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Molecular identification and characterization of indigenous bacterial strains isolated from soils around some oil refineries in Iran

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ABSTRACT

One of the main causes of soil pollution is the release of crude oil into the environment. Indigenous microorganisms with the capability of biodegrading hydrocarbon components can be used to improve the efficiency of microbial bioremediation technology. Here, oil-contaminated soils were collected from five oil refineries (Abadan, Isfahan, Tehran, Tabriz and Shiraz) for screening and isolation of geographically adaptive indigenous oil-degrading bacteria. Bacterial colonies from oil-contaminated soil samples were isolated, which were able to grow in a medium containing crude oil, light, and heavy diesel, as a sole carbon source. Twelve strains were isolated, purified, and identified, using the 16S rRNA gene sequencing analysis. The isolates belonged to five species, including *Achromobacter spanius*, *Klebsiella quasipneumoniae*, *Ochrobactrum intermedium*, *Citrobacter amalonaticus*, *Pseudomonas aeruginosa*. These bacterial strains were capable of growing in media containing crude oil, light and heavy diesel for 7 to 9 days and had the potential for biosurfactant production. These bacterial strains can be considered as geographically adaptive bacteria, creating preliminary data for further research to utilize their bioremediation potential.

Keywords: Bacteria, Bioremediation, Biosurfactant production, Petroleum hydrocarbons, 16S rRNA.

1. Introduction

Oil pollution is a worldwide environmental challenge, which may occur either accidentally or operationally due to production and transportation processes. Therefore, there is a high demand for an effective and affordable technology for the treatment of oil-contaminated sites (Xu et al., 2018).

To remediate oil-contaminated soils, several soil treatment approaches have been developed, including chemical, biological, and physical techniques. Such methods are able to remove a wide range of contaminants; however, high energy utilization and the need for supplementary chemicals are the major disadvantages of these methods (da Silva et al., 2012). Biological methods such as bioremediation are utilizing microorganisms to improve the elimination of harmful substances from a contaminated area. Bioremediation is considered a cost-effective method, applicable in large areas where microorganisms, especially bacteria, are key players in the process (da Silva et al., 2012; Guerra et al., 2018).

Biodegradation of organic pollutants, such as petroleum products, using indigenous microorganisms has been considered to be a useful and promising technique for large-scale remediation (Chandra et al., 2013; Das and Chandran, 2011). More than 79 genera of bacteria with the capability of degrading petroleum hydrocarbons have been identified (Tremblay et al., 2017). Four of such bacterial genera including *Pseudomonas*, *Rhodococcus*, *Mycobacterium,* and *Alcanivorax* were capable of degrading petroleum hydrocarbons in various environments such as contaminated sediments and seawater (Abed et al., 2014; Isaac et al., 2013; Salam et al., 2014) as well as contaminated soils (Romina et al., 2012; Roy et al., 2014). Therefore, the first step for bioremediation of pollutant areas is to identify indigenous species with the biodegradation potential.

Iran is one of the major producers of crude oil and several oil refineries have been constructed around the country. Therefore, oil pollution is widespread in the country during production and also transportation activities (Hassanshahian et al., 2012; Soleimani et al., 2013). Although biological approaches (e.g. bioremediation and phytoremediation) are considered useful for soil and water remediation (Soleimani et al., 2010; Soleimani et al., 2013), little information is available about geographically adaptive oil degrading bacteria in industrial zones of Iran. Therefore, the current study aimed to identify bacterial strains which were capable of growing in mediums containing crude oil, light and heavy diesel and had the potential for biosurfactant production in soils of five oil refineries in

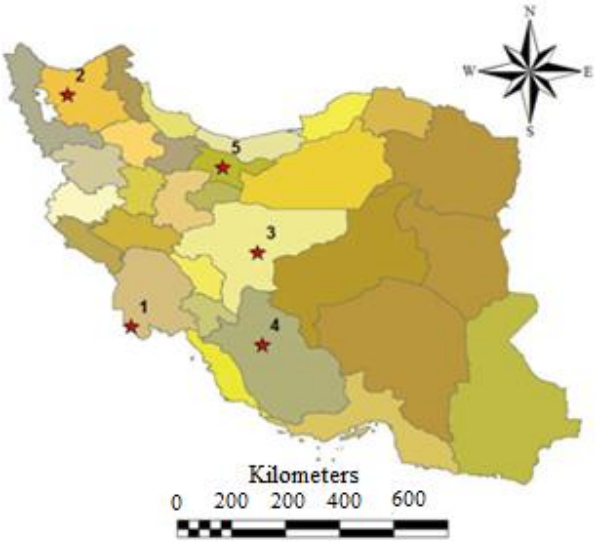

Fig. 1. Geographic locations of the five refineries in Iran sampled in the current study.1: Abadan, 2: Tabriz, 3: Isfahan, 4: Shiraz, and 5: Tehran refinery.

