academicJournals

Vol. 11(10), pp. 400-407, 14 March, 2017
DOI: 10.5897/AJMR2016.8413
Article Number: 1D4BF2963151
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Degradation of diesel-oil by a newly isolated *Kocuria sediminis* DDK6

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Received 14 December, 2016; Accepted 24 February, 2017

A bacterial strain named DDK6 was isolated from diesel-contaminated soil from a petrol station in Al-Hofuf city, after enrichment on diesel oil. The strain DDK6 formed a reddish-pink colony with a 2 to 3 mm diameter after two days of incubation at 30°C. Cells were Gram-positive coccoid and formed no endospores. Phenotypic identification by the automated bacterial system, Vitek compact II, identified the DDK6 as *Kocuria* sp. at 95% probability level. The 16s rRNA gene sequencing analysis confirmed the identity of the strain as *K. sedimins* at an identity level of 99.15%. Results of Gas chromatography-mass spectrometry (GC-MS) revealed that the DDK6 degraded the C14-C19 compounds in diesel. In addition, the DDK6 strain consumed the majority (68%) of the carbon sources tested, including monosaccharides, disaccharides, polysaccharides, and sugar alcohols as noticed by biochemical characterization using the API 50CH. The cultural, biochemical, and molecular characteristics were in general agreement with the strain identification. The results confirmed the metabolic versatility of the strain DDK6, in addition to its ability to degrade diesel oil, thereby providing ecological and environmental merits for its application in bioremediation of hydrocarbon pollutants.

Key words: Bioremediation, hydrocarbon, *Kocuria*, diesel oil.

INTRODUCTION

There is an increasing concern globally about potential environmental consequences arising from contamination by accidental petroleum release during storage, transport, or exportation. Saudi Arabia is one of the largest oil-producing countries worldwide, therefore marine and terrestrial biota are negatively affected. Diesel oil is a common fuel for diesel engines. Chemically, it is a mixture of aliphatic and aromatic hydrocarbons produced during petroleum separation by fractional distillation. Leakages of diesel oil can occur from storage tanks when the oil seeps into soils and groundwater causing severe environmental problems (Das and Chandran, 2010). The ecological effects of diesel on plants growing on diesel-contaminated soils result in reduction of seed germination,

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plant growth and productivity. Additionally, diesel sticks with soil particles causing bad soil aeration, leading to a decline in microbial biodiversity. Consequently, the ecosystem functioning is negatively affected and the biogeochemical cycling of essential elements become truncated (Ciric et al., 2010).

One promising and efficient strategy to remove diesel oil from soil and water is via microbial degradation. Bioremediation is an ecofriendly, cost-cheap, versatile and efficient treatment of hydrocarbons. It has been confirmed in many laboratories worldwide that several bacterial groups possess the catabolic machinery for diesel oil. Bacterial species, which belong to *Acinetobacter, Bacillus, Citrobacter, Corynebacterium, Flavobacterium* and *Micrococcus* are representatives of oil-degrading bacteria (Jirasripongpun, 2002; Das and Chandran, 2010). Furthermore, hydrocarbon-degrading bacteria that have recently been documented in different localities in Saudi Arabia, include *Stenotrophomonas maltophilia* (Arulazhagan et al., 2017), *Cupriavidus taiwanensis*, *Ochrobactrum intermedium*, *Pseudomonas aeruginosa* and *P*. *citronellolis*, (Oyehan and Al-Thukair 2017).

