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Isolation and characterization of diesel-degrading *Pseudomonas* strains from diesel-contaminated soils in Iran (Fars province)

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ABSTRACT: In this study, among the 21 diesel-degrading bacteria that were isolated from an oil-polluted area in Fars (Iran), 6 bacterial strains were tested for their capability to metabolize and grow on diesel oil by degrading its hydrocarbons content. The biochemical characteristics and 16S rRNA sequence analysis of diesel-degrading bacteria showed that these strains were related to the genus *Pseudomonas.* Among the six isolates, five strains (L1, I2, D1, D2, and G1) were clustered with *Pseudomonas aeruginosa*, whereas only one strain (K3) was clustered with *Pseudomonas fragi*. Gas chromatographic (GC) analysis of the diesel oil that was remaining in the culture medium after 10 days of culture at 30°C showed that *P. aeruginosa* I2 presented the highest growth rate and diesel-oil degradation (88%) between all isolates. *P. aeruginosa* I2 also presented the best emulsification activity, but the best hydrophobicity was seen in *P. aeruginosa* G1. By applying these bacteria in bioremediation processes, diesel oil contamination in soil can be counteracted.

Keywords: biodegradation, bioremediation, diesel oil, polluted soil, *Pseudomonas*

INTRODUCTION

Diesel oil is a complex fuel mainly composed of saturated hydrocarbons (primarily paraffins including *n*-, iso-, and cycloparaffins) and aromatic hydrocarbons (including naphthalene and alkylbenzene) obtained from the middle-distillate gas– oil fraction during petroleum separation (Zanaroli *et al*., 2010). Nowadays, diesel oil pollution is an environmental problem of increasing importance. Diesel oil spills from pipeline ruptures, tank failures, various production storage problems, and transportation accidents are the most frequent causes of soil and groundwater pollution (Lee *et al*., 2006). Until now, a variety of methods have been developed to treat diesel oil contamination. While many of the established physical and chemical methods are efficient, they are also expensive and can cause recontamination via secondary contaminants (Hong *et al*., 2005).

Remediation of oil spill-affected areas by the use of microorganisms can be a cost-effective solution for restoring the ecosystem for ensuring clean groundwater supplies. Several studies have reported microorganisms with enhanced oil-degrading abilities isolated from natural habitats that are historically contaminated with oil (Hess *et al*., 1997). Bioremediation is significantly affected by the following factors: the inherent capability of the microorganisms to metabolize oil; the ability of the microorganisms to overcome the limitations of bioavailability in multiphase environmental scenarios (oil–water– soil); and the effect of various environmental factors such as temperature,

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pH, nutrients, and electron acceptor availability of the microorganisms (Mukherji and Vijay, 2002). Some *Pseudomonas* species can metabolize chemical pollutants in the environment; therefore, they can be used in the process of bioremediation. Moreover, the *Pseudomonas* species, in general, grow rapidly and are particularly renowned for their ability to metabolize an extensive number of substrates such as toxic organic chemicals, including aliphatic and aromatic hydrocarbons (Edward *et al*., 2006).

The aim of this study was to study the microbial strains isolated from diesel-polluted soils of five regions of Fars province, Iran for their characteristics such as morphology, capability to metabolize and grow on diesel, hydrophobicity, and extracellular emulsifying activity.

MATERIALS AND METHODS

Sampling

For the isolation of diesel-degrading bacteria, soils samples were collected from five contaminated stations in the Fars province (Iran): Greenhouse's Moshkan (Fars, 37°30′N; 49°15′E; Sample D), White Cement Factory Neyriz (Fars, 47°30′N; 31°15′E; Sample G), Diesel Tanker's Shahrak (Moshkan, Fars, 24°10′N; 34°18′E; Sample I), Chemical Industry Shiraz (17°50′N; 22°19′E; Sample K), and Diesel Tanker's Moshkan (Fars, 67°10′N; 69°19′E; Sample L). Soils samples (50 g) were taken from 1 to 10 cm below the surface of land using a sterile knife. The samples were collected in sterile jars, placed on ice, and transported immediately (within 30 min) to the laboratory for further analysis.