Iran (Abadan, Isfahan, Tehran, Tabriz and Shiraz). The selected refineries are located in different regions which may provide a diversity of bacterial species with the capability of petroleum hydrocarbons biodegradation.

2. Material and Methods

2.1. Sampling and data collection

Five oil refineries in different geographical locations in Iran were selected as sampling regions (Fig. 1). Among them, Isfahan, Tehran, and Shiraz refineries are located in the semi-arid environment of the central part of Iran with almost similar climatic conditions. Tabriz refinery is located in the north-west with a cold and semi-arid climate, and Abadan is located in the south-west of the country with a hot and dry climate (Fig. 1).

Petroleum contaminated soil samples were collected from the 20 cm beneath the soil surface, because the first 3-5 cm was heavily weathered (Soleimani et al., 2013). Due to restrictions imposed by the officials, 4 soil samples were collected within each site. Then, composite samples of four subsamples were obtained from each site and transferred to the laboratory for bacterial isolation.

To remove large debris before the experiment, soil samples were crushed, mixed, and sieved through a 2 mm pore size sieve. Soil characteristics such as nitrogen, phosphorous, calcium carbonate equivalent contents (Soil and Plant Analysis Council 1999) and microbial respiration were measured (Paul and Clark, 1996). Heavy metals including Cd, Pb, Ni, and Cr were measured based on the method described by Kasassi et al., (2008). Total petroleum hydrocarbons (TPHs) were measured in soil samples following the gravimetric method (Schwab et al., 1990). Briefly, 3 g of each soil sample was mixed with 70 mL n-hexane and the mixture was heated and refluxed for 3 h using a Soxhlet extractor. Then, the solvent was evaporated in an oven and the weight of TPHs was measured.

2.2. Isolation of oil degrading bacteria

We used a medium of synthetic Bushnell Haas Mineral Salts (BHMS) for the isolation of oil-degrading stains. BHMS medium contained (g L^{-1}) KH_2PO_4, 1; K_2HPO_4, 0.2; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.02; NH_4NO_3, 1; and 0.004 of $FeCl_3$ and the pH adjusted to 7.0 (Cappello et al., 2012). A portion (10 g) of each soil sample was added to 250 mL Erlenmeyer flasks containing 100 mL BHMS medium and the flasks were incubated for 10 days at 30 °C on a rotating shaker operating at 180 rpm. Then 5 mL aliquots were transferred into a fresh BHMS medium. Following enrichment, 100 µL were plated onto BHMS medium containing nutrient agar having 1.5% w/v of crude oil, and incubated at 30 °C for 2-4 days. Colony growth was monitored and dissimilar colonies from each plate were stored in BHMS medium. This procedure was repeated and only pure colonies with the maximum growth in crude oil were isolated and used for further analyses.

2.3. Taxonomic identification of isolates

The 16S rRNA gene was used for the taxonomic identification of the isolates. The bacterial DNA extraction kit (Qiagen) was used to extract the total DNA. The 16S rRNA gene was amplified by a forward primer (27f: 5'-GAGTTTGATCCTGGCTCAG-3') and a universal reverse primer (1541r: 5-AAGGAGGTGATCCAGCCGCA-3') (James, 2010). All amplifications were conducted in a final volume of 25 µL containing 100 ng DNA, 1X PCR buffer (Qiagen), 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 250 pm of each primer, and 2 U of Taq polymerase (Qiagen). Thermocycling was carried out, using an initial denaturation cycle of 95 °C for 3 min and 35 cycles of denaturation at 93°C for 45 s, annealing at 58°C for 60 s; extension at 72°C, 90 s. All amplified products were purified using Qiaquick PCR Purification Kit and sequenced on an automated ABI 3730XL DNA Analyzer. To estimate the degree of similarity and define the phylogenetic affinities of sequences, Ribosomal Database Project RDP (Cole et al., 2005) and Eztaxon (Chun et al., 2007) were used.

BioEdit (version 7.0.5.2) was used to edit and align sequences. Phylogenetic analysis of partial 16S rRNA fragments from the isolated strains was conducted using neighbor joining algorithms in MEGA version 5.0. The robustness of the phylogenetic tree was examined by bootstrap analysis with 1000 iterations. In order to root the phylogenetic tree, the 16s rRNA fragment of *Pseudomonas fluorescens* was used as an out-group.

2.4. Growth and crude-oil, heavy, and light diesel removal assay

The growth of bacterial isolates in the medium containing different concentrations of crude oil, heavy and light diesel (1, 2.5, 3, and 5% v/v) agitated by an orbital shaker incubator (180 rpm, at 30 °C for 1 week), was regularly checked by measuring the turbidity ($OD_{600\ nm}$), using a UV–visible spectrophotometer (JASCO/N-530). An analysis of variance was performed, using SPSS 17.0, to evaluate the significant differences between growths of different bacterial isolates, based on Duncan's multiple comparison test ($P = 0.05$).

