

Characterization and Potentials of Indigenous Oil-Degrading Bacteria Inhabiting the Rhizosphere of Wild Oat (*Avena Fatua* L.) in South West of Iran

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ABSTRACT

Background: The contamination of ecosystem with petroleum and its derivatives is considered as one of the most crucial environmental threat in Iran. Application of microorganisms has been demonstrated as an appropriate and more practical alternative to clean-up petroleum hydrocarbons in the contaminated environments.

Objectives: The objectives of this study were isolating rhizosphere-inhabiting indigenous oil-degrading bacteria in wild oat grown in petroleum-polluted areas and *in vitro* evaluating the efficiency of oil biodegradability by microbial isolates.

Materials and Methods: Bacteria were isolated from rhizosphere of wild oat grown on contaminated sites in Khuzestan and were identified based on 16S rRNA gene sequencing. The catabolic genes were detected using PCR and hybridization analysis. Hydrocarbon degradation in liquid culture was evaluated by gas chromatography-mass spectrometry (GC-MS).

Results: 23 indigenous oil-degrading bacterial strains were isolated from the wild oat rhizosphere, grown in severely oil contaminated soil in Khuzestan. Based on 16S rRNA gene sequence analyses, isolated strains were classified to Genera *Acinetobacter*, *Pseudomonas*, *Enterobacter*, *Stenotrophomonas*, *Bacillus*, *Achromobacter*, *Ochrobactrum*, *Paenibacillus*, *Microbacterium*, *Curtobacterium* and *Sphingobacterium*. Catabolic genes *alkM*, *alkB* and *xylE*, responsible for biodegradation of the alkanes and aromatic petrochemical compounds were detected in bacterial community inhabiting rhizosphere of the wild oat. The GC-MS analysis indicated that consortium of these bacteria was capable of reducing crude petroleum in the liquid culture by 40.5%, after 10 days. The results of the present study revealed the adaptability of microbes to the rhizospheric area and subsequently their great potential to be exploited for cleaning up hydrocarbon contaminated sites.

Conclusions: This study might be an important step towards the development of a phytoremediation strategy in the South of Iran.

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► Implication for health policy/practice/research/medical education:

This research introduced some petroleum-degrading bacteria as suitable indigenous strains for bioremediation of aged -petroleum contaminated soil.

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1. Background

Iran is one of the world's major oil producers and since the discovery and extraction of petroleum in the South West of the country in 1908, the environment of this vast area has encountered serious petroleum pollution. Besides this direct pollution, the occasionally pipeline leaks, transportation accidents, storage tank ruptures and refining petroleum is further intensifying the pollution of this area. Most of these compounds are considered as carcinogenic, mutagenic and potent immunotoxicants and classified as priority environmental pollutant by the US Environmental Protection Agency (1-4). Constituents of this contaminant have long-term effects on ecosystems and increase the presence of toxicants in organisms towards the top of the food chain. Destroyed ecosystems and health risks have emphasized on the necessity for clean up and remedies for soil and water, which have prompted many researchers to develop environmentclean up strategies, such as bioremediation (5, 6). Crude petroleum and its associated products contain many kinds of organic compounds, dominated by aliphatic and aromatic hydrocarbons. Biodegradation of hydrocarbon contaminated soils, using the potentials of microorganisms to degrade or detoxify these contaminants, has been demonstrated as cost-effective and an environmentally sound remedy (2). Petroleum-degrading microorganisms have enzyme systems and metabolic capabilities, enable them to withstand adverse environmental conditions. Bacteria abundance, their fast growth and wide spectrum of molecules, have allowed them to be considered as a main bioremediation tool. Up to now, many bacterial strains, such as *Pseudomonas*, *Alcanivorax*, *Acinetobacter*, *Rhodococcus*, and *Bacillus* (7-9) have been isolated from soil and water, and were effectively used for the bioremediation of petroleum contaminants.

It is well established that many microbial catabolic pathways are involved in the degradation of petroleum (8). For example, the alk pathway in *Rhodococcus* sp. Q15, which degrades C8 to C32 n-alkanes (10); *Acinetobacter* sp. M-1, which can degrade n-alkanes ranging from C13 to C44 (11); the OCT plasmid in *Pseudomonas putida* GP01, which uses n-alkanes (12) and naphthalene (nah gene); or the *xylE* gene of *P. putida* involved in the meta-cleavage of catechol in aromatic compounds (13) have been documented in the literature. Molecular identification of the genes responsible for biodegradation processes using Polymerase Chain Reaction (PCR) and hybridization analyses may allow monitoring oil biodegradation at the DNA level (14). For an efficient bioremediation of contaminated sites, the first step is the isolation and characterization of indigenous strains that are adapted to the particular environmental conditions, such as temperature, water availability, oxygen and nutrients (7, 15). Also, long-term presence of petroleum contaminants in soil can develop microorganisms that are able to use organic pollutants as the sole carbon and energy source (6, 8, 10). Rhizosphere,

