The first peak in the graph is in the range of 3000-3200 cm^{-1}. This peak of the graph shows several connection bands O-H regards to alcohol compounds. The altitude of these peaks declines after bacterial treatments under some of the environmental situations. The next peak that was in the range of 2800-3000 cm^{-1}, related to strong connections of aldehydes with several CH bonds. Our study revealed these groups of compound are also prone to bacterial cracking in various situations in contrast with previous studies in which aldehyde compounds remain unaltered [21, 29]. Next peak ranged from 1600-1800 cm^{-1}, revealed the aromatics and alkenes connections in the structure of asphaltene. These bonds are weak. These connections could also transform bacterial activity. The range of 1400-1600 cm^{-1}, refers to amine groups in asphaltene that often in comparison to other bonds and connections are weak. Some of these connections were attacked by bacteria, and caused a decline in its amount in asphaltene. In general, by comparing the results of our research with other researches, bacteria attack OH

groups the most but aldehydes were the most (Figure 7-12). This study indicated the high potent of *B.toyonensis* BCT- 7112 in breaking all the asphaltene fractions down including alcohol, alkenes, aldehydes and amines [20, 21 and 29].

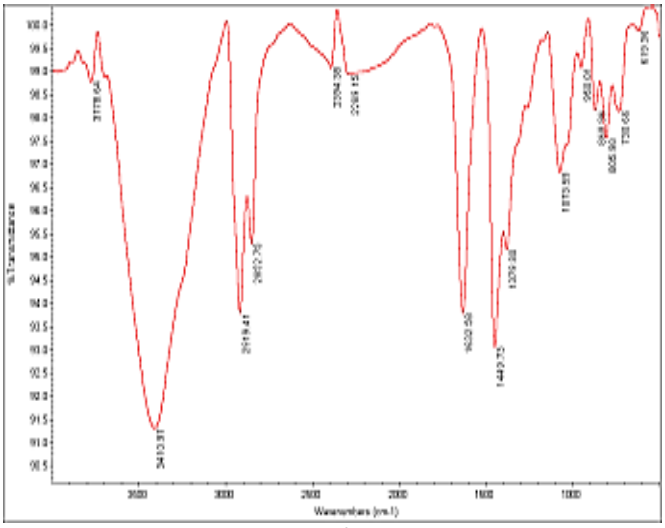

Figure 6. FT-IR spectra of natural asphaltene

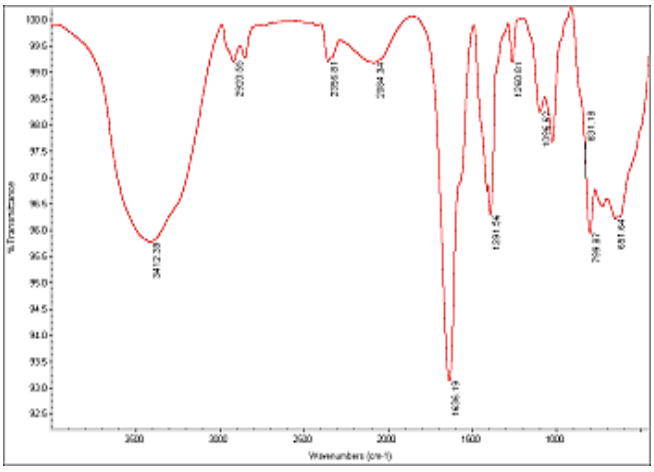

Figure 7. FT-IR spectra of asphaltene after 2 d at 25 °C

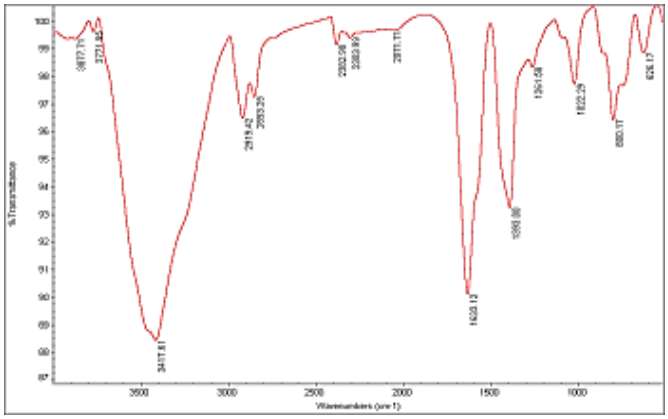

Figure 8. FT-IR spectra of asphaltene after 2 d at 45°C.