As constructing a consortium of native microorganisms can increase their bioremediation capability, a consortium including *Achromobacter spanius*, *Ochrobactrum intermedium*, *Klebsiella quasipenumoniae, Pseudomonas aeruginosa* and *Citrobacter amalonaticus* was used based on the five isolated bacteria and the bacterial growth and removal ability of the constructed consortium was measured in the medium containing different concentrations (1, 2.5, 3, and 5% v/v) of heavy diesel as a recalcitrant compound with the same method described above.

2.5. Assessment of emulsifying activity of biosurfactants and bacterial adhesion to hydrocarbons

The biosurfactant production of isolates was assessed by drop collapsing and oil displacement tests, based on the method described by Maneerat and Phetrong (2007). In this method, the biosurfactant is confirmed by oil emulsification in growth medium. In this regard, the emulsification activity (E24) was measured by adding hexadecane to the same amount of cell-free culture broth, mixing with a vortex for two minutes, and letting the mixture stand for 24 hours. To analyze bacterial adherence to hydrocarbon, after adding oil to the tubes containing bacterial suspension and incubation for 10 min in ambient temperature and agitation for 2 min, the solution was stood for 15 min and finally, the turbidity was measured and compared to the amount before the treatment (Pruthi and Cameotra, 1997).

3. Results

3.1. Soil characteristics

Soil characteristics, heavy metals, and TPH concentrations are given in Table 1. TPH concentrations were the highest in soils from Shiraz and Isfahan refineries (154.4 ± 32.3 and 137.7 ± 78.2 g kg^{-1}, respectively) and the lowest TPH concentration (11.7 ± 0.5 g kg^{-1}) was recorded in soils from Tehran refinery. Maximum microbial respiration was recorded in soil samples from Tehran refinery (9 ± 1.41 mg CO_2 $g.day^{-1}$) and the minimum amount was obtained from Shiraz and Isfahan refineries (1.63 ± 0.3 and 1.76 ± 0.4 mg CO_2 $g.day^{-1}$, respectively).

Among the heavy metals, Cd had the lowest concentration, while Cr and Ni showed the highest concentration in soils (Table 1). Overall, Tehran refinery had the lowest concentrations of TPH and heavy metals in comparison to the other sites (Table 1).

3.2. Isolation and identification of bacteria

From the enrichment cultures, fifty crude oil-degrading bacterial strains were isolated. Twelve strains with the highest growth in crude oil were chosen for further analysis. Molecular identification showed that these isolates belonged to five major taxa including: *Achromobacter spanius* (34%), *Ochrobactrum intermedium* (33%), *Klebsiella quasipenumoniae* (17%), *Pseudomonas aeruginosa* (8%) and *Citrobacter amalonaticus* (8%) (Table 2 and Fig. 2). *Achromobacter spanius* was identified from three refineries, including Isfahan, Tabriz and Shiraz. *Ochrobactrum intermedium* was identified in soils from four refineries, including Tehran, Isfahan, Shiraz, and Abadan. *Klebsiella quasipneumoniae* was identified in soils of Isfahan and Tehran refineries. *Citrobacter amalonaticus* and *Pseudomonas aeruginosa* were only present in Shiraz and Tabriz, respectively. *Achromobacter spanius,* and *Ochrobactrum intermedium* were the two most common bacteria in the studied sites. DNA sequences were submitted to GenBank (https:// www.ncbi.nlm.nih.gov/genbank/) under the accession numbers: KY635397 to KY635408.

3.3. Growth of bacterial isolates and hydrocarbon decomposition

All bacterial strains were capable of growing in mediums containing crude oil, light and heavy diesel for 7 to 9 days. The most favorable concentration, allowing high growth of bacterial strains was 1% (v/v) of all tested hydrocarbon components (Fig. 3). The *K. quasipneumoniaein* and *A. spanius* strains had the highest growth in light diesel and were, respectively, capable of removing 82 and 67 percent of the diesel in 9 days. On the other hand, *P. aeruginosa* had a low growth and the lowest percentage of light diesel removal (50%). The *O. intermedium* could degrade all tested hydrocarbons. However, the best growth of this strain was observed for diesel (52 %) in 9 days of incubation. The strain *C. amalonaticus*, which was isolated only from the soil samples of the Shiraz refinery, has the highest growth in crude oil and could remove 55 percent of the oil in 9 days.

3.4. Assessment of emulsifying activity of biosurfactants and bacterial adhesion to hydrocarbons

Qualitative tests of drop collapsing and oil displacement

Table 1. Selected soil parameters of soils from five refineries in Iran. Values are reported as mean ± standard errors (SE).