Kocuria is Gram-positive cocci arranged in pairs, short chains, tetrads, cubical packets of eight and irregular clusters. *Kocuria* belongs to the phylum *Actinobacteria*, class *Actinobacteria*, order *Actinomycetales*, sub order *Micrococcinae* and family *Micrococcaceae*. The genus *Kocuria* was coined by Stackebrandt et al. (1995) and separated from *Micrococcus* based on chemotaxonomic and phylogenetic features (Stackebrandt et al., 1995). Currently, there are more than 18 species of *Kocuria* identified based on the 16S rRNA phylogenetic studies. In general, *Kocuria* spp. are non-pathogenic however, some species have been isolated from infected superficial and deep human tissues. At the time of writing, 20 different species with validated names are included under the genus *Kocuria.* The completely detailed list of the species is outlined in http://www.bacterio.cict.fr/k/kocuria.html. Most of these bacterial species have been isolated from diverse ecological niches. For example, *K. dechangensis* obtained from saline and alkaline soils (Wang et al., 2015), *K. salsicia* from salt-fermented seafood (Yun et al., 2011), *K. gwangalliensis* from seawater (Seo et al., 2009), *K. palustris* and *K. rhizophila* from rhizoplane of *Typha angustifolia* (Kovács et al., 1999), *K. pelophila rhizosphere* of a mangrove (Hamada et al., 2016). Additionally, *K. sedimins* has been isolated from a sediment sample from Kerala, India and described by polyphasic approaches (Bala et al., 2012) but its ability to degrade diesel oil was not investigated. Researchers have showed the role of *Kocuria* spp. in bioremediation of hydrocarbon (Esmaeil et al., 2009), removal of copper from copper- contaminated soils (Achal et al., 2011) and production of probiotics (Sharifuzzaman et al., 2014), biocontrol agents (Sharifuzzaman and Austin 2010), plant-growth-promoting activities (Egamberdieva, 2008). Members of the genus *Kocuria* have been shown to produce, kocurin, a novel thiazolyl peptide antibiotic, which exhibited anti-bacterial activities against clinically relevant strains (*Bacillus subtilis*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii* and *Candida albicans*) (Palomo et al., 2013).

Hydrocarbon-degrading bacteria that have recently been investigated in different localities in Saudi Arabia, include *Stenotrophomonas maltophilia* (Arulazhagan et al., 2017),

Cupriavidus taiwanensis, *Ochrobactrum intermedium*, *Pseudomonas aeruginosa* and *P*. *citronellolis*, (Oyehan and Al-Thukair 2017). Jeddah, Khobar and Duhran. However, little is known about the biodegradability of diesel-oil in Al-Houf, eastern region, Saudi Arabia. In addition, the search for local diesel-oil-degrading bacteria from soils exposed to the prevailing conditions in Al-Houf city has a pivotal importance in bioremediation approaches. Therefore, the current study aimed at isolation and characterization of a bacterial strain, designated DDK6, which was able to grow on diesel as a sole energy and carbon sources. To fulfill this aim, a soil sample was collected from a diesel-contaminated soil from a petrol station.

MATERIALS AND METHODS

Collection of soil sample

A diesel contaminated soil sample was collected a petrol station in Al-Hofuf Saudi Arabia, in a sterilized screw-capped test tube. In the laboratory, 0.5 g of soil sample was enriched with diesel oil (1%v/v) as a sole carbon source in a 200 ml conical flask containing 50 ml mineral salts (MS) medium with the composition; 1 g $(NH4)_2SO_4$, 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2{\cdot}2H_2O$, and 5 mg $FeSO_4{\cdot}7H2O$ in 1 L distilled water, pH 7) (Wu et al., 2013). Flasks were placed in a shaking-incubator at 100 rpm for three weeks. To ensure oil degradation ability, subsequent transfer of inoculum (3%) into a fresh MS medium was carried out.

Isolation, purification and preservation of the strain

Aliquots (100 µl) from the diesel-enriched media were streaked into MS agar plates and sprayed with diesel on the surface. Plates were incubated at 30°C for 7 days. Single colonies were picked and sub-cultured into fresh MS agar amended with diesel.

Morphology of colony and cells

The morphological characteristics of the colony; pigmentation, diameter, elevation and transparency was determined visually of 24 h old colony growing on soya agar medium and incubated at 30°C. Cell shape, arrangement and reaction to the gram staining were assessed.

Table 1. Cultural and cellular characteristics of the strain DDK6.

Feature	Description
Size	2 mm
Shape	Circular
Pigmentation	Reddish pink
Texture	Smooth
Elevation	Convex
Edge	Entire
Cells	Coccoid
Gram Reaction	Positive
Endospore formation	None

Biochemical characterization using the API50Ch strip kit

In order to investigate the biochemical characteristics of the strain DDK6 API50Ch strip kit (Biomerieux, France) was used following the guidelines of the manufacturer. Results were recorded after 48 h of incubation of API50Ch.

Identification of the strain using Vitek compact II

The bacterial strain DDK6 was identified using the automated system for identification of bacterial strains, Vitek compact II. Gram-Positive Card was used and the data analysis were carried out using the Software version: 05.02 (Biomerieux, Mary L'Etoile, France).