Total count of heterotrophic and diesel-degrading bacteria in collected samples

For the enumeration of heterotrophic and diesel-degrading bacteria, soil samples were serially diluted and plated (100 µl) on Nutrient Agar (NA) and Bushnell Hass (BH) agar media, respectively. All plates were incubated at 30 ± 1 °C. After 2 days, the numbers of colonies grown were counted (Hassanshahian *et al*., 2013). Results were expressed as colony forming units per one gram of soil (CFU gr^{-1}).

Isolation and selection of diesel-degrading bacteria

BH medium with 1% (v/v) diesel oil (Iranian diesel) as a sole carbon and energy source was used for the isolation of diesel-degrading bacteria. BH medium contained (per liter of distilled water): 0.2g $MgSO_4 7H_2O$, 0.02g $CaCl_2 2H_2O$, 1.0g KH_2PO_4, 1.0g K_2HPO_4, 1.0g NH_4NO_3, 0.05g $FeCl_3$. For preparing the solid media, Bacto Agar (Difco) (15 g/l) was added to the BH medium (Atlas, 1995). A portion of each soil sample (5g) was added to Erlenmeyer flask containing 100 ml of the BH medium, and the flasks were incubated for 10 days at 30±1°C on rotary shaker (180 rpm, INFORS AG, Switzerland). From this soil slurry, 5 ml aliquots were transferred into the fresh medium. After a series of two further subcultures, inoculum from each flask was streaked out and later, the phenotypically different colonies from the BH agar medium were purified by culturing on BH agar medium. The phenotypically different colonies obtained from the culture plates were transferred to a fresh medium with and without diesel oil to eliminate autotrophs and agar-utilizing bacteria. This procedure was repeated and the isolates that exhibited pronounced growth on diesel oil-containing medium were stored in a stock media containing glycerol at −20°C for further characterization (Hong *et al*., 2005; Hassanshahian *et al*., 2012b).

Identification of isolates

Biochemical characterization

To identify and characterize the isolated bacteria, biochemical tests such as Gram staining, oxidation/fermentation, production

of acid from carbohydrates, tests for oxidase and catalase activity, and production of gas were carried out according to the Bergey's manual of determinative bacteriology (Holt *et al*., 1998).

Molecular identification

The taxonomic characterization of isolated strains was performed by analyzing 16S rRNA. Total DNA extraction of the isolated bacterial strains was performed by the *cetyl trimethyl ammonium bromide* (CTAB) method (Winnepenninckx *et al*., 1993). The bacterial 16S rRNA loci were amplified using the forward domain-specific bacterial primer, Bac27-F (5′-AGAGTTTGATCCTGGCTCAG-3′) and universal reverse primer Uni-1492R (5′-TACGYTACCTTGTTACGACTT-3′). The amplification reaction was performed in a total volume of 25 µl consisting, 2 mM $MgCl_2$ (1 µl), 10× PCR reaction buffer (200 mM Tris; 500 mM KCl) (2.5 µl), 2 mM each dNTP (2 µl), 0.15 mM each primer (1 µl), 1U (0.5 µl) Taq DNA polymerase (Qiagen, Hilden, Germany), and 2 µl of template DNA (50 p). Distilled water (15 µl) was added to the reaction mixture.

Amplification for 35 cycles was performed in a GeneAmp 5700 (PE Applied Biosystem, Foster City, CA, USA) thermal cycler. The temperature profile for PCR was 95°C for 5 min (1 cycle), 94°C for 1 min, 72°C for 2 min (35 cycles), and 72°C for 10 min after the final cycle (Troussellier *et al*., 2005). PCR products were sequenced by Macrogen (Korea). Similarity rank from the Ribosomal Database Project (RDP) (Maidak *et al*., 1997) and FASTA Nucleotide Database Query were used to determine partial 16S rRNA sequences to estimate the degree of similarity to other 16S rRNA gene sequences. Analysis and phylogenetic affiliates of the sequences was performed according to the previously described protocols (Yakimov *et al*., 2006).

Growth and diesel oil removal assay

The bacterial isolates were grown at 30±1°C for 10 days on a rotary shaker (180 rpm INFORS AG, Switzerland). Growth curves of the isolates were routinely assessed indirectly by turbidity measurement at an optical density (OD) of 600 nm in a UV–visible spectrophotometer (Shimadzu UV-160, Japan) (Rahman *et al*., 2004).