Refinery	N (%)	P (mg Kg^{-1})	$CaCO_3$ (%)	Microbial Respiration ($mgCO_2$ $g.day^{-1}$)	EC‡ ($dS\ m^{-1}$)	pH	TPH† (g kg^{-1})	Heavy metals (mg kg^{-1})			
								Pb	Cr	Ni	Cd
Abadan	0.138 ± 0.04	177.89 ± 97.5	61±2.8	3.93 ± 2.4	40.73 ± 17.3	7.0	45.78 ± 30.3	9.95 ± 10.6	83.77 ± 17.4	75.73± 14.75	1.8 ± 0.64
Isfahan	0.408 ± 0.30	31.07 ± 14.8	31±15	1.63 ± 0.8	8.8 ± 2.3	7	137.72± 78.3	9.1 ± 5.1	98 ± 25	122 ±43.3	4.4 ± 1.1
Shiraz	0.188 ± 0.04	113.37 ± 29.3	38±8.4	1.76 ± 2.2	0.44 ± 0.2	7.3	154.4 ± 32.3	13.38 ± 8.5	212.8 ±137	77.42 ±17.6	2.2 ±0.4
Tehran	0.054 ± 0.05	6.79 ± 0.3	36±1.6	9 ± 1.4	1.08 ± 0.01	7.5	11.7 ± 2.3	6.1± 0.5	61 ± 1	46.5 ± 1	1.3 ± 0.4
Tabriz	0.435 ± 0.32	192.03 ±143.4	26±6.9	3.1 ± 1.5	4.03 ± 1.5	7.2	115.1 ± 74.9	5.41 ± 4.4	52.4 ± 26.8	76.31 ± 46	2.7 ± 0.6

† Total Petroleum Hydrocarbons, ‡EC Electrical Conductivity

Table 2. Identification of 12 hydrocarbon-degrading bacterial isolates from soils of five oil refineries in Iran.

Isolates	Code	Refinery	Accession numbers	16S rRNA sequence comparison	
				Species as close relative	Similarity (%)
1	Ta3	Tabriz	KY635400	*Achromobacter spanius LMG 5911T*	100
2	Ta6	Tabriz	KY635407	*Pseudomonas aeruginosa DSM 50071T*	100
3	Th2	Tehran	KY635408	*Klebsiella quasipneumoniae 01A030T*	99.86
4	Th6	Tehran	KY635406	*Ochrobactrum intermedium LMGT 3301*	100
5	Is2	Isfahan	KY635403	*Ochrobactrum intermedium LMGT 3301*	99.42
6	Is3	Isfahan	KY635402	*Klebsiella quasipneumoniae 01A030T*	99.64
7	Ab2	Abadan	KY635404	*Ochrobactrum intermedium LMGT 3301*	99.39
8	Ab4	Abadan	KY635398	*Achromobacter spanius LMG 5911T*	100
9	Sh1	Shiraz	KY635405	*Ochrobactrum intermedium,LMGT 3301*	99.32
10	Sh2	Shiraz	KY635399	*Achromobacter spanius LMG 5911T*	99.91
11	Sh3	Shiraz	KY635401	*Citrobacter amalonaticus CECT 863T*	99.91
12	Sh4	Shiraz	KY635397	*Achromobacter spanius LMG 5911T*	100

