

RESEARCH PAPER

Isolation and characterization of alkane hydrocarbons-degrading *Delftia tsuruhatensis* strain D9 from petroleum-contaminated soils

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How to cite

Eren, A. & Güven, K. (2022). Isolation and characterization of alkane hydrocarbons-degrading *Delftia tsuruhatensis* strain D9 from petroleum-contaminated soils. *Biotech Studies*. 31(1), 36-46. <http://doi.org/10.38042/biotechstudies.1103695>

Article History

Received 30 October 2021
Accepted 06 April 2022
First Online 14 April 2022

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Keywords

Alkane hydrocarbon-degrading bacterium
Oil pollution
16S rRNA gene sequence analysis
GC-MS analysis
Delftia

Abstract

A bacterial strain from petroleum-contaminated soil in south-eastern Turkey was isolated and characterized to determine the potential of alkane hydrocarbon biodegradation. Phenotypic characteristics and the sequence analysis of the 16S rRNA gene revealed that the strain D9 is a member of the *Delftia* genus and most similar to *Delftia tsuruhatensis* (100%). The optimum pH and temperature values for the growth of *D. tsuruhatensis* strain D9 were found to be 9.0-10.0 and 35°C, respectively. The strain was found to grow in some single, medium and long-chain hydrocarbons such as decane, hexadecane, and squalene, tested by short-time incubation in basal medium (BM) in the presence of 1% hydrocarbon concentrations under optimum conditions. After incubation for 3 days, 65% of the single hydrocarbon hexadecane was degraded by the *D. tsuruhatensis* strain D9, revealed by GC-MS analysis. The biodegradation of petroleum hydrocarbons by *D. tsuruhatensis* strain D9 isolated and characterized in the present study shows that it can be a good candidate in the bioremediation process.

Introduction

Petroleum hydrocarbons are well known to cause pollution over the world and oil spills cause a great danger to various ecosystems (Head et al., 2006; Emtiazi et al., 2009). Due to petroleum being an important resource of the global economy with several million tons of crude oil, its transport, manipulation, and exploitation can lead to accidental spills (Malatova 2005; Crone & Tolstoy, 2010; Mapelli et al. 2017). When petroleum enters the natural environment, all biotic communities are exposed and affected due to the oil hydrocarbons and toxic fumes (Peterson et al., 2003; Fingas, 2011). Several techniques are possible for hydrocarbon pollution removal: Physical, chemical, or biological (Costes & Druelle, 1997; Chu & Kwan, 2003; Hamme et al., 2003; Koma et al., 2003; Parales & Haddock, 2004; Pieper et al., 2004; Malatova, 2005; Mittal & Singh, 2009). Mechanical, evaporation,

dispersion, burying, and washing are the main technologies commonly used for soil remediation, although these technologies are expensive and can result in insufficient decomposition of contaminants (Medina-Bellver et al., 2005). Bioremediation using the microorganisms degrading hydrocarbons has major advantages to remove contaminants from environments (Adams et al., 2015) which is regarded as a productive, environmental-friendly, and low-cost technology (Liu et al., 2014). Bioremediation, which is the process of using microorganisms to detoxify or remove pollutants due to their diverse metabolic capabilities is a developing method also for the elimination and degradation of pollutants arising from the petroleum industry (Medina-Bellver et al., 2005). Recently, a wide variety of microorganisms from oil-contaminated soils, which can degrade hydrocarbons within petroleum were studied. Among microorganisms including, bacteria, archaea, and fungi, the bacteria are

