Bitlis Eren Üniversitesi Fen Bilimleri Dergisi
BİTLİS EREN UNIVERSITY JOURNAL OF SCIENCE
ISSN: 2147-3129/e-ISSN: 2147-3188
VOLUME: 11 NO: 2 PAGE: 630-639 YEAR: 2022
DOI: 10.17798/bitlisfen.1071785

Isolation and Characterization of Alkane Hydrocarbons-Degrading *Enterobacter ludwigii* strain D8 from Petroleum-Contaminated Soils

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Keywords: Oil pollution, Bacterial characterization, 16S rRNA gene sequence analysis, GC-MS analysis, Bioremediation.

Abstract
A bacterial strain has been isolated from petroleum contaminated soil with in southeastern Turkey. This isolated strain was characterized to determine its hydrocarbon biodegradation potential. Phenotypic features and 16 S gene sequence analysis of rRNA revealed that strain D8 belongs to the *Enterobacter* genus and most closely resembles *Enterobacter ludwigii* (100%). The optimum temperature and pH values for the growth of *E. ludwigii* D8 were found to be 30°C and 5.0, respectively. This bacterial strain grew in long and medium chain hydrocarbons such as 1% decane, pentadecane and squalene separately at the end of 3 days incubation in the basal medium (BM) under optimum conditions. It was shown that *E. ludwigii strain* D8 degraded about 27% of crude oil incubated for 5 days, while it degraded 29% of pentadecane after 3 days of incubation determined by Gas chromatography-MS analysis. The biodegradation potential of petroleum hydrocarbons of *E. ludwigii* strain D8 isolated and characterized in this study indicates that this strain may play a role in the bioremediation process.

1. Introduction

The most common environmental pollutants are petroleum hydrocarbons [1]. Oil spill can cause serious environmental pollution and harm all organisms including plants, animals, aquatic animals and humans [2]. Although oil is a very important resource in the world economy, the operation, transportation and manipulation of oil may result in spills that damage the ecosystem [3]. The accidental discharge and leakage may happen during the extraction, refining and distribution of petrochemical products as well as due to the accidents such as damaged pipes, blasting wells and fire, broken tanks, sinking ships, and wars. Crude oil spills and fires can also pollute ground water and air. Thus, the pollution caused should be treated and cleaned for a safe environment [4]. To clean up crude oil-contaminated sites is an important and preferential task for the maintenance of the nature and mainly succeeded by physical and chemical methods. On the other hand, the uses of these methods are known to be very expensive and need site restoration [5]. There have been many physicochemical and biological methods to assess treatment of environments contaminated with petroleum. However, biological treatment methods are mostly preferred rather than physico-chemical methods using chemical agents, because it is more reliable, capable of succeeding high remediation levels and cost-effective, as well as being simple for low-energy design, setting-up and operation [6,7].

Bioremediation using biological tools is well known to be an efficient, economical and environmentally friendly approach for treatment of oil-contaminated sites [8, 9]. Recently, although a wide variety of microorganisms including archaea, bacteria and fungi that can degrade hydrocarbons within petroleum have been isolated from contaminated soils, bacterial strains are the most

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Received: 11.02.2022, Accepted: 22.03.2022

plentiful and significant in microbial ecosystems [10-12]. Recent research has identified more than 79 bacterial species capable of degrading petroleum hydrocarbons [13]; *Bacillus* sp., *Acinetobacter* sp., *Alcanivorax* sp., *Achromobacter* sp., *Enterobacter* sp., *Pseudomonas* sp., *Stenotrophomonas* sp., *Ochrobactrum* sp., *Cronobacter* sp., *Nocardia* sp., *Micrococcus s*p., *Pantoea* sp., *Klebsiella* sp. [14-19]. *Enterobacter* genus is Gram-negative, facultative aerobic, rod-shaped, non-spore-forming, which belongs to the family *Enterobacteriaceae*. *Enterobacter* species are universal bacteria that live in various aquatic and terrestrial environments (soil, water, food and sewage), as well as being important human opportunistic pathogens. *Enterobacter ludwigii* belongs to the *Enterobacter cloacae* complex [20-22]. There have been a few reports on *E. cloacae* strains degrading hydrocarbons. *E. cloacae strain* S1:CND1 isolated from oil-contaminated soil has been found to degrade alkanes, such as n-hexane and n-hexadecane, polyaromatic naphthalene, as well as diesel and crude oil [23,24]. Moreover, *E. cloacae* was found to degrade highly hexadecane, heptadecane, tridecane, and docosane in diesel oil after two-week incubation by 99.71%, 99.23%, 99.66%, and 98.34% respectively [25].