the area of soil under the direct influence of roots, could be an appropriate microsite for in situ degradation of petroleum contaminated soils. Microbial density, diversity and activity in the rhizosphere soil are greater and more effective for bioremediation than non rhizosphere soils, likely due to the growth stimulation by the exudation of chemical compounds from the roots (16). It has been shown that microbial populations in the rhizosphere may enhance a plant's adaptation to petroleum hydrocarbons through detoxifying contaminated soils as a result of direct mineralization of these organic contaminants (17). Wild oat (*Avena fatua* L.) is a member of grass family (Poaceae), with a fibrous root system which grows and completes its life cycle in the petroleum-contaminated soils of South West of Iran. It is hypothesized that wild oat might be able to benefit specific rhizobacterial communities in its highly branched root system, with a good potential for petroleum degradation. We focused on: (I) isolating rhizosphere-inhabiting indigenous oil-degrading bacteria in wild oat grown in petroleum-polluted areas; (II) characterizing isolates based on 16sRNA gene sequence. (III) monitoring catabolic pathways, and finally (IV) evaluating the efficiency of oil biodegradability in vitro by microbial isolates with chemical methods.

2. Objectives

Present study aimed to make appropriate consortium for rhizosphere inoculation in newly contaminated sites feared to be further polluted in near future.

3. Materials and Methods

3.1. Soil Sampling and Analyzes

Wild oat rhizosphere-surrounding soil samples were gathered in persistently antecedently contaminated sites in Khuzestan (arid climate with less than annual 250 mm rainfall; Max.: 48°C and Min.: 4°C). Additionally, soil showed hyperthermic regime. Seven root samples were collected per plant, seven soil samples were gathered from contaminated uncovered areas. Soil-adhered roots were shaken 4 to 5 times, soil remained adhered to the roots was considered as rhizosphere soil (18). Non contaminated soil samples were used as control. Soil samples were sieved with a 2 mm mesh, stored at -20°C. Certain soil characteristics were measured such as pH, electricity conductivity (EC), and total petroleum hydrocarbon (TPH). 10 g of each contaminated soil sample was mixed with 10 g anhydrous Na₂SO₄, extracted with dichloromethane for 12 h using a Soxhlet apparatus (1), finally, the extract was evaporated and remaining weighed to quantify TPH.

3.2. Isolation and Characterization of Bacteria

One gram of each soil sample (roots, adhering rhizosphere) was added to the minimal Bushnell-Haas medi-

um (BH) which contains followings (g/L): MgSO₄ 0.2, CaCl₂ 0.02, KH₂PO₄ 1.0, K₂HPO₄ 1.0, NH₄NO₃ 1.0 and FeCl₃ 0.05 (19), supplemented with 2% to 5% (w/v) crude petroleum (API gravity: 31, Tehran Refinery) as a carbon source and selective substrate. Cultures were incubated at 30°C on a rotary shaker at 180 rpm for 7 days, and 20 µL of the resulting culture was sub-cultured in fresh BH medium. After five subsequent subcultures, cultures were purified on diesel agar and nutrient agar (20). The identification of isolates was performed based on 16S rRNA gene sequencing. The isolated strains were grown in nutrient broth and harvested from overnight cultures. DNA extraction was performed following the CTAB method (21). A 1500 bp region of 16S rDNA from each strain was amplified with 27F and 1492R degenerated primers (20). The PCR mixtures (50 µL) contained 1.5 mM MgCl₂, 200 mM concentration of each deoxynucleoside triphosphate (dNTP), 50 pmol of each primer, 2.5 U of Taq DNA polymerase and 10 ng of DNA template. PCR amplification was performed using a PeQ STAR thermocycler (Biotechnologie GmbH, Germany). Forty cycles were performed at the following temperatures and times: 94°C for 5 min, 94°C for 30 sec, 56°C for 45 sec, 72°C for 1 min and 72°C for 10 min. The amplicons were analyzed by electrophoresis in a 1% (w/v) agarose gel and purified using the Agarose Gel DNA Extraction Kit (Roche, Germany), then ligated into the pTZ57RT vector (K1214, Fermentas, Germany). The constructed plasmids were subsequently introduced into *Escherichia coli* DH5α competent cells and spread onto X-gal plates. After 17 h incubation at 37°C, white colonies were picked, and the DNA was amplified by colony PCR. Positive colonies were grown in Luria Bertani (LB) liquid culture, and plasmids extracted using the Plasmid Extraction Kit (Roche), and then sequenced for at least three times. 16S rRNA sequencing data of each isolate was compared to the sequences of reference strains available in the Gen Bank (NCBI) databases using the BLASTn search tool (<http://blast.ncbi.nlm.nih.gov>) to determine the most similar sequences in the Gen Bank database. The sequences from this study were deposited to Gen Bank under the accession numbers of GU586300 to GU586322. Sequences of strains with the maximum homology were selected to identify the isolates. A phylogenetic tree was constructed based on the UPGMA of Mega4 software version 4 (www.megasoftware.net).