the most plentiful and significant in ecosystems (Acer et al., 2020). To date, more than 79 genera of bacteria have been identified that can degrade hydrocarbons in crude oil (Tremblay et al., 2017) including *Acinetobacter*, *Achromobacter*, *Alkanindiges*, *Arthrobacter*, *Alteromonas*, *Dietzia*, *Burkholderia*, *Enterobacter*, *Mycobacterium*, *Marinobacter*, *Kocuria*, *Pseudomonas*, *Streptococcus*, *Streptobacillus*, *Staphylococcus*, and *Rhodococcus* which play significant roles in the destruction of petroleum hydrocarbons (Margesin et al., 2003; Chaerun et al., 2004; Jin et al., 2012; Nie et al., 2014; Varjani & Upasani, 2016; Acer et al., 2016; Sarkar et al., 2017; Varjani, 2017; Xu et al., 2017; Xu et al., 2018; Acer et al., 2020). In recent studies, *Delftia* species have been reported to degrade various contaminants such as chlorobenzene degraded by *D. tsuruhatensis* (Ye et al., 2019), Dimethylphenols degraded by *Delftia* sp. LCW (Vásquez-Piñeros et al., 2018) and diesel oil degraded by *Delftia* sp. NL1 (Lenchi et al., 2020). The *Delftia*, described in the year 1999 within the class Betaproteobacteria, is a group that contains only five characterized species: *Delftia acidovorans* (Wen et al., 1999), *D. tsuruhatensis* (Shigematsu et al., 2003), *D. lacustris* (Jørgensen et al., 2009), *D. litopenaei* (Chen et al., 2012) and most recently identified *D. deserti* (Li et al., 2015). *Delftia* spp. are known to have a wide geographic distribution in marine and fresh-water, rhizosphere, soil, plants, and clinical samples (Wen et al., 1999).

However, there are few studies regarding the degradation of petroleum hydrocarbons by various *Delftia* sp. strains. They report different effectiveness and range of degradable hydrocarbons suggesting that there is still a pool of unexplored information. Therefore, more studies are needed on the degradation of petroleum hydrocarbons by *Delftia* sp. In the current study, we isolated and identified *D. tsuruhatensis* strain D9 from petroleum-contaminated soils and investigated its potential for petroleum hydrocarbon biodegradation. It is expected that the result of this work will contribute towards improving biodegradation efforts of petroleum-contaminated sites.

Materials and Methods

Collection of Samples

For the isolating bacteria which degrade petroleum-hydrocarbons, a soil sample was collected from petroleum-contaminated soil around the petrol well at areas of Southern Raman (237. petroleum station, Batman).

The Basal medium (BM) consisted, per liter: 5.0 mL phosphate buffer, 1.0 mL solution of calcium chloride, 3.0 mL solution of magnesium sulfate, 1.0 mL solution of ferric chloride, as well as 1.0 mL mineral elements at trace levels which contain ZnSO₄.H₂O, MnSO₄ and (NH₄)₆MoO₂₄.4H₂O. 1% crude oil was filtered (0.2 mm pore size) and then transferred into the medium as the sole carbon source to get the energy to

determine the bacteria biodegrading the petroleum hydrocarbons. The crude oil used in the experiments was provided from a petroleum-contaminated site. 1 g of the soil samples were transferred into 100 mL BM composed of 1% crude oil and samples were incubated in a shaker water bath at 30°C at 120 rpm for 5 days. After incubation, 1 mL aliquots were taken from each sample and transferred to freshly prepared BM containing 1% of crude oil. Following two subculture cycles, the bacterial cells were cultivated on Nutrient agar at 30°C for 5 days. Phenotypically different colonies on the plates were transferred to a fresh Nutrient agar.

Morphological, Biochemical, and Physiological Characteristics

Aspect, color, consistency, elevation, shape, size, and surface, etc. characteristics were used for phenotypical characterization of selected colonies. Gram staining to confirm the gram reaction was investigated using a light microscope according to the Dussault method (Dussault, 1955). Motility was determined by the hanging drop method. For the presumptive identification, data of phenotypic characteristics from the conventional methods such as catalase, citritase, indole, oxidase, and urease activity, utilization of starch, and gelatine were used.

Antibiotic Resistance

The antimicrobial susceptibility profiles of the isolate were determined using the antibiotic disk diffusion method. Antibiotic multi-disks were comprised of ampicillin (10 µg), fucidic acid (10 µg), lincomycin (15 µg), neomycin (10 µg), novobiocin (5 µg), kanamycin (5 µg), chloramphenicol (30 µg), nystatin (100 units), gentamicin (10 µg), tilmicosin (15 µg), streptomycin (10 µg), penicillin (2,10 units), bacitracin (10 units), tetracycline (30 µg). Following the inoculation of bacterial *D. tsuruhatensis* strain D9 on Nutrient agar in Petri dishes, the antibiotic disks were replaced on Nutrient agar. The agar plates were placed into an incubator at 30°C for 48 h. Following incubation, the evaluations were made by measuring the diameters of the zone of inhibition.

