QATAR UNIVERSITY

Graduate Studies

College of Art & Sciences

ISOLATION AND SCREENING OF HYDROCARBON DEGRADING BACTERIAL STRAINS FOR BIOREMEDIATION OF PETROLEUM POLLUTION IN QATAR

A Thesis in

Department of Biological and Environmental Sciences

By

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Submitted in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science in Environmental Science

June 2013

THESIS APPROVAL

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Abstract

Pollution, due to activities related to the oil industry, represents a serious threat to the natural environment. The application of biotechnological methods provides much safer and sustainable alternatives for bioremediation of polluted areas, using microorganisms. Several techniques for the isolation of hydrocarbon degrading bacteria have been investigated and published worldwide. A wide range of biological activities was shown. However, local hydrocarbon degrading strains and the factors affecting bacterial and strains variability were not studied deeply.

In this study, we showed that the isolation and screening strategy affected a lot, the selection of the strains. We combined the bacterial tolerance to hydrocarbon toxicity, assessed by the growth parameters, and the bacterial degradative activities, assessed by the degradation of a wide range of petroleum hydrocarbons via Gas Chromatography analysis.

The main investigations and findings of the present work are:

- A collection of 39 bacterial isolates from the Qatari environment was set up and a new isolation and screening program was proposed.
- The Growth conditions and the activity of pre-selected strains
- Shift of the activity of the selected strains from a range of hydrocarbons to another by the effect of the nitrogen source, C/N ratio and organic nitrogen source
- 70% hydrocarbon removal, achieved with several strains in 2 weeks.
- Amongst 12 identified isolates and by molecular ribotyping-DNA sequencing, 3 *Pseudomonas* strains isolated from a polluted area in Qatar, are available for

bioremediation of highly polluted soils, tolerating high toxicity and may be adapted to a variety of low or high molecular weight hydrocarbons.

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Acknowledgement

Honestly, it is very difficult to find words to express my acknowledgement and appreciation.

Completing about 2 years of my Master's program, wrapped up by writing and submitting this project, has been one of the most significant challenges in my life. I would not have reached this far without the unconditional support, love and understanding of my husband, Subhi Al Disi.

I also extend my deepest gratitude to the committee members and my project supervisors, Prof. Nabil Zouari and Prof. Samir Jaoua for their unrelenting efforts, commitment, encouragement, guidance, support and for being so precise in providing great assistance towards my understanding and conducting the research.

Introduction

Environmental pollution is one of the greatest problems the world is facing today; wherein, pollution associated with the oil industry presents serious threats to the natural environment. Consequently, risks due to contamination from oil industry spills, storage containers and accidental leakages cause increased national and global concerns.

Qatar is one of the largest oil and natural gas producers. The Qatari economic policy is focused on developing Qatar's non associated natural gas reserves and increasing private and foreign investment in non-energy sectors. However, oil and gas still account for more than 50% of the GDP, roughly 85% of export earnings and 70% of government revenues. Oil and gas have made Qatar the world's highest per-capita income country as well as the country with the lowest unemployment rate (CIA-The World FactBook, 2013).

In the recent years and due to increasing industrialization, significant social and economic changes have been taking place at an alarming rate, putting at risk the natural and cultural resources of Qatar. However, Qatar is in a unique position, given the availability of financial resources and her forward thinking leadership, to be amongst the first countries ready to take advantage of the next economic revolution: the green revolution (Richer, 2009).

Promoting environmental sustainability is a key component of Qatar's National Development Strategy, 2011-2016. The country's economic boom, based on the hydrocarbon industry, is putting a strain on other scarce resources. The government is therefore developing a forward-looking plan to manage Qatar's environmental challenges (Hukoomi , 2013).

The Emerging enhancements in biotechnology methods provide alternative safe and sustainable methods for bioremediation of polluted areas by using microorganisms.

Due to the harsh weather conditions in the Arabian Gulf region that result in prolonged periods of time for microorganism selection and adaptation, studying these microorganisms and selecting candidates for bioremediation would give local biological tools for implementing bioremediation, modified to suit the country and the region.

It would be valuable to investigate these particular potentialities of local microbial strains that would be utilized for the remediation of polluted areas by using available biotechnological tools and techniques, that is, balanced micro-ecosystems consisting of different kinds of species: anaerobic and micro-aerobic.

Several techniques for isolation of hydrocarbon degrading bacteria have been investigated in various scientific researches and published worldwide. The presented project would contribute to the establishment and development of practical bioremediation processes, in which local microorganisms will be applied; hence, this would facilitate such applications to be extended to the whole gulf region and similar areas.

Isolation and Screening of diesel biodegrading bacterial strains from the local Qatari environment will be investigated and discussed in this research work.

Literature Review

1. Introduction

Industrialization resulted in large contaminated areas in the highly industrialized countries. Among them is soil contamination, resulting from several industrial activities such as mining and production of oil, steel, coke and chemicals or manufacturing paint among others.

Signs of pollution and soil contamination have been identified and characterized and these include: contamination by metals (copper, zinc, lead, cobalt, nickel, arsenic and cadmium); light hydrocarbons (oil, gasoline and diesel) and heavy hydrocarbons (lubricants, heavy oil and crude oil); halogenated solvents and other more complex molecules (aromatic hydrocarbons polycyclic and PAHs among others).

Currently, the massive use of petroleum products, in addition to activities related to extraction and transportation of crude oil, are major sources of water and soil pollution (Ronald & Hazen, 2011). This emphasizes the importance of the studies on the biodegradability of oil products, in order to evaluate the potential for natural attenuation in soil or define strategies for bioremediation (Leahy & Colwell, 1990; Prince, 1993).

Global concerns arose as a result of the increased soil contamination incidents, mainly in the industrialized and developed countries, as well as in developing countries due to relocation of industrial and manufacturing sites. Weak mechanisms in the implementation of international norms and environmental standards expose these countries to the risk of soil pollution. Thus, there is an

urgent need to develop strategies for the rehabilitation of land, to preserve agricultural soils and also prevent the spread of harmful elements and their leakage into the groundwater and consequently into the food (Ronald & Hazen, 2011).