Identification of the strain DDK6 using 16S rRNA gene sequencing

PCR amplification of the 16S rRNA gene

16S rRNA gene was amplified using the universal primers; 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-TACGGYTACCTTGTTACGACTT-3' (Weisburg et al., 1991) in 20 µl total PCR reaction. Genomic DNA was extracted from the strain using the InstaGene Matrix (Bio-Rad, USA) following the instructions of the manufacturer. PCR conditions were adjusted as previously described (Khalifa et al., 2015).

16S rDNA sequencing and construction of phylogenetic tree

For sequencing of the 16S rRNA gene, the Big Dye terminator cycle sequencing kit (Applied BioSystems, USA) was used. Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

Blast search and calculations of similarity values of pairwise nucleotide 16s rRNA gene sequence were performed using EzTaxon server (http://www.eztaxon.org/; Chun et al., 2007). Multiple alignments with sequences of the most closely related recognized *Kocuria* strains and construction of phylogenetic tree were carried out by using the Neighbor-joining method based on the Tamura-Nei model (Tamura and Nei, 1993). Evolutionary analyses were conducted in MEGA5.02 (Tamura et al., 2011). The 16s rRNA gene sequence of strain DDK6 was deposited in the NCBI database under the accession number KY307788.

Detection of diesel oil degradation using the Gas chromatography-mass spectrometry (GC-MC)

The ability of the strain DDK6 to degrade diesel oil was further detected using GC-MS. DDK6 was inoculated into 50 ml MS medium containing 1% (V/V) diesel oil and incubated at 30°C with shaking 150 rpm for 5 days. Non-inoculated flask containing the same growth medium was used as control. After incubation period, diesel oil was extracted using equal volume of dichloromethane and analyzed using a Shimadzu GCMS –QP2010 SE instrument. The flow rate of the Helium as a carrier gas was set at 6.0 ml/min. The temperatures of injector and detector were adjusted at 250 and 300°C, respectively. The temperature program was as followings: 2-min hold at 60°C, ramp to 300°C at 20°C/min and 6-min hold at 300°C.

RESULTS AND DISCUSSION

K. sediminis strain DDK6 was isolated from diesel-contaminated soils from a petrol station in Al-Hofuf. The cultural characteristics are outlined in Table 1. DDK6 formed a reddish-pink, convex colony, with a diameter of 2 to 3 mm, and entire margin. Cells of the strain DDK6 were coccoid, formed no endospores and exhibited positive reaction to the Gram staining. These features were typical to the type strain of *K. sediminis* FCS-11^T (Bala et al., 2012) confirming the identity of the DDK6 strain.

Biochemical characterization using the API50Ch strip kit

The biochemical characteristics of the strain DDK6 using the API50Ch (Biomerieux, France) were shown in Table 2. API 50 CH is a phenotypic-based system, comprising 50 biochemical tests designated to estimate the carbohydrate metabolism ability of a microorganism. The system is exploited in the current study as a fast, effective a reliable tool to study the metabolic versatility of the strain DDK6 towards different sources of carbohydrate.

Table 2. API 50Ch for bacterial strain DDK6.

Number	Test	Result
0	Control	-
1	Glycerol	+
2	Erythritol	-
3	D-arabinose	+
4	L-arabinose	+
5	D-ribose	+
6	D-xylose	+
7	L-xylose	+
8	D-adonitol	+
9	Methyl-BD-xylopyranoside	+
10	D-galactose	+
11	D-glucose	+
12	D-fructose	+
13	D-mannose	+
14	L-sorbose	+
15	L-rhamnose	-
16	Dulcitol	+
17	Inositol	+
18	D-mannitol	+
19	D-sorbitol	+
20	methyl-αD-mannopyranoside	+
21	Methyl-aαD-glucopyranoside	+
22	N-acetylglucosamine	+
23	Amygdalin	+
24	Arbutin	+
25	Esculin	+
26	Salicin	+
27	D-cellobiose	+
28	D-maltose	+
29	D-lactose	+
30	D-melibiose	+
31	D-saccharose	+
32	D-trehalose	+
33	Inulin	-
34	D-melezitose	-
35	D-rafinose	-
36	Starch	+
37	Glycogen	+
38	Xylitol	-
39	Gentibiose	+
40	D-turanose	-
41	D-lyxose	-
42	D-Tagatose	-
43	D-Fucose	-
44	L-Fucose	-
45	D-arabitol	-
46	L-arabitol	-
47	potassium gluconate	-
48	potassium 2- ketogluconate	-
49	potassium 5-ketogluconate	-

