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HIGH POTENTIAL OF *Enterobacter* sp. CASPIAN1394 IN DIESEL OIL BIODEGRADATION

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Abstract

Diesel oil (DO) as a mixture of alkanes and aromatic compounds causes damage to the ecosystem in terrestrial and aquatic habitats. One of the best methods of treating contaminated areas is to use microorganisms in a bioremediation method that can degrade pollutants. The aim of this study was to isolate indigenous bacteria with high biodegradation potential of DO from oil-contaminated wastewater. Bacterial isolation was performed using a mineral medium containing contain 1% DO as the sole source of carbon and energy. The isolates were identified using differential biochemical tests and *16S rDNA* gene analysis. The effects of 2, 3, 4 and 5% concentrations of DO on isolate growth were evaluated. Gas chromatography (GC) was used to determine the quantity of degraded DO. *Enterobacter* sp. CASPIAN1394 (EC strain) could degrade 99.44% of 1% DO over two weeks as the studied bacterium. Alkanes C11, C12, C16-C18 and C29-C32 had been completely degraded. The amount of total petroleum hydrocarbon (TPH) at 2, 3, 4 and 5% DO concentrations were 93.25%, 86%, 95.7% and 86.49%, respectively. Increasing DO concentration had no toxic effect and did not reduced bacterial growth, even to some extent increased its growth. With regard to the rate of degradation of different DO concentrations by EC strain and the good potential of this strain for the degradation of different DO hydrocarbons, EC strain may be a candidate for bioremediation in DO-contaminated wastewater. However, the environmental variables need to be studied affect the degradation of DO by this strain.

Keywords: bioremediation, diesel oil, *Enterobacter sp.*, gas chromatography, wastewater

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1. Introduction

The main issue in petroleum-contaminated soils is that they reach groundwater sources and affect the quality of surface water. Activities such as the extraction, transport, refining, storage, use and ultimate disposal of these non-aqueous phase liquids composed of a large amount of hazardous components pose a significant threat of contamination of the environment. Accidental leakage of ships, cars, trucks, underground storage tanks, pipelines and illegal disposal leads to contamination of the marine and underground environment (Kingston, 2007).

Petroleum hydrocarbon components include volatile and non-volatile aromatic compounds, as well as aliphatic fractions (Stroud et al., 2007). Diesel oil (DO) is a complex hydrocarbon pollutant that is a mixture of alkanes and aromatic compounds (Gallego et al., 2001). DO consist of four major hydrocarbon structural groups, including n-alkanes or n-paraffins, isoalkanes or isoparaffins, cycloalkanes or naphthens and aromatics. Diesel oil consists mainly of aliphatic hydrocarbons (> 90%) and lower levels of aromatic hydrocarbons (< 5%) (Stroud et al., 2007). DO contain between 2000 and 4000 hydrocarbons, which cannot be completely segregated by gas chromatography. In reality, this technique can separate only n-alkans and a few branched hydrocarbons (Olson et al., 1999). To achieve remediation objectives, however, it is important to address the removal of large quantities of aliphatic hydrocarbons accumulated in the soil. Furthermore, DO mid-length aliphatic hydrocarbons

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are less biodegradable due to non-polar, non-volatile, water-insoluble and limited bioavailability properties (Abbasian et al., 2015; Stroud et al., 2007). Recent studies have shown that in unsaturated soil conditions, C18-C22 hydrocarbons of DO are not readily degraded compared to other aliphatic hydrocarbons (Bajagain et al., 2018).

Mechanical, burial, evaporation, dispersion and washing techniques are extensively used for soil remediation. However, these techniques are costly and can lead to incomplete pollutant degradation (Medina-Bellver et al., 2005).

One of the best methods to restore contaminated soil or wastewater is to use microorganisms that can degrade toxic compounds in a method of bioremediation (Medina-Bellver et al., 2005). Bioremediation is a cost-effective strategy to removing petroleum hydrocarbons from contaminated regions because it is easy to maintain, applicable across large fields and leads to complete pollutant destruction (Makkar and Rockne, 2003). Microorganisms inherent ability to overcome the constraints of bioavailability in multi-phase environmental situations (oil–water–soil) and environmental variables including temperature, pH, nutrients and accessibility of electron acceptors have a significant impact on these pollutants (Makkar and Rockne, 2003).

Under aerobic conditions, the most fast and complete degradation of most organic pollutants occurs (Fritsche and Hofrichter, 2000). Oxidizing reactions have been shown to use the degradation pathways of a number of petroleum hydrocarbons (e.g. aliphatics and polyaromatics). However, these pathways differ greatly because of the specific oxygenases found in different bacterial species (Tremblay et al., 2017). Other procedures involved are (1) binding of microbial cells to substrates and (2) biosurfactant production (Hommel, 1990).

