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# Isolation, Screening and Activity of Hydrocarbon-Degrading Bacteria from Harsh Soils

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***Abstract*** **-** The study of microbial communities in relation to their ecology, interaction, and genetics must be covered in Qatar with special soil, weather and water. Microorganisms of an environment never having been exposed to such factors do not have necessarily the ability to resist. However, when exposed to such factors, they are often able to adapt, that is to say the potential to acquire metabolic potentialities by recruitment vertical or horizontal specific genes, or recruitment of metabolic pathways present in the genome but not expressed. In polluted environments with oil hydrocarbons, the phenomena of horizontal/vertical transfers, mutations and adaptation to environmental stressors increase the metabolic capabilities of bacteria, including expanding their range of substrates, and allow their adaptation to new substrates. Extensive research on such fields is often focusing on genetic studies, meaning that the genes pools and expression as well as species occurrence are considered as basis. However, from the biotechnological point of view, the cell is considered, as an analogy, as factory. All its components (physical structure and the functions of the organelles and metabolic activities) are strictly regulated by regulatory machineries strictly controlled by the microenvironment. This approach will help answer the question on the different microbial communities that are available in Qatar soil and how they are unique in some biological activities. Indeed, the focus of our research is to characterize and demonstrate the diversity of biological activities within bacterial communities and strains of the same species. Here, we consider the bacterial activities related to polluting oil hydrocarbon-degrading communities, including those which are biosurfactant-producers,

Here, we showed that the isolation and screening strategy affected a lot, the selection of the hydrocarbon-degrading bacteria, by combining the bacterial tolerance to hydrocarbon toxicity to the hydrocarbons degradative activities. We demonstrated that several Qatari isolates are able to shift their activity from a range of hydrocarbons to another by the effect of the nitrogen source, C/N ratio and organic nitrogen source. By application of Factorial Investigation of Strains' Potentiality to Degrade Hydrocarbons, which is original in the attempt to integrate the study of the most essential nutritional elements as well as their interactions in one experimental frame, we demonstrated diversity of nutritional requirements among the same bacterial group of *Pseudomonas aeruginosa* strain, isolated in Qatar. Strains' response to altered growth conditions varied substantially; although they were from the same taxonomical group.

***Keywords*:** Bioremediation, hydrocarbons, Diesel, petroleum, polluted soils; biodegradation

## 1. Introduction

Remediation of contaminated soil and groundwater is vital to minimize the effects of accidental hydrocarbon releases and to protect these limited resources in many countries [1]. The oil polluting compounds are light hydrocarbons (oil, gasoline, diesel), heavy hydrocarbons (lubricants, heavy oil, crude oil), halogenated solvents, and other more complex molecules (aromatic hydrocarbons polycyclic, PAHs, etc.) [2]. Organic compounds are involved in nearly 75% of pollution of such polluted sites.

Microbial remediation is typically the preferred method for remediating subsurface hydrocarbon contamination, though it is often limited by low hydrocarbon aqueous solubility and strong sorption to soil [3]. On the other hand, it is obvious that exposure of hydrocarbons to the environment at polluted sites subjects them to various chemical, physical and biological degradation mechanisms such as oxidation, biodegradation and photo degradation [4]. Such factors make it difficult to predict and estimate the type of petroleum products in the polluted soil [3]. In addition, the physical and chemical characteristics of soils as well as weather conditions, affect a lot the chemical stability of the pollutants but also their availability to biodegradation. The hydrocarbons' composition, structure and occurrence depend on the pollution

history, soil properties and weather conditions, affecting the occurrence, stability and biological activities of hydrocarbon-degrading bacteria [5, 6]. In fact, this represents the main origin of failure of most of the bioremediation applications for cleaning polluted areas with hydrocarbons. It has been shown that the capacity of the microorganisms to degrade pollutants is not a result of just adding any type of species to polluted ecological sites, but the capacity of individual strains to produce appropriate biosurfactants and form a strong biodegrading association, and their ability to survive in the soil under natural conditions and with limited available nutrients and water [7, 8]. However, the extreme weather in certain regions leads over prolonged periods of time to the selection of adapted microorganisms to harsh conditions, but also to acquire new potentialities to remediate special composition of organics from soil or water [6, 9]. We consider that bioprocesses of natural remediation in Qatar, an Arabian Gulf Country, is a suitable model to study hydrocarbons-degrading bacteria and how they deal with high variable composition and structure of hydrocarbons, especially under the extreme environmental conditions, and dry soils in Qatar [10].