This study aims to investigate and characterize the bacterium *Enterobacter ludwigii* strain D8 isolated from petroleum-contaminated soils and to determine its potential of petroleum hydrocarbon biodegradation.

2. Material and Method

2.1. Sample Collection and Bacterial Isolation

For the isolation of bacterial strain that degrade petroleum hydrocarbons, a soil sample was taken from oil-contaminated soil around the oil well in Southern Raman (South Raman, petrol station1, Batman) region.

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 $ZnSO_4.H_2O$, $MnSO_4$ and $(NH_4)_6MoO_{24}.4H_2O$. 1% of Crude oil was filtered (0.2 mm pore size) and transferred into the medium for determination of the bacterial biodegradation of the hydrocarbons in petroleum. Crude oil used in the experiments was provided from oil refinery in Batman. Contaminated soil (1 g) was transferred into 100 mL BM with1% crude oil and samples were placed in a shaker water bath for incubation at 120 rpm at 30 °C for 5 days. After incubation, 1 mL aliquots were taken from each sample and transferred to freshly prepared BM with crude oil (1%). Following two subculture cycles, the bacterial cells were grown on nutrient agar (NA) at optimum temperature for 5 days. Colonies differed phenotypically on the plates were transferred to NA, and then pure cultures were transferred to BM in the absence or presence of crude oil to determine degradation.

2.2. Morphological, Biochemical and Physiological Characteristics

Gram staining was carried out following the method of Dussault [26]. For growth in liquid medium, the temperature used was in the range between 10 °C and 45 °C, while the pH range was between 4.0 and 12.0. The initial pH of media was calibrated using acidic and basic solutions. Urease test was carried out according to Lanyi [27]. Procedures described within Bergey et al. [28] and Claus and Berkeley [29] were followed to determine different biochemical properties such as indole, oxidase, citrate, catalase and urease activity as well as motility of the studied isolate.

2.3. Antibiotic Resistance

Antibiotic susceptibility of D8 strain was determined using disk diffusion method. Antibiotic multidisc ampicillin (10 µg), lincomycin (15 µg), neomycin (10 µg), novobiocin (5 µg), kanamycin (5 µg), chloramphenicol (30 µg), fucidic acid (10 µg), gentamicin (10 µg), tilmicosin (15 µg), streptomycin (10 µg), penicillin (2-10 units), bacitracin (10 units), nystatin (100 units), tetracycline (30 µg). After the bacterial strain D8 was inoculated on NA, antibiotic discs were placed on NA. Agar plates were placed in an incubator for 48 hours at 30 °C. After incubation, results were obtained by measuring of inhibition zone diameters.

2.4. Phylogenetic Analysis

Identification of the strain D8 was carried out by 16S rRNA gene sequencing. 16S rRNA sequence analysis of the strain was performed by BMLabosis (Ankara). The 16S rRNA gene sequence for strain D8 was determined (D8: 1413). A BLAST search GenBank database to compare sequences (http://www.ncbi.nlm.nih.gov/blast/) was used. 16S rRNA gene similarities were retrieved from the database by identifying the most closely related strains. The phylogenetic tree was built using the Mega X software package.

2.5. Growth of Bacterial Cells in Crude Oil and Single Hydrocarbons

A fresh culture was obtained, which was grown in NB liquid medium for 24 hours. Then it was centrifuged to recover from NB and the pellet was washed with BM. This process was repeated 3 times. Bacteria were incubated in crude oil for 5 days and in single hydrocarbons (1% hexane, heptane, octane, decane, pentadecane, hexadecane, squalene and toluene) for 3 days at 30°C at 120 rpm under optimum growth conditions. Following incubation, the bacterial growth was measured at OD 600 nm. All of these hydrocarbons were obtained from (Sigma-Aldrich, USA).