Hydrocarbons in the environment are mainly biodegraded by bacteria, yeast, and fungi. Bacteria are the most effective agents in the degradation of petroleum. They operate in the environment as main degraders of spilled oil. It is even known that several bacteria feed solely on hydrocarbons (Hommel, 1990). The species of *Acinetobacter*, *Pseudomonas*, *Rhodococcus*, *Alcanivorax*, *Bacillus*, *Stenotrophomonas*, and *Arthrobacter* have been widely applied for the degradation of aliphatic hydrocarbons (Chandra et al., 2013; Stroud et. al 2007; Varjani 2017). Several studies have shown that there are a large number of hydrocarbon degrading bacteria in oil-rich areas, such as oil spills and oil reservoirs (Yang et al., 2015), and that their frequency and quantity are closely related to the types of petroleum hydrocarbons and the environmental factors surrounding them (Fuentes et al., 2015; Varjani and Gnansounou, 2017).

Bioremediation of petroleum spills has two primary strategies: (a) bioaugmentation, adding known oil degrading bacteria to supplement the current microbial population, and (b) biostimulation, stimulating the growth of native petroleum degraders by adding nutrients or other growth-limiting cosubstrates (Medina-Bellver et al., 2005).

This study focused on DO and selective n-alkanes biodegradation. There are no previous reports on the ability of *Enterobacter* spp. to bioremediation of wastewater contaminated with DO hydrocarbon residual fractions.

The main aims of the present study are (1) to isolate, identify and describe the DO hydrocarbons-degrading strain, (2) to determine the trend of biodegradability and to identify the most biorecalcitrant aliphatic residual fraction of DO, and (3) evaluate the degradation of hydrocarbons of DO using the newly isolated bacterial strain.

2. Material and methods

2.1. The wastewater samples

The contaminated wastewater was sampled from effluents of petroleum exporting company in Rasht, Guilan province (N37/26268OE/6117O). Wastewater samples were collected in 1000 ml amber glasses, placed on ice after sampling and subsequently transported to the laboratory for further study of total petroleum hydrocarbon (TPH) and poly aromatic hydrocarbons (PAHs) as well as biological oxygen demanded (BOD) (APHA, 2011; US EPA, 1986).

TPH was extracted with 125 ml of dichloromethane. The program of extraction and concentration was as follows: the heating plate temperature ramped up to 140 °C, followed by 50 min of hot extraction and six concentration intervals, each of which lasted 3.5 minutes. Then, the extract was filtered through a 0.2 μm nylon membrane and assayed using a gas chromatograph (GC) (Shimadzu GC-2010) equipped with a flame ionization detector (FID) (Shimadzu). TPH was measured in accordance with US EPA 8015B method guidelines (US EPA, 1986). The column and analytical parameters were as follows: column of chromatography (Restek Rxi-1HT, 30m×0.25mm×0.25μm). Oven temperature program: initial 60 °C, hold time 1 min, ramp of 8°C/min to 290°C, hold time 30 min; total run time: 59.75 min; sample/autosampler injection: 1μL; carrier gas, hydrogen (H_2) at 3 mL/min; oxidiser, air at 400 mL/min; fuel, hydrogen at 32 mL/min; injector, AOC-20i autoinjector with a split/splitless (SPL) injection unit, injection port temperature: 285°C; and FID temperature: 315°C (US EPA, 1986).

TPH has been characterized as the collective concentration of all C10 to C35 hydrocarbon compounds. Aliphatic hydrocarbon calibration was created by diluting a standard alkane mixture C10-C35 at five or six concentrations.

An Agilent 6890N GC equipped with an Agilent 7683B Injector, HP-5MS capillary column coated with 5% phenyl-methylsiloxane (film thickness 0.25 μm) and an Agilent 5975 mass selective detector (MSD) was applied to separate and quantify the PAHs. The samples were injected at a temperature of 300°C

in the splitless mode. Initially, the column temperature was held at 40°C for 1 min, raised to 120°C at 25°C/min, then to 160 °C at 10 °C/min, and finally at 5°C/min at the final temperature for 15 min. The temperature of the detector was held at 280°C. Helium was used at a constant flow rate of 1 mL/min as a carrier gas. Mass spectrometry was acquired using modes of electron ionization (EI) and selective ion monitoring (SIM). The identification of PAHs in the samples was confirmed by the retention time and abundance of quantification ions in the authentic PAH standards. Sixteen priority PAHs were quantified using the corresponding internal standards response factors based on the five-point calibration curve for individual compounds (Chen and Chen, 2011).

To determine the rate of degradation and use of PAHs by isolated strain, BOD was analyzed using standard methods (APHA, 2011).

2.2. Isolation and screening of DO-degrading bacteria

One ml of wastewater sample spread on the surface of nutrient agar plates and the plates incubated at 37°C for 24 h. To obtain the pure colonies, the colonies obtained from the agar plates were further sub-cultured. The bacterial species were screened based on the degradation of DO Studies of degradation were performed in Mineral Salt Medium (MSM), which contains DO as the sole source of carbon. The isolated bacterial species were cultured in MSM with filtered sterile DO 1% and incubated at 37°C for 3 days. The growth of the isolated bacterial species was controlled by measuring the optical density at 600 nm at regular intervals. The best degrader of DO was selected based on the growth of bacterial species on DO degradation (Palanisamy et al., 2014; Ramasamy et al., 2017).

2.3. Identification of DO-degrading bacteria

DO-degrading bacteria have been identified by gram stain, biochemical tests according to Bergey's bacteriology handbook and confirmed by *16S rDNA* sequencing (Holt et al., 1994).