The diesel oil removal assay was carried out by the addition of 5 ml of semi-logarithmic culture to Erlenmeyer flasks containing 75 ml of nutrient broth medium and 15 ml of diesel oil and incubated at 30±1°C for 10 days. The samples were compared with control, and the results were reported qualitatively (Nisha *et al*., 2013).

Extraction and analyses of residual diesel oil

Qualitative– quantitative analysis of hydrocarbons was analyzed by a GC-FID Varian 3800 model (USA) equipped with a SE-54 capillary column (25m × 0.32mm) and flame ionization detector (FID). Helium was used as the carrier gas (30 ml min^{-1}). After acidification, the samples were extracted at room temperature on a shaking table by using dichloromethane (CH_2Cl_2, Sigma-Aldrich, Milan; 10% v/v). This procedure was repeated three times, and the CH_2Cl_2 phase was combined and treated with anhydrous sodium sulfate (Na_2SO_4, Sigma-Aldrich, Milan) to remove any residual diesel oil. The samples were then quantified according to the previously described protocol (Hassanshahian *et al*., 2014).

Measure of emulsification activity and bacterial adhesion to hydrocarbon (BATH)

The emulsification activity (E24) was determined by the addition of *n*-hexadecane to an equal volume of cell-free culture broth and then mixing it with a vortex mixer for 2 min. The mixture was then allowed to stand for 24 h.

Emulsification activity was determined as the percentage of height of the emulsified layer (mm) divided by the total height of the liquid column (mm). Furthermore, the BATH test at the oil-water interface was carried out according to the procedure of Pruthi and Cameotra (1997).

Growth of selected strains on different concentrations of diesel oil

The effect of different concentrations of diesel oil on the growth of selected bacterial strains was measured. For this purpose, BH medium was supplemented with various concentrations of diesel oil (1, 2.5, 4, and 5.5%). The flasks were incubated for 10 days at 30±1°C on a rotary shaker, operating at 180 rpm (Shaker INFORS AG, Switzerland). Growth was routinely assessed indirectly by turbidity measurement at $OD_{600\ nm}$ in a UV-visible spectrophotometer (Shimadzu UV-160, Japan) (Hassanshahian *et al*., 2012a).

RESULTS AND DISCUSSION

The quantity of heterotrophic and diesel-degrading bacteria in collected samples

The quantity of heterotrophic and diesel-degrading bacteria was determined in all soil samples. As shown in Table 1, soil sample indicated as "I" has the greatest number of diesel-degrading bacteria (94×10^4 CFU gr^{-1}) compared to all soil samples. The highest quantity for heterotrophic bacteria relate to soil sample named "G" (119×10^6 CFU gr^{-1}).

Table 1. Enumeration (CFU gr^{-1}) of heterotrophic and diesel oil-degrading bacteria present in soil samples in study

sample	Quantity of diesel oil degrader bacteria (CFU gr^{-1})	Quantity of heterotrophic bacteria (CFU gr^{-1})
D	25×10^4	86×10^4
G	100×10^3	119×10^6
I	94×10^4	20×10^5
K	119×10^3	130×10^4
L	80×10^3	60×10^4

It is necessary to study the diesel degradation abilities of microbes to clear oil contamination in soil. Bacteria are the main agents responsible for the degradation of diesel fuel. This research describes the study on the isolation and characterization of diesel-degrading bacterial strains from contaminated soils (Marchal *et al*., 2003).

Diesel- degrading bacteria have been isolated from different environments such as wastewater and oil-contaminated soils. For example, Nisha *et al.* (2013) isolated diesel-degrading bacteria from Ernakulum District in India. They concluded that five bacterial strains isolated in their research may be useful in bioremediation of sites that are highly contaminated with diesel oil. Zanaroli *et al*. (2010) characterized two diesel- degrading microbial consortia from petrochemical wastewater in Italy. These consortia belong to the genera *Acinetobacter* and *Pseudomonas.*