Phylogenetic Analysis

The final isolate namely *D. tsuruhatensis* strain D9 chosen as the petroleum hydrocarbon degrader were identified using a gene sequence of 16S rRNA. The 16S rRNA sequence analysis of the isolate was conducted by BM Laboratory system (Technocity/Ankara). Universal 16S rDNA primers (27 F, 5-AGAGtttGATcAtGGctcAG-3 and 1492 R, 5-tAcGG ttAccttGttAcGActt-3) were applied for bacterial identification through PCR technique. PCR conditions was designed as 95°C for 5 min; 40 cycles at 95°C for 45 s, 57°C for 45 s, 72°C for 60 s, and final step at 72°C for 5 min. 1.5% of Agarose gel was applied for detecting amplified amplicon of 16S rDNA fragment which eluted (ExoSAP-IT™ PCR Product Cleanup Reagent, ThermoFisher Scientific, USA), sequenced by

ABI 3730XL Sanger Sequencing Equipment and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The 16S rRNA gene sequence for *D. tsuruhatensis* strain D9 was determined (*D. tsuruhatensis* strain D9:1405). The GenBank database using a BLAST search (<http://www.ncbi.nlm.nih.gov/blast/>) was used to compare the sequences. The 16S rRNA gene similarities were retrieved from the database, determining the most closely related strains. A phylogenetic tree was obtained by using the software package CLC Sequence Viewer 8 (Qiagen, Denmark).

Growth of the Strain in the Medium Supplemented with Hydrocarbons

The bacterial cells grown overnight in nutrient broth liquid medium was washed several times with BM to determine the growth in BM supplemented with hydrocarbons (hexane, heptane, octane, decane, pentadecane, hexadecane, squalene, and toluene; all of these hydrocarbons were obtained from Sigma-Aldrich, St. Louis, MO, USA). The bacterial strain was cultivated in 25 mL BM containing 1% hydrocarbons in 100 mL Erlenmeyer flasks and incubated at pH 7.0, 30°C, and 120 rpm for 3 days in hydrocarbons. After incubation, bacterial growth was measured by using a spectrophotometer at 600nm. Each data point represents the mean of at least three experiments.

Analysis of Degradation of Hexadecane by the Strain

The degradation activities of alkane hydrocarbons by *D. tsuruhatensis* strain D9 were determined by Gas Chromatography-Mass Spectrometry (GC-MS). Pre-cultured bacterial strain incubated for at least 12 hours at 30°C was transferred to 25 mL of BM plus with 1% hydrocarbon hexadecane in a 100 mL flask. After incubation, the bacterial cells were removed and the remaining hexadecane in the medium was used for calculating the degradation ratio of the hydrocarbon tested. The remaining hexadecane fractions were analyzed by GC-MS with a flame ionization detector

(Hewlett Packard, Wilmington, USA; FID; HP 6850) and also equipped with a PONA quartz capillary column (100 m x 0.250 mm [inner diameter, i.d.] x 0.50 m). Helium was utilized as a carrier gas to perform split injections. The increase of column temperature was from 60°C to 320°C with a rate of 3°C per minute. The injector and detector temperatures used were 300°C and 350°C, respectively. Individual hexadecane fraction was determined, using an authentic standard for matching the retention time (70.528 min).

Results and Discussion

Collection of Samples

For the isolation of alkane hydrocarbon-degrading *D. tsuruhatensis* strain D9 strain, soil samples were collected from petroleum-contaminated soil around the petrol well at areas of Southern Raman (237. petroleum station, Batman).