In spite of several methods and techniques being used today, further innovation efforts are necessary in order to cope with the diversity of industrial activities.

2. Bioremediation of pollution areas

Biological techniques have been known and used for many years that make use of the natural power of decontaminating microorganisms or plants that usually grow on contaminated soils. While some of these techniques have been implemented since the 80s and 90s and come to maturity, others are still the subject of research and development (Leahy & Colwell, 1990). The implementation of these techniques is considered practical and much cheaper due to the fact that almost no excavation or transport is required.

These biological techniques are mainly divided into two broad categories (Meagher, 2000). The first one is bioremediation by microorganisms (bacteria) and the second is phytoremediation by using the photosynthesis properties of plants (such as shrubs and trees).

There are several known bioremediation techniques, which include: Biodegradation, Bioimmobilisation, Bioleaching, Bioslurry (treatment bioreactor), Biorehabilitation, Bioaugmentation and Biostimulation. Each of these techniques has been designed to deal with a particular pollution situation.

Several techniques are already in the market, while others are still under research and development (Ronald & Hazen, 2011). Concurrently, researchers are investigating and testing the possibility of combining several biological techniques in order to increase performance while reducing the number of treatments.

3. Bioremediation of petroleum components

Bioremediation, basically functions through biodegradation, which may refer to the complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds and cell protein or the transformation of complex organic contaminants to other simpler organic compounds by biological agents like microorganisms. Many original microorganisms in water and soil are capable of degrading hydrocarbon contaminants (Nilanjana & Preethy, 2011). Many oil-degrading bacteria have been isolated and their degradation potential investigated. However, most of these bioremediation studies have been carried out using pure-cultures and the roles of these bacteria in a natural environment remain substantially unknown (Sutiknowati, 2007).

Petroleum hydrocarbons can be divided into four classes:

1. Saturates;
2. Aromatics;
3. Asphaltenes (phenols, fatty acids, ketones, esters and porphyrins) and
4. Resins (pyridines, quinolines, carbazoles, sulfoxides and amides.

The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes. Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all (Bragg, 2009).

Bioremediation utilizes the biodegradative potentials of organisms in an effective technology in which detoxification and volume reduction can be achieved. Besides, this bioremediation technology is believed to be non-invasive and relatively cost-effective (April, et al., 2000).

Biodegradation of petroleum hydrocarbons is a complex process that depends on the nature and amount of the hydrocarbons. This process is affected by various factors such as the hydrocarbons' availability to microorganisms, since petroleum hydrocarbon compounds bind to soil components and thus would be difficult for them to be removed and/or degraded.

Physical limiting factors such as temperature play an important role in the biodegradation of hydrocarbons by directly affecting the chemistry of the pollutants as well as affecting the physiology and diversity of the microbial flora. (Atlas, 1997) Found that, at low temperatures, the viscosity of oil increased while the volatility of the toxic low molecular weight hydrocarbons was reduced, thus delaying the onset of biodegradation. Temperature also affects the solubility of hydrocarbons. Although hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with decreasing temperatures.

Physicochemical parameters such as pH, temperature and nutrients have been examined in several studies, according to (Coulon, et al., 2005) and confirmed,

from an applied perspective that heating soil as well as nutrient amendment appear to be effective means of accelerating bioremediation of hydrocarbon-contaminated sub- Antarctic soils.

Moreover, nutrients are a very important ingredient for the successful biodegradation of hydrocarbon pollutants, especially nitrogen, phosphorus and in some cases iron. Of great importance also, is oxygen availability, which may pose multiple problems with respect to strictly controlled laboratory conditions. One of the most effective ways of increasing the bioavailability (or solubility) of petroleum hydrocarbon pollutants in soil is by use of surfactants to enhance desorption and solubilization of petroleum hydrocarbons, thereby facilitating their assimilation by microorganisms (Lai, et al., 2009).

Diesel contains highly concentrated toxic materials that can negatively impact on soil and plants (Hong, et al., 2005). Many organisms have the ability to utilize hydrocarbon as the sole carbon source, such as the hydrocarbon degrading bacteria (mostly by pseudomonas species), as reported in various studies (Jyothi, et al., 2012; Lou, et al., 2012). However, the hydrocarbon degrading strains introduced to polluted sites are not able to metabolize efficiently all the oil components due to lack of necessary nutrients. Biostimulation, by addition of nutrients, has been the most widely practiced bioremediation strategy (Huang, et al., 2008).

Shukor, et al., (2008) conducted further studies on several diesel degrading isolates in order to optimize their cellular growth and hence promote enhanced degrading of diesel. It was found that degradation is unfavorable at concentrations higher than 3.5% (v/v) of diesel for the isolated strains.

4. Microorganisms used for bioremediation of petroleum components

Most of the hydrocarbon-degrading bacteria with the highest ability of degradation have been studied and their genomes sequenced. Among the completely sequenced organisms are *Pseudomonas putida* KT2440 (Nelson, et al., 2002), *Alcanivorax borkumensis* (Golyshin, et al., 2003), *Acinetobacter sp.* DR1 (Jung, et al., 2010) and several others.

Although bioremediation field trials have often been carried out, there is lack of information on the indigenous microbial communities that catalyze oil degradation under in situ conditions at spill sites (Kostka, et al., 2011). Most of the isolated and investigated hydrocarbon-degrading bacteria that were involved in the in situ bioremediation are poorly understood; in fact, less than 5% of the soil bacteria are cultured in the laboratory (Mittal & Singh, 2009).