In this study, we intend to isolate, screen and select bacterial strains able to degrade petroleum hydrocarbons specifically in recently or continuously polluted soils as well as area polluted over a very long period of time, all at the Qatari conditions. Ability of these bacteria to interact with a variable composition of pollutants is investigated through their growth, tolerance to toxicity and range of removed hydrocarbons.

## 2. Material and Methods

### 2.1. Soil Samples

Several amples were collected from various oil-contaminated sources, including the polluted seashore, near gas stations and in automotive workshops, using a sterile spatula at a tillage depth of 1-2 cm, randomly from different points.
The soil samples were collected into sterilized glass bottles, properly sealed, labeled and warped with foil to prevent any further light reactions. All collected samples were temporally stored in an icebox at 4°C and then transferred to the laboratory for further analysis. Temperature of collected soils ranged from 35-36°C.

### 2.2. Culture Media

For erichment Cultures and purification of isolates, Luria Broth (LB) medium) was used. Biodegradation experiments were performed in MSM liquid medium containing per liter (pH 7.2): Nitrogen source either ($NH_4NO_3$ , $NH_4Cl$, or $NaNO_3$) 4.0 g; $Na_2HPO_4$, 2.0 g; $KH_2PO_4$, 0.53 g; $K_2SO_4$, 0.17 g, $MgSO_4$, $7H_2O$, 0.10 g, and 1ml trace element solution (per 100 ml) : EDTA, 0.1 g; $ZnSO_4$, 0.042; $MnSO_4$, 0.178 g; $H_3BO_3$, 0.05; NiCl, 0.1 g.
All media were sterilized at 121ºC for 20 minutes.

### 2.3. Isolation of hydrocarbon degrading Bacteria

Luria Broth (LB) as used of enrichment medium with 2.5 g from each sample suspended in 25 ml. The liquid cultures were incubated at 30º C in a rotating shaker set at 300 rpm for 3 days. After the incubation period, 2 ml from each liquid culture were transferred to 25 ml MSM liquid medium supplemented with 1ml crude oil (or diesel) as the sole carbon source. This step of adaptation of the microorganisms to oil/diesel as the sole carbon source was repeated twice to enrich the media only with microorganisms able to grow by using oil and diesel components as the carbon source. The spray plate technique [11] was also used to spread 100µl of LB-cultures (liquid MSM-cultures) on MSM agar medium and then 100µl crude oil/Diesel was spread on the surface of the MSM agar.

### 2.4. Screening of diesel degrading isolates based on biomass production

The best candidates for the degradation of petroleum components, were first cultured into 10 ml LB broth for 48 hours at 30°C. Then, adaptation was performed by pouring into the respective sterilized 20 ml MSM-diesel 10% or MSM-petroleum 10% liquid media supplemented with 10% diesel, crude oil respectively, and incubated for up to 2 weeks at 30°C. The growth was then evaluated by spreading 100µl of serial dilutions in MSM medium on LB plates. Colony Forming Units (CFU) was used after enumeration as cell counts.

### 2.5. Statistical analysis

Three replicates were used throughout the experiments, and the mean values with standard deviation were calculated using Microsoft Excel 2013.

### 2.6. Molecular Identification of isolates

Extraction of DNA was done from cells after overnight growth in LB plates. The cells were suspended in 0.5 ml of distilled water, boiled for 10 min in a water bath and then centrifuged for 10 min at 13,000 rpm. The supernatant (total DNA) was placed in a new tube for PCR amplification. The 16s rDNA fragment (1500 bp) was amplified using universal primers; RibS73sp 5'-AGAGTTTGATCCTGGCTCAG-3', and RibS74sp 5'-AAGGAGGTGATCCAGCCGCA-3' [12]. The sequencing of bacterial 16S rDNA amplicons was performed after purification, using Applied Biosystems 3500 Series Genetic analyzer system. The obtained 16S rDNA sequence of each isolate was used to determine the most closely related sequence of available sequences in Gene Bank database using the Blast server at NCBI.