Isolation and identification of diesel oil degrading bacteria

Twenty one diesel-degrading bacteria were isolated from enrichment soil cultures that were established at 30 ± 1°C for 2 weeks. From a total of 21 isolates, 6 isolates (L1, I2, D1, D2, G1, and K3) that showed highest growth rate in diesel oil-containing medium were selected for further study. These strains were first identified using classical biochemical tests (Table 2). According to Table 2, only strain L1 and K3 present motility. We amplified and sequenced the 16S rRNA gene and compared them to the database of known 16S rRNA sequences to identify the molecular characteristics of the isolates. The result shows that the isolated bacterial strains G1, L1, I2, D1, and D2 belonged to *Pseudomonas aeruginosa*, whereas strain K3 belonged to *Pseudomonas fragi*. The sequences of these strains were submitted to gene sequence database at the National Center for Biotechnical Information (NCBI). Gene bank ID of these strains in

NCBI is as follows: LK391625 (strain I2), LK391626 (strain G1), LK391631 (strain D1), LK391632 (strain L1), and LK391633 (strain D2). Figure 1 shows the phylogenic tree of these bacteria.

Table 2. Biochemical identification of isolated strains

Biochemical test	L1	I2	D1	D2	G1	K3
Motility	+	-	-	-	-	+
H_2S production	-	-	-	-	-	-
Indole test	-	-	-	-	-	-
Glucose fermentation	-	-	-	-	-	-
Catalase	+	+	+	-	+	+
Oxidase	+	+	+	-	+	+
O/F	-/-	-/-	-/-	-/-	-/-	+/+
Gas production	-	-	-	-	-	+

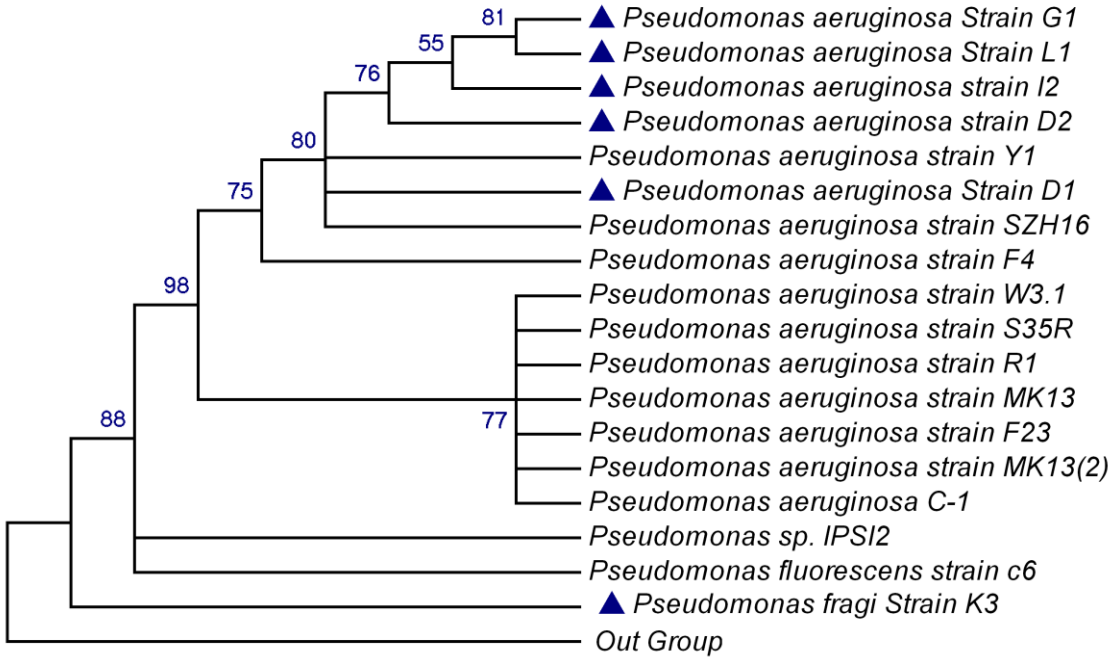

Fig. 1. Phylogenetic tree of 16S rDNA sequences of the bacterial isolates obtained from contaminated soils. The tree was constructed using sequences of comparable region of the 16S rDNA gene sequences available in public databases. Neighbour-joining analysis using 1,000 bootstrap replicates was used to infer tree topology. The bar represents 0.1% sequence divergence. Sequenced data showing the location of selected isolated strains.