Some soil microorganisms, particularly bacteria, are able to obtain energy metabolism via soil pollutants such as PAH (Mittal & Singh, 2009). They are able to develop mechanisms of using these molecules as a source of carbon and energy, providing detoxification of the environment, and/or transforming them into substrates metabolized by other microorganisms, hence resulting in decontamination of polluted areas without disturbing the integrity of the ecosystem. These bacteria, through specific metabolic pathways, take the central role of the phenomena of hydrocarbon biodegradation. Some of them have developed a system of signaling, chemotaxis, to facilitate access to hydrocarbons. Chemotaxis is of selective advantage to the degradative bacteria

for guiding them to sense and locate pollutants that are present in the environment (Pandey & Jain, 2002).

These mechanisms operate via chemoreceptors and signaling pathways that cause the relocation of bacteria, according to the pollutant's gradient concentration. The Bacteria then accumulate at the interface between the hydrophobic pollutant and the hydrophilic medium, thus increasing desorption of these compounds and eventually the rate of their degradation (Kastner, et al., 1994). Chemotaxis allows microorganisms to move towards areas of the soil where pollutants are inaccessible (Johnsen, et al., 2007). Some microorganisms are able to adsorb to the pollutant's rich particles through their hydrophobic wall (Lotfabad & Gray, 2002) and form biofilms (Johnsen, et al., 2007). Other microorganisms produce surfactants. These molecules have the property of increasing the solubility of hydrophobic compounds and therefore improve accessibility (Lai, et al., 2009).

In the case of hydrocarbons that are very recalcitrant to biodegradation, such as benzo [alfa] pyrene, mutual processes can be put in place; for instance fungi, known for their ability to degrade high molecular weight polyaromatic hydrocarbons PAHs initiate degradation (Mrozik, et al., 2003), then bacteria use the degradation products which are more hydrophilic as carbon sources (Santos, et al., 2008). Similarly, the interaction of bacteria with plant roots often favors the activity of degradation of hydrocarbons in the diffusion zone of root exudates or of oxygen. The co-metabolism of the carbon sources derived from extracts of roots increases the degradation of benzo [alfa] pyrene by a strain of *Sphingomonas yanoikuyae* (Supaka, et al., 2001)

Somehow, the microbial communities are able to degrade pollutants under the influence of environmental parameters, considering properties of pollutants and micro-flora in the soil (Doylep, et al., 2008). Microorganisms of a soil that has never been exposed to contaminating compounds do not necessarily have the ability to metabolize pollutants. However, when exposed to such compounds, they are often able to adapt, that is, to acquire the potential of metabolic degradation of these pollutants by vertical or horizontal recruitment of specific genes (Hamme, et al., 2003). Recruitment of metabolic pathways present in the genome but not expressed, may be done by the events of mutation, genetic rearrangement or transposition (vertical recruitment) (Weissenfels, et al., 1991). Microorganisms can also acquire catabolic gene clusters via mobile elements transferred from a donor to a receiving host (horizontal transfer). In bacteria, such transfer phenomena are best known through the combination of plasmids, transformation or transduction (Woo, et al., 2004). These phenomena of horizontal transfers increase the metabolic capabilities of bacteria, including expanding their range of substrates and allowing adaptation of microbial populations to new contaminants (W. Xue, 2005; Yu, et al., 2005).

5. Factors affecting microbial bioremediation of polluted soils with petroleum

Very often, natural conditions are not favorable enough; for example, the lack of nutrients, pH, redox potential, oxygen, and/or suitable bacteria. In such situations, the process can be improved by optimizing the necessary factors. For example, in 1989, during the oil spill occasioned by the supertanker

accident, Exxon Valdez spilled about 201,000 m^3 of oil and in order to accelerate the degradation of oil hydrocarbons, the required nutrients were spread over 1000 miles along the coast of Alaska (Leahy, 2011). In the field of bioremediation, it will be more likely to observe the speed of natural biodegradation and intervene only if the natural activity is not sufficient to eliminate the pollutant fairly quickly (Kostka, et al., 2011). Biodegradation is influenced by abiotic factors such as pH, redox potential, temperature, moisture, oxygen, nutrient availability and soil salinity. The composition of the mixture of hydrocarbons determines their solubility and resistance to degradation. Similarly, the interaction of pollutants with soil components determines the effectiveness of bioremediation. In soils exposed over long periods, hydrocarbons are strongly adsorbed to particles and/or incorporated in very fine pores, making them inaccessible to microorganisms (Doylep et al., 2008). Bioavailability of hydrocarbons is a key factor that may often limit the effectiveness of bioremediation processes (Samanta, et al., 2002), especially bacterial consortia. Under specific conditions, a process of biopolymerization of compounds via the process of bioautoxidation, producing intermediate degradation products, might occur, resulting in the production of non-biodegradable polymers.

Approach/Methodology

The objective of this study is to isolate and screen hydrocarbon degrading bacterial strains from different polluted sites in Qatar and hence, assess the crude oil/diesel biodegrading potentiality of these stains under *in vitro* conditions. The potentiality of degradation of diesel hydrocarbons is to be examined at various conditions to help find out the most interesting hydrocarbon degrading strains, which could be used in future bioremediation studies.

1- Research Strategy

- Sample collection
- Enrichment culture
- Isolation and purification
- Screening of diesel degrading isolates
- Selection based on biomass production
- Screening based on diesel biodegradation potentiality/efficiency
- Investigation of best nutrition requirements for higher biodegradation efficiency
- Identification of selected isolates

2- Soil Sampling

Soil samples are collected from various contaminated sites. Sampling is then done using a sterile spatula at a tillage depth, randomly from different points.

For this study, nine samples were collected from various oil-contaminated sources including the seashore, near gas stations and in automotive workshops.

The samples were then transported to the laboratory under sterile conditions. The location from where sampling was done is indicated with results.

