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Isolation and characterization of an *n*-hexadecane degrading *Acinetobacter baumannii* KSS1060 from a petrochemical wastewater treatment plant

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Abstract Hydrocarbons are widespread in the environment, but because of the massive utilization of petroleum products, they are nowadays strongly involved in environmental pollution. Bioremediation is the obliging technology for the treatment of hydrocarbon-contaminated sites. Therefore, to investigate the potential of petrochemical hydrocarbon (HC)-degrading indigenous microorganisms in wastewater samples collected from Fajr petrochemical wastewater treatment plants, a strain of *Acinetobacter baumannii* was isolated from this hydrocarbon-contaminated wastewater and examined for its ability to utilize hexadecane. This strain was capable to grow on *n*-hexadecane as the sole source of carbon and energy. The ability of the isolate to degrade *n*-hexadecane was assessed by growth assays and gas chromatography/mass spectrometry analysis. Using GC analysis, it was shown that the strain KSS1060 was able to degrade 62 % of *n*-hexadecane within 6 days, which mostly (51.6 %) occurred within the first 24 h. Identification of this hexadecane-degrader bacterium was carried out using 16S rDNA sequence analysis. Additionally, characterization of chemical composition of wastewater samples by the use of gas chromatography/mass spectrometry analysis indicated the presence of Hexanal, Benzene methanol, Indanol, 1,2-benzenedicarboxylic acid diethyl ester, diisobutyl phthalate, and Phenol,4,4'-(1-methylethylidene) in the major constituents of wastewater. In conclusion, this study can focus on more cost-efficient applications of native bacterial strains for the large-scale biodegradation of wastewater samples from petrochemical plant in industry, where it causes disturbing problems due to its harmful effects on different organisms and human beings.

Keywords Bioremediation · Hydrocarbons · *Acinetobacter baumannii* · *n*-Hexadecane · GC/MS analysis · Petrochemical wastewater

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Introduction

Hydrocarbons are the main sources of energy in our life (Bouchez-Naitali et al. 2001). They are the major biological organic contaminant with toxic, mutagenic, and carcinogenic effects (Das and Chandran 2011). Moreover, these contaminants can be absorbed into the soil and penetrate into the surface and ground waters and cause numerous problems to living organisms because of their long durability in the environment (Dehghani et al. 2013).

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The structure of hydrocarbons is composed of carbon and hydrogen atoms where the number of carbon atoms determines their physical properties. Medium-chain alkanes are the major soil contaminants, and among this class of alkanes, n-hexadecane (CH_3 $(CH_2)_{14}CH_3$; 226.44 g mol^{-1}) has been used as a model contaminant of diesel oil by many researchers (Bouchez-Naitali et al. 1999; Noordman et al. 2002) and has been chosen because of its low water solubility (0.9 μg l^{-1}) (Dehghani et al. 2013), as well as rapid degradability by numerous microorganisms (Dashti et al. 2008). Hexadecane causes skin problems such as dryness, irritation, and cracking of skin, and when ingested can be harmful and even be fatal. It also stimulates mucous membranes and upper respiratory system inhalation and even causes pulmonary injury (Bouchez-Naïtali and Vandecasteele 2008; Wentzel et al. 2007).

Soil and water remediation by non-biological technologies are expensive and incomplete (Medina-Bellver et al. 2005; Siddiqui and Adams 2002). Moreover, bioremediation technology, the use of microorganisms to remove or detoxify contaminations, is a cost-effective and noninvasive method (Siddiqui and Adams 2002) and is highly efficient and can remove even trace levels of contaminants (Okoh 2006). However, it has been revealed that natural populations of microorganisms are able to remove environmental hydrocarbon pollutants including the products of petroleum industry, due to their diverse metabolic capabilities (Barin et al. 2014). Additionally, numerous scientific articles have studied the factors influencing the rate of hydrocarbon biodegradation. As Das et al. reported that the presence of microorganisms with high metabolic capacity can be the most significant factor, environmental factors like temperature, pH, oxygen, and nutrient availability can affect the rate of bioremediation (Das and Chandran 2011). Also, it has been found that among physical factors, temperature plays an important role in the rate of hydrocarbon biodegradation due to its direct effect on the diversity of the microbial flora and the chemical structure of pollutants (Jain et al. 2011). Also the optimal growth rate of microorganisms and hydrocarbon biodegradation is achievable by providing the adequate concentrations of nutrients and oxygen in pH ranging from 6 to 9 (Das and Chandran 2011). Although biodegradation of hydrocarbons can occur in a wide range of temperature changes, but lower temperatures increase the viscosity of the hydrocarbons and reduce their solubility and thus decrease the rate of biodegradation (Atlas 1975; Foght et al. 1996). Jung et al. (2011) showed that

the high temperature (37 °C) inhibits the hexadecane biodegradation of *Acinetobacter oleivorans* DR1 and observed the highest biodegradation of hexadecane at 30 °C. Biodegradation can occur under a wide range of pH; however, optimal pH for biodegradation in most terrestrial and aquatic environments has been reported between 6.5 and 8.5 (Cases and de Lorenzo 2005).