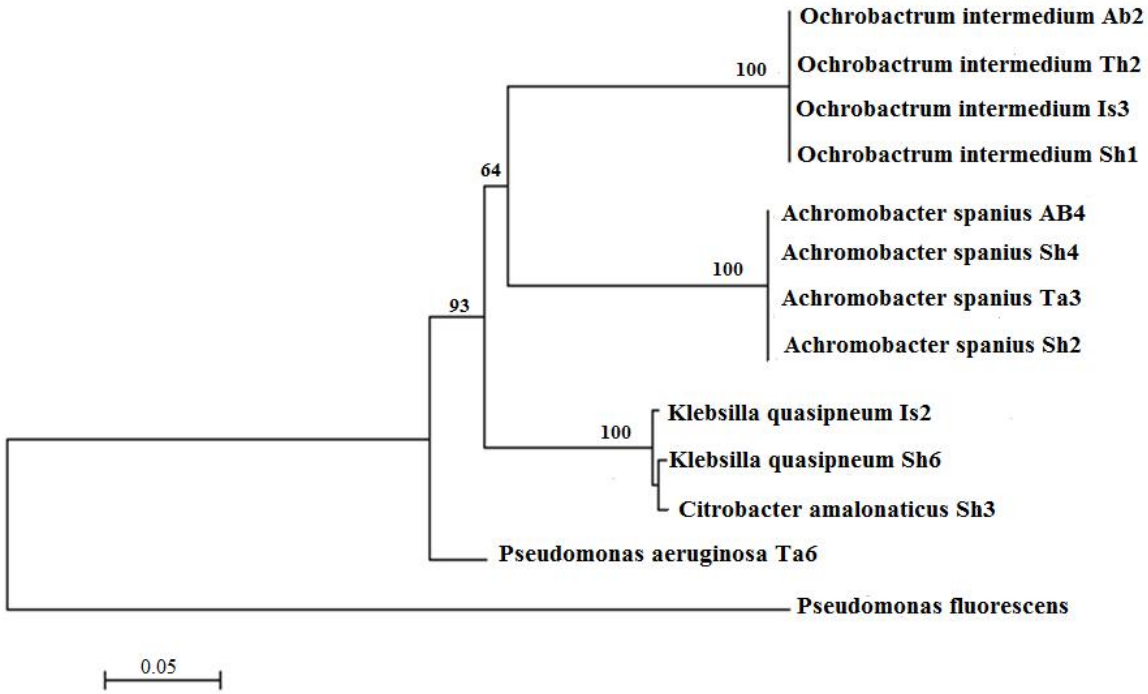

Fig. 2. A phylogenetic tree of 16S rDNA sequences of the bacterial strains isolated from oil-contaminated soils in the five oil refineries (Sh: Shiraz, Is: Isfahan, Ab: Abadan, Ta: Tabriz, Th: Tehran, using a neighbor-joining approach with 1000 bootstrap replications. *Pseudomonas fluorescens* (accession no. HE86228) was used as the out-group to root the phylogeny. The bar represents 0.05% sequence divergence and the numbers at nodes are bootstrap values.

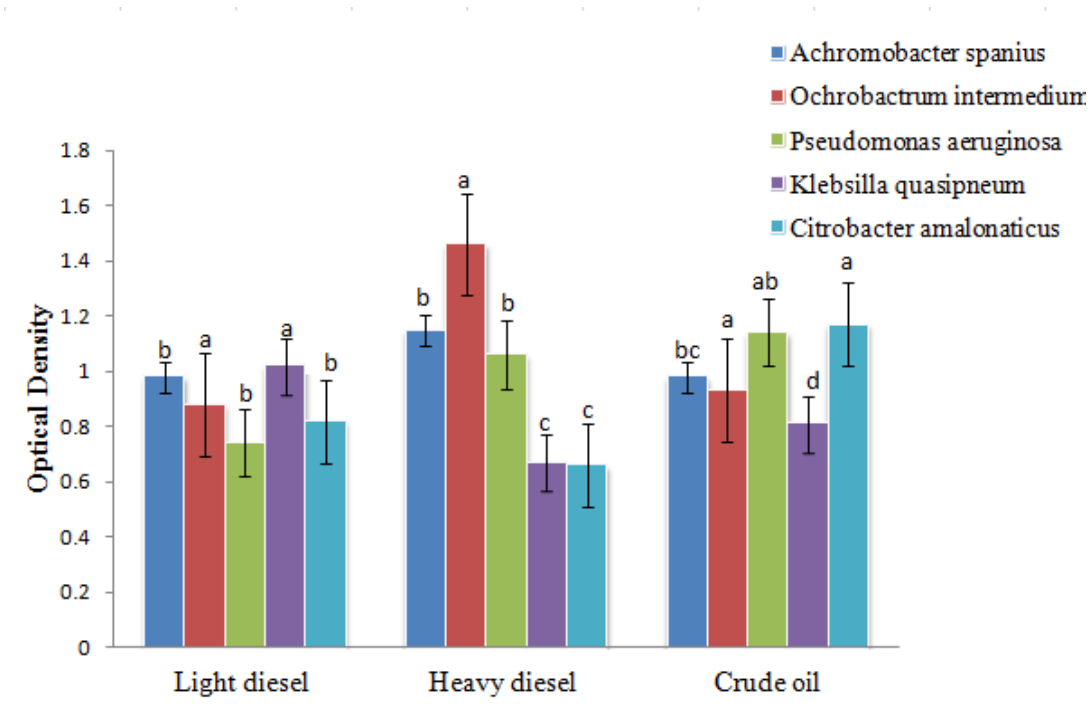

Fig. 3. Bacterial growth comparison in light diesel, heavy diesel, and crude oil, separately. Different letters in each growth medium indicate significant differences (Duncan test, $P < 0.05$).