3.3. Detection of Catabolic Genes by PCR and Hybridization Analysis

Primer sets include *alkM* (Alkane monooxygenase, F: 5'-CGIGIGCIACICCTGAAGATCCAGC-3' and R: 5'-ITTAT-TITCCAICTATGCTCTGG-3', (22), *alkB* (Alkane hydroxylase, F: 5'-TGGCCGGCTACTCCGATGATCGGAATCTGG-3' and R: 5'-CGCGTGGTGATCCGAGTGCCGCTGAAGGTG-3', (15), *xylEa* (Catechol 2,3-oxygenase, F: 5'-TCGAGTTCGCTGGCCTGATCG-3' and R: 5'-CCC GCAGAACACTTCGTTGCG-3', (23), *xylE* (F: 5'-GCGAGATAGAAGCGCTCTTG-3'

and R: 5'-GTATTGATACCTGGGAGGAAG-3', (15), C230 (Catechol 2,3-oxygenase, F: 5'-CAAGCCCCACGACGTGCNNT-3' and R: 5'-CGGTIACCGGACGGGTGCGAAGAAGT-3', (24), *Niada* (Pyrenedioxygenase, F: 5'-ATCTTCGGGGCGCGCTGGGTGTTTCTCGG-3' and R: 5'-AATTGTGCGCGGCTGTCTCCAGTTCGC-3', (22), to *dCl* (Toluene dioxygenase, F: 5'-GCGAGATAGAAGCGCTCTTG-3', R: 5'-GTATTGATACCTGGGAGGAAG-3' (15) and *nahAC* (Naphthalene dioxygenase, F: 5'-AAGCACCTGATTCATGGCGATGA-3' and R: 5'-GAACTCAGCCAGTTGGAGCTGCTG-3', (4) were selected to determine the presence of certain catabolic genes that encode enzymes involved in hydrocarbon degradation pathways. DNA was extracted from isolated strains as described above and screened by PCR. The PCR fragments were cloned as mentioned above, and then sequenced. Each sequenced PCR product that showed similarity to the target catabolic gene was used for the synthesis of the probe. Hybridization reaction was performed to detect catabolic genes in other bacterial isolates. DNA of 23 isolates was denatured and spotted onto a Hybond nylon membrane (Roche). The membranes were hybridized with DNA probes specific for the genes *alkB*, *alkM*, and *xylE* using high-stringency prehybridization, hybridization, and washing conditions at 65°C. The probes were labeled with the digoxigenin (DIG) non-radioactive nucleic acid labeling and detection system, using the DIG DNA Labeling and Detection Kit (Roche), according to the manufacturer's instructions.

3.4. Analysis of Hydrocarbon Degradation in Liquid Culture

Bushnell-Haas liquid culture with 2.5% (w/v) crude oil as carbon source was inoculated by the bacterial consortium at 25°C on a rotary shaker at 150 rpm for 10 days. Control flasks devoid of inoculum, were incubated under the same conditions. Residual TPH in liquid culture was extracted with dichloromethane, dried and purified with silica gel to remove polar compounds such as microbial matter and products (25). The GC-MS analysis of extracted TPH was performed using a Fisons instruments gas chromatograph 8000 (Italy) with a CP-Sil 5CB column (30 m × 0.25 mm, 0.25µm film thickness), connected to a mass detector (TRIO 1000) operating at an ionization energy of 70 eV, and using helium as the carrier gas with a split ratio of 1:20. The oven temperature was programmed at 50°C for 5 min and then increased to 280°C at 5°C per min, and held at this temperature for 20 min. The compounds were identified by comparison of their mass spectra with the mass-spectral libraries.

4. Results

4.1. Isolation and Characterization of Bacteria

The soil samples taken from the polluted locations were very viscous and dark brown to black. BS9 and RS1 soil samples had the highest (33.1 ± 0.55%) and lowest (4.5 ±

Table 1. Some Chemical and Microbiological Properties of Collected Soil Samples

Soil Sample	TPH, Mean \pm SD	pH	ECa, dsm ⁻¹	Culturable Oil-Degrading Colonies in Agar Plate
BS1	21.50 \pm 0.288 ^b	5.63	3.78	-
BS2	21.72 \pm 0.364	5.26	4.93	-
BS3	24.00 \pm 0.057	6	4.42	P, Ac, B, O
BS4	12.96 \pm 1.291	7.1	1.49	A, E, P, Ac, B, O
BS5	17.43 \pm 0.425	6.75	4.13	A, P, Ac, B
BS6	19.96 \pm 1.155	6.18	2.07	E, P, Ac, St (<i>maltophilia</i>), O
BS7	33.10 \pm 0.55	5.99	1.74	P, Ac, St (<i>maltophilia</i>), B
RS1	4.500 \pm 0.288	7.32	0.91	E, P, Sp, Ac, St (<i>maltophilia</i> & <i>rhizophilia</i>), B, C, M
RS2	26.60 \pm 1.833	6.74	4.51	E, P, Sp, Ac, Pa, St (<i>maltophilia</i> & <i>rhizophilia</i>), B, C, M
RS3	11.73 \pm 0.371	7.02	2.71	A, E, P, Ac, Pa, St, B, O, C
RS4	11.33 \pm 0.902	7.41	2.18	A, E, P, Sp, Ac, St, M
RS5	4.963 \pm 0.031	7.04	0.79	E, P, Sp, Pa, St (<i>maltophilia</i> & <i>rhizophilia</i>), B, M
RS6	18.96 \pm 0.548	7.48	1.07	A, E, P, Ac, Pa, St, C, M
RS7	17.50 \pm 0.763	7.47	3.33	E, P, Sp, Ac, St (<i>maltophilia</i> & <i>rhizophilia</i>), B, C, M
Control	-	7.3	0.80	P