Since alkanes are saturated hydrocarbons and nonpolar molecules with very low chemical activity (Labinger and Bercaw 2002), their degradation by microorganisms has been faced major challenges due to their low water solubility, excessive accumulation in cell membranes, and higher activation energies (Labinger and Bercaw 2002; Rojo 2009). Therefore, interest in the study of biodegradation of hydrocarbons by bacteria has increased because of their potential for degrading hydrocarbons and to transform them to easily metabolizable substrates. To get maximum benefits from this kind of bacteria, the selection of the most effective hydrocarbon-biodegrading bacteria is a prerequisite. Therefore, the isolation and study of native strains that are adapted to their environment may contribute to the formulation of a microbial inoculant to be used for the bioremediation of regional polluted environments. Therefore, this study was designed to determine the ability of indigenous bacteria isolated from wastewater samples that were taken from the Fajr Petrochemical Company in Mahshahr, Iran to degrade n-hexadecane in order to identify potent strains to be used as candidate bacteria in biological treatment of wastewaters.

Materials and methods

Sampling site and isolation conditions

Wastewater samples were taken from Fajr Petrochemical Company located in Mahshahr, Khouzestan province, Iran. Samples were collected in September of 2008. The mean annual maximum temperature and atmospheric humidity were, respectively, 45 °C and 50 % during the year 2008. In this plant, wastewater is subjected to three treatment steps: physical, biological, and chemical treatments. The biological treatment plant consists of three stages, namely primary (oil separation, equalization, and stripping), secondary (biological treatment and settling), and tertiary (chlorination). Twenty-five wastewater samples of 12 activated sludge tanks were taken from influent and effluent in sterile bottles and were stored at 4 °C for microbial experiments.

Bacterial counts and isolation

Microbial counts of cultivable aerobic heterotrophic microorganisms were determined using the plate count methods (pour plate and spreading on agar). Sample suspensions were prepared by homogenizing 10 ml of sample in 90 ml of Tween 80 (0.1 % w/v) plus 0.85 % (w/v) saline solution and serially diluted (tenfold) in the same normal saline solution. Total heterotrophic bacteria were enumerated by using plate count agar (PCA) (Merck, UK). Plates were incubated at 30 °C for 48 h (Rahman et al. 2003). Single bacterial colonies, growing on the medium, and different colonies were isolated and purified from cultivated plates and repeatedly streaked onto nutrient-agar (NA) plates in order to obtain pure cultures after 24 h of incubation at 30 °C. The number of counted microorganisms was reported as cfu ml^{-1}. Total *Pseudomonas* bacteria were enumerated using cultures of serially diluted samples on cetrimide agar (CA) after 48 h of incubation at 30 °C.

The active bacterial cells were slanted on NA and preserved at 4 °C. For long-term preservation, pure cultures were prepared as skim-milk stocks (10 %) and maintained at −70 °C in a deep freezer.

PCR amplification and 16S rRNA gene sequencing

Genomic DNA of KSS1060 was extracted by phenol–chloroform method (Sambrook and Russell 2001). The approximately 850-bp 16S rRNA gene fragment was amplified using the universal primers corresponding to *E. coli* 16S rRNA gene fw (5′-CCTACGGGAGGCAG-CAG-3′) and rw (5′-GACGTCRTCCNCDCCTTCCT-3′) (Khalaj-Kondori et al. 2007). PCR was performed in a Thermo cycler (company TECHNE). The PCR program comprised initial denaturation 94 °C for 1 min, followed by 35 cycles each of 94 °C for 1 min; 61 °C for 30 s; 72 °C for 50 s; 72 °C for 4 min; and incubation at 4 °C for 10 min. PCR products were purified with DNA extraction kit (Bioneer, South Korea). Both strands of the PCR product were sequenced by dideoxy chain termination method.