### 2.7. Analysis of diesel degradation by Gas Chromatography (GC)

Analysis of diesel degradation by done by Gas Chromatography (GC) analysis, performed after the incubation periods using Perkin Elemer- Clarus 680 GC, FID detector at 150°C injector temperature. The diesel layer was carefully extracted using micropipette, placed into sterilized Eppendorf tube, centrifuged for 1 min at 13,000 rpm to separate any remaining liquid medium, and then the pure diesel layer was transferred to new sterilized Eppendorf tube and used for GC analysis. The biodegradation of diesel was concluded by the reduction in the area under the hydrocarbon peaks in the chromatograms when compared to that of the abiotic control, suggesting the removal of diesel componentsThe Removal Efficiency (RE) based on the decrease in peak area of selected hydrocarbons from the chromatogram of fresh diesel, was evaluated by using the following expression: $RE (\%) = 100 - (As \times 100/Ac)$; Where: As is the total area of the peak in each sample; Ac is the total area of the peak in the control and RE (%) is the efficiency of biodegradation.

## 3. Results

### 3.1. Isolation of hydrocarbon degrading bacteria

Following the strategy designed in our work to isolate hydrocarbon-degrading bacteria both with high growth and biological activity, 39 purified bacterial isolates were recorded amongst hundreds of isolated ones at the first step of the program.

### 3.2. Screening of bacterial isolates based on biomass production in 10% diesel

Evaluation of the activity of all isolates was done for their ability to grow in the mineral salt medium (MSM) with 10% diesel as the sole carbon source. This diesel concentration of almost 75 g/l hydrocarbons may be considered high, leading to the selection of bacterial strains with the highest ability to grow at such a concentration. The criterion of selection was the cells' biomass (cell counts) after 1 week and 2 weeks of incubation. The cell counts and growth rates were correlated to the ability of the isolate to use the diesel hydrocarbons (sole carbon source) at the experimental conditions. Results (not shown) show interesting isolates based on the reported selection criteria. Out of the preselected 23 isolates, 15 isolates showed relatively high growth, while 8 others showed moderate to low cell counts after 1 or 2 weeks of incubation.

### 3.3. Screening of the strains based on their ability to degrade diesel hydrocarbons

Biodegradation of diesel hydrocarbons was monitored by gas-chromatographic (GC) analysis, with FID detector. The diesel fraction separated from the cultural media (MSM medium) is fractionated by GC. Diesel from the control (culture not inoculated with cells) was used to select representative peaks covering a wide range from the low molecular weight (MW) hydrocarbons to high MW hydrocarbons. The degradation of the corresponding hydrocarbons was calculated as a reduction in the corresponding area from the chromatograms obtained similarly to that of control-diesel. It was expressed as a percentage of area reduction corresponding to the degraded hydrocarbon. This is in relation with the amount of the remaining fractions in the appropriate abiotic control samples as described by Michaud et al. [13]. The peaks were characterized by their retention time in the chromatogram. The Removal Efficiency (RE) of several isolates on 10% diesel is shown in Fig.1.