Abbreviations: A, *Achromobacter*; Ac, *Acinetobacter*; B S, Bulk soil; B, *Bacillus*; C, *Curtobacterium*; E, *Enterobacter*; M, *Microbacterium*; P, *Pseudomonas*; Pa, *Paenibacillus*; RS, Rhizosphere soil; St, *Stenotrophomonas rhizophila*; Sp, *Sphingobacterium*; O, *Ochrobactrum*

^a EC and pH in 1:2.5 1:2.5 soil extract

^b Mean \pm SE (n=3)

Table 2. Degradation Capabilities in Single and Mix Bacterial Cultures

Culture	Symbol	Time consuming, h (OD 660nm 1)	Attenuation ^a , Mean \pm SD
<i>Acinetobacter sp.</i> (GU586302)	A	48	29.40 \pm 3.27 ^b
<i>Pseudomona ssp.</i> (GU586316)	P1	72	16.20 \pm 2.15 ^c
<i>Stenotrophomona ssp.</i> (GU586311)	S	48	29.60 \pm 3.28 ^b
<i>Paenibacillu ssp.</i> (GU586318)	P2	72	9.21 \pm 1.49 ^c
Consortium ASc	A (GU586302 + GU586303) + S	24	40.14 \pm 2.45 ^a
Consortium ASPP	A+ P1+S+ P2	24	43.70 \pm 2.41 ^a

^a Incubation was for 10 days at 25 °C, (Mean \pm SE, n=3).

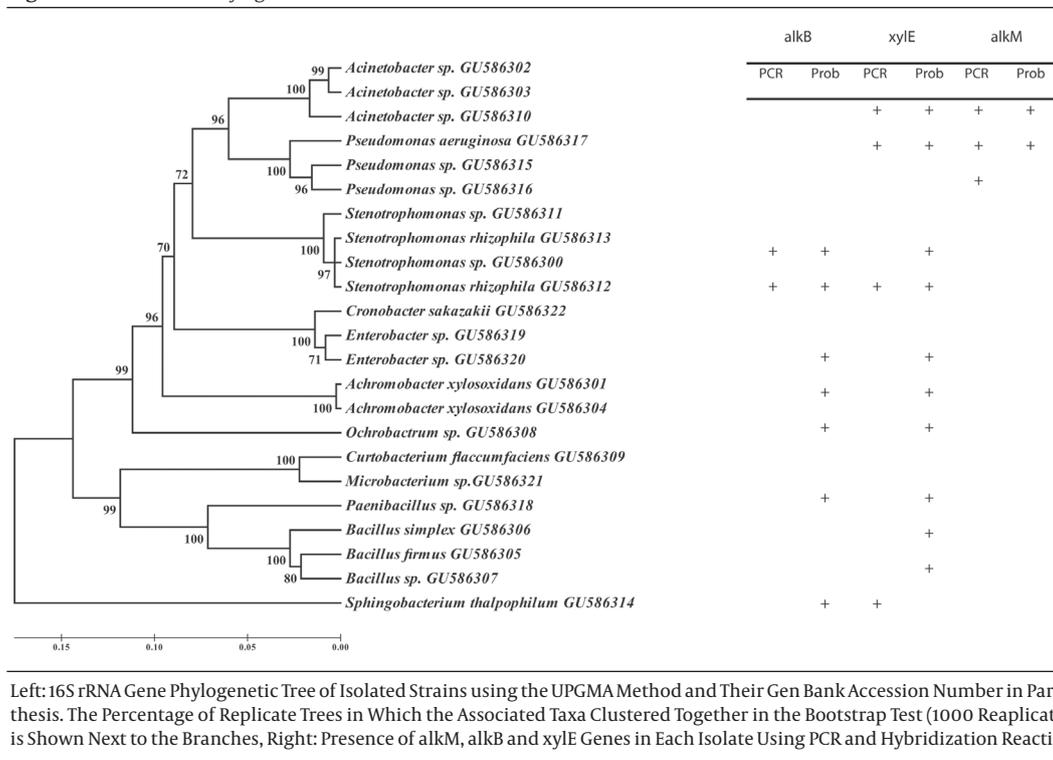
^b Similar letters indicate no significant difference among cultures at P < 0.05.

^c The related GC-MS analysis has been shown in Figure 2.