Pseudomonas strains have been previously described in various researches as diesel- degrading bacteria. For example, Hong *et al*. (2005) isolated *P. aeruginosa* IU5 from oil-contaminated soil in Korea and identified it as diesel-degrading bacterium. This strain degraded up to 60% of the applied diesel (8,500 mg/kg) over 13 days in a soil-slurry phase. Furthermore, Shukor *et al*. (2009) characterized a *Pseudomonas* diesel-degrading strain from Antarctica that can grow optimally between 10 and 15°C, pH 7.0, and 3.5% (v/v) diesel. In our study, 21 bacterial strains capable of diesel oil degradation were isolated. Among them, 6 strains that showed high growth rate and diesel-degradation potential were selected and identified by 16S rRNA sequence analysis.

We found that these strains belonged to the genus *Pseudomonas*.

Phylogenetic tree of 16S rRNA sequences of the bacterial isolates obtained from the contaminated soils of Fars province, Iran. The tree was constructed using sequences of comparable region of the 16S rRNA gene sequences available in public databases. Neighbor-joining analysis using 1,000 bootstrap replicates was used to infer tree topology. The bar represents 0.1% sequence divergence. The sequenced data showing the location of selected isolated strains.

Growth rate and diesel oil removal by strains

All bacterial strains were grown in 1% (v/v) diesel oil for 1 week with shaking. After 1 week, the microbial growth and diesel oil biodegradation were analyzed using GC. As reported in Table 3, all strains except D1, have the high percentage of diesel oil biodegradation, although strain I2 has the highest growth and diesel oil degradation (~88%) capacity between all isolated strains. The GC chromatograms for all strains were compared with the chromatograms with the blank that were used to estimate the percentages of diesel oil degradation. Table 3 shows that strain I2 has the highest diesel oil degradation capacity whereas strain D1 showed the lowest. Other investigators, over a period of 10 days of culture, have found 80% (Nikhi *et al*., 2013) and 50% (Mukherji and Vijay, 2002) diesel degradation capacity.

Emulsification activity (E24%) and BATH in diesel-degrading bacteria

The results of emulsification activity and BATH test are shown in Table 4. According to this table, diesel-degrading bacterial strains have good emulsification and hydrophobicity characteristics. Strain I2 showed the best emulsification activity, whereas strain G1 showed the best hydrophobicity. In general, there was a direct relationship between emulsification activity and diesel oil biodegradation capacity. Strain I2 had high emulsification activity; therefore, it may be possible to have a better diesel oil biodegradation capacity.

Table 3. Growth and diesel oil removal by *Pseudomonas* strains. All strains were cultured in Bushnell Hass (BH) agar medium with diesel oil (1%) with shaking (180 rpm) for 1 week at 30 ± 1 °C.

Strain	Qualitative degradation	Value of O.D600	Rate of diesel oil removal (%)
L1	+ +	1.911	42
K3	+ + +	1.114	76
D1	+ +	1.602	32
D2	+ +	1.440	63
G1	+ + +	1.663	48
I2	+ + +	2.054	88

Table 4. Emulsification activity (E24%) and bacterial cell surface hydrophobicity (measured as bacterial adhesion to hydrocarbons, BATH %) in isolated strains of the genus *Pseudomonas*

Strain	Emulsification activity (E24%)	Cell surface hydrophobicity (BATH%)
L1	46	67
K3	56	39
D1	30	76
D2	41	10
G1	49	83
I2	75	69

The miscibility of diesel oil in water is less (Marchal *et al*., 2003). Therefore, it is supposed that bacteria must have some mechanisms to take up and use this hydrophobic substrate for energy and metabolism. Moreover, the cell surface hydrophobicity and concomitant production of emulsifier may be the two mechanisms for better uptake and degradation of diesel oil in liquid solution. The results of this study indicate that there is a direct relationship between cell surface hydrophobicity and emulsification activity for diesel oil biodegradation. Because strain I2 has high cell surface hydrophobicity and emulsification activity, its diesel oil degradation capacity is more than the other strains. This demonstrates that when a bacterial strain has high cell surface hydrophobicity, it can produce more emulsifier that will in turn enhance diesel oil biodegradation. In our previous study (Hassanshahian and Emtiazi, 2008), we reported a clear correlation between emulsification activity, bacterial cell adherence to hydrocarbon, and growth rate of the crude oil-degrading bacteria in crude oil media.