DNA extraction was performed with one colony suspended in 100 µL of distilled water (95°C for 10 min) followed by cell suspension centrifugation. The PCR reactions were conducted by the Thermal Cycler PCR system (BioRad, USA) (Lane, 1991). The *16S rRNA* gene was amplified using universal *16s rDNA* primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5'TACGGYTACCTTGTTACGACTT3').

The PCR products were purified by gel extraction kit and were sequenced (Macrogen Company) and the sequences data obtained were edited (CLC Genomic workbench 5) and edited sequences were compared with bacterial *16S rDNA* sequences in GenBank using BLAST program. Neighboring phylogenetic trees (Saitou and Nei, 1987) were constructed using version 7 of the program Molecular Evolutionary Genetics Analysis (MEGA) (Tamura et al., 2007). To estimate the reliability of phylogenetic reconstructions, Bootstrap analysis (5000 replicates) was performed (Saitou and Nei, 1987).

2.4. Biodegradation of DO

2.4.1. Growth rate and biodegradation

The conical flask of 250 mL containing 100 mL MSM supplemented with 1% v/v filter-sterilized DO as the source of carbon and energy, inoculated with 1 mL of DO-degrading bacterial suspension to determine the growth curve (Rahman et al., 2004). For the determination of the biodegradation potential of DO by bacteria, 1 mL of 0.5 MacFarland bacterial suspension was added to 250 mL conical flask containing 100 mL of MSM with different concentrations of 1%, 2%, 3%, 4% and 5% v / v DO as carbon and energy source (Palanisamy et al., 2014).

2.4.2. Extraction and analysis of residual diesel/n-alkanes by GC

The residual diesel oil in the liquid culture was extracted three times with dichloromethan on a separate funnel. The organic phase was then dehydrated with anhydrous sodium sulfate (Na_2SO_4), and 1 µL of the organic phase was analyzed by GC apparatus (Agilent 6890) equipped with a flame ionization detector (FID), a splitless injector and the HP5 capillary column (0.32 m in length; 0.32 mm in internal diameter; 0.25 µm in film thickness). Nitrogen was used as a carrier gas, the injection volume was 2 mL, and a splitless injection mode was applied. The programming of the oven temperature was as follows: initial temperature 50°C, with holding time 1 min; ramping at 10°C/min up to 150°C, with holding time 1 min; ramping at 5°C/min up to 200°C, with holding time 1 min; and the final ramping at 25°C/min up to 280°C, with holding time 15 min; total running time was 41.2 min. The temperature of injector was set at 200°C while the temperature of detector was set at 280°C. An n-alkane reference calibration mixture containing 25 n-alkanes over the range C10-C35 was used for both qualitative and quantitative analysis. Calibration curves were prepared from calibration mixture dilutions over the range of 0.01–0.5 mg/mL for individual n-alkane (Luo et al., 2013; Mohanty and Mukherji, 2008).

3. Results and discussions

3.1. Wastewater analysis

One of the main soil and groundwater pollutants are fuels due to underground storage tank leaks and transfer line defectiveness (Marchal et al., 2003). DO is one of the main fuels and contains rich lightweight hydrocarbons and PAHs (Ramasamy et al., 2017). DO has been reported as contaminant from seawater and soil (Palanisamy et al., 2014). DO components are potentially carcinogenic and

environmentally toxic, and then the existence of DO in seawater and soil will have severe effects on the marine ecosystem (Prathyusha et al., 2016). Bioremediation is the best practice to remove DO spills in seawater and soil, taking into account latest findings. Some bacterial strains from a DO-contaminated region can produce degrading enzymes and use DO as a source of carbon and energy (Patil et al., 2012). The quantity of TPH analyzed by GC-FID in wastewater was 23.38 mg/L and the hydrocarbon chromatogram spectrum was shown in Fig. 1 and Table 1. The types and quantities of PAHs measured by GC-MS in wastewater were shown in Fig. 2 and Table 2. As shown in Table 2, naphthalene content was at least twenty times that of the other compounds. Wastewater BOD was 3743 mg/L. The wastewater sample used in this study was highly contaminated by oil tankers as confirmed by the quantity of TPH, PAHs and BOD.