The 16S rRNA gene sequence of the KSS1060 was compared with those in the NCBI/EZtaxon/Ribosomal Database Project (RPD)/EMBL nucleotide sequence databases by using the BLAST (blastn) program (http://www.ncbi.nlm.nih.gov/BLATS/), and all of the sequences were aligned using the ClustalW program (Thompson et al. 1994). A phylogenetic tree and neighbor-joining phylogeny were constructed by using the with MEGA software package version 4.0 (Tamura et al. 2007), and bootstrapping was used to estimate the reliability of the phylogenetic reconstructions (1,000 replicates).

HC utilization fingerprints and degradation abilities

To determine the growth and HC utilization fingerprints of the isolates, each strain was incubated at 28 °C with shaking (120 rpm) in batch culture of 250 ml Erlenmeyer flasks containing 50 ml of mineral salts medium (MSM) supplemented by 2 ml of hexadecane (4 % w/v) for 7 days. The medium was contained (g l^{-1}): NH$_4$Cl (4.0), K$_2$HPO$_4$(2.5), NaCl (0.5), 0.3 g of MgSO$_4$.7H$_2$O, 0.03 g of FeCl$_3$.6H$_2$O, 0.01 g of CaCl$_2$, 0.01 g of MnCl$_2$.4H$_2$O, and pH adjusted to 7.0. Control plates without the HC were also inoculated. To prepare inoculums, a loopful of cells from NA pure cultures was first transferred into a test tube containing 10 ml of Tryptic Soy Broth (TSB) and incubated at 30 °C for 24 h. Then, 1.5 ml of the overnight bacterial culture was harvested by centrifugation at 12,000g for 10 min at 4 °C, when the desired optical density at 600 nm (OD600 of 0.5) was obtained.

The pellet was washed twice in phosphate-buffered saline (PBS) solution pH 7.4 and then suspended in MSM medium. The cultures were incubated on a rotary shaker at 120 rpm for 7 days at 28 °C (Chayabutra and Ju 2000). Control flasks without inoculation were incubated in parallel under the same conditions, to demonstrate reliability of the tests.

Bacterial growth was monitored regularly by measuring of absorbance at 600 nm. Changes in pH values were also measured regularly.

GC analysis of *n*-hexadecane

The biodegradation of *n*-hexadecane for selected bacteria was analyzed using gas chromatography (GC) after chloroform–methanol (3:1) extraction was performed. Chloroform–methanol (30 ml) was added to 100 ml of bacterial culture to extract the residual oil; the extracted sample was used for GC analysis and measurement of the waste-oil degradation rate. All measurements were performed in duplicate.

A Varian CP-sil 5CB capillary column (50 m × 0.32 mm) and a Varian GC system (CP-3800), fitted with a Flame Ionization Detector (FID) and 1079 PTV injector, were used for the analysis of waste-oil degradation. The conditions for GC were as follows:

injection temperature, 230 °C; column temperature, 200 °C; and detection temperature, 250 °C. We also used pentadecane as an Internal Standard (IS) for calibration.

GC and GC–MS analysis of wastewater samples

The collected wastewater samples (100 ml) from influent and effluent streams of the activated sludge tanks were extracted twice by 50 ml dichloromethane (DCM), after filtering with a 0.45-μm pores filter to eliminate the suspended particles. The extraction phase was concentrated in a vacuum evaporator to reach around 2 ml and then cold-dried by flow of nitrogen gas. The residue was diluted in 1 ml of DCM and analyzed by gas chromatography/mass spectrometry (GC/GC–MS).

A sample of 0.5 μl of the residue solution was injected into a GC system (Model: HP5890) equipped with FID and a HP-5 capillary column (30 m × 0.53 mm × 1.5 μm) to determine the existing chemical compounds. Helium was used as the carrier gas with flow rate of 1 ml min^{-1}. The oven temperature was maintained at 50 °C for 5 min, increased to 250 °C at a rate of 8 °C min^{-1}, and kept in this temperature for 10 min. The temperature was thereafter increased to 285 °C with an increase rate of 20 °C min^{-1} and kept for 5 min. The injection port and detector temperatures were 270 and 285 °C, respectively.

Also, GC–MS analysis was performed using a GC–MS system (Hewlett–Packard Model: HP6890N) equipped with a HP-5793 series mass selective detector and a HP-5 fused silica capillary column (5-methylsyloxane; 30 m × 0.25 mm × 0.32 μm film thickness) connected to an ion-trap detector (ITD). The scan frequency was 0.6 times s^{-1}, and the mass range scanned was 50–700 amu. The electron impact source temperature was 230 °C with electron energy of 70 eV.

The temperature conditions for GC–MS system was similar to that used in the GC apparatus.

Accession number

The nucleotide sequence of the 16S rRNA gene was deposited in the GenBank database under accession number KC424637.