The effect of different concentrations of diesel oil on the growth rate of selected bacterial strains

For determining the effect of various concentrations of diesel oil on bacterial growth, four strains (D1, G1, L1, and K3) were selected and grown in different concentrations of diesel oil (1, 2.5, 4, and 5.5%, v/v) and then the absorbance was read at 600 nm for each strain. As shown in Figure 2, when the concentrations of diesel oil were increased, the growth rates of the bacteria decreased. For example, strain L1 showed an OD of 0.9 in 1% diesel oil; however, this value decreased to 0.3 in 5.5% diesel oil. The concentration of diesel oil that allowed the maximum growth of bacteria was different for different strains. For example, strains D1 and G1 showed maximum growth in 4% diesel oil, whereas strains L1 and K3 had maximum growth at 2.5% diesel oil. The lowest growth in different concentration of diesel oil in all bacterial strains belonged to strain K3 with an OD of 0.1 at 5.5% diesel oil.

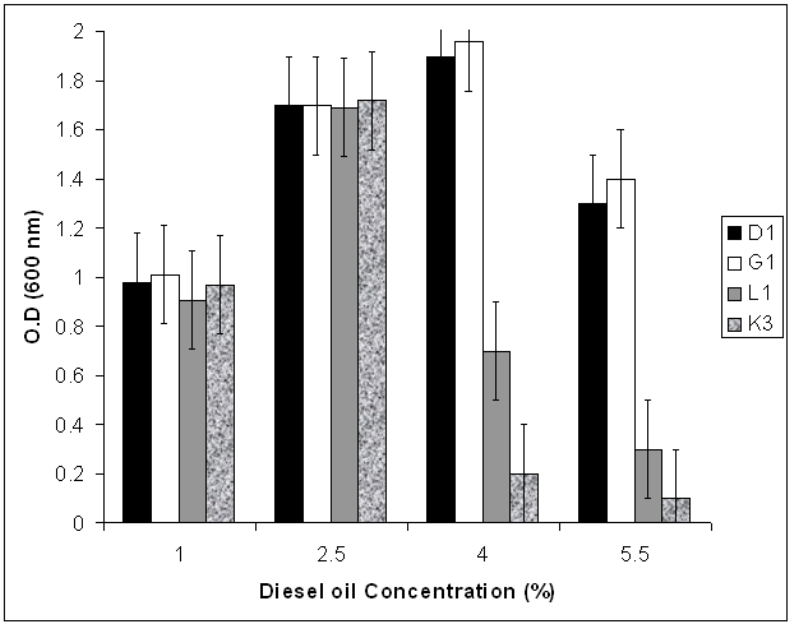

Fig. 2. Measurement of microbial abundance [as Optical Density (OD) at 600 nm] in *Pseudomonas* strains (D1, G1, L1, and K3) during growth with different diesel oil concentrations (from 1 to 5.5%). All strains were grown for 10 days at 30 ± 1°C

Most researchers have studied biodegradation of diesel oil in concentrations less than 4% in liquid system (Marchal *et al*., 2003). In this study, 1 to 5.5% diesel oil was tested on the growth of isolated strains. When the concentration of diesel oil increases, the growth rate of bacteria decreases; however, this decrease is not the same for all strains. The optimum concentration of diesel oil to grow was between 2.5 and 4%.

CONCLUSION

In this study, the removal of diesel oil by selected isolated bacterial strains was studied. After 1 week of culture at 30±1°C, diesel oil concentration decreased by approximately 50% by all strains; however, two strains had more degradation capacity (more that 70%).

The results described in this paper show that diesel-degrading bacteria have more diversity and ability for biodegradation in soils of Iran. In particular, *Pseudomonas* genus has more potential for degradation of diesel oil that was established in this research. Furthermore, we showed a correlation between emulsification activity, bacterial cell adherence to hydrocarbon, and growth rate of the isolated bacteria in diesel-containing medium. By applying these bacteria for bioremediation purposes, we can manage diesel oil contamination.

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