Table 3. Measurements of emulsifying activity of biosurfactants *(E_{24}%)* and bacterial adhesion to hydrocarbons(BATH%) of the five bacterial strains isolated from soils of five Iranian refineries

Bacterial strains	Biosurfactant production		E_{24} (%)	BATH (%)
	Crude oil spread (mm)	Drop collapse		
Achromobacter spanius	17.5	++	7	34.12
Ochrobactrum intermedium	11.33	++	9.75	39.79
Klebsiella quasipneumonia	14.5	++	5.9	25.73
Pseudomonas aeruginosa	12	+++	5	29.90
Citrobacter amalonaticus	20	++	13.3	48.08

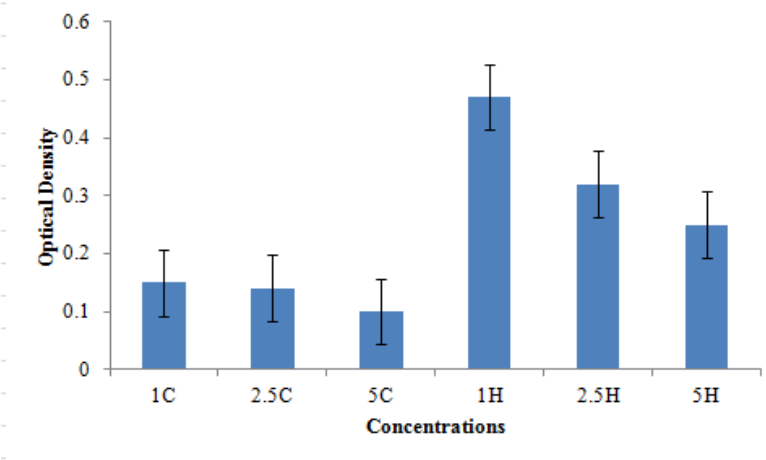

Fig. 4. Growth of the microbial consortium in the media containing 1, 2.5 and 5% w/w petroleum hydrocarbons as crude oil (C) and heavy diesel (H).

tests and further E_{24} and BATH confirmed the biosurfactant producing capability of all the five studied bacterial strains (Table 3). The strains *C. amalonaticus* and *O. intermedium* exhibited the highest emulsification activities (13.3% and 9.7 $E24_{\%}$) and the cell surface hydrophobicity (48.08 and 39.79 BATH %) (Table 3). However, *P. aeruginosa* had the lowest emulsification activity (5%) and hydrophobicity (29.9 %) among all strains. Figure 4 shows the growth of the microbial consortium in the media containing 1, 2.5 and 5% w/w petroleum hydrocarbons.

4. Discussion

The success of bioremediation of oil-contaminated environments depends upon the ability of microbial communities to degrade hydrocarbon components and the key to this success is the identification of indigenous species present in contaminated soils. In the current research, twelve oil-degrading bacteria were extracted from the soils of five refineries in Iran. Also, degradation capability for different hydrocarbon components was reported.

Bacterial strains with the greatest growth in crude oil, light, and heavy diesel were *C.amalonaticus*, *K. quasipneumoniae* and *O. intermedium,* respectively. *C. amalonaticus* is reported for the first time in this study with the capability to degrade crude oil. This strain had the highest growth and removal capability in the medium containing crude oil and the lowest growth in heavy diesel. All other isolates have been previously reported as hydrocarbon-degrading bacteria in various environments (Bezza et al., 2015; Deng et al., 2014; Hassanshahian et al., 2013; Ismail et al., 2013). This indicates that the decomposition of hydrocarbons depends on the types of hydrocarbons and microorganisms (Kumari et al., 2012; Megharaj et al., 2011).

Bacteria have several mechanisms to absorb and utilize hydrocarbon components. The production of emulsifiers and cell surface hydrophobicity might be two of the mechanisms that bacteria use for better uptaking and degrading of hydrocarbon components. In the current study, all the five isolated strains were able to produce surface-active agents and emulsifiers; however, C. *amalonaticus* and *O. intermedium* produced higher amounts of biosurfactant and showed higher cell surface

hydrophobicity. A direct relationship was found between the cell surface hydrophobicity, emulsification activity, and petroleum hydrocarbon biodegradation. Previous research also showed such a direct relationship (Hassanshahian et al., 2013; Isaac et al., 2013).

Biodegradation of different hydrocarbon components needs a variety of metabolic and physiological factors. All of such requirements are not gathered into one microorganism. Furthermore, microorganisms in a contaminated environment may adjust themselves to the contaminants. Therefore, constructing a consortium of native microorganisms can increase their bioremediation capability. In this work, a consortium was constructed from bacterial isolates, showing a high growth and hydrocarbon removal ability in heavy diesel. The presence of *C. amalonaticus* in this consortium with a high capability of producing surface-active agents and emulsifiers might have positive effects on its removal ability. Mixed bacterial consortiums have been reported to substantially increase the efficiency of biodegradation of hydrocarbon components such as lube oil (Wang et al., 2010) and crude oil (Tao et al., 2017; Wang et al., 2018). Bacterial enrichment together with the supplement of nutrients (e.g. nitrogen and phosphorous) has been proposed for enhancing the bioremediation of total petroleum hydrocarbons in contaminated soils (Soleimani et al., 2013; Wang et al., 2018).