Results and discussion

Microbial count of wastewater samples

A total average of 3×10^7 cfu ml^{-1} of mesophilic aerobic bacteria and 2.4×10^2 cfu ml^{-1} of pseudomonads were counted in wastewater samples.

According to morphological examinations, 117 different morphotypes were isolated.

HC utilization fingerprint and degradation abilities

Ninety-one isolates were capable to grow on minimal mineral medium containing 1.5 % of n-hexadecane as a sole source of carbon. After regularly monitoring of pH changes and bacterial growth by measuring of the absorbance at 600 nm during 7 days, eight isolates out of the total number showed the high ability to resist and grow at the presence of 4 % of hexadecane. Further studies were resulted in the selection of the strain KSS1060.
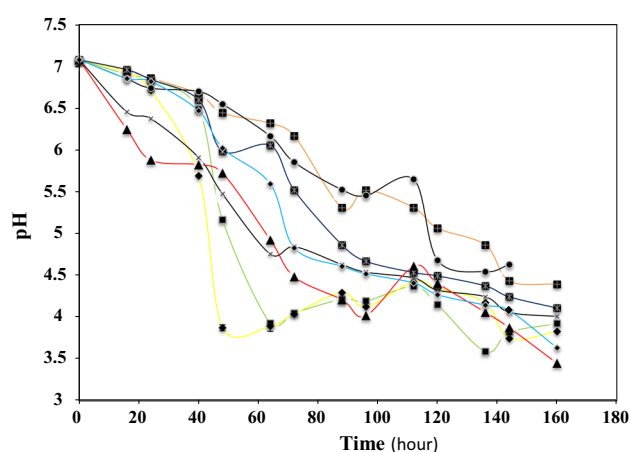

Fig. 1 Changes in pH of culture medium containing 4 % (w/v) of n-C$_{16}$ in eight isolates (Incubation time: 7 days); *Filled triangle* is for KSS1060 isolate
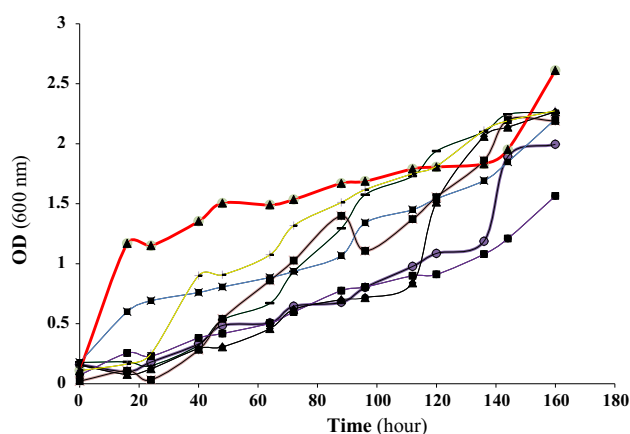

Fig. 2 Changes in OD of culture medium containing 4 % (w/v) of n-C$_{16}$ (4 % w/v) in eight isolates (Incubation time: 7 days); *Filled triangle* is for KSS1060 isolate

Fig. 3 Neighbor-joining tree, based on 16S rRNA gene sequences (850 bases), shows the phylogenetic relationship between strain KSS1060 and other species of the genus *Acinetobacter*. *Numbers above branches* represent bootstrap values (1,000 replicates) and *superscript T's* indicate type strains. Accession numbers are given in *brackets*
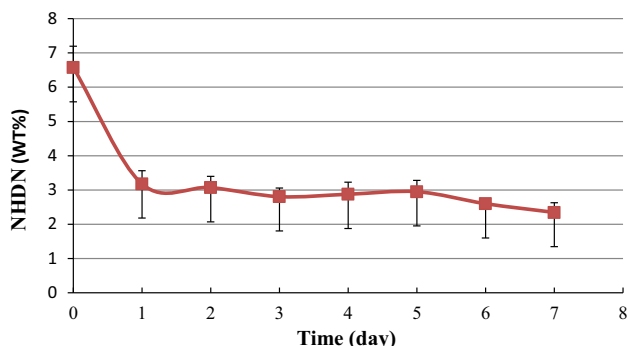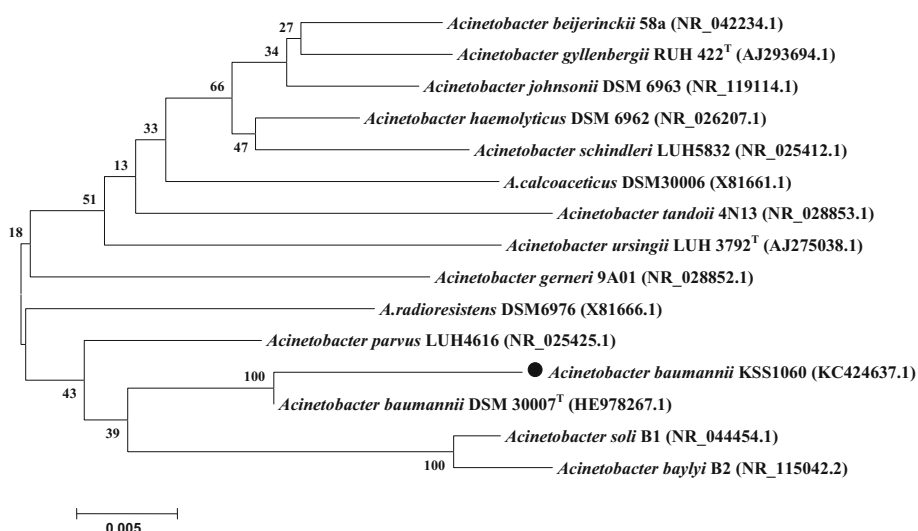