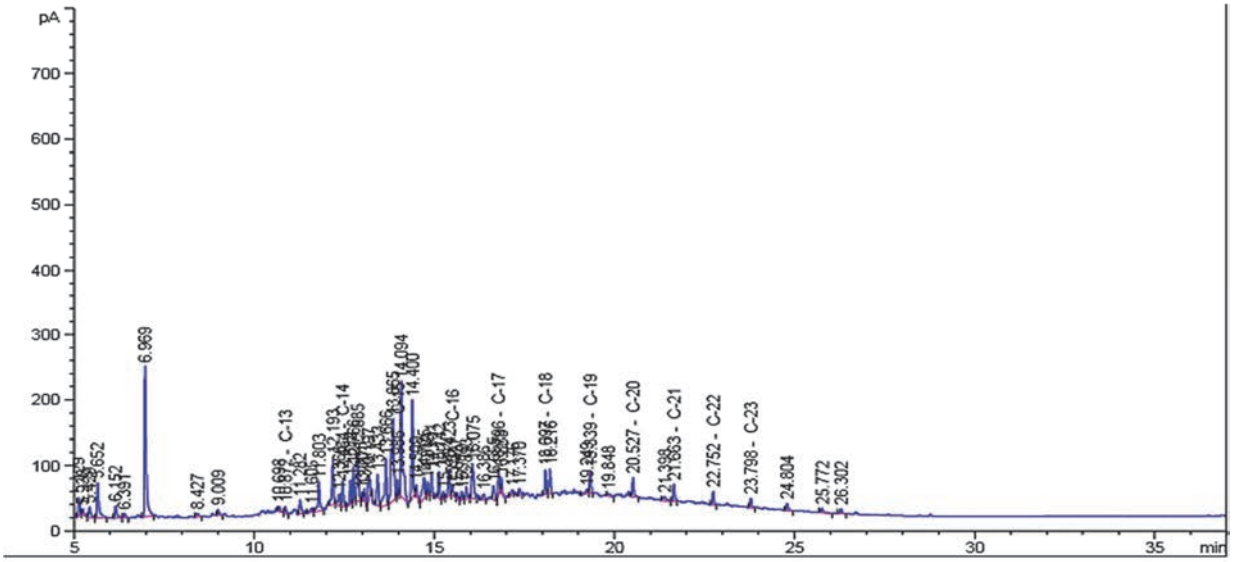


Fig. 1. The chromatogram of TPH from wastewater. The peaks show hydrocarbon compounds with the number of carbon atoms

Table 1. GC-FID profiles of TPH obtained from wastewater

Name	*RT (min)*	*Area*	*Amount (mg/L)*
C-10	5.719	-	-
C-11	7.477	-	-
C-12	9.234	-	-
C-13	10.872	33.61066	1.11954
C-14	12.464	112.15997	3.01531
C-15	13.986	64.13596	1.79202
C-16	15.511	51.28268	1.45496
C-17	16.796	84.18380	2.31201
C-18	18.097	114.83054	3.05093
C-19	19.339	104.55750	2.79497
C-20	20.527	99.02407	2.58439
C-21	21.663	80.47615	2.15779
C-22	22.752	64.45078	1.76572
C-23	23.798	44.90230	1.33087
C-24	24.943	-	-
C-25	25.914	-	-
C-26	26.849	-	-
C-27	27.752	-	-
C-28	28.626	-	-
C-29	29.469	-	-
C-30	30.194	-	-
C-31	31.078	-	-
C-32	31.846	-	-
C-33	32.626	-	-
C-34	33.634	-	-
C-35	34.822	-	-
			TPH= 23.37850