3- Culture Media

The following culture media were used:

- Luria Broth (LB) liquid and solid medium.
- Mineral salt medium (MSM) liquid composed per liter (pH 7.2): NH_4NO_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 trace element solution (per 100ml) ; EDTA 0.1g, $ZnSO_4$ 0.042, $MnSO_4$ 0.178g, H_3BO_3 0.05, NiCl 0.1g, solid MSM was prepared by adding agar (20 g/L).
- Liquid minimal medium (MM) containing (per liter): $Na_2HPO_4 \cdot 2H_2O$ 8.5 g, KH_2PO_4 3.0 g, NaCl 0.5 g, NH_4Cl 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCl_2$ 14.7 mg. MM also contained trace elements as follows (per liter): $CuSO_4$ 0.4 mg, KI 1.0 mg, $MnSO_4 \cdot H2O$ 4.0 mg, $ZnSO_4 \cdot 7H_2O$ 4.0 mg, H_3BO_3 5.0 mg, $H_2MoO_4 \cdot 2H_2O$ 1.6 mg, $FeCl_3 \cdot 6H_2O$ 2.0 mg.
- All media were sterilized by autoclave at 121°C for 20 minutes.

4- Isolation of hydrocarbon degrading Bacteria

Hydrocarbon degrading bacteria were isolated from our soil samples using Enrichment Cultures as a first step.

2.5g from each sample was suspended in 25 ml of Luria Broth (LB) as enrichment medium. The liquid cultures were incubated at 30° C in a rotating shaker set at 300 rpm for 3 days. After the incubation period, 2 ml from each

liquid culture was transferred to 25 ml MSM medium and MM liquid medium supplemented with 1ml crude oil (or diesel) as the sole carbon source. They were called MSM-diesel or MSM-petroleum. This step of adaptation of the microorganisms to oil/diesel as the sole carbon source was repeated 2-folds to enrich the media only with microorganisms able to grow by using oil and diesel components as the carbon source. The spread plate technique was also used to spread 100µl of LB-cultures on MSM agar medium and then 100µl Crude oil/Diesel was spread on the surface of the MSM agar.

5- Isolation and purification of strains from enrichment cultures

At the end of the adaptation step, aliquots of the enrichment cultures were spread on LB-agar medium; isolates exhibiting distinct colonial morphologies were isolated and transferred to separate LB-agar plates.

The best diesel and/or petroleum degrading bacteria were purified on solid MSM agar plates containing petroleum or diesel respectively, as the sole carbon and energy source, by spray-plate technique (Survery, et al., 2005). The plates were wrapped with aluminum foil and incubated in the dark at 30°C for 2 weeks. Colonies forming clear zones on the coated solid MSM medium were selected. The isolates were purified using a sub-culturing by streaking on LB-agar plates.

6- Screening of diesel degrading isolates based on biomass production

The selected isolates, considered the best candidates for the degradation of petroleum components, were first cultured into 10ml LB broth for 48 hours at 30°C. Then, adaptation was performed by pouring into the respective sterilized

20 ml MSM-diesel or MSM-petroleum liquid media and incubated for up to 2 weeks at 30°C. The cells' biomasses were then evaluated by placing them on LB-agar plate 100µl of serial dilutions in MSM medium. Colony Forming Units (CFU) was used after enumeration as cell counts.

7- Analysis of hydrocarbons in diesel by Gas Chromatography (GC)

A GC analysis was performed after the incubation periods, to investigate the degradation of diesel. The biodegradation of diesel was verified 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 components. The raw diesel, obtained from Um-Saeed Refinery, is composed of hydrocarbons containing 11 to 25 carbon atoms (personal communication). All strains showed a reduction in the area under the hydrocarbon peaks corresponding to 11 to 25 carbon atoms (C11 to C25) when compared to the abiotic control.

The degradation of the diesel oil as a whole was expressed as the percentage of the diesel oil degraded in relation to the amount of the remaining fractions in the appropriate abiotic control samples.

The 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 x 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. (Michaud, et al., 2004)

8- Identification of the selected hydrocarbon Degrading Bacterial Isolates

The new isolates were identified based on the morphological and physiological, combined with molecular identification.

8.1. Sequence determination of 16s rDNA:

The 16S rRNA is a section of prokaryotic DNA found in all bacteria. It codes for an rRNA. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been, by far, the most common housekeeping genetic marker used due to: its presence in almost all bacteria, the stability of its function and its validity for informatics purposes. (Janda & Abbott, 2007)

8.2. Sequence determination of PCR amplified product:

The Polymerase Chain Reaction (PCR) has rapidly become a standard laboratory technique. With the continuous development of PCR technology, there has been a growing need for PCR product quantitation.

PCR is a revolutionary method developed by Kary Mullis in the 1980s. It is based on using the ability of DNA polymerase to synthesize a new strand of DNA complementary to the offered template strand. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons).

Indeed, the amplification of the 16S ribosomal RNA gene through the polymerase chain reaction (PCR) technique allows one to get enough of the DNA product to be sequenced and blasted against known sequences in the NCBI database, to provide species identification.

8.3. Genomic DNA Extraction

DNA was obtained from cells grown overnight in (LB) plates. The cells were suspended in 0.5ml of distilled water, boiled for 10 minutes in a water bath and then centrifuged for 10 minutes at 13000 rpm. The supernatant total DNA was placed in a new tube for PCR protocol.

8.4. PCR Amplification:

The 16s rDNA region (1500 bp) of the strains were amplified using universal primers; RibS73sp AGAGTTTGATCCTGGCTCAG, and RibS74sp AAGGAGGTGATCCAGCCGCA, (Xiao & Zhang, 2011).

Each PCR reaction was carried out in a total volume of 25µl PCR buffer containing 1.5mM $MgCl_2$, 0.8µM dNTP, 1.35µM of both forward and reverse primers, 10-20 ng genomic DNA of the isolates used as template for PCR reactions and 0.5IU Taq DNA.

The thermocycler program for each PCR reaction began with an initial denaturation step at 94 °C for 3 minutes followed by 35 cycles of a 45 second denaturation step at 94 °C, a 45 second annealing step at 50 °C, and a 45 second elongation step at 72 °C, then one final 2 minute extension step at 72 °C.

To examine the amplified DNA, 5 µl of each reaction was run on a 1% agarose gel containing ethidium bromide and visualized using ultraviolet light.

8.5. DNA Purification:

DNA purification was made using QIAquick Gel extraction Kit.