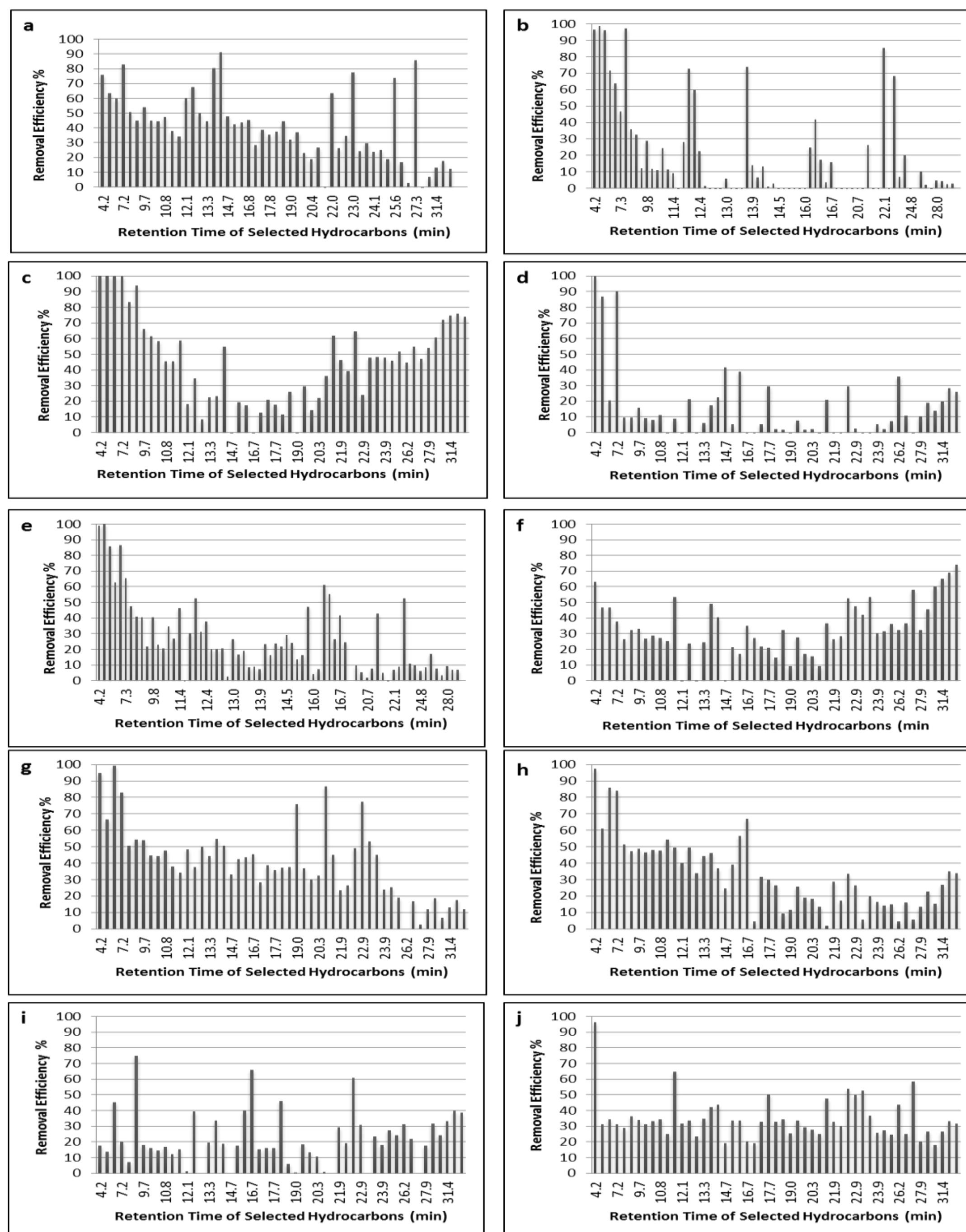


Fig. 1: Removal Efficiency (RE) of selected hydrocarbons on 10% Diesel (indicated) by their retention time in their corresponding GC chromatograms of isolates: a) QDD1, b) QDD2, c) QDD3, d) QDD4, e) QDD5, f) QDD6, g) QDD7, h) QDD8, i) QDD9, and j) QDD10.

### 3.4. Involvement of nitrogen source and C/N ratio in the degradation of diesel hydrocarbons by selected strains

The effect of the composition of the nitrogen source as well as the C/N ratio on the biological activity of the selected strains was investigated. Three different nitrogen sources which are ammonium nitrate ($NH_4NO_3$), ammonium chloride ($NH_4Cl$), and Sodium Nitrate ($NaNO_3$), were used at different C/N ratios (60/1, 80/1 and 120/1 respectively) using 10% diesel corresponding to 75 g/l total hydrocarbons. This may be considered actually, as a comparison between different conditions of growth. The results (not shown) 3 were obtained with three representative strains QDD8, QDD9 and QDD10.