5. Conclusion

With the advance in technology, biological control solutions have become more common in removing hazardous materials from the environment. The microbial remediation process is a safe and low-cost technique with high public acceptance to remove petroleum hydrocarbons from aquatic or terrestrial ecosystems. Results described here showed that some crude oil degrading bacteria are region specific with substantial potential for production of biosurfactants and the capability of decomposing crude oil, light, and heavy diesel. These bacteria may be potentially used for bioremediation of oil contaminated soils. However, finding the efficiency and the effect of environmental conditions on bioremediation of oil-contaminated soils and the limiting factors of the method (e.g. time) should be considered.

References

Abed, R. M. M., Al-Sabahi, J., Al-Maqrashi, F., Al-Habsi, A. & Al-Hinai, M. (2014). Characterization of hydrocarbon-degrading bacteria isolated from oil-contaminated sediments in the Sultanate of Oman and evaluation of bioaugmentation and biostimulation approaches in microcosm experiments. *International Biodeterioration & Biodegradation, 89*, 58-66.

Bezza, F. A., Beukes, M. & Chirwa, E. M. N. (2015). Application of biosurfactant produced by *Ochrobactrum intermedium* CN3 for enhancing petroleum sludge bioremediation. *Process Biochemistry, 50*(11), 1911-1922.

Cappello, S., Santisi, S., Calogero, R., Hassanshahian, M. & Yakimov, M. M. (2012). Characterization of oil-degrading bacteria isolated from bilge water. *Water, Air, & Soil Pollution, 223*(6), 3219-3226.

Chandra, S., Sharma, R., Singh, K. & Sharma, A. (2013). Application of bioremediation technology in the environment contaminated with petroleum hydrocarbon. *Annals of Microbiology, 63*(2), 417-431.

Chun, J., Lee, J. H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y. W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *International Journal of Systematic and Evolutionary Microbiology, 57*(10), 2259-2261.

Cole, J. R., Chai, B., Farris, R. J., Wang, Q., Kulam, S. A., McGarrell, D. M., Garrity, G. M. & Tiedje, J. M. (2005). The ribosomal database project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Research, 33*, 294-296.

da Silva, L. J., Alves, F. C. & de França, F. P. (2012). A review of the technological solutions for the treatment of oily sludges from petroleum refineries. *Waste Management & Research, 30*(10), 1016-1030.

Das, N. & Chandran, P. (2011). Microbial degradation of petroleum hydrocarbon contaminants: an overview. *Biotechnology Research International, 2011*(1), 941810.

Deng, M. C., Li, J., Liang, F. R., Yi, M., Xu, X. M., Yuan, J. P., Peng, J., Wu, C. F. & Wang, J. H. (2014). Isolation and characterization of a novel hydrocarbon-degrading bacterium *Achromobacter* sp. HZ01 from the crude oil-contaminated seawater at the Daya Bay, southern China. *Marine Pollution Bulletin, 83*, 79-86.

Guerra, A. B., Oliveira, J. S., Silva-Portela, R. C., Araujo, W., Carlos, A. C. & Vasconcelos, A. T. (2018). Metagenome enrichment approach used for selection of oil-degrading bacteria consortia for drill cutting residue bioremediation. *Environmental Pollution, 235*, 869-880.

Hassanshahian, M., Ahmadinejad, M., Tebyanian, H. & Kariminik, A. (2013). Isolation and characterization of alkane degrading bacteria from petroleum reservoir waste water in Iran (Kerman and Tehran provenances). *Marine Pollution Bulletin, 73*(1), 300-305.

Hassanshahian, M., Emtiazi, G. & Cappello, S. (2012). Isolation and characterization of crude-oil-degrading bacteria from the Persian Gulf and the Caspian Sea. *Marine Pollution Bulletin, 64*(1), 7-12.

Isaac, P., Sánchez, L., Bourguignon, N., Eugenia Cabral, M. & Ferrero, M. (2013). Indigenous PAH-degrading bacteria from oil-polluted sediments in Caleta Cordova, Patagonia Argentina. *International*

Biodeterioration & Biodegradation, 82, 207-214.

Ismail, W., Alhamad, N. A., El-Sayed, W. S., El Nayal, A. M., Chiang, Y. R. & Hamzah, R. Y. (2013). Bacterial degradation of the saturate fraction of Arabian light crude oil: biosurfactant production and the effect of ZnO nanoparticles. *Journal of Petroleum and Environmental Biotechnology, 4*, 163-170.