Biochemical, Physiological, and Morphological Characteristics

The results of the biochemical and morphological tests on the isolated *D. tsuruhatensis* strain D9 were used for identification which is presented in Table 1. As can be seen from Figure 1, the cells are rod-shaped and gram-negative. *D. tsuruhatensis* strain D9 is aerobic and able to utilize oxidase, catalase, starch hydrolysis, citrate, urease, as well as positive for indole test and negative gelatine test.

[Lenchi et al. \(2020\)](#) found that *Delftia* sp. NL1 strain was gram-negative, not-motile, rod-shaped, oxidase-positive, and catalase-negative. Moreover, several other studies on *Delftia* strains showed that they are gram-negative, motile, rod-shaped, catalase, and oxidase-positive ([Carro et al., 2017](#); [Jørgensen et al., 2009](#); [Shigematsu et al., 2003](#)).

The time-course experiments showed that the growth of *D. tsuruhatensis* strain D9 was observed to be maximum up to 8 h, while the growth is constant from 12 h to 48 h, followed by the death phase up to 72 h (Figure 2).

Table 1. The phenotypical characteristics of the *Delftia tsuruhatensis* strain D9 in comparison with other *Delftia* species

Characteristic	<i>D. tsuruhatensis</i> strain D9	<i>Delftia</i> sp. strain NL1	<i>D. tsuruhatensis</i> strain T7 ^T	<i>D. lacustris</i> strain 332 ^T	<i>D. rhizosphaerae</i> strain RA6T	<i>D. acidovorans</i> strain ACM 489
Cell shape	R	R	R	R	R	R
Gram staining	-	-	-	-	-	-
Motility	+	-	+	+	ND	+
Oxygen requirement	A	ND	ND	ND	A	A
Growth temperature (°C)	20-40	20-50	10-40	3-37	4-30	4-41
Optimum Growth temperature (°C)	35	30	35	25	28	30
Growth pH	5.0-10.0	5.0-9.0	5.0-9.0	5.0-10.0	6.0-10.0	ND
Optimum growth pH	9.0-10.0	8.0	7.0	6.0-7.0	7.0	ND
Starch hydrolysis	+	-	-	-	-	-
Gelatin hydrolysis	-	-	ND	+	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	-	+	+	+	+
Citrate	+	ND	+	+	ND	ND
Urease	+	ND	+	ND	-	ND
Indole	+	ND	ND	ND	-	+
NaCl resistance (%)	3	2	ND	0.1	1	0.5-1.5
References	This study	(Lenchi et al., 2020)	(Shigematsu et al., 2003)	(Jørgensen et al., 2009)	(Carro et al., 2017)	(Wen, 1999)

+ = positive; - = negative; ND= no data available; R= rod; A= aerobic.

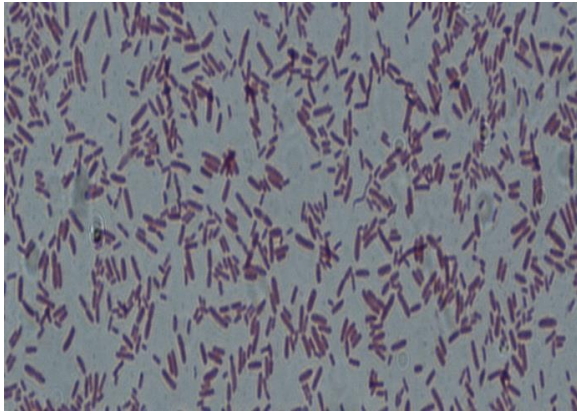


Figure 1. The Gram-staining of *D. tsuruhatensis* strain D9 observed under a light microscope ($\times 1000$ magnification).

To determine optimum pH and temperature values, pH, *D. tsuruhatensis* strain D9 was cultivated in Nutrient broth for 24 h, after which bacterial growth was measured by using a spectrophotometer at 600nm.

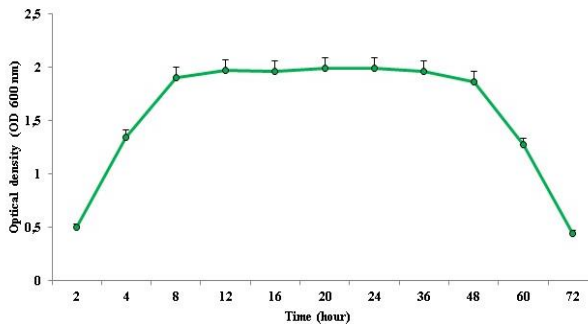


Figure 2. Effects of cultivation time on the growth of *D. tsuruhatensis* strain D9. The cells were incubated at 30°C, pH 7.0 for 72 h.