It was clear that QDD8 and QDD9 exhibit the highest growth rates when using Ammonium Nitrate as nitrogen source, while QDD10 has the highest growth with Ammonium Chloride. All strains have the lowest growth rates with Sodium Nitrate. It is clear that for each strain, there is a corresponding optimal C/N or a range within which the growth is optimal. This growth dependence on C/N ratio may vary from one nitrogen source to another at 10% diesel. The hydrocarbon degradation in diesel by each strain at all the conditions was evaluated by the GC analysis and calculation of the removal efficiency of representative hydrocarbons (Fig.2 to Fig.4).

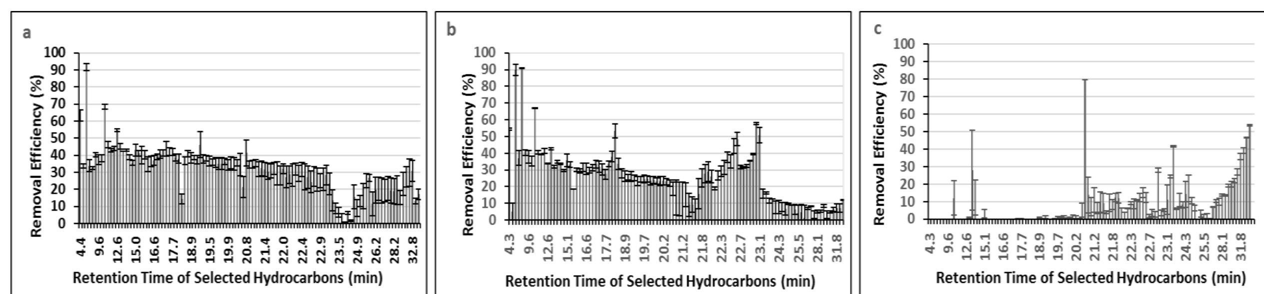


Fig. 2: Hydrocarbon Removal Efficiency QDD8 with nitrogen sources, a) $NH_4NO_3$, b) $NH_4Cl$, and c) $NaNO_3$.

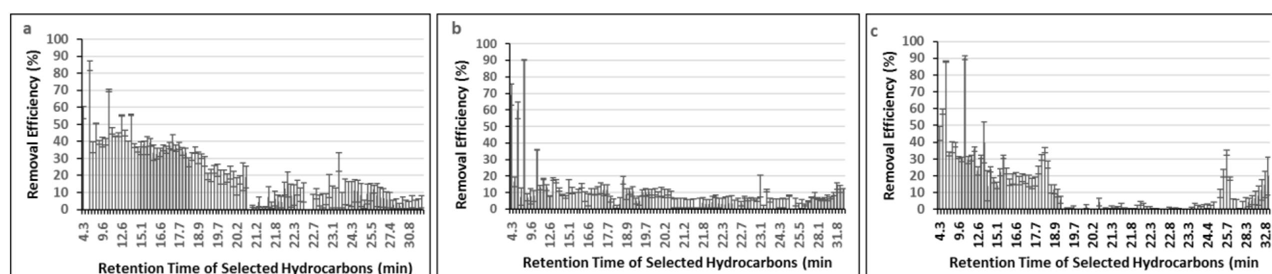


Fig. 3: Hydrocarbon Removal Efficiency QDD9 with nitrogen sources, a) $NH_4NO_3$, b) $NH_4Cl$, and c) $NaNO_3$.