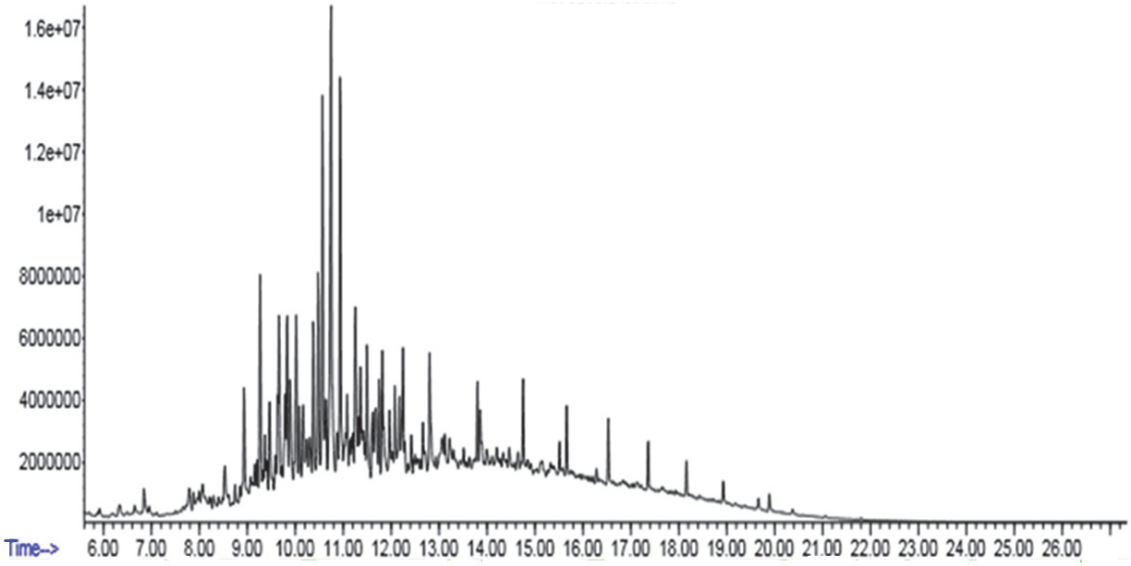


Fig. 2. The chromatogram of wastewater. The peaks show different PAHs

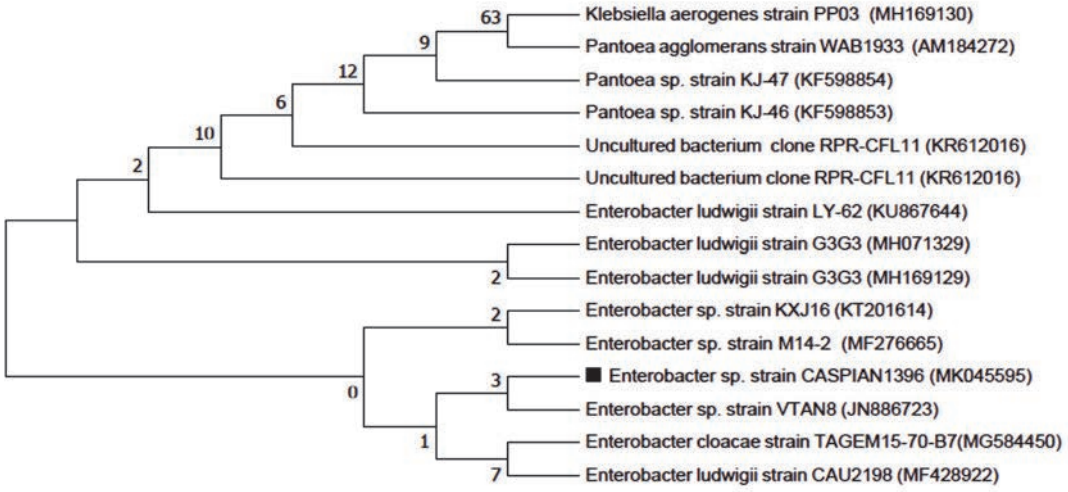


Fig. 3. Phylogenetic tree was constructed from the GenBank database using a neighbor-joining method based on the EC strain's *16S rDNA* gene sequence and associated bacteria. Bootstrap probabilities are shown close the nodes and parentheses are provided for GenBank accession numbers. The strain isolated and identified with the solid black square is shown (■)

Table 2. The PAHs from wastewater measured using GC-MS

PAHs	***(µg/L)***
Naphthalene	14
Acenaphthalene	<0.7
Acenaphthylen	<0.7
Fluorene	<0.7
Phenanthrene	<0.7
Anthracene	<0.7
Fluoranthene	<0.7
Pyrene	<0.7
Chrysene	<0.7
Benzo[a]anthracene	<0.7
Benzo[b]fluoranthene	<0.7
Benzo[k]fluoranthene	<0.7
Dibenz[ah]anthracene	<0.7
Indeno[1,2,3-cd]pyrene	<0.7
Benzo[ghi]perylene	<0.7
Benzo[a]pyrene	<0.7

3.2. Isolation of DO-degrading bacteria

Based on the capacity to use DO, isolated bacterial strains were screened. There were isolated three strains of DO-degrading bacteria. After 14 days of incubation, the growth and biodegradation rate of DO by one strain (EC) was 99.4%, because of more growth in a MSM containing 1% DO as the sole source of carbon and energy. Consequently, this strain was identified in more detail and selected for further research of its DO degrading properties.

3.3. Identification of DO-degrading bacteria

EC strain was described by morphological and biochemical tests and by an analysis of *16S rDNA*. This strain was identified as Gram-negative, straight-shaped and non-endospore-forming and had positive tests for the production of motile, indole, methyl-red,

CO_2 and lactose, sucrose, malonate and citrate and negative biochemical tests for the production of Voges Proskuaer and H_2S. The EC strain could not be identified on the basis of these results. The EC strain's *16S rDNA* gene sequence was sequenced and used for further analysis to construct a phylogenetic tree. The *16S rDNA* gene sequence was a continuous sequence of 1354 bp.The similarities between this strain *16S rDNA* sequence and the bacterial sequences deposited in the GenBank databases were calculated, and the EC sequence showed 100% similarity to those of *Enterobacter* and *Pantoea* genera. The EC strain was categorized in the *Enterobacter* genus based on the phylogenetic analysis (1387 unambiguous bases aligned). A phylogenetic tree was constructed based on the neighbor-joining method, indicating that the nearest EC strain relative was *Enterobacter* sp. VTAN8 (Fig. 3). Therefore, the EC strain was identified and associated with *Enterobacter* sp. CASPIAN1394 and *16S rDNA* sequence is available under the accession numbers MK045595 of GenBank

3.4. Biodegradation of DO

3.4.1. Growth rate and biodegradation

To study the growth rate and extent on DO, this survey was performed for 28 days. The use of the n-alkanes was followed by the growth of culture. On DO, the EC strain showed good growth. The lag phase seemed to be long and lasted about 8 days. From the eighth to the twentieth day, it has been slow in the exponential phase, but since the twentieth day the rate of growth has increased rapidly. A threefold increase in culture concentration was observed over a period of 20 days (Fig. 4).

Biodegradation rate and extent were interpreted on the basis of GC chromatograms for residual DO. The peak identities were first confirmed in the chromatograms for residual DO extracted at time zero with the external reference calibration mix for n-alkanes by matching the relative retention time (in regards to the internal standard). In the DO chromatograms, the resolved n-alkane peaks were found to be in the range of carbon numbers C10-C35 (Fig. 5). All alkanes in DO were degraded except for C33-C35, but the last compounds were either very low or not in the control. Alkanes C11, C12, C16-C18 and C29-C32 have been completely degraded (Table 3). The relative composition of n-alkanes in DO is expressed as a percentage of the total diesel range or TPH of n-alkanes. Table 3 showed the rate of degradation of each alkan by the EC strain in the control and the samples tested. The resolved chromatogram peaks (Figs. 5a and b) represented residual DO in control and experimental flasks after 15 days (end of the biodegradation study) and showed modifications due to biodegradation.