DDK6 exhibited the ability to utilize a wide range of different carbon including monosaccharides (e.g., D-galactose and L-arabinose) disaccharides (e.g., D-maltose and D-lactose), polysaccharides (e.g., Starch and Glycogen) and sugar alcohols (e.g., D-galactose, D-mannitol). Out of 49, DDK6was able to utilize 33 (~67%) different carbon compounds tested. However, 33% of the carbon compounds were not metabolized by the strain under study. D-turanose, D-lyxose, D-Tagatose, D-Fucose, Arabitol, potassium gluconate and Inulin are representatives of carbon sources that were not consumed by the strain DDK6. The biochemical profile of the strain DDK6 is shown by the API 50CH kit strip is typical to that of the *K. sediminis* (Bala et al., 2012) providing another evidence to the reliable identification of the strain DDK6. Genome sequences of members of *Kocuria* genus such as *K. marina* SO9-6 (Castro et al., 2015) and *K. rhizophila* strain TPW45 (Tan et al., 2016) revealed the existence of many gene clusters involved in catabolic pathways of carbohydrate. The ability of the strain DDK6 to grow using a wide range of different biochemical compounds. This range of metabolic versatility could provide an explanation about the ubiquity of *Kocuria* sp. in diverse ecological niches particularly, in oil-contaminated ecosystems.

Identification of the strain using mass spectrometry technology

Vitek II is a powerful and accurate automated platform with an expanded identification database for rapid microbial identification, and antibiotic susceptibility testing based on biochemical analysis using colorimetry. The bacterial strain DDK6 was identified as *Kocuria* sp. (at 98%) by using the Vitek compact II indicating that this tool is efficient for bacterial identification at the genus and specific levels. The efficiency of this system to correctly identify *Staphylococcus* spp. and other bacterial genera has been proven previously (Chatzigeorgiou et al., 2011; Paim et al., 2014). Nonetheless, discordance between VitekII and 16S rRNA gene sequencing for bacterial identification has been reported. For example, VitekII identified a bacterial strain from human blood as *K. kristinae* (score of 98%) whereas 16s rRNA sequencing identified it as *Rothia amarae* (Abouseada et al., 2016).

16S rDNA sequencing and of phylogenetic analysis

The 16S rRNA gene sequencing is a well-established technique for a robust and accurate bacterial identification and for inferring phylogenetic relationships among species. Comparative analyses of the 16S rRNA gene sequencing revealed that the strain DDK6 was related to the genus *Kocuria*. Sequence analysis showed that DDK6 was most closely related to *K. sediminis* strain FCS-11^T (99.15% identity), followed by *K. flava* HO-9041^T (98.9%), *K. turfanensis* HO-9042^T (98.9%), *K. dechangensis* NEAU-ST5-33^T (98.41%), *K. polaris* CMS 76or^T (98.28%), *K. rosea* DSM 20447^T (98.27%), *K. aegyptia* YIM 70003^T (98.28%), *K. himachalensis* K07-05 ^{T} (97.51%), *K. atrinae* P30 ^{T} (97.1%) at similarity levels of gene sequences. As can be seen in Figure 1, the neighbour-joining phylogenetic tree (Figure 1) clearly highlighted that the strain DDK6 grouped with the *K. sediminis* strain FCS-11. The 16S rRNA gene sequencing confirmed phenotypic identification of the strain DDK6 based on Vitek II. Similar results have been obtained by Hassan et al. (2016) who showed that both Vitek2 and 16S rRNA gene sequencing correctly identified *K. kristinae* (99%) (Hassan et al., 2016).