Fig. 4 Changes of *n*-C16 in culture medium of KSS1060 isolate (Incubation time: 7 days)

Beilen and Funhoff 2007). This enzyme is very typical in aerobic oil-degrading bacteria (Rojo 2009). Therefore, the primary products of hexadecane degradation include hexadecanol (alcohol) and hexadecanoic acid (fatty acid), respectively. Fatty acids are conjugated to CoA and further processed by B-oxidation to produce acetyl-CoA (Van Hamme et al. 2003; Wentzel et al. 2007).

According to Fig. 1, the pH changes in the bacterial culture medium were not obviously significant during the first 2 days. Therefore, it is concluded that bacteria could not produce acid during the first 2 days. The optimum pH for the activity of hydrocarbon-degrading enzymes is neutral range (not acidic) (Cases and de Lorenzo 2005). Accordingly, the environmental conditions were favorable for enzymes activity during the first 2 days, and bacteria could consume hexadecane as carbon and energy source and grow easily (Fig. 2). A considerable decrease in pH was observed after 2 days (reduced from pH 6 to 3). These results suggest that acid production has been begun after the second day, and thus, there was no significant reduction in the concentration of hexadecane after second day.
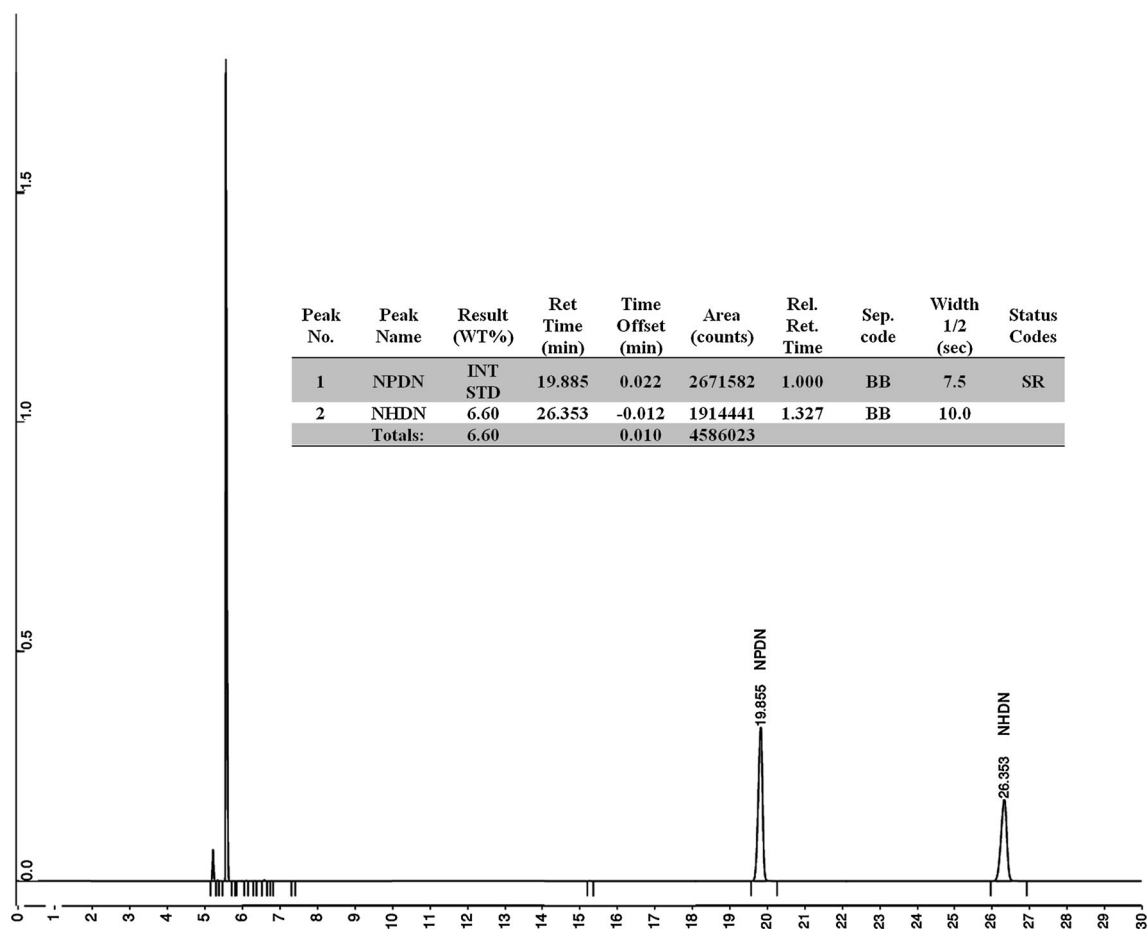
The minimum pH value was observed in the culture medium of strain KSS1060 within 7 days during a dramatically decrease trend (Fig. 1). So, this isolate was able to grow better than other bacterial isolates in the presence of hexadecane, as well as the highest biodegradability. Also, the highest OD belonged to the strain KSS1060 was observed during the first 2 days (Fig. 2). After the second day, the trend continued almost steadily, which is totally in correlation with the utilization of hexadecane in bacterial culture medium (described later).