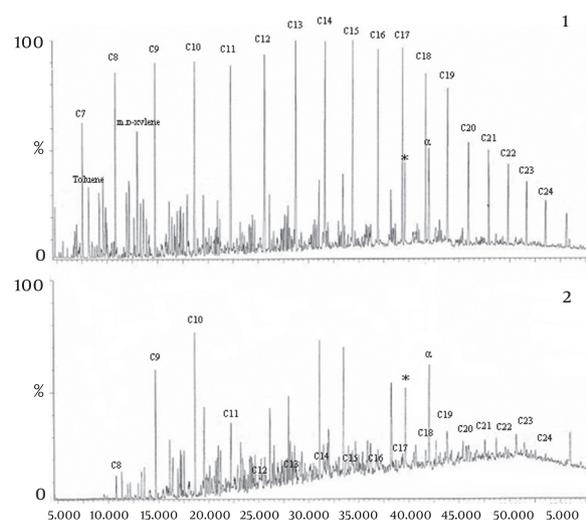
0.29%) TPH concentrations, respectively (Table 1). Generally, TPH concentrations in the plant-free soils were greater than that in the rhizospheric soils. However, the number of different oil-degrading colonies showed a reverse trend, being greater in the rhizosphere soil samples than in the plant-free soils. This may indicate the availability of root exudates of wild oat to the bacteria inhabiting the rhizosphere, and thereby enhancing bacterial composition and diversity (Table 1). Similar results have been previously reported by Liste and Prutz (18). It appears that increasing TPH concentrations, decreased soil pH at polluted locations. In general, soil samples showed relatively high EC values (up to 4.93 dSm⁻¹ in 1:2.5 soil extract, Table 1), probably due to the geographical location and the high water table. Totally, 150 bacterial colonies, capable of growing in presence of 10% (w/v) crude oil as the sole car-

bon source were isolated from the wild oat rhizosphere using serial enrichment method. The areas where the soil samples were collected usually exposed to an extreme temperature (above 50°C) during midsummer; thus, all isolated colonies were considered as thermo tolerant (data not shown). The twenty three fastest growing bacterial isolates were selected to characterize their 16S rRNA gene sequences, and deposited in GenBank database under accession numbers of GU586300 to GU586322.

Figure 1 shows a maximum likelihood tree constructed based on 16S rRNA gene sequences. The twenty three sequences were affiliated, with 98-100% identity, to twelve different genera including *Acinetobacter*, *Pseudomonas*, *Enterobacter*, *Cronobacter*, *Stenotrophomonas*, *Achromobacter*, *Ochrobactrum*, *Paenibacillus*, *Bacillus*, *Microbacterium*, *Curtobacterium* and *Sphingobacterium*.

Figure 1. 16S rRNA Gene Phylogenetic Tree of Bacterial Isolates

Left: 16S rRNA Gene Phylogenetic Tree of Isolated Strains using the UPGMA Method and Their Gen Bank Accession Number in Parenthesis. The Percentage of Replicate Trees in Which the Associated Taxa Clustered Together in the Bootstrap Test (1000 Reaplicates) is Shown Next to the Branches, Right: Presence of alkM, alkB and xylE Genes in Each Isolate Using PCR and Hybridization Reaction.

Figure 2. GC-MS Profile of Crude Petroleum in Liquid Culture, Upper graph: control flask and Lower Graph: Bacterial Consortium (*Acinetobacter sp.* (GU586302), *Acinetobacter sp.* (GU586303), *Stenotrophomonas ssp.* (GU586311)).

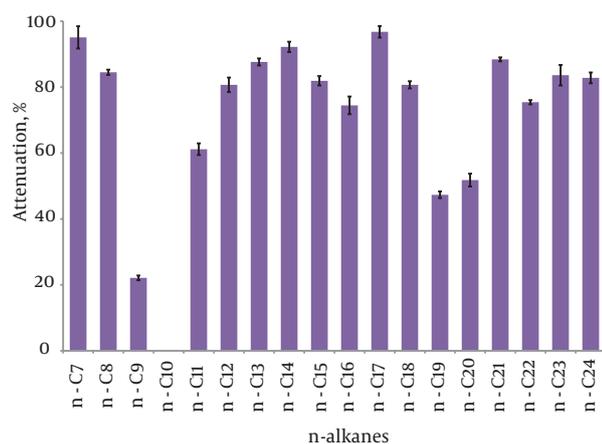
*, α unknown alkane
C n-alkane as term of carbon number

Among the twenty three isolates, sixteen sequences were affiliated to Class *Proteobacteria*, four sequences to Class *Firmicutes*, two sequences to Class *Actinobacteria*, and one sequence to the *Bacteroidetes* class. In the Class *Proteobacteria*, thirteen sequences were affiliated to the gamma subclass, two sequences to the beta and one to the alphaone. Gamma *Proteobacteria* dominated rhizosphere-inhabiting oil-degrading populations; *Bacteroidetes*, *Actinobacteria* and *Firmicutes* were found at lower amounts. 65% of the effective isolates contained Gram-negative bacteria.