James, G. (2010). Universal bacterial identification by PCR and DNA sequencing of 16S rRNA gene. In M., Schuller, T.P., Sloots, G.S., James, C.L., Halliday & I.W.J., Carter (Eds.), *PCR for Clinical Microbiology*, (pp. 209-214). Springer Netherlands.

Kasassi, A., Rakimbei, P., Karagiannidis, A., Zabaniotou, A., Tsiouvaras, K., Nastis, A. & Tzafeiropoulou, K. (2008). Soil contamination by heavy metals: measurements from a closed unlined landfill. *Bioresource Technology, 99*(18), 8578-8584.

Kumari, B., Singh, S. N. & Singh, D. P. (2012). Characterization of two biosurfactant producing strains in crude oil degradation. *Process Biochemistry, 47*(12), 2463-2471.

Maneerat, S. & Kulnaree, P. (2007). Isolation of biosurfactant-producing marine bacteria and characteristics of selected biosurfactant. *Applied Microbiology, 29*, 783–791.

Megharaj, M., Ramakrishnan, B., Venkateswarlu, K., Sethunathan, N. & Naidu, R. (2011). Bioremediation approaches for organic pollutants: a critical perspective. *Environment International, 37*(8), 1362-1375.

Paul, E. A. & Clark, F. E. (1996). *Soil Microbiology and Biochemistry*. 2nd ed. Academic Press. London. 63 pp.

Pruthi, V. & Cameotra, S. S. (1997). Rapid identification of biosurfactant-producing bacterial strains using a cell surface hydrophobicity technique. *Biotechnology Techniques, 11*(9), 671-674.

Romina, L., Ruberto, L., Hernández, E., Vázquez, S., Alfredo, B., Del Panno, M. & Cormack , M. (2012). Bioremediation of an aged diesel oil-contaminated Antarctic soil: Evaluation of the on site biostimulation strategy using different nutrient sources. *International Biodeterioration & Biodegradation, 75*, 96-103.

Roy, A. S., Baruah, R., Borah, M., Singh, A. K., Deka Boruah, H. P., Saikia, N., Deka, M., Dutta, N. & Chandra Bora, T. (2014). Bioremediation potential of native hydrocarbon degrading bacterial strains in crude oil contaminated soil under microcosm study. *International Biodeterioration & Biodegradation, 94*, 79-89.

Salam, L., Obayori, O. & Olatoye, N. (2014). Biodegradation of anthracene by a novel actinomycete, Microbacterium sp. isolated from tropical hydrocarbon-contaminated soil. *World Journal of Microbiology and Biotechnology, 30*(1), 335-341.

Schwab, J., Wetze, L. & Bank, S. M. (1990). Extraction of petroleum hydrocarbons from soil by mechanical shaking. *Environmental Science & Technology, 33*, 1940-1945.

Soil and Plant Analysis Council. (1999). *Soil Analysis Handbook of Reference Methods*. CRC Press, Boca Raton, USA. 264 pp.

Soleimani, M., Afyuni, M., Hajabbasi, M. A., Nourbakhsh, F., Sabzalian, M. R. & Christensen, J. H. (2010). Phytoremediation of an aged petroleum contaminated soil using endophyte infected and non-infected grasses. *Chemosphere, 81*(9), 1084-1090.

Soleimani, M., Farhoudi, M. & Christensen, J. H. (2013). Chemometric assessment of enhanced bioremediation of oil contaminated soils. *Journal of Hazardous Material, 254-255*, 372-381.

Tao, K., Liu, X., Chen, X., Hu, X., Cao, L. & Yuan, X. (2017). Biodegradation of crude oil by a defined co-culture of indigenous bacterial consortium and exogenous *Bacillus subtilis*. *Bioresource Technology, 224*, 327-332.

Tremblay, J., Yergeau, E., Fortin, N., Cobanli, S., Elias, M. & King, T. L. (2017). Chemical dispersants enhance the activity of oil-and gas condensate-degrading marine bacteria. *ISME Journal, 11*, 2793-2808.

Wang, C., Liu, X., Guo, J., Lv, Y. & Li, Y. (2018). Biodegradation of marine oil spill residues using aboriginal bacterial consortium based on Penglai 19-3 oil spill accident, China. *Ecotoxicology & Environmental Safety, 159*, 20-27.

Wang, H., Xu, R., Li, F., Qiao, J. & Zhang, B. (2010). Efficient degradation of lube oil by a mixed bacterial consortium. *Journal of Environmental Science, 22*(3), 381-388.

Xu, X., Liu, W., Tian, S., Wang, W., Qi, Q., Jiang, P., Gao, X., Li, F., Li, H. & Yu, H. (2018). Petroleum hydrocarbon-degrading bacteria for the remediation of oil pollution under aerobic conditions: a perspective analysis. *Frontiers in Microbiology, 9*, 2885.