Changes in pH and OD accompanied with *n*-C$_{16}$ degradation for eight isolates were shown in Figs. 1 and 2, respectively.

As shown in Figs. 1 and 2, pH of the culture medium of all eight bacterial isolates decreased with increasing of bacterial OD. The complete degradation of hydrocarbons mainly occurs under aerobic conditions (Liu et al. 2012). Degradation of *n*-alkanes needs the activation of the substrates by molecular oxygen with the help of oxygenases, which overcomes the low reactivity of the alkane molecules using O$_2$ as a reactant. Oxidation of alkanes leads to the formation of a primary alcohol, which is subsequently converted to aldehyde and then to organic acid. Alkane hydroxylase is one of the key enzymes in aerobic degradation of aliphatic alkanes, which converts aldehyde to organic acid by the removal of two hydrogens and two electrons, and the addition of water to an aldehyde (van

Table 1 Biochemical characteristics of KSS1060 isolate in comparison with other species of the genus *Acinetobacter*

Test	A. junii	A. haemolyticus	A. johnsonii	A. baumannii	KSS1060
Catalase	+	+	+	+	+
Oxidase	−	−	−	−	−
Mac Conkey (Mac)	G	G	G	G	G
NO3 → NO2	−	−	−	−	−
Growth at 37 °C	G	G	NG	G	G
Growth at 41 °C	V	NG	NG	G	G
Citrate (Simmons)	V	V$^+$	+	+	+
Esculin hydrolysis	−	−	−	−	−
Gelatine liquefaction (22 °C)	−	V$^+$	−	−	−
Glucose	−	V	−	V$^+$	+
Malonate	−	−	V	V$^+$	+
Phenylalanine deaminase	−	−	−	V	−

+ positive reaction, − negative reaction, *G* positive growth, *NG* negative growth, *V* variable reaction, *V*$^+$ variable reaction (almost positive)

Peak No.	Peak Name	Result (WT%)	Ret Time (min)	Time Offset (min)	Area (counts)	Rel. Ret. Time	Sep. code	Width 1/2 (sec)	Status Codes
1	NPDN	INT STD	19.885	0.022	2671582	1.000	BB	7.5	SR
2	NHDN	6.60	26.353	-0.012	1914441	1.327	BB	10.0	
	Totals:	6.60		0.010	4586023				

Fig. 5 Chromatogram of residual *n*-hexadecane (NHDN) in inoculated control medium

Bacterial identification

Biochemical analysis

The biochemical analysis revealed that the bacterium KSS1060 was an oxidize-negative, non-fermentative, Gram-negative aerobic, coccoid bacterium, and it was not fastidious and able to grow in simple culture media. It used hexadecane as the sole carbon source. Through its biochemical characteristics, was identified (at 99 % consistency index) to be *Acinetobacter baumannii*. The results of biochemical analysis were shown in Table 1.