4.2. Detection of Catabolic Genes

Presence of catabolic genes was investigated with PCR tests and DNA-DNA hybridization. Results are shown in Figure 1. The *alkB* gene belonged to *Pseudomonas sp.* (GU586315) showed 89% nucleotide sequence identity with *P. oleovorans alkB* gene, and *alkB*-derived amino acid sequences indicated 94% similarity with *alkB* belonged to *P. oleovorans* TF4-1L. Amplified fragments of *alkM* from *Acinetobacter sp.* (GU586302) showed 82% identity with *alkM* belonged to *Acinetobacter sp.* strain 69-V; amino acid sequence of corresponding gene was 90% similar to that of *alkM*. The *xylEa* nucleotide sequence of *Pseudomonas sp.* (GU586316) revealed 99% nucleotide sequence and 99% amino acid identities with those of *P. putida*, while the nucleotide sequence of *xylE* from *Acinetobacter sp.* (GU586302) showed 98% nucleotide sequence and amino

Figure 3. Attenuation of N-Alkanes as Term of Carbon Number in Bacterial Consortium, *Acinetobacter* sp. (GU586302), *Acinetobacter* sp. (GU586303), *Stenotrophomona* ssp. (GU586311) After 10 Days Incubation, Bars Indicate Standard Errors (n=3)



acid identities with those of *P. putida*.

Hybridization probes were PCR products of *alkB* (*Pseudomonas* sp.), *alkM* (*Acinetobacter* sp.) and *xylEa* (*Pseudomonas* sp.). Specific hybridization experiments were performed to detect aforementioned catabolic genes in the case of unsuccessful amplifications as well as confirmation of positive isolates. No significant cross-hybridization was observed.

4.3. Microbial Degradation of Petroleum Hydrocarbon in Liquid Culture

GC-MS profiles of residual crude petroleum in inoculated and control media are depicted in Figure 2. Three isolates including *Acinetobacter* sp. (GU586302), *Acinetobacter* sp. (GU586303) and *Stenotrophomonas* sp. (GU586311) were selected as a consortium for the biodegradation assays due to their fast growth in the presence of hydrocarbons (Table 2), reflecting corresponding catabolic pathways (Figure 1). GC-MS analysis of residual hydrocarbons in treated and untreated cultures showed decreased TPH after 10 days of incubation in the presence of bacterial consortium. N-alkane fractions removed in terms of in carbon number has been shown in Figure 3. The carbon number in alkane chains ranged from C7 to C24. GC-MS profile of oil residual components in liquid culture was compared to the control and showed that all components were highly reduced in inoculated culture; however, the consortium degraded C7-C24 n-alkanes in different quantities (Figure 3). Degraded aromatic compounds (PAHs) included C7 (toluene), C8 (ethylbenzene, m-, p- and o-xylene), C9, C10 (naphthalene), C12 and C18 (chrysene or benzo-anthracene). In cultures inoculated

with the bacterial consortium, crude oil amounts generally decreased about 40% after 10 days of incubation (Table 2). With the exception of C9 and C10, GC-MS analyses revealed that removal rate of n-alkane components, i.e. C7-24 was more than 80% in the consortium culture. C10 was the least degraded component in maximum removal rate of approximately 2%. The overall removal rates of oil hydrocarbons (aliphatic, aromatic, naphthenic, olefin) by the AS and ASPP consortia were 40.14% and 43.7% after 10 days, respectively (Table 2).

5. Discussion

Iran is the world's fifth crude oil producer (www.wikipedia.org). Environmental pollution is a severe challenge which oil producing countries face due to crude oil exploitation, refining, transportation, storage as well as accidents. Khuzestan is Iranian oil producing center of excellence where soil has been heavily contaminated over the past 80 years, threatening local ecosystems such as wetlands and rivers. Numerous efforts have been made to isolate indigenous oil-degrading bacteria in oil-contaminated soil and waters in Mideast oil rich countries such as Kuwait and Bahrain (19, 26, 27). In the present research, previously isolated rhizosphere-inhabiting bacterial strains in wild oat plants growing in contaminated sites were screened and introduced through a series of oil-enriched cultures as the sole carbon and energy sources based on their oil degrading efficacies and capabilities. The strains belonged to different genera previously reported as oil degrading species (8, 9, 20, 28, 29). Studies suggested that hydrocarbon contamination enriches oil degrading bacteria (6, 10). *Proteobacteria* and *gamma Proteobacteria* are commonly found in microbial communities exposed to hydrocarbon pollution (30, 31). We also found class *Proteobacteria* and *gamma Proteobacteria* highly prevalent belonged to r-strategists group, such as *Pseudomonas* (22). Kaplan and Kitts (32) suggested that Gram-negative bacteria dominate oil-contaminated areas. The rhizosphere is a dynamic environment where the roots, soil and microorganisms interact. Branched root systems provide organic substrate for microbial communities and consequently might accelerate degradation of soil organic matter (SOM) and organic pollutants (33), a process leading to the soil priming effect (25, 34). Beneficial root effects and long-term petroleum contamination can develop a very specific highly potent microbial community capable of detoxifying pollutants. Major isolates belonged to *Acinetobacter* and *Pseudomonas*, while others were only found in rhizosphere-bound soil samples including *Curtobacterium*, *Paenibacillus*, *Sphingobacterium* and *Microbacterium* sp., *Stenotrophomonas rhizophila*. Haichar et al. (33) reported that Sphingomonadales was specifically associated with monocotyledons such as wheat and maize, whereas *Enterobacter*-like bacteria were considered as generalists colonizing both mono and dicoty-