DNA quality was evaluated by 1% agarose gel electrophoresis and ethidium bromide staining.

Implementation/Research Findings

The findings are distributed into four main sections:

Section 1: Program of isolation of bacterial isolates growing on petroleum hydrocarbons as the sole carbon and energy source: Collection of strains;

Section 2: Screening of the isolates based on growth and hydrocarbon degradation;

Section 3: Investigation of the bacterial activities in several nutritional conditions

Section 4: Molecular identification of the pre-selected isolates.

Section 1: Program of isolation of bacterial isolates growing on petroleum hydrocarbons as sole carbon and energy source: Collection of strains

3.1. Strategy of isolation and screening of hydrocarbon degrading bacteria

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. 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. The strategy followed in our work is described in Figure 1.

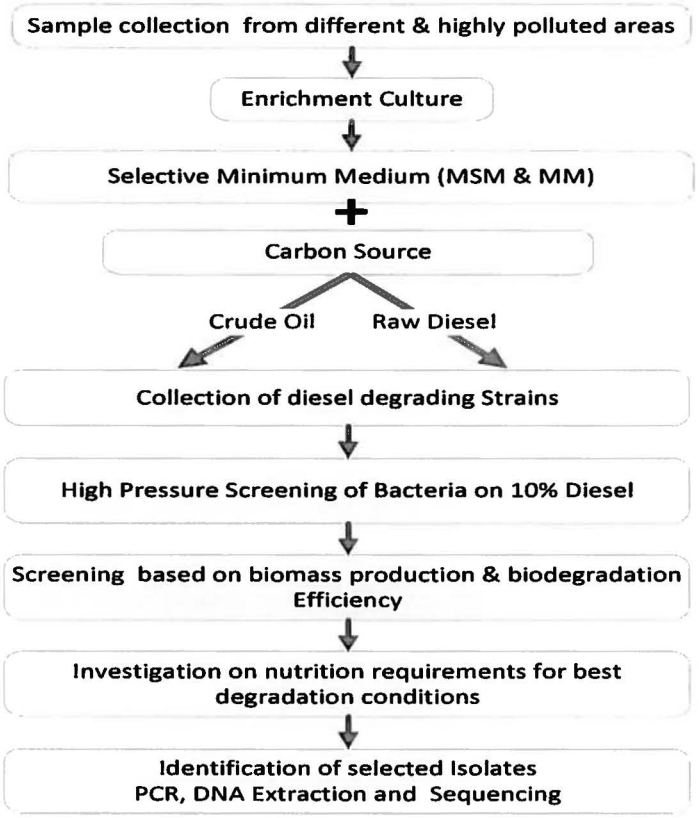

Figure 1: Steps of Isolation, Screening and Identification of bacteial isolates based on their hydrocarbon degrading activities

In our isolation and screening program, we preferred to use two different media, named MSM and MM, the most used media for isolation of hydrocarbon-degrading bacteria. Moreover, to increase the chance of isolating interesting bacteria, diesel and crude oil were used as the sole hydrocarbon sources, at 10% (v/v) corresponding to almost 75 g/l hydrocarbons. 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 interesting isolates, most suitable for the bioremediation of oil hydrocarbons.

On the other side, 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. Indeed, hydrocarbons are characterized by chemical instability with modifications in their structures due to oxidation and light occurring in nature.

Following this program, 39 purified bacterial isolates were recorded amongst hundreds of isolated ones at the first step of the program. 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 (Oluwafemi & Lateef, 2010).

3.2. Isolation of hydrocarbon degrading bacterial strains

At the end of the steps of adaptation to diesel/oil and purification on LB-agar medium, the 39 isolates exhibiting distinct colonial morphologies were recorded and coded. Table 1 gives the list of the coded isolates and their origins. It may be clearly noticed that most of the isolates were isolated using the MSM medium, supplemented with crude oil or diesel. Results of Table 1 show that it was interesting to diversify media and conditions in the isolation program. Using the MM medium, isolation of stable isolates was only obtained with one sample, coming from automotive workshops. We can conclude that diversification of samples as well as media and hydrocarbons led to a potential large variety of isolates with biological activities which may be different.

Table 1: List and origin of the coded 39 isolated isolates and media used for isolation

Sample	Origin	MSM Solid + Crude Oil	MSM liquid + Crude Oil	MSM Liquid + Diesel	MM liquid + Crude Oil
S1	Auto-workshop		ZA11, ZA19	ZA7	
S2	Auto-workshop			ZA1, ZA10	
S3	Auto-workshop		ZA8, ZA9, ZA36, ZA37	ZA6	ZA3, ZA12, ZA15, ZA18
S4	Auto-workshop	ZA4, ZA5, ZA16, ZA17	ZA2, ZA13, ZA14, ZA20		
S5	Soil (in depth)			ZA26, ZA27, ZA28, ZA32, ZA34	
S6	Beach Soil	ZA25	ZA22	ZA33	
S7	Surface Soil		ZA21, ZA23, ZA24	ZA29, ZA30, QDD1, QDD2	
S8	Beach Soil			ZA31	
S9	Surface Soil			ZA35	

Section 2: Screening of the isolates based on growth and hydrocarbon degradation

3.3. Screening of the isolated strains on MSM-oil solid medium

As the first step in the screening program, all the isolates were spread on MSM-oil solid medium. Indeed, the best diesel and/or petroleum degrading isolates were purified on solid MSM agar plates containing petroleum or diesel respectively, as the sole carbon and energy source, by spray-plate technique (Survery, et al., 2005). The plates were wrapped with aluminum foil, incubated in the dark at 30 °C for 2 weeks. Colonies forming clear zones on the coated solid MSM medium were selected (Figure 2). According to the results, amongst the 39 retained isolates, only 23 were able to form clear zones on the coated solid MSM medium. They were selected for further screening programs for the hydrocarbon degradation capability. The selected strains are coded: QDD1, QDD2, ZA1, ZA2, ZA3, ZA5, ZA6, ZA7, ZA8, ZA9, ZA10, ZA11, ZA13, ZA14, ZA16, ZA17, ZA18, ZA19, ZA20, ZA31, ZA34, ZA36 and ZA37.