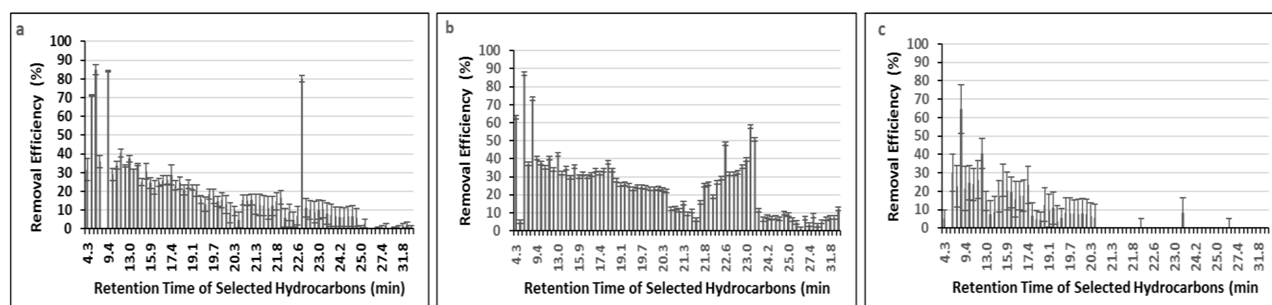


Fig. 4: Hydrocarbon Removal Efficiency QDD10 with different nitrogen sources, a) $NH_4NO_3$, b) $NH_4Cl$, and c) $NaNO_3$.

Results of Fig.5 and Fig. 6, show high variability in the biological activity of the three strains at different conditions. Indeed, with ammonium, QDD8 was more active on high MW hydrocarbons at high a C/N ratio of 50/1. At higher C/N ratio of 60, the overall activity was much higher, but less on high MW hydrocarbons.

With the strain QDD8, by using ammonium nitrate, the activity towards all the hydrocarbons was higher at C/N of 60. However, at C/N of 70/1, only high MW hydrocarbons were degraded. The strain QDD10, is not able to efficiently degrade medium MW hydrocarbons, but it is effective towards high MW ones at all conditions. The highest activity was obtained using ammonium nitrate at a C/N ratio of 60/1.

### 3.5. Identification of the selected strains

The most interesting isolates, showing high growth and removal efficiency of diesel hydrocarbons were identified based on sequencing of bacterial 16S rDNA amplicons, after purification. The obtained 16S rDNA sequence of each isolate was used to determine the most closely related sequence of available sequences in Gene Bank database using the

Blast server at NCBI. Table 1 lists the identified isolates. 3 of our isolates are *Pseudomonas aeruginosa*, which are the most effective strains. There are also several strains of *Arthrobacter*, *Citrobacter*, *Bacillus* and *Klebsiella*. Unexpectedly, one of our isolates belongs to *Klebsiella* species.