ledons. We found that *Sphingobacterium* was associated with the wild oat roots (*A. fatua*) and could use petroleum hydrocarbon. *Microbacterium sp.* was found by Supaphol et al. (35) in tropical soil samples contaminated with petroleum, we recorded the species in the crude oil-contaminated wild oat rhizosphere in Iran for the first time. In Iran, most bioremediation works have focused on degradation rate of contaminant in oil-polluted soil and sediments, and we are the first to report molecular mechanisms probably involved in hydrocarbon degradation. Identifying catabolic genes in hydrocarbon-degrading bacteria is a common approach to evaluate hydrocarbon-contaminated sites for bioremediation processes (36). The specific probes of *alkB*, *alkM*, *xylE* genes, catabolic genes involved in hydrocarbon degradation, were used to determine the potential of bacterial isolates. Dot-blot hybridization experiments revealed the presence of these catabolic genes in several bacterial strains. Frequent occurrence of specific hybridization between sequenced DNA belonged to the isolates and their corresponding probes reflected high sequence identity between some isolates and corresponding regions of the target catabolic genes. The *xylE* gene involved in the aromatic hydrocarbon degradation pathway (*xylE*, BTEX degradation), was found in 10 of the 23 bacterial isolates (13). In addition to the *Pseudomonas* strains, the *xylE* gene was detected in other *Proteobacteria* isolates, such as *Acinetobacter*, *Achromobacter*, *Enterobacter* and *Stenotrophomonas*. In fact, hybridization and PCR amplification confirmed the presence of *xylE* in half of the isolates. Aromatic hydrocarbons are more resistant against biodegradation than aliphatic compounds, they often cause serious problems during bioremediation (3, 8). TOL catabolic plasmid carries the gene (13); thus, these bacteria could obtain the gene through horizontal transfer rather than independent evolution of their degradation capabilities (6, 18, 23). Root exudates (e.g. phenolics) selectively enriched bacterial flora, regulates the expression of PAHs catabolic genes in certain plant species (16). For instance, dioxygenase-expressing bacteria were the most abundant ones in contaminated rhizosphere in mustard, oat, and cress attributed to phenolic root exudates (16). Old persistent crude oil contamination as a most important environmental factor has driven bacterial population selection, followed by horizontal gene transfer between microbial communities diverged them to survive. Partial sequences analysis of 16S rRNA gene revealed a level of diversity mainly associated with two bacterial divisions; *Proteobacteria* and *Firmicutes* (Figure 1). Certain hydrocarbon-degrading strains, i.e. *Stenotrophomonas*, *Achromobacter*, *Pseudomonas*, *Enterobacter* and *Acinetobacter* possess both aromatic and aliphatic catabolic pathways suggesting that a single strain is capable of degrading both aromatic and aliphatic hydrocarbons. Two degradation pathways were previously reported in a single microorganism (4, 8). Some isolates carried none of

the studied genes; however, they grew in the presence of crude oil implying the existence of other biodegradation pathways in an indigenous population. Certain bacterial species occasionally provide degrading pathways when acting synergistically (37); diverse root exudates promote the assembly of these synergistic communities (16). Although all isolates grew in mineral media with crude petroleum as the carbon source, but 5 of the 23 isolates showed the most significant growth rates. These 5 isolates were identified as different species of *Stenotrophomonas*, *Pseudomonas* and *Acinetobacter* (Table 2). In fact, these strains grew in media with high quantities (up to 10%) of crude petroleum effectively, confirming that these strains are capable of using n-alkanes. GC-MS experiment revealed hydrocarbons with carbon numbers as high as C24 suggesting that these strains possibly use longer chain n-alkanes. *Acinetobacter sp.* strain M-1 has reportedly degraded a variety of n-alkanes, including very long chain n-alkanes (or paraffin wax) with carbon chain lengths up to C44 that are under a solid state (38). A combination of *Acinetobacter sp.* and *P. putida* has been found to degrade Arabian light crude oil containing 40% saturates and 21% aromatics (8). In the present study, crude petroleum at 25 g/L (2.5%) was degraded up to 40% during 10 days in the presence of bacterial consortia. *Stenotrophomonas spp.* and *Stenotrophomonas maltophilia* have been reported to use alkanes (39) and polycyclic aromatic hydrocarbons (29). The results also confirmed the presence of *alkB* and *xylE* genes in *Stenotrophomonas* species bearing ecological role in mineral cycle in nature, promoting plant growth via biological control of fungal diseases, and degrading a wide range of pollutants. Thus, it might be used potentially for bioremediation and phytoremediation (40). Using aromatic compounds by *Enterobacteria* has also been reported previously (41). *Pseudomonas* and *Enterobacter* strains, PAH-degrading microorganisms, have been isolated in highly oil-polluted soil, along with an *Enterobacter* strain capable of degrading naphthalene (42). Genetic study of PAH degradation demonstrated the presence of a highly efficient pathway for the degradation of naphthalene and phenanthrene, in *Pseudomonas* (29). Also, it has been reported that these species act as plant growth promoting *rhizobacteria* enhancing its tolerance to contaminants (43). During the incubation period, emulsification of crude petroleum was observed in the culture broth inoculated with consortia, suggesting that the production of extracellular bio surfactants may be one of the underlying mechanisms implemented by the isolates for using crude oil. Actually, production of bio surfactants is one of the ways that microorganisms take up hydrophobic substrates (2, 9). It has been frequently reported that the *Acinetobacter spp.* Produce bio-surfactants/bioemulsifiers indicating the presence of a hydrophobic exterior enabling cellular contact with hydrocarbons (2). Production of biosurfactants has also been reported in other isolates, such as *Pseudomonas ae-*