2.6. GC-MS Analyses of crude oil and hydrocarbon degradation by strain D8

The degradation activities of crude oil and single n-alkanes by the strain were analysed using Gas Chromatography-Mass Spectrometry (GC-MS). The bacterial strain was pre-cultured in Nutrient broth overnight at 30°C. They were then replaced in to 100 mL flasks comprising 25 mL of BM plus 1% single hydrocarbon (pentadecane) or crude oil and incubated under optimum conditions. After incubation, the cells were removed, and the remaining hydrocarbons in NB medium were used for calculating degradation ratio of crude oil and hydrocarbons tested.

3. Result and Discussion

3.1. Collection of Samples and Bacterial Isolation

Oil-contaminated soil sample was taken from near oil well in South Raman (South Raman petrol station1, Batman) area. Soil samples was transferred to BM with 1% filtered-crude oil and incubated for 5 days. After serial dilutions and growth observations on both BM and NB media, one bacterial strain was found to use and grow within crude oil. Figure 1 shows the strain degrades and grow in crude oil (decreasing petroleum layer and increasing bacterial density compared to control).

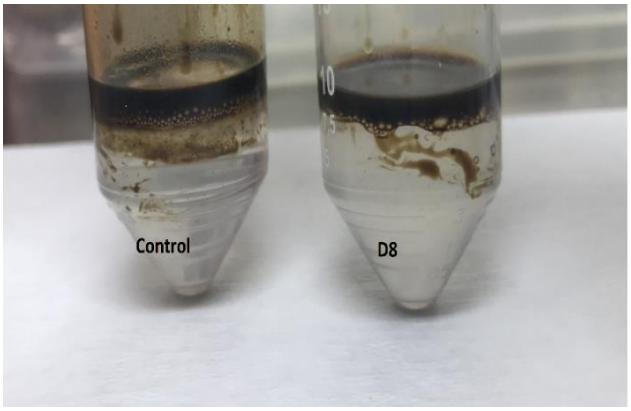


Figure 1. Demonstration of growth of strain D8 at 1% crude oil as carbon source, compared to control.

3.2. Morphological, Biochemical and Physiological Characteristics

The results of the biochemical and morphological tests of isolated strain D8 can be seen in Table 1.

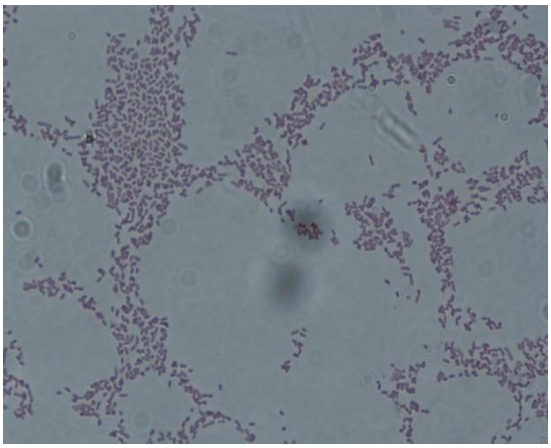


Figure 2. The Gram-staining of the strain D8 observed under light microscope (×1000 magnification).

As seen in Figure 2, the cells are rod-shaped and gram negative. Strain D8 was aerobic, spore forming, motility and starch hydrolysis, catalase, citrate, urease positive and gelatine, oxidase and indole negative (Table 1).

Table 1. The phenotypical characteristics of the *E. ludwigii* strain D8 compared to other related species.

Characteristics	1	2	3	4	5	6
Cell shape	R	NDA	R	R	NDA	R
Gram staining	(-)	(-)	(-)	(-)	(-)	(-)
Spore	(+)	NDA	NDA	(+)	NDA	(-)
Motility	(+)	NDA	NDA	NDA	(+)	(+)
Growth pH	3.0-11.0	NDA	5.0-8.0	6.0-10.0	NDA	NDA
Optimum growth pH	5.0	NDA	6.0	7.0	7.0	NDA
Growth temperature (°C)	20-40	NDA	25-40	28-44	NDA	NDA
Optimum Growth temperature (°C)	30	NDA	30	37	40	NDA
Oxygen requirement	A	NDA	NDA	NDA	NDA	A
Gelatin hydrolysis	(-)	NDA	(-)	(+)	(-)	NDA
Starch hydrolysis	(+)	NDA	(+)	(-)	(+)	(+)
Oxidase	(-)	(-)	(-)	NDA	NDA	NDA
Urease	(+)	(-)	(+)	NDA	NDA	NDA
Citrate	(+)	(+)	(+)	(+)	(+)	NDA
Catalase	(+)	(+)	(+)	NDA	(+)	(+)
Indole	(-)	(-)	(-)	NDA	(-)	(-)
NaCl resistance	7	NDA	NDA	4-12	NDA	NDA

+ = positive; - = negative; NDA= no data available; R=Rod; A= aerobic.