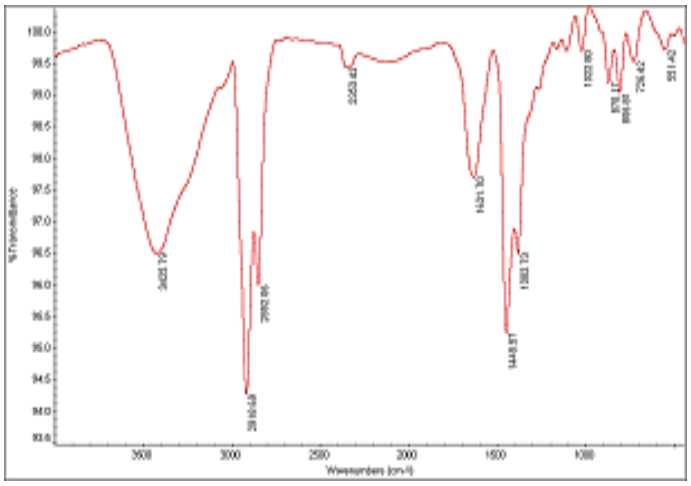

Figure 9. FTIR spectra of asphaltene after 20 d at 25 °C

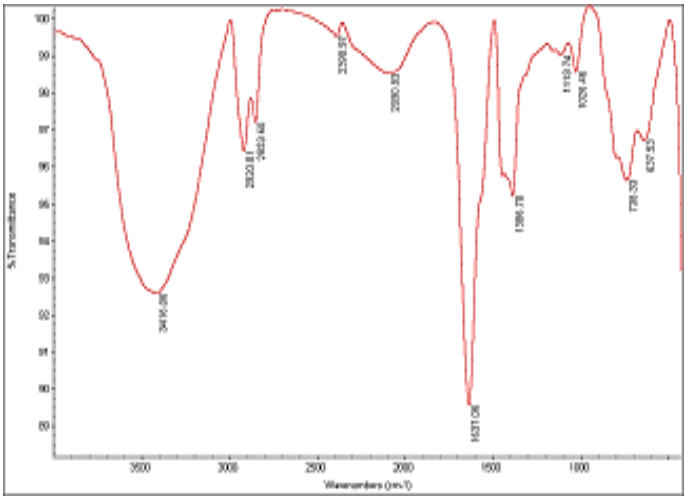

Figure 10. FT-IR spectra of asphaltene after 20 d at 45 °C

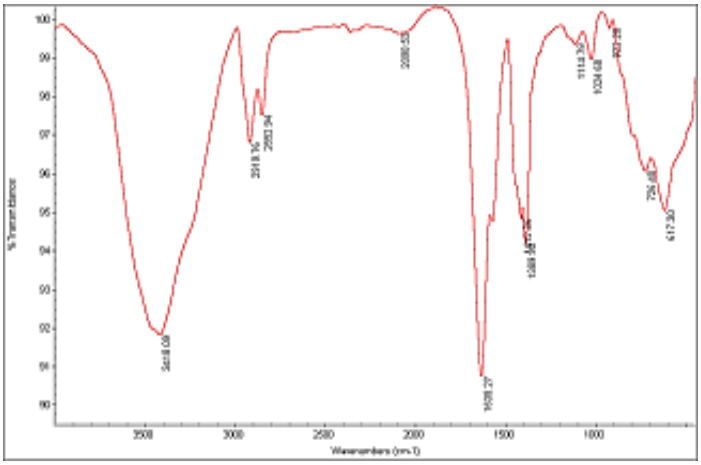

Figure 11. FT-IR spectra of asphaltene after 50 d at 25 °C.

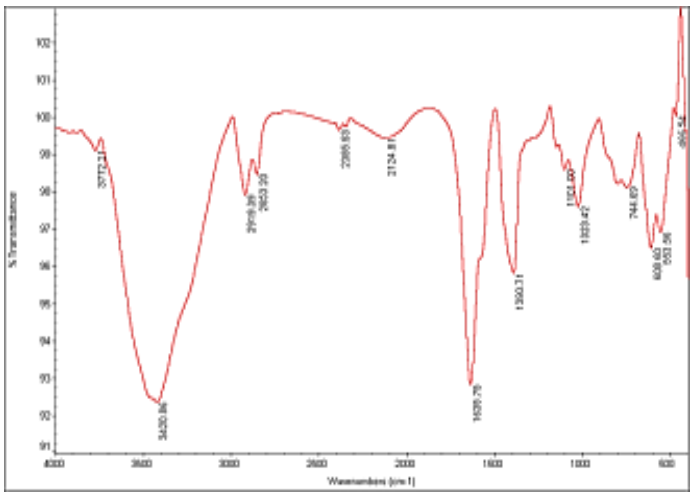

Figure 12. FT-IR spectra of asphaltene after 50 d at 45°C.

CONCLUSIONS

Since asphaltene is the main problem in crude oil extraction and processing; and consequently, it imposes a great financial burden to our petroleum industry, introducing an indigenous strain, Bacillus toyonensis, which degrades asphaltene efficiently, is a novel applicable approach in the oil industry. This bacterium degrades 64.8% of asphaltene at 25°C and 60% of it at 45 °C, which is remarkably higher than existed records and powerful tool to bio-upgrade heavy crude oils and resolve environmental issues.

ACKNOWLEDGMENTS

We have to express our appreciation to the cooperation of the Research Department of the Islamic Azad University Science and Research Branch.

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