ruginosa, *Acinetobacter* spp., *Acinetobacter radioresistens*, *Acinetobacter calcoaceticus* and *Bacillus polymyxa* (8, 44). Presence of alkanes in control cultures revealed by GC-MS suggested that n-alkanes (C7-24) were removed up to 80% in consortium culture except for C9 and C10. The least degraded component was C10, which showed a maximum removal of 2% approximately. The overall removal of petroleum hydrocarbons (aliphatic, aromatic, naphthenic, olefin) by the AS and ASPP consortia was 40.14% and 43.7% after 10 days, respectively (Table 2). This study showed that longer chain petroleum hydrocarbons were degraded more effectively. Our results also revealed that C8 to C19-hydrocarbon chains dominated the crude petroleum samples, indicating that it is important to develop a more efficient consortium procedure to degrade shorter chain hydrocarbons. Studied strains used both long and shorter chain alkanes (Figure 2). As a substrate for bacterial enrichment, using crude oil has often led to isolation of microorganisms metabolizing n-alkanes (6). GC-MS analyses demonstrated that n-alkanes (C12-C24) were preferentially degraded by the consortium, when compared to PAHs present in the crude petroleum. Jain et al., (9) reported that most of n-alkanes can be significantly biodegraded within the first 10 days of incubation. The present work showed that the consumption of the substrates is faster and more efficient by mixed cultures in comparison to pure ones (Table 2). Thus, to achieve efficient crude petroleum biodegradation, a rather large consortium of the effective species would be needed. However, the higher growth rates of *Stenotrophomonas*, *Pseudomonas* and *Acinetobacter*, might be related to higher breakdown and using petroleum hydrocarbons compared to other isolated strains. We isolated a wild oat rhizosphere-inhabiting oil-degrading bacterial spectrum. Some of the studied species have been named as plant growth promoting rhizobacteria which might help plants to grow and withstand in severely polluted soil. Therefore, our findings highlighted the role of such isolates to be considered in further studies. The results of microbial isolation and identification corroborated literature data about the presence of aerobic bacteria in petroleum-contaminated rhizosphere in wild oat support the hypothesis that these bacteria might play a role in oil biodegradation processes in situ. Phytoremediation with such a spectrum of oil degraders would be the preferable choice of treatment at a demonstrative scale. Crude petroleum is a complex mixture of hydrophobic components, assemblies of mixed populations with overall broad enzymatic capacities are required for increasing the rate and extent of TPH biodegradation. Bio and phytoremediation techniques success relies on inoculating the right environment with the right microbial consortia. Therefore, an in-depth understanding of processes that occur, the responsible microorganisms and molecular mechanisms involved in the degradation process would provide more chance to tailor techniques to site-specific remediation. Long-term

exposure to widespread crude oil pollution in southwestern of Iran facilitates the selection and co-evolution of bacteria and plants. The findings of this study affirmed the adaptability of microbes of the rhizosphere and their great potential to be exploited for cleaning up hydrocarbon contaminated sites, either through inoculation of specifically isolated microbial communities or targeted stimulation of the selected species *in situ* in the presence of wild oat. Isolation, identification of bacteria and detection of some catabolic pathways from wild oat rhizobacteria is the first study of this kind in Iran and thus might be considered as an important step towards the development of phytoremediation strategies for sites contaminated with these pollutants.

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Authors' Contribution

The work presented here was carried out in collaboration between all authors. SMS and SR defined the research theme. SR and SMS designed methods and experiments, carried out the laboratory experiments, analyzed the data, interpreted the results and wrote the paper. JR, BR and FR co-designed experiments, discussed analyses, interpretation, and presentation. All authors have contributed to, seen and approved the manuscript.

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