1. *E. ludwigii* strain D8, 2. *E. hormaechei* strain KA6 [30], 3. *Enterobacter* species strain ALK-23 [31], 4. *Enterobacter* sp. strain F3 [32], 5. *E. ludwigii* strain LY-62 [33], 6. *E. ludwigii* strain MS32 [34].

In a similar study, *E. ludwigii* strain LY-62 was gram negative and motile, the starch hydrolysis, catalase, citrate were positive, while gelatin hydrolysis and indole was found to be negative [33]. In addition, studies on most *Enterobacter* strains have shown that they are gram-negative, rod-shaped and motile. It was also found that catalase positive while oxidase was negative in isolated strains studied [30-34].

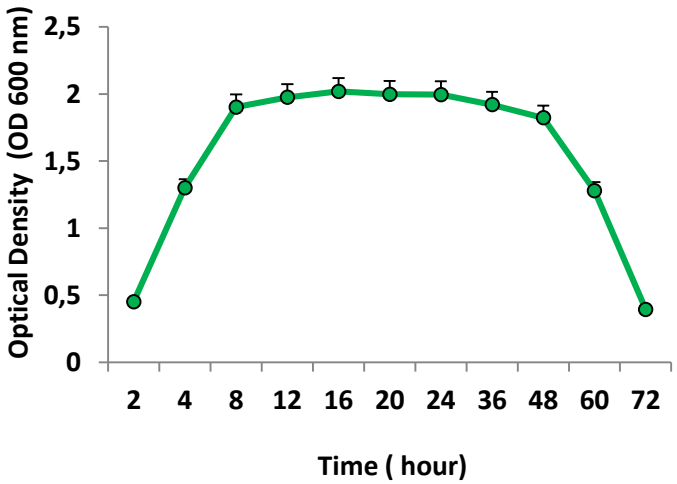


Figure 3. Effect of cultivation time on the strain growth.
Bacterial cells were grown at optimum temperature and pH for 72 hrs.

It was observed that the growth of the D8 strain was maximum between 8 to 24 hours. The growth was then found to decrease up to 72 hours (Figure 3).

a)