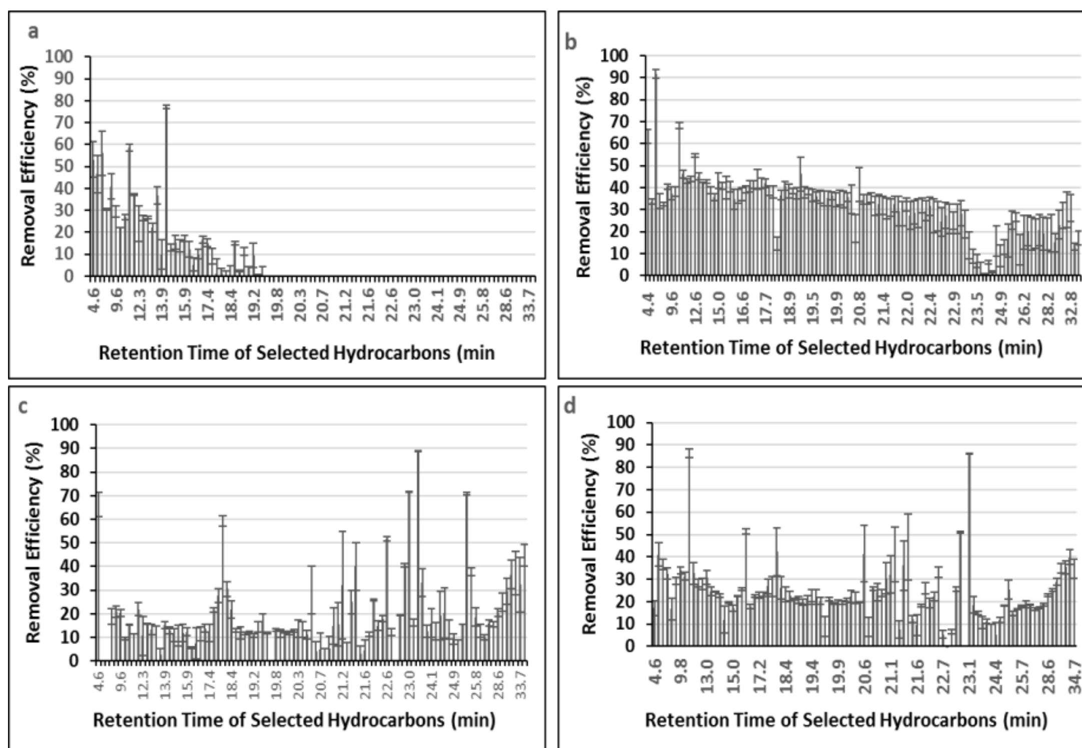


Fig. 5: Hydrocarbon Removal Efficiency of QDD8 with $NH_4NO_3$ at different C/N ratios: a) 70/1, b) 60/1, c) 50/1, and d) 40/1.

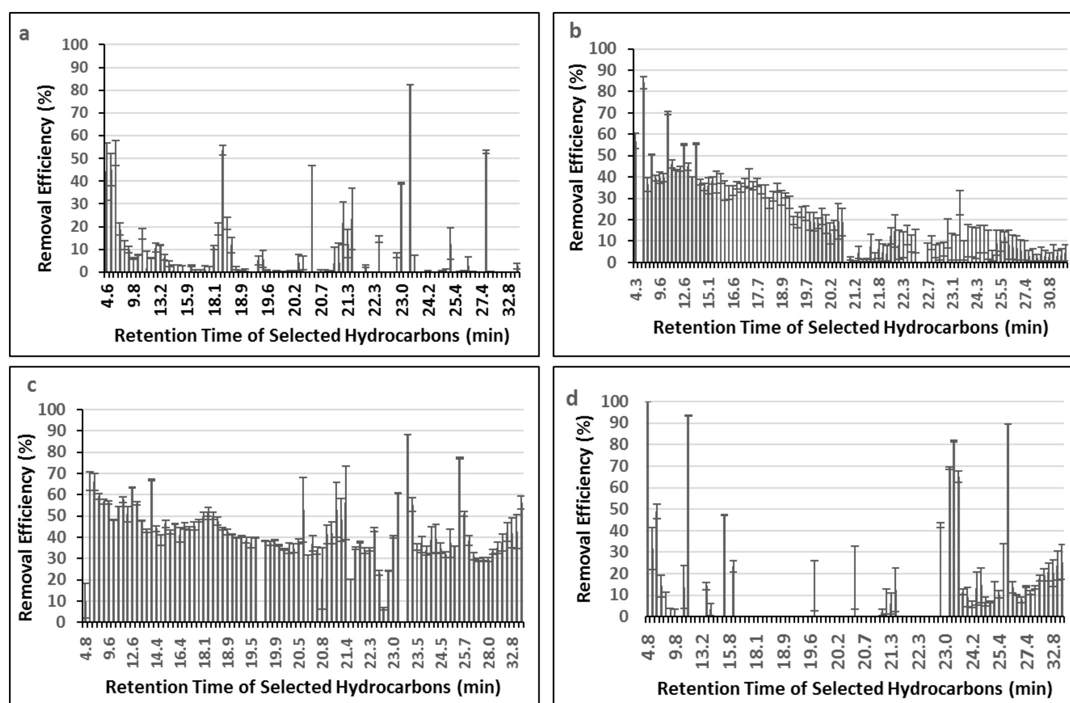


Fig. 6: Hydrocarbon Removal Efficiency of QDD9 with $NH_4NO_3$ at different C/N ratios: a) 70/1, b) 60/1, c) 50/1, and d) 40/1.