PCR amplification and 16S rRNA gene sequencing

The almost complete 16S rRNA gene was amplified and sequenced (850 bp), and the analysis clearly demonstrated

that strain KSS1060 was a member of the genus *Acinetobacter* and exhibited maximum similarity with the 16S rRNA sequence of *A. baumannii* DSM 30007[T] (99.3 % sequence similarity). Also, the phylogenetic tree was constructed by neighbor-joining method and showed that *A. baumannii* KSS1060 and *A. baumannii* DSM 30007[T] were placed in the same clade and both, in higher level, were placed with other strains of *Acinetobacter* in similar group (Fig. 3).

Finally, this isolated bacterium was identified as a member of the genus *Acinetobacter* by biochemical, 16S rDNA sequence, and phylogenetic analysis and designated as *A. baumannii* KSS1060.

Hassanshahian et al. (2012) isolated 25 petroleum degrading bacteria from petroleum contaminated sites in Iran. Additionally, most reports have enforced that *n*-alkane-degrading bacteria isolated from oil-contaminated
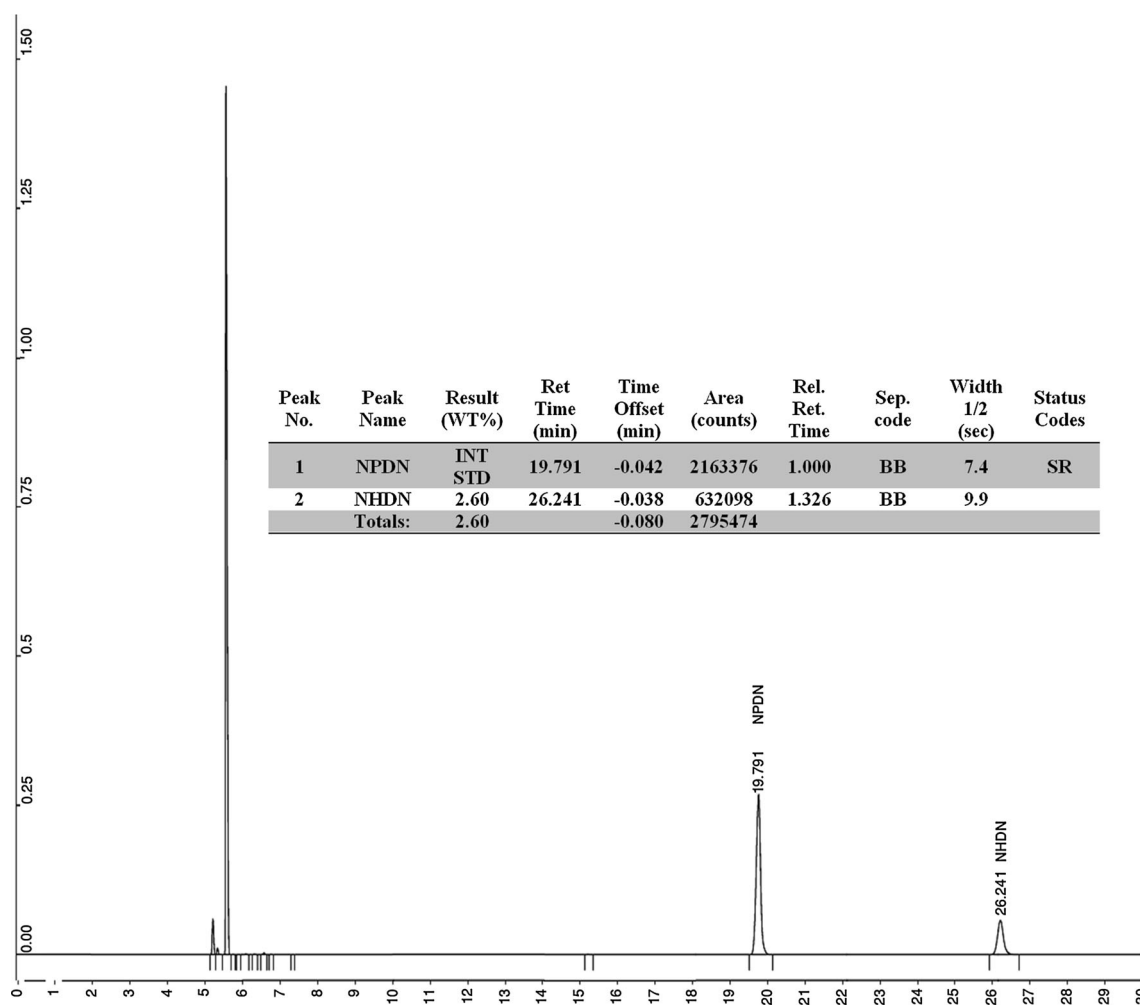