The range of growth for *D. tsuruhatensis* strain D9 was determined to be between 20°C and 40°C with an optimum of 35°C (Figure 3a). Furthermore, *D. tsuruhatensis* strain D9 grew at pH ranging from 5.0 to 10.0 with a wide optimum value of pH 9.0-10.0 (Figure 3b). *D. tsuruhatensis* strain D9 was also found to tolerate NaCl up to 3%. In a previous study by [Shigematsu et al. \(2003\)](#), strain *D. tsuruhatensis* strain T7^T also grew at 10-40 °C, with 35°C of optimum growth. Similarly, the range of pH for growth was from 5.0 to 9.0, with an optimum value of pH 7.0. [Jørgensen et al. \(2009\)](#) reported that cells *D. lacustris* strain 332^T grow between 3 and 37°C, with optimum growth at 25°C, as well as observing growth at a pH range of 5.0 to 10.0, with an optimum of pH 6.0-7.0. In a recent study, optimal growth for the strain *Delftia* sp. NL1 strain was observed at 30°C and pH 8.0 ([Lenchi et al., 2020](#)). The reason of different and such a high optimum pH for *Delftia tsuruhatensis* strain D9 compared to other strains described above is probably due to different culture media utilised with different initial pH and composition, as bacteria tend to release acidic products into their environment, which can interfere with their growth by changing the pH of the culture media.

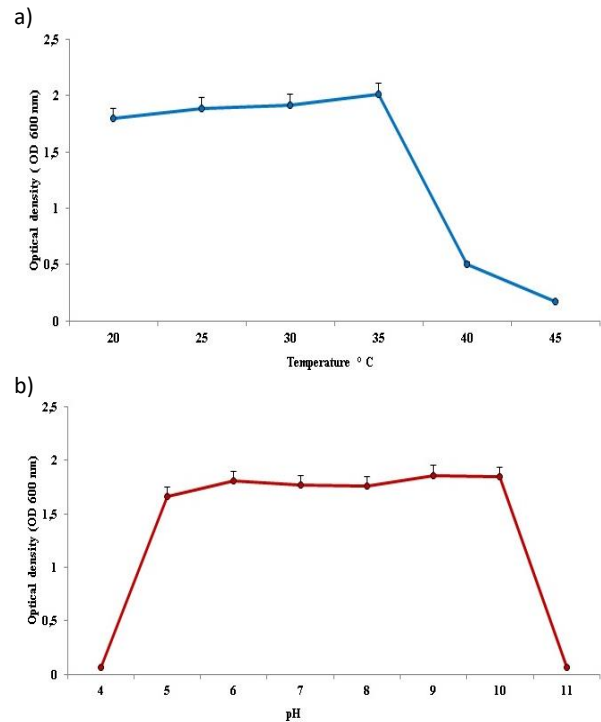


Figure 3. Effects of temperature (a) and pH (b) on the growth of *D. tsuruhatensis* strain D9. The bacterial strain was cultivated in Nutrient broth for 24 h, after which the growth was measured by using a spectrophotometer at 600nm.

Antibiotic Resistance

In Table 2, the effects of various antibiotics on *D. tsuruhatensis* strain D9 were determined by measuring the zone (mm) of inhibition of antibiotics. *D. tsuruhatensis* strain D9 was resistant to ampicillin, gentamicin, streptomycin, penicillin, bacitracin, fusidic acid, novobiocin, lincomycin, neomycin, kanamycin, nystatin, however, it was semi-sensitive to chloramphenicol, tilmicosin, sensitive to tetracycline. There have been several studies on the effects of antibiotics on *Delftia* sp. For example, [Lenchi et al. \(2020\)](#) reported that strain *Delftia* sp. NL1 was resistant to ampicillin, but it was sensitive to fusidic acid, gentamicin, and kanamycin. *Delftia tsuruhatensis* strain TR1180 was also found to be resistant to ampicillin, gentamicin, tetracycline and streptomycin, but sensitive to chloromphenicol ([Cheng et al., 2021](#)).