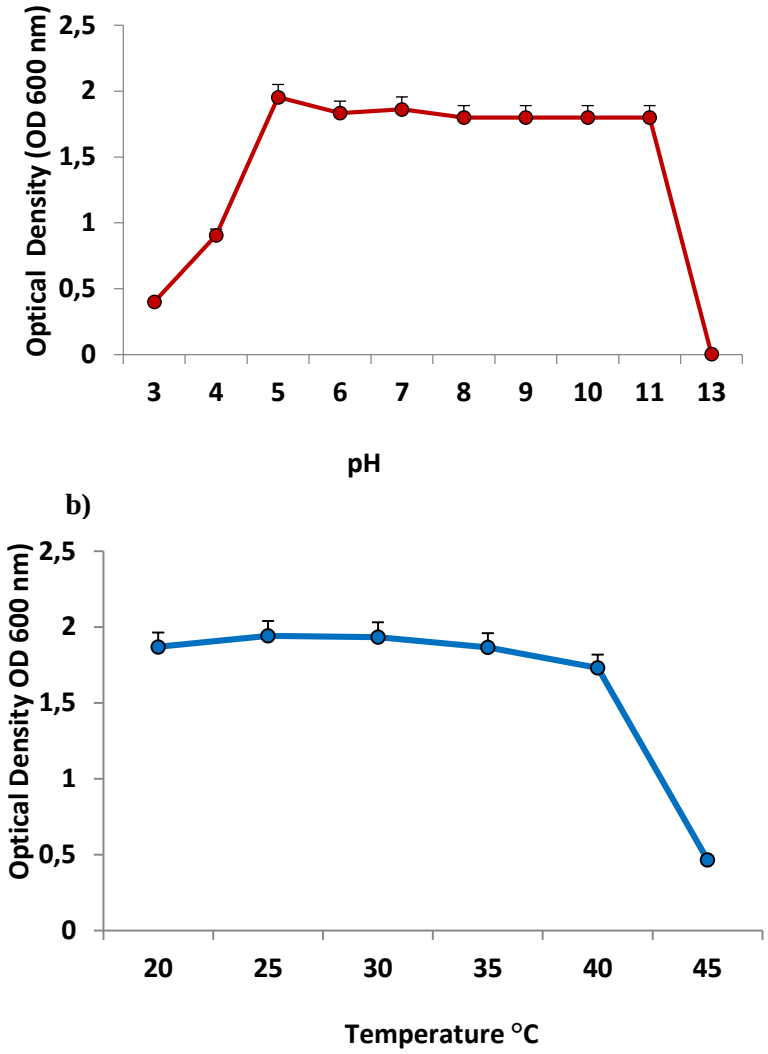


Figure 4. Effects of pH (a) and temperature (b) on the bacterial growth.

For strain D8, the growth range was determined to be between 20-40°C with optimum of 30°C (Figure 4b). In addition, the strain was found to grow in the pH range between 3.0-11.0 (Figure 4a) with optimum value of 5.0. The strain D8 was also found to tolerate up to 7% of NaCl. In a previous study, *Enterobacter* species strain ALK-23 was found to grow at an optimum 30°C in the range of 25-40°C. Also, the pH range in which the strain grows was 5.0 to 8.0 with an optimum pH of 6.0. [31].

3.3. Antibiotic Resistance

D8 was found to be ampicillin, fucidic acid, novobiosin, bacitracin, streptomycin, lincomycin, penicillin, neomycin, kanamycin, nystatin resistant, gentamicin, tilmicosin semi-susceptible, chloramphenicol, tetracycline sensitive (Table 2). *E. ludwigii* CCUG 51323^T strain was also found to be sensitive to gentamicin, but resistant to ampicillin [35].

Table 2. Antibiogram results of D8 (mm: zone of inhibition).

Antibiotics	D8

Novobicin (5 µg)	0
Fucidic acid (10 µg)	0
Kanamycin (5 µg)	12
Bacitracin (10 units)	0
Gentamicin (10 µg)	15
Tilmicosin (15 µg)	18
Ampicillin (10 µg)	0
Streptomycin (10 µg)	12
Chloramphenicol (30 µg)	30
Lincomycin (15µg)	0
Penicillin G (2 units)	0
Tetracycline (30 µg)	33
Neomycin (10 µg)	6
Penicillin G (10 units)	0
Nystatin (100 units)	0

3.4. Phylogenetic Analysis

The gene sequence analysis of 16 S rRNA showed that isolated strain D8 was a member of *Enterobacter* genus. After phylogenetic analysis, phylogenetic dendrogram was drawn, as seen in Figure 5. The bacterial strain designated as D8 was found to be most similar to *E. ludwigii* strain LY-62 (100%). GenBank accession number: MT374261.

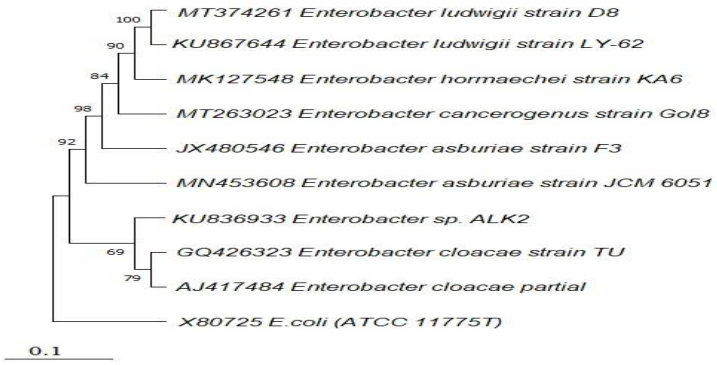


Figure 5. The 16S gene sequence analysis of the strain D8.