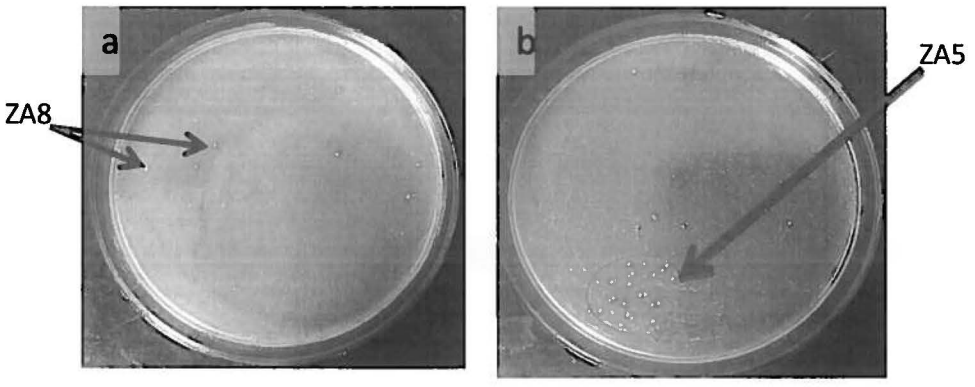

Figure 2: Screening of the isolates based on their ability to form clear zones on the coated solid media with Diesel/Oil, a) Isolate ZA8, b) Isolate ZA5. The arrows show the clear zones surrounding colonies.

3.4. Screening of bacterial isolates based on biomass production on 10% diesel

The 23 bacterial isolates were tested for their ability to grow in the mineral salts 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 the 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 are correlated to the ability of the isolate to use the diesel hydrocarbons (sole carbon source) at the experimental conditions.

Out of the preselected 23 isolates, 15 strains showed relatively high growth, while 8 strains showed moderate to low cell counts after 1 or 2 weeks of incubation. The results are shown in Table 2.

In total, 15 isolates producing cell counts above 10^7cfu/ml were selected for further screening programs except 4 of them, ZA13, ZA14, ZA17 and ZA19. These four were characterized by a decrease in their cfu after 2 weeks of incubation as compared to 1 week incubation. These results may reflect the expected variability within the ability of our collected isolates to degrade diesel hydrocarbons.

Table 2: Growth in terms of cfu counts of bacterial isolates 1 week and 2 weeks of incubation on MSM media supplemented with 10% Diesel

No.	Strain	1 week incubation 10^6 cfu/ml	2 weeks incubation 10^6 cfu/ml
1.	**QDD1**	160	260
2.	**QDD2**	20	20
3.	**ZA1**	0.6	0.3
4.	**ZA2**	230	200
5.	**ZA3**	5	20
6.	**ZA5**	400	110
7.	**ZA6**	9.50	0.1
8.	**ZA7**	1	3
9.	**ZA8**	280	330
10.	**ZA9**	210	340
11.	**ZA10**	590	110
12.	**ZA11**	340	82
13.	**ZA12**	5	30
14.	**ZA13**	13	1
15.	**ZA14**	89	8
16.	**ZA17**	12	1
17.	**ZA18**	180	300
18.	**ZA19**	88	15
19.	**ZA20**	4.0	200
20.	**ZA31**	0.5	0.4
21.	**ZA34**	0.6	1.4
22.	**ZA36**	150	230
23.	**ZA37**	1.1	20

3.5. Screening of bacterial isolates based on biomass production on 5% diesel

In our screening program, the best isolates to be selected as candidates for field application must not only grow at high hydrocarbon concentrations, but also have an acceptable growth rate, which is also a criterion for selection. We chose to compare the growth rate of the 11 pre-selected strains on 5% diesel while avoiding

interference with potential toxicity needing variable adaptation phases from one strain to another. The 11 pre-selected stains were incubated in MSM with 5% diesel. Results are shown in Table 3.

Table 3: Growth in terms of cfu counts of bacterial isolates 1 week and 2 weeks of incubation on MSM media supplemented with 5% Diesel

No.	Strain	3 days of incubation 10^6 cfu/ml	1 week of incubation 10^6 cfu/ml	2 weeks of incubation 10^6 cfu/ml
1.	ZA2	43	320	200
2.	ZA5	160	100	500
3.	ZA8	270	190	240
4.	ZA9	330	140	300
5.	ZA10	100	110	660
6.	ZA18	50	240	450
7.	ZA19	60	100	60
8.	ZA20	28.9	460	190
9.	ZAF36	380	130	100
10.	QDD2	6.3	160	40
11.	QDD1	2.00	1.50	200

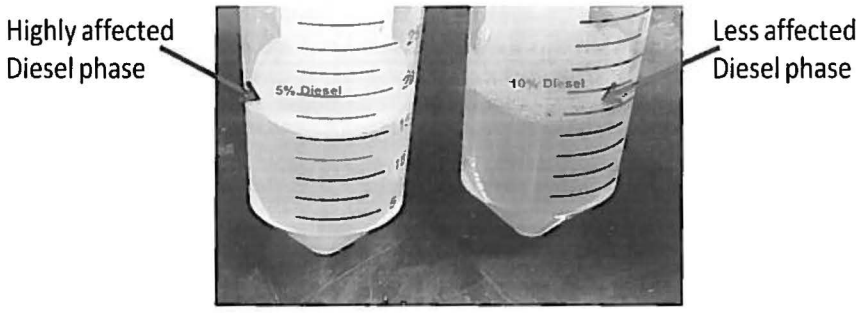

Figure 3: Observable change in physical appearance of 5% & 10% Diesel after 1 week of incubation with ZA9

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

Biodegradation of diesel hydrocarbons was monitored by gas-chromatographic (GC) analysis. 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 of the low molecular weight (MW) hydrocarbons and 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 to the amount of the remaining fractions in the appropriate abiotic control samples as described by Michaud (2004). The Removal efficiency (RE) was evaluated by using the following expression:

$$RE\% = 100 - (As\ x100/Ac)$$

Where; As is the total area of the peak in each sample while *Ac* is the total area of the same peak in the control. The peaks are characterized by their retention time in the chromatogram.