Table 2. Antibiogram results of *D. tsuruhatensis* strain D9

Antibiotics	<i>D. tsuruhatensis</i> strain D9 (mm: zone of inhibition)
Novobicin (5 µg)	0
Fucidic acid (10 µg)	0
Kanamycin (5 µg)	0
Bacitracin (10 units)	0
Gentamicin (10 µg)	0
Tilmicosin (15 µg)	13
Ampicillin (10 µg)	0
Streptomycin (10 µg)	0
Chloramphenicol (30 µg)	12
Lincomycin (15µg)	0
Penicillin G (2 units)	0
Tetracycline (30 µg)	33
Neomycin (10 µg)	0
Penicillin G (10 units)	0
Nystatin (100 units)	0

Phylogenetic Analysis

The sequence analysis of the 16S rRNA gene indicated that *D. tsuruhatensis* strain D9 belonged to the genus *Delftia*. Following the phylogenetic analysis, similarity calculations revealed that the closest relative of *D. tsuruhatensis* strain D9 was *Delftia* sp. NLI. As can be seen in Figure 4, the constructed phylogenetic dendrogram shows *D. tsuruhatensis* strain D9 identified as *D. tsuruhatensis* strain D9 (100%) (GenBank accession number: MT374262).

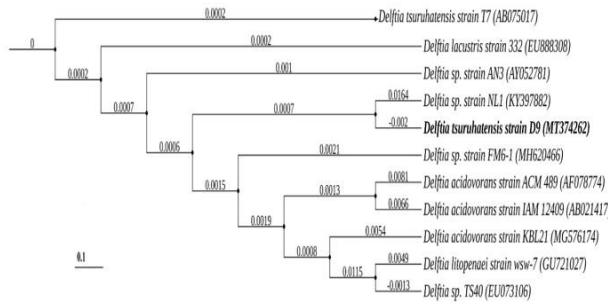


Figure 4. The 16S gene sequence analysis of the *D. tsuruhatensis* strain D9.

Growth of the Bacterial Strain in Various Hydrocarbons

Figure 5 shows that *D. tsuruhatensis* strain D9 uses alkane hydrocarbons to grow. For studying the growth of alkane-degrading bacteria, a basal mineral salt medium (BM) modified from [Nie et al. \(2010\)](#) was used. *D. tsuruhatensis* strain D9 was found to biodegrade and grow in medium and long-chain alkanes including decane, hexadecane, and squalene, rather than the short-chain alkanes. Hexadecane was found to be the most preferred hydrocarbon to grow effectively. In a previous study, *Delftia tsuruhatensis* B7 strain was also found to grow in crude oil and hexadecane ([Roy et al., 2014](#)). Moreover, *Delftia lacustris* strain LZ-C was grown in the mineral salt medium with PAHs as sole carbon source including naphthalene, 2-methylnaphthalene, anthracene, phenanthrene, pyrene, naphthol, benzene, phenol or toluene ([Wu et al., 2016](#)).