3.5. Growth of the Bacterial Strain in Various Hydrocarbons

Figure 6 shows that strain D8 uses alkane hydrocarbons to meet carbon and energy needs. Bacterial strain D8 was found to grow and degrade both medium and long chain alkanes such as decane, pentadecane, hexadecane and squalene instead of short chain alkanes. It was found to degrade pentadecane mostly for effective growth.

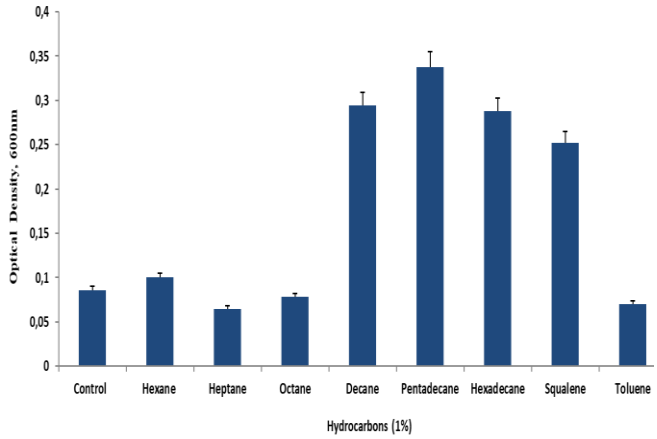


Figure 6. Growth of strain D8 at 1% single hydrocarbon concentrations. Each data represents the mean of at least 3 different experiments.

3.6. GC-MS Analyses of Degradation of Crude Oil and Single Hydrocarbons by the strain D8

Figure 7(A, B) shows gas chromatography-mass spectroscopy and degradation of n-alkanes (C_{11}-C_{33}) by strain D8 in 1%crude oil. GC-MS analysis showed that after 5 days of incubation, strain D8 degraded about 27 % hydrocarbons in crude oil. The total petroleum hydrocarbon degradation values were calculated by using decreased values, compared to abiotic control (Figure 7).

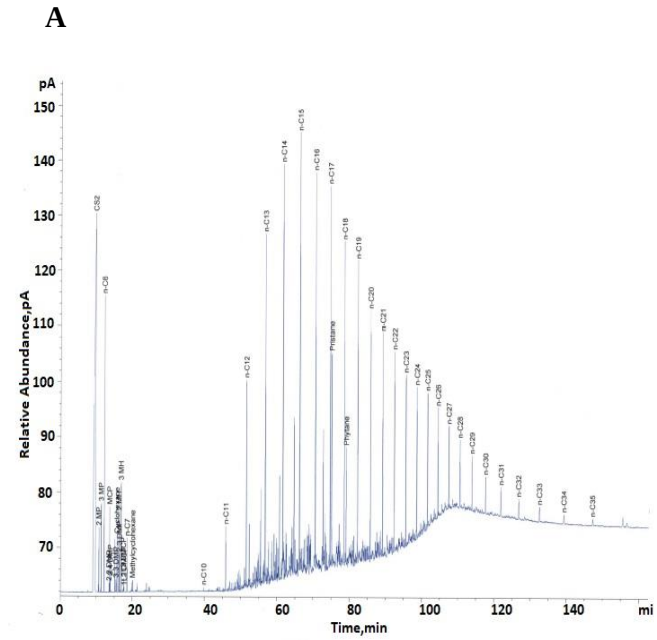
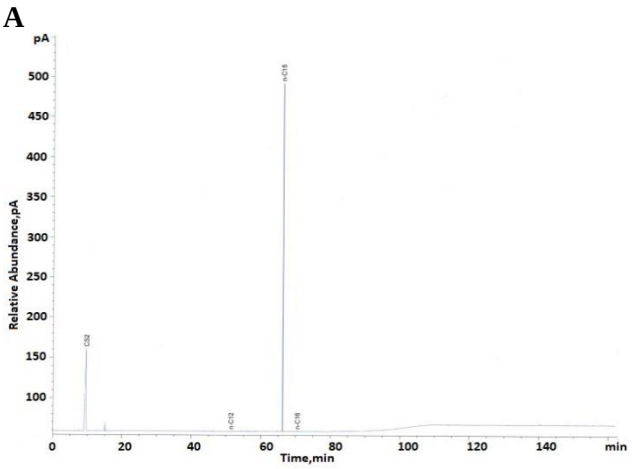


Figure 7. GC profiles of crude oil A) Abiotic control B) After degradation by strain D8. C_{11}-C_{33} represents n-alkanes.