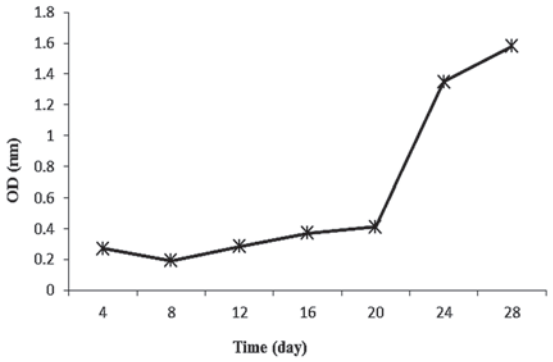
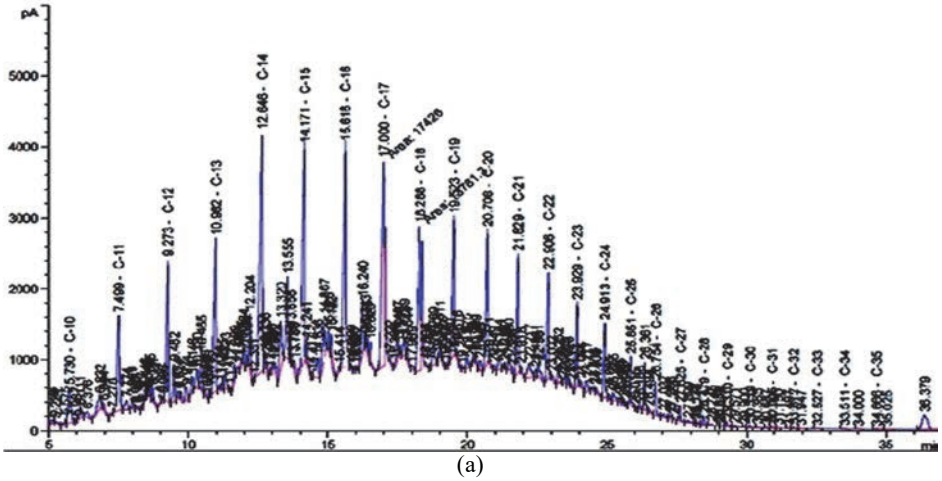
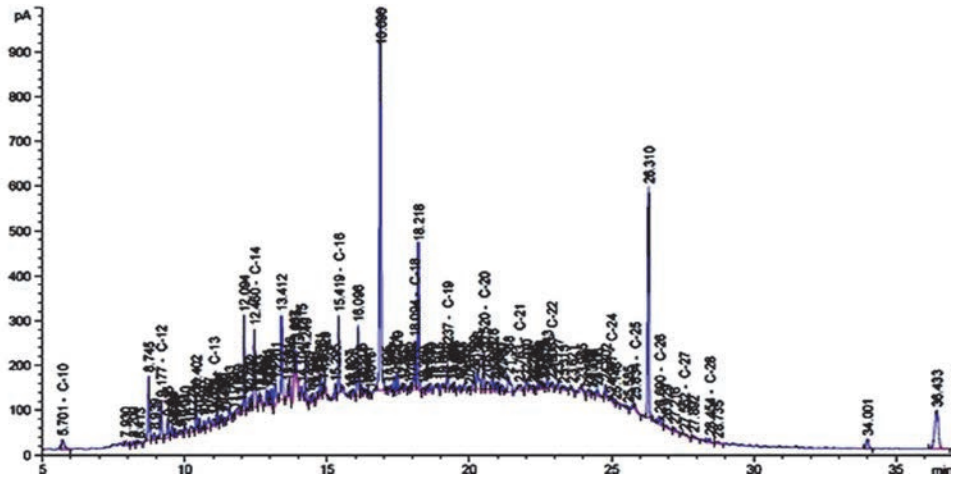


Fig. 4. The EC strain growth curve in MSM contains 1% DO as the sole source of carbon and energy

Within two weeks, this strain was able to degrade DO by 99.4% and degrade hydrocarbons with a wide range of carbon numbers simultaneously. This strain efficacy was good in DO degradation, enabling it to completely degrade some hydrocarbons, including C11, C12, C16-C18 and C29-C32, and approximately 100% others. No correlation existed between the degradation rate of hydrocarbons and the number of carbon atoms. In other words, there was a uniform rate of hydrocarbon degradation. Moreover, the degradation of many hydrocarbons occurred with increasing concentrations up to 5%.