The Removal Efficiency (RE) of several isolates on 10% diesel is exhibited in Figure 4 - Figure 6.

Figure 7 shows results obtained for the selected isolates with 5% diesel.

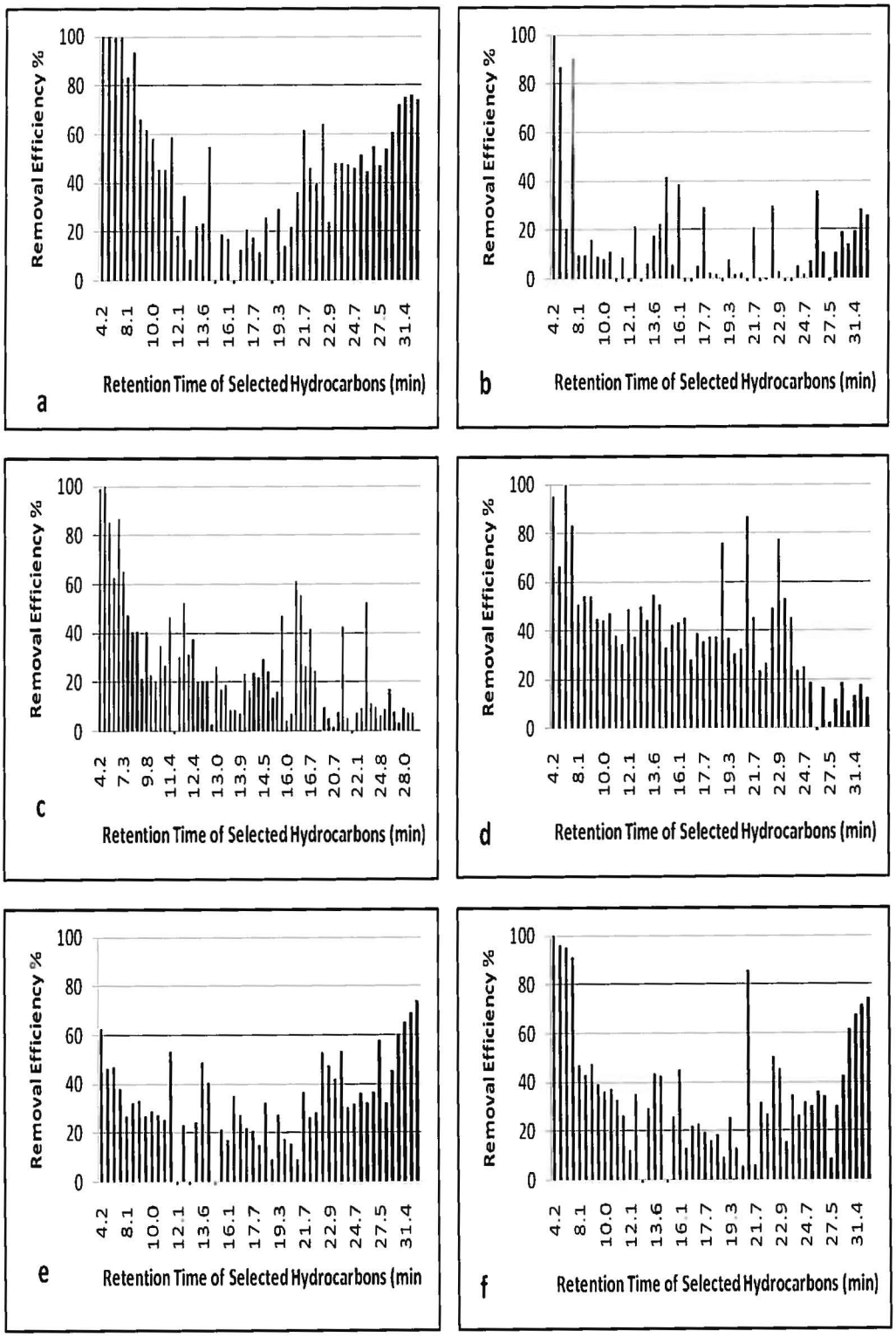

Figure 4: Removal Efficiency (RE) of selected hydrocarbons on 10% Diesel (indicated) by their retention time in their corresponding GC chromatograms of isolates:
a) ZA2, b) ZA3, c) ZA4, d) ZA5, e) ZA8, f) ZA9