Peak No.	Peak Name	Result (WT%)	Ret Time (min)	Time Offset (min)	Area (counts)	Rel. Ret. Time	Sep. code	Width 1/2 (sec)	Status Codes
1	NPDN	INT STD	19.791	-0.042	2163376	1.000	BB	7.4	SR
2	NHDN	2.60	26.241	-0.038	632098	1.326	BB	9.9	
	Totals:	2.60		-0.080	2795474				

Fig. 6 Chromatogram of residual *n*-hexadecane (NHDN) in inoculated medium after 6 days

Table 2 Composition of wastewater samples

Compound	Formula	Retention time (min)
Hexanal	$C_6H_{12}O$	2.835
Benzene methanol	$C_{13}H_{12}O$	8.445
Indanol	$C_9H_{10}O$	9.712
1,2-benzenedicarboxylic acid diethyl ester	$C_{12}H_{14}O4$	15.838
Diisobutyl phthalate	$C_{16}H_{22}O4$	19.535
Phenol,4,4′-(1-methylethylidene)	$C_{15}H_{16}O2$	23.206

sites are mesophilic strains belonging to different bacterial species such as *Acinetobacter* (Koma et al. 2001), *Nocardia* sp. CF8, *Planococcus alkanoclasticus* (Engelhardt et al. 2001) and *Ochrobactrum* (Yuan et al. 2005). Wang et al. (2006) isolated a novel thermophilic *Bacillus* strain degrading long-chain *n*-alkanes.

In addition, some bacterial species are highly specialized in degrading hydrocarbons and therefore called hydrocarbonoclastic bacteria. They play fundamental role in the removal of hydrocarbons from the contaminated environments (Head et al. 2006). Mahjoubi et al. (2013) showed that *Acinetobacter* were found to be one of the most abundant hydrocarbonoclastic species.

GC analysis

Using the GC method, it was shown that the KSS1060 was able to degrade 62 % of hexadecane within 6 days, which mostly (51.6 %) occurred within the first 24 h (Fig. 4). Chromatograms of residual *n*-hexadecane (NHDN) in inoculated control and after 6 days treatment are shown in Figs. 5 and 6, respectively.

Medium-chain alkanes are major soil contaminants, and among this class of alkanes, *n*-hexadecane (CH_3 $(CH_2)_{14}CH_3$) has been used as a model contaminant of diesel oil by many researchers (Bouchez-Naitali et al. 1999; Noordman et al. 2002).

Members of *Acinetobacter* genus were considered to be a significant part of the alkane-degradative bacteria. Many bacterial strains capable of degrading petroleum hydrocarbons have been isolated (Lu et al. 2006). Further investigations have shown 37 and 86.4 % hexadecane biodegradation after 31 days of culture, by *Aspergillus niger* (Setti et al. 1993) and *Pseudomonas* spp. (Volke-Sepulveda et al. 2003) at initial concentrations of 80 and

12 g l^{-1}, respectively. In addition, Sivaraman et al. (2011) reported that *Pseudomonas mendocina* is able to degrade effectively hexadecane leaving a residual concentration of about 54 % in 120 h.

This finding, together with our recent data, indicates that *A. baumannii* KSS1060 was more effective in degrading hexadecane than others. Therefore, *A. baumannii* KSS1060 seems ideally suited for the remediation of alkane-contaminated areas.

GC and GC–MS analysis

According to the results of GC and GC–MS analysis, the composition of wastewater samples is outlined in Table 2.

The characterization of the chemical composition of wastewater samples by the use of GC–MS analysis indicated the presence of Hexanal, Benzene methanol, Indanol, 1,2-benzenedicarboxylic acid diethyl ester, diisobutyl phthalate, and Phenol,4,4′-(1-methylethylidene) in the major constituents of the wastewater. Additionally, GC–MS results indicated the absence of *n*-hexadecane in the wastewater samples; thus, these results provide evidences that *A. baumannii* KSS1060 alone or in combination with other microorganisms have a high performance on biodegradation of *n*-hexadecane.

Conclusion

In conclusion, this study can focus on more cost-efficient applications of native bacterial strains for the large-scale biodegradation of wastewater samples from petrochemical plant in industry, where it causes disturbing problems due to its harmful effects on different organisms and human beings.

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