As can be seen in Figure 2, the major components of the diesel oil were C14-C19 alkanes. The peak areas of the C14-C19 were significantly lower than those in the control indicating that DDK6 was able to degrade this fraction of diesel oil (Figure 2). Our reseults are in accordance with those obtained by Mariano et al. (2007) who demonstrated that *K. palustris* was able to efficiently degrade diesel oil (Mariano et al., 2007). Microbial biodegradation of diesel oil and/or its components is a common process in terrestrial and aquatic ecosystems (Austin and Groves et al., 2011). Two main classes of metalloenzymes are involved in this multi-step process. The first class comprises membrane-associated enzymes such as alkane hydroxylase and the latter composed of cytoplasmic –soluble enzymes such as cytochrome P540. The existence of the alkane hydroxylase and cytochrome P540 in the strain DDK6 is consistent with previous studies on other bacterial species such as *Acinetobacter* sp. (Hou et al., 2013), *Enterobacter cloaecea* (Ramasamy et al., 2017), *Pseudomonas aeruginosa* and *Bacillus subtilis* (Safdari et al., 2017). Both enzymes exhibited wide range of alkane substrates and could have synergistic effect. The ability of other Kocurial species to grow on oil and other hydrocarbon as a sole carbon and energy sources has been documented. For example, *K. flava* and *K. rosea* were shown to degrade naphthalene, phenanthrene and fluoranthene and crude oil (Tumaikina et al., 2008). Apparently, the efficiency of local bacterial strains in hydrocarbon-degradability was found to be substantially higher than that of the introduced strains (Wu et al., 2013), this could be attributed to outcompeting, biotic and/or abiotic interacting factors. Therefore, isolation of new bacterial strains adapted to the local conditions of a particular area is crucial for the efficiency of hydrocarbon clean up in that area. It has been reported that production of biosurfactants enhances the bacterial oil degradation (Matvyeyeva et al., 2014). Biosurfactants are active molecules that lower the interfacial tension between two immiscible liquids. Sarafin et al. (2014) have highlighted

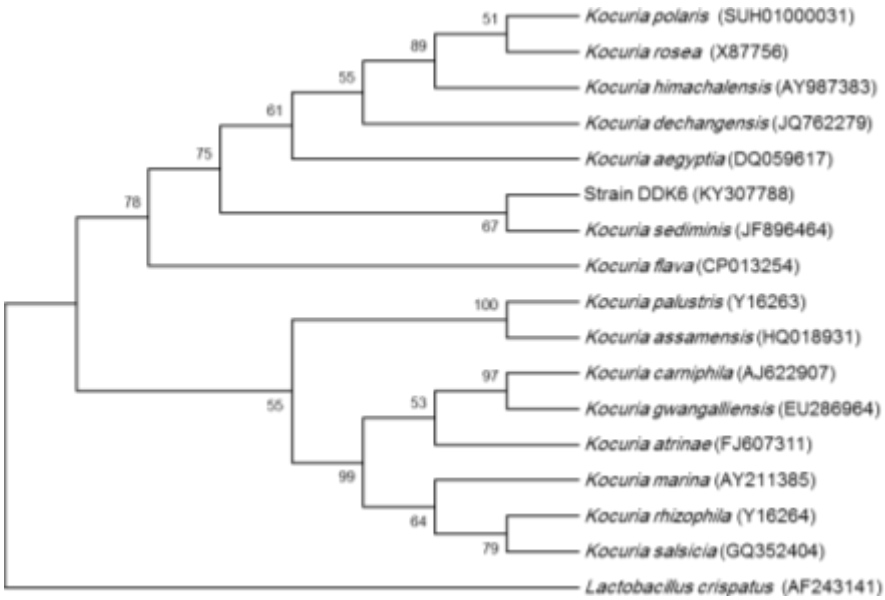

Figure 1. Neighbor-joining tree based on 16S rDNA gene sequences revealing the phylogenetic relationships between *K. sediminis* strain DDK6 accession number KY307788 and other closely related bacterial species. Accession number is given between parentheses after each bacterial species. The percentage numbers above each branch indicate the 567 levels of bootstrap support (>50%) for the branch point based on 1,500 568 resamplings. The bar represents 0.02 substitutions per site.