(a)



(b)

Fig. 5. Chromatogram of DO extracted from (a) control and (b) experimental flask at 15th day in biodegradation study with EC strain

Table 3. Relative composition and retention time of n-alkane components from DO in control and of reference calibration mixture

	DO components (control)			*DO components (14 days)*			
Names	***RT (min)***	***Area***	***Amount (mg/L)***	***RT (min)***	***Area***	***Amount (mg/L)***	***Degradation rate (%)***
C-10	5.729	3613.62573	96.68988	5.701	145.50696	3.81418	96.05
C-11	7.499	7970.16406	212.66424	7.500	-	-	100
C-12	9.273	9694.69434	251.05516	9.270	-	-	100
C-13	10.982	1.12855e4	296.42239	11.026	73.51593	1.87969	99.36
C-14	12.646	2.04769e4	537.03396	12.613	20.98314	4.30185e-1	99.91
C-15	14.171	1.73434e4	440.67265	14.104	78.24102	1.93329	99.56
C-16	15.616	1.85722e4	465.64621	15.610	-	-	100
C-17	17.001	1.64018e4	416.56190	17.000	-	-	100
C-18	18.289	1.31281e4	331.01029	18.280	-	-	100
C-19	19.523	9264.90527	233.23905	19.486	54.96503	1.33072	99.42
C-20	20.708	8919.57617	220.71895	20.611	122.76286	2.98190	98.64
C-21	21.829	7671.92822	190.86646	21.763	85.61726	2.07822	98.91
C-22	22.906	6661.69629	164.43230	22.903	53.43309	1.26562	99.23
C-23	23.929	4752.18652	117.01686	23.965	69.06590	1.64647	98.59
C-24	24.913	4294.04834	105.11059	24.997	41.44620	9.44712e-1	99.10
C-25	25.851	2805.04297	69.42492	25.834	42.22247	9.92929e-1	98.57
C-26	26.754	1769.09229	42.98792	26.690	97.51480	2.31809	94.60
C-27	27.625	805.49292	20.87773	27.562	37.77682	8.88974e-1	95.78
C-28	28.479	503.42191	12.30297	28.454	39.93691	9.10387e-1	92.60
C-29	29.310	309.36273	7.81970	29.310	-	-	100
C-30	30.119	195.15681	4.59043	30.100	-	-	100
C-31	30.905	127.64489	3.03970	30.900	-	-	100
C-32	31.677	85.43870	2.00240	31.670	-	-	100
C-33	32.359	-	-	32.359	-	-	-
C-34	33.694	-	-	33.694	-	-	-
C-35	34.295	-	-	34.295	-	-	-
			TPH= 4242.18666			**TPH=23.41538**	99.44

The amount of TPH DO at different concentrations including 2, 3, 4 and 5% was 93.25%, 86%, 95.7% and 86.49%, respectively. The observations showed that the increase in DO concentration had no toxic effect and did not reduce the growth of the bacteria; even to some extent increased their growth (Fig. 6). As shown in Fig. 6, by increasing the DO concentration from 2% to 3%, the sub-peak area or the degradation efficiency slightly decreased but increased again at concentration 4%. However, EC strain was able to tolerate 5% of the DO concentration and degrade it. Mohanty and Mukherji (2008) reported that isolated *Exiguobacterium aurantiacum* and *Burkholderia cepacia* could degrade 1% DO by 52.93% and 51.37%, respectively and some of the fewer hydrocarbons, such as C9, C17-C19 and C26 were also degraded completely. These strains showed uniform degradation rates for a broad spectrum of n-alkanes from C12 to C26 (Mohanty and Mukherji, 2008).