Table 1: List of identified diesel degrading strains.

| Isolate | Identity | Accession No. |
|---|---|---|
| QDD1 | *Pseudomonas aeruginosa* | JX962696.1 |
| QDD2 | *Klebsiella pneumoniae* | LC093517.1 |
| QDD3 | *Arthrobacter sp.* | GU451067.1 |
| QDD4 | *Citrobacter sp.* | KR063563.1 |
| QDD5 | *Citrobacter amalonaticus* | KC689297.1 |
| QDD6 | *Enterobacter helveticus* | JN255127.1 |
| QDD7 | *Cronobacter muytjensii* | JN255123.1 |
| QDD8 | *Pseudomonas aeruginosa* | KC776528.1 |
| QDD9 | *Pseudomonas aeruginosa* | JF919950.1 |
| QDD10 | *Citrobacter amalonaticus* | KC689297.1 |
| QDD11 | *Bacillus cereus* | LN890172.1 |

## 4. Discussion

It is always recommended to follow an isolation and screening program to select microorganisms with expected biological activity, suitable to the objective of the application [14]. Here, the objective of the isolation program is to build a collection of bacterial strains exhibiting biological activity for the degradation of hydrocarbons from oil-polluted area. Most of the lost isolates were in the phase of purification, as a result of losing hydrocarbon degrading activity, known to be exhibited by plasmidic genes that lack several growth conditions [15]. All the retained isolates are supposed to be more genetically stable. It was interesting to diversify media and conditions in the isolation program. Two different media, named MSM and MM, the most used media for isolation of hydrocarbon-degrading bacteria were used [11]. Moreover, to increase the chance of isolating interesting bacterial strains, diesel and crude oil were used as the sole hydrocarbon sources, at 10% (v/v) corresponding to almost 75 g/l hydrocarbons (Um-Saeed Refinery, Qatar personal communication). This may be considered as a high hydrocarbon concentration, with a potential toxicity towards most of the microorganisms. The isolates, purified through this program should tolerate high hydrocarbon concentrations and their potential toxicity. Indeed, the high selection pressure should lead to the isolation of suitable isolates for the bioremediation of oil hydrocarbons [16].
On the other hand, the isolation was performed from a recently polluted area and from an area polluted over a very long period of time. This may provide both, the adaptation of bacteria to the pollution potential of hydrocarbons (natural selection of microorganisms) and to different compositions of the hydrocarbons [17]. Indeed, hydrocarbons are characterized by chemical instability with modifications in their structures due to oxidation and light occurring in nature [18]. Diversification of samples as well as media and hydrocarbons led to a potential large variety of isolates with biological activities, which may be different [19]. To explain these results, we can postulate two hypotheses:

Hypothesis 1: The nitrogen sources are responsible for providing nitrogen for the synthesis of cells' components. This may be performed differently from one nitrogen source to another. Since, in the used MSM media, there are no supplemental amino acids, the addition of amino acids to the media may increase the rate of growth, depending on the nitrogen source.

Hypothesis 2: In the three media, there is the same carbon source concentration (10% diesel corresponding to almost 75 g/l hydrocarbon concentration) but different nitrogen concentrations supplied by the different nitrogen sources. The C/N ratios are of 60/1 in MSM1, 80/1 in MSM2 and 120/1 in MSM3. The study of the effect of C/N ratio on the growth was necessary. The effect of the composition of the nitrogen source as well as the C/N ratio on the biological activity of the selected strains showed that the biological activity of each strain might shift from a range of hydrocarbons MW to another. This finding is of importance, since it is possible to implement the isolated bacterium for the degradation of the available hydrocarbons in the polluted site. 3 of the isolates are Pseudomonas aeruginosa, which are the most effective strains [20-22]. This result is expected due to the fact that this species is the most involved in bioremediation, especially of hydrocarbons. There are also several strains of *Arthrobacter*, *Citrobacter*, *Bacillus* and *Klebsiella*. These species are also reported in literature for their biological activities for bioremediation of hydrocarbon [20-22]. Unexpectedly, one of our isolates belongs to *Klebsiella* species. Up to know, it is not reported how *Klebsiella* which is normally pathogenic, exhibits such biological activity. Several strains of Bacillus cereus and *Bacillus thuringiensis* are also shown with some activity of

degradation of hydrocarbons. In literature, it is not reported that *Enterobacter helveticus* or *Cronobacter muytjensii*, are involved in degradation of hydrocarbon [21, 22] It was clearly observed that the biological activity for each strain was affected and shifted towards higher molecular weight hydrocarbons by changing the nitrogen source and altering C/N ratios. This is a result of high adaptation of these bacteria to the local environmental conditions, in term of chemicals composition and harsh conditions.

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