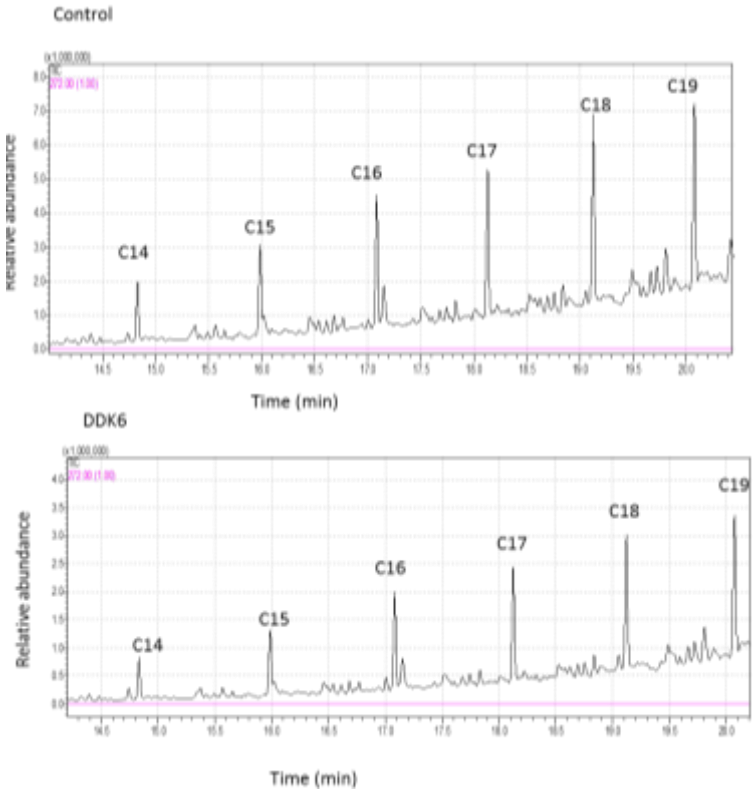

Figure 2. The GC-MS chromatogram of diesel oil extracted from culture; control (top graph) uninoculated culture and DDK6 culture (Bottom graph).

Table 3. A list of *Kocuria* spp. that possess the cytochrome P450.

Number	Name	Accession number
1	*K. rhizophila*	WP_012399225.1
2	*K. salsicia*	WP_055082043.1
3	*K. rhizophila*	WP_059281539.1
4	*K. rhizophila*	WP_019310814.1
5	*K. rhizophila*	WP_039100753.1
6	*K. rhizophila*	WP_047979332.1
7	*K.* sp. HMSC066H03	WP_070637452.1
8	*K. varians*	WP_068469792.1
9	*K. rhizophila*	WP_058954974.1
10	*K. marina*	WP_035965726.1
11	*K.* sp. ICS0012	WP_064845916.1
12	*K. flava*	WP_058857146.1
13	*K.* sp. UCD-OTCP	WP_017833470.1
14	*K. polaris*	WP_035928135.1
15	*K. polaris*	WP_058873945.1
16	*K.* sp. SM24M-10	WP_047802943.1
17	*K. turfanensis*	WP_062735211.1

that *K. marina* BS-15 produced biosurfactants reflecting its efficiency in oil degradation via increasing its water solubility to facilitate enzyme attack.

Generally, microbial degradation of diesel oil and other hydrocarbon is attributed to cytochrome P450, haem-thiolate monooxygenases, which catalyze the oxidative addition of atomic oxygen to the C-H or C-C bond of the organic compound (Van Beilen and Funhoff, 2007). Data mining for the Kocurial species that possess Cytochrome P450 showed that 17 different putative Cytochrome P450 proteins were found in 11 species (Table 3). Testing these species is crucial to confirm the ability to degrade diesel. Other enzymes such as soluble and particulate methane monoxygenases are involved in hydrocarbon degradation (Das and Chandran, 2011).

In conclusion, the cultural, biochemical and molecular characteristics were in general agreement for the strain DDK6 identification as *K. sedimins*. DDK6 exhibited a metabolic versatility and ability to degrade diesel oil indicating ecological and environmental merits for its application in bioremediation of hydrocarbon pollutants. Nonetheless, further studies are required to investigate the efficiency of the strain DDK6 for biodegradation of different organic pollutants and enzymes involved in this process at the molecular level, to explore the potentialities of the strain for biotechnological exploiting. Bearing in mind the efficiency of local bacterial strains in hydrocarbon-degradability was found to be substantially higher than that of the introduced strains (Wu et al., 2013), this could be attributed to outcompeting, biotic and/or abiotic interacting factors. This is the first report addresing *K. sediminis* as an oil-degrading bacterium isolated from an oil-contaminated soil exposed to the prevailing conditions in Al-Hofuf, Saudi Arabia.

CONFLICT OF INTERESTS

The authors has not declared any conflict of interests.

ACKNOWLEDGMENT

Thanks to Dr. Adnan Aldeghaish for the technical assistance in GC-MS analysis.

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