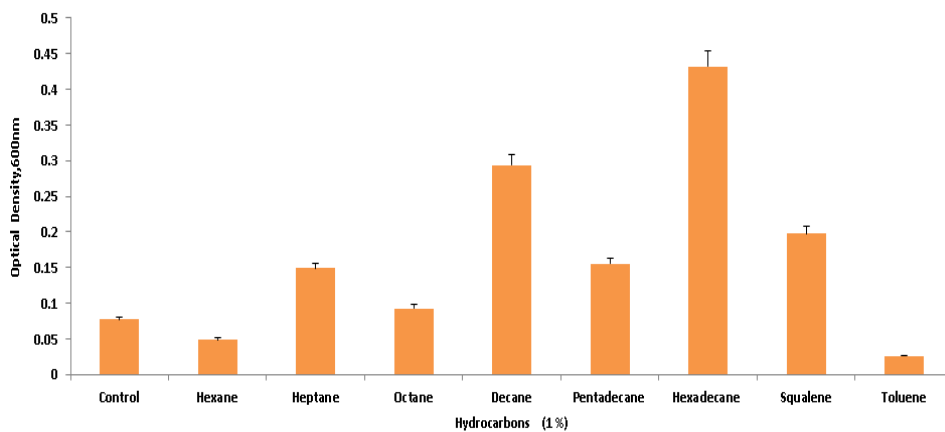


Figure 5. Growth of *D. tsuruhatensis* strain D9 at 1% concentration of different hydrocarbon sources. Each data represents the mean of at least 3 different experiments.

GC Analysis of Degradation of Hexadecane by *D. tsuruhatensis* strain D9

The gas chromatography analysis of hexadecane degradation by the strain incubated at a growth temperature of 30°C in BM with 1% (v/v) hexadecane for 3 days under constant shaking is shown in Figure 6b, compared to abiotic control in Figure 6a. The average hexadecane (1%) degradation rate was found to be as much as 65% after 3 days incubation time.

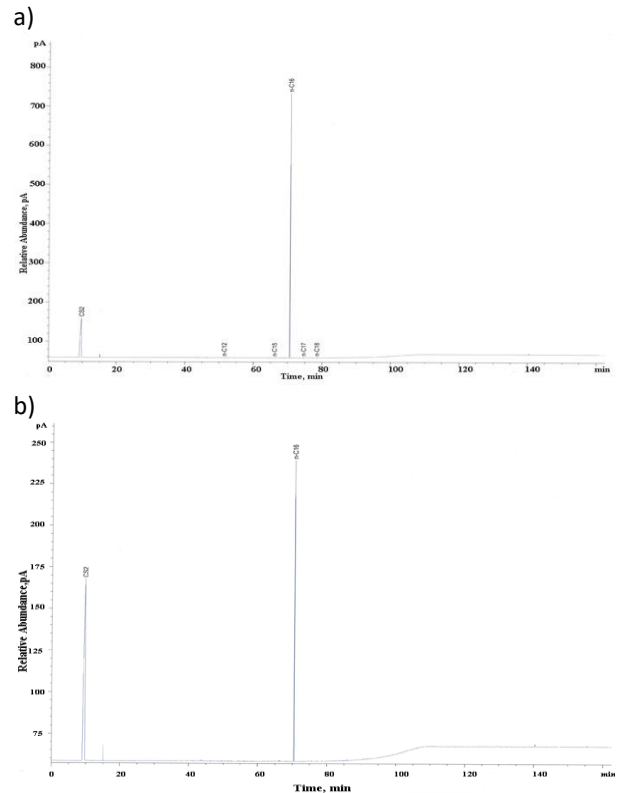


Figure 6. GC Analysis of hexadecane degradation. (a) Abiotic control of 1% hexadecane. (b) After degradation of 1% hexadecane by *D. tsuruhatensis* strain D9.

Oil hydrocarbons have been the most dangerous pollutants in the environment, and seem to continue to be a major energy and chemical source ([Peterson et al.,](#)