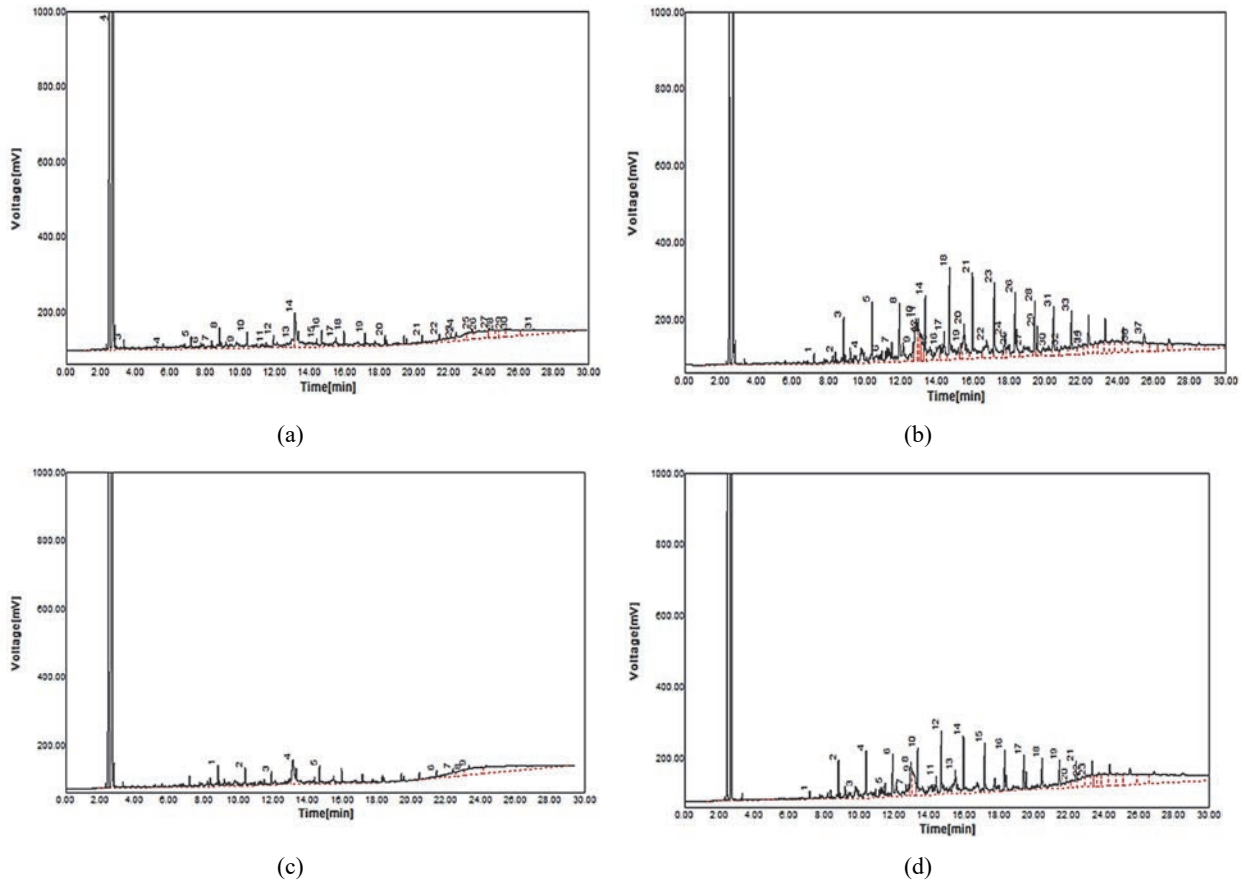


Fig. 6. Chromatographic profiles of residual hydrocarbons at various concentrations of DO including 2% (a), 3% (b), 4% (c) and 5% (d) extracted from experimental flasks at the 15th day of the EC strain biodegradation study

Acinetobacter baumannii isolated from DO-contaminated soil had the highest growth rate at 4% concentration among different concentrations of DO (1%, 2%, 3%, 4% and 5%) (Palanisamy et al., 2014). *Acinetobacter* isolated from oil-contaminated wastewater could degrade almost entirely all components (C11-C21) in 2% DO with a degradation ratio of up to 80% after 10 days of incubation under optimal conditions. By increasing the DO concentration to 2.5% and 3%, the degradation rate decreased (Luo et al., 2013).

The *Nocardia* sp. H17-1 isolated from oil-contaminated soil could degrade more than 95% of the aliphatic compounds after 6 days of incubation, while less than 25% of the aromatic compounds were degraded. This strain was able to break down the C12-C26 n-alkanes, but the degradation rate decreased considerably from C-26 to higher (Baek et al., 2006).

Contrary to our study, Raju et al. (2017) observed a rapid exponential growth in microorganisms isolated from DO-polluted soil. The lag phase of two species of *Bacillus* sp. studied by Raju et al. (2017) lasted 12 hours and the exponential phase continued for 6 days. In their research, the highest growth rate of both strains was observed at a concentration of 2% DO and growth decreased considerably with increased concentration to 2.5%.

Biodegradation was observed for n-alkanes (C14-C18) by *Pseudomonas fluorescens*, up to 65% in 8 d. Abiotic losses have also shown that these compounds are less affected. In the first 20 d of incubation, the majority of n-alkanes were significantly degraded (Sepic et al., 1996).

Different treatments for DO biodegradation undertaken by Mariano et al. (2007) in sandy soil with an elevated rate of contamination originated from DO leakage. Their trials showed that hydrocarbon biodegradation from C11-C14 were greater in most treatments than other hydrocarbons (Mariano et al., 2007).

Biotic losses have generally been shown to be higher for aliphatic compounds than for aromatic compounds. In the case of DO and its aromatic fraction, abiotic losses were smaller than in the case of a single standard compound or a mixture of standards (Sepic et al., 1996).

Lee et al. (2006) demonstrated that *Rhodococcus baikonurensis* DSM 44587T isolated from oil-contaminated soil could degrade 100%, 64.9%, 60.7% and 30.5% of the concentrations of DO including 0.1%, 0.5%, 1% and 2% respectively, after 7 days of incubation. In their study, the most significant degradation occurred during the first 3 days and degradation reached a plateau between days 4 and

7 (Lee et al., 2006). *Pseudomonas* sp. strain DRYJ3 isolated from Antarctica grew optimally in 3.5% DO and cellular growth dramatically decreased at diesel concentrations higher than this (Shukor et al., 2005).

4. Conclusions

The findings indicate that the degradation of the DO in the quantities used does not depend on their concentration. EC Strain has a good potential for the degradation of aliphatic hydrocarbons in DO, and the high biodegradation of these hydrocarbons does not seem to result from abiotic losses such as a volatilization, and perhaps a small percentage of these hydrocarbons are removed by abiotic processes.

It was also proved that the first 15 d of incubation were the most important for biodegradation, during which the majority of degradable aliphatic hydrocarbons were degraded. Regarding the rate of degradation of various and high concentrations of DO by EC strain, and also the good potential of this strain for the degradation of various hydrocarbons in this fuel, EC strain may be a candidate for bioremediation of this fuel in DO-contaminated wastewaters.

However, it is necessary to study the environmental factors affects DO degradation by this strain.

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