In the present study, it was found that the strain grew better in 1% pentadecane in the basal medium. As seen in Figure 8A and B, the strain also degraded single pentadecane as much as 29% using the gas chromatographic analysis after 3 days of short incubation time.



B

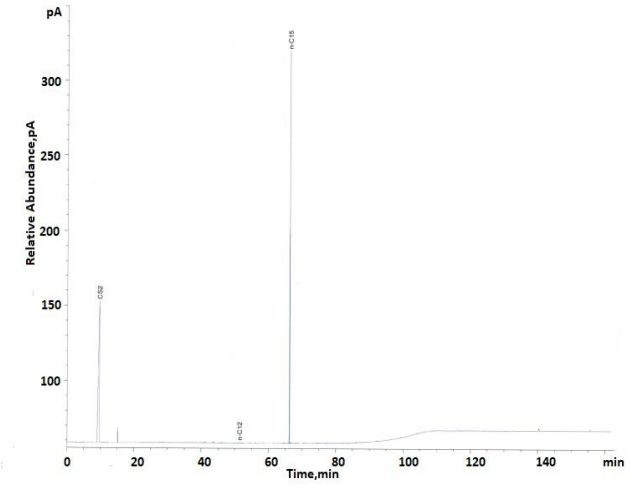


Figure 8. GC-MS Analysis of pentadecane degradation. A) Abiotic control of 1% pentadecane. B) After degradation of 1% pentadecane by strain D8.

Acinetobacter spp. is known that n-alkanes with different chain lengths (C_{10}-C_{40}) are used as the sole carbon source [36]. *Acinetobacter baumannii* MKS2 was found to degrade about 43 % of crude oil after 7 days [37]. In a recent study, GC-MS analysis of the hydrocarbon compounds indicated that the studied crude oil possessed alkanes in the range of C_{20}-C_{44} and *Enterobacter* ALK-23, *B. methylotrophicus* ALK16 and *Alcaligenes* ALK-14 were found to degrade maximum 5.59%, 8.11% and 11.65%, respectively [31]. *Pseudomonas aeruginosa* found 49.93% degradation of 0.5% diesel oil after 20 day incubation period [4]. They found that *P. aeruginosa* showed 34.4% degradation in diesel oil within 10 days [38]. *E. cloacae* TU was found to use n-hexadecane as sole carbon source [23]. However, studies on members of *Enterobacter* sp are mainly focused on degradation of aromatic hydrocarbons rather than n-alkanes degradation. *Enterobacter* sp. strain F3 was used to evaluate naphthalene degradation in petroleum-contaminated soil and a reduction of 61.11% in initial concentration was observed [32]. Kafilzadeh and Khezri [39] revealed that *E. ludwigii* KH-A5 showed highest degradation rate (96%) which is likely to be due to high levels of enzyme production responsible for aniline biodegradation under favourable growth conditions, similar to those of other bacteria reported previously [40,41].

4. Conclusion and Recommendations

Different microorganisms, including bacteria that can break down hydrocarbons in petroleum or single hydrocarbons, have been isolated from soils contaminated with petroleum. Bacteria are most dominant microorganisms in microbial ecology, degrading petroleum hydrocarbons. In the present study, 16S rRNA gene sequence analysis and phenotypic characteristics of the isolated strain D8 from oil-contaminated soil showed that it most closely resembles *Enterobacter ludwigii*. The strain D8 was found to degrade 27% of 1% crude oil incubated for 5 days, whereas it degraded 29% of 1% pentadecane after 3 days of incubation. The strain D8 in the current work appears to break down both crude oil and single hydrocarbons in shorter incubation times, and further microbial consortium studies with the strain of interest may have a good advantage in bioremediation of petroleum-contaminated soils.

Acknowledgements

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

Authors' 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.

Conflicts of Interest

The authors declare no conflict of interest.

Statement of Research and Publication Ethics

The study is complied with research and publication ethics.

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