2003; Macaulay & Rees, 2014; Khan et al., 2018). It has been more than a century that the biodegradation of hydrocarbons by microbial activity has been recognized (Muthukamalam et al., 2017). In many studies, petroleum hydrocarbon-degrading bacteria have been described from various environments including oil-contaminated soils and waste-water (Cappello et al., 2012; Jurelevicius et al., 2012). The capacity of native bacterial populations is well known to mineralize hydrocarbons in crude oil-contaminated soil sites (Ojo, 2006). Wang et al. (2011) studied *Dietzia* sp. determining that bacteria use n-alkanes (C₆-C₄₀) as a carbon source. Throne-Holst et al. (2007) determined that *Acinetobacter* strain can use carbon sources in the range of decane and tetracontane long-chain n-alkanes. Razak et al. (1999) isolated *Acinetobacter* sp. degrading more than 60% crude oil in 15 days. In another study, diesel-oil alkanes were degraded an average of 58.1% by *Acinetobacter baumannii* in 10 days incubation (Nkem, 2016). Among the various groups of bacteria, *Pseudomonas* species and strains were found to be the most potent oil-degrading bacteria, having a wide distribution at the petroleum contaminated sites. Zhang et al. (2011) found that the strain *Pseudomonas* DQ8 degraded crude oil, n-alkanes, and polycyclic aromatic hydrocarbons (PAH). *Pseudomonas aeruginosa* was found to be a degradation of 49.93% of 0.5% diesel oil at the end of 20 days incubation period (Panda et al., 2013). *P. aeruginosa* had shown 34.4% of diesel oil degradation in 10 days (Simaria et al., 2015). Godini et al. (2018) found that three strains, namely *Staphylococcus arlettae* (strain 3), *Pseudomonas* sp. (strain 8), *Pseudomonas* sp. (strain 5) utilized crude oil containing various compounds, as carbon and energy source. In a recent study, GC-MS analysis of the hydrocarbon compounds indicated that the studied crude oil possessed alkanes in the range of C₂₀-C₄₄. *Enterobacter* species ALK-23, *Bacillus methylotrophicus* ALK16 and *Alcaligenes* species ALK-14 were found to degrade maximally 5.59%, 8.11%, and 11.65%, respectively (Dwivedi et al., 2019). There have also been some studies on the use of members of *Delftia* genus in bioremediation processes. Liu et al. (2002) found that *Delftia* sp. strain (named AN3) for its sole carbon, nitrogen, as well as energy sources utilizes aniline or acetanilide. *D. tsuruhatensis* AD9 obtained from the soil site near a textile dyeing plant were also found to degrade aniline (Geng et al., 2009). Similarly, several *Delftia* strains degrading aniline were also reported (Kahng et al., 2000; Urata et al., 2004; Sheludchenko et al., 2005; Xiao et al., 2009).

Moreover, phenanthrene degrading *D. acidovorans* strain was collected and characterized from soil contaminated with PAH (Vacca et al., 2005). Moreover, *D. lacustris* strain LZ-C was found to degrade compounds such as naphthalene and 2-methylnaphthalene (Wu et al., 2016). In recent studies, *Delftia* species have been reported to degrade various contaminants such as chlorobenzene degraded by *D.*

tsuruhatensis (Ye et al., 2019), dimethylphenols degraded by *Delftia* sp. LCW (Vasquez-Pineros et al., 2018). Most recently, more than 66.76% of diesel oil was found to be degraded by *Delftia* sp. NL1 in only 7 days (Lenchi et al., 2020).

Conclusion

A wide variety of microorganisms including bacteria that can degrade petroleum hydrocarbons were isolated from oil-contaminated soils. Bacteria are the most dominant microorganisms determined in microbial ecology that can degrade petroleum hydrocarbons. Among bacteria, *Delftia* is recently found to be the most common genera that are capable of degrading hydrocarbons. A bacterial strain designated as D9 from petroleum-contaminated soil was isolated and characterized by phenotypic characteristics and 16S rRNA gene sequence analysis, which was found to be most similar to *Delftia tsuruhatensis* (100%). The optimum pH and temperature values for the growth of *D. tsuruhatensis* strain D9 were found to be 9.0-10.0 and 35°C, respectively. Although *D. tsuruhatensis* strain D9 was found to grow in some single, medium and long-chain hydrocarbons, it should be emphasized that *D. tsuruhatensis* strain D9 is a very effective degrader of hexadecane as much as 65% in a short incubation time (3 days). Further studies on *D. tsuruhatensis* strain D9 and microbial consortium studies with the related strains may provide a good advantage in the bioremediation process of oil-contaminated soil.

Funding Information

This study, a part of Ayse EREN's Ph.D. project, was supported by Dicle University Scientific Research Projects Coordination Unit (Project number: FEN.19.014).

Author Contributions

AE: Conceptualization, Data Curation, Formal Analysis, Visualization, and Writing-original draft; KG: Funding Acquisition, Project Administration, Resources, Data Curation, Formal Analysis, Investigation, Methodology, Writing-review, and editing.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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