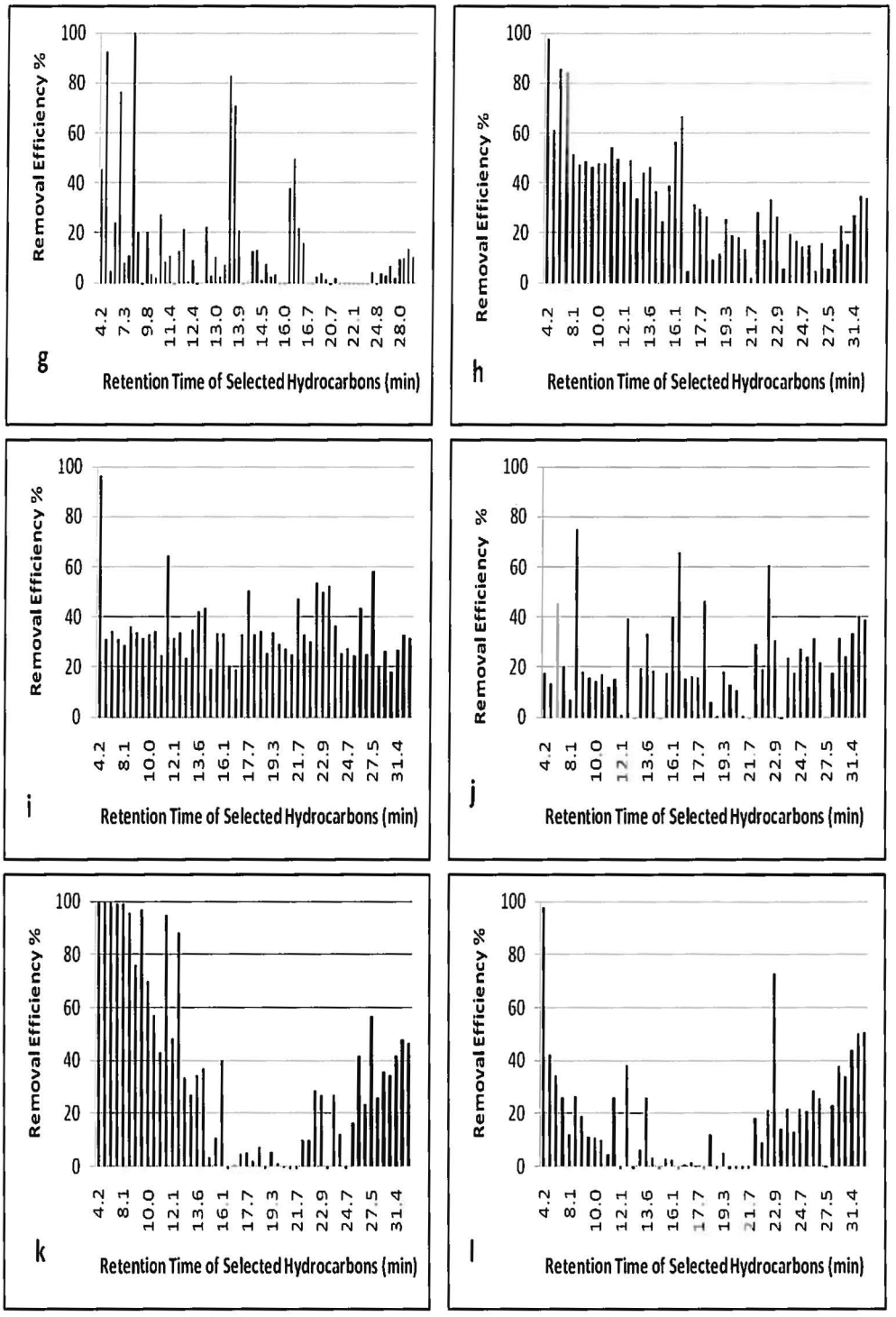

Figure 5: Removal Efficiency (RE) of selected hydrocarbons on 10% Diesel (indicated) by their retention time in their corresponding GC chromatograms of isolates: g) ZA10, h) ZA11, j) ZA12, i) ZA18, k) ZA20, l) ZA36.

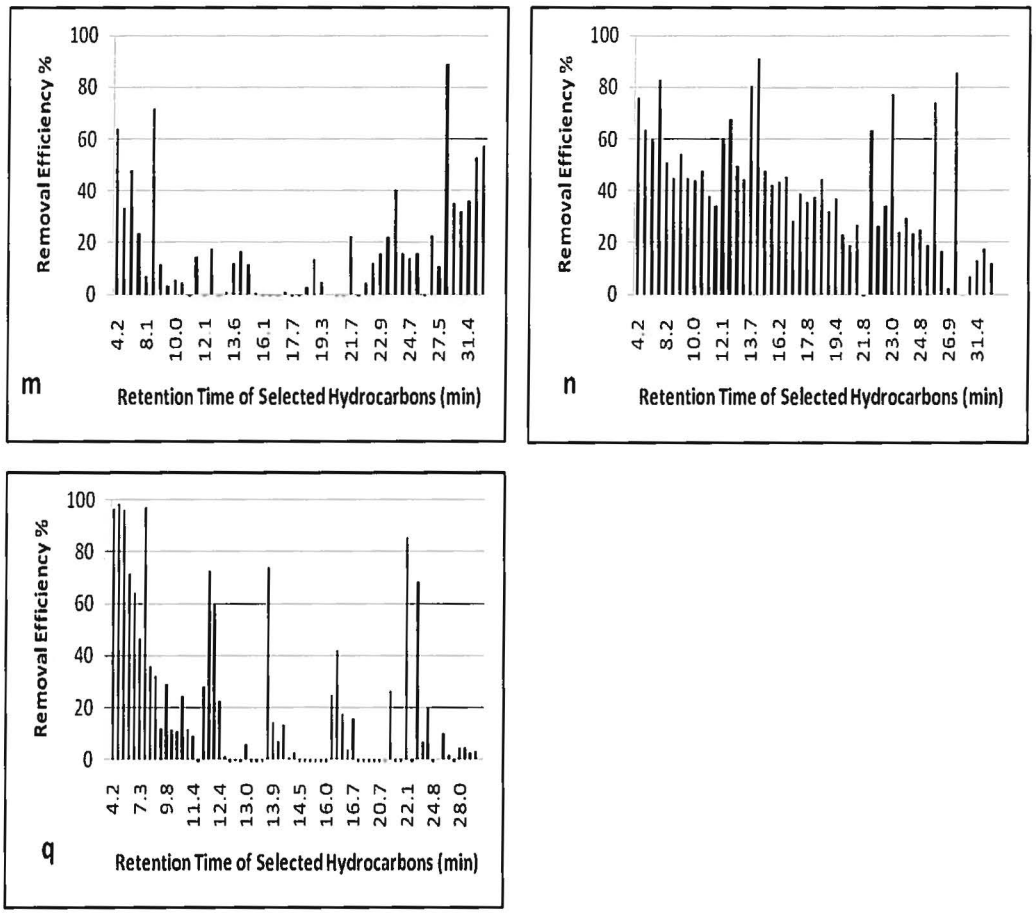

Figure 6: Removal Efficiency (RE) of selected hydrocarbons on 10% Diesel (indicated) by their retention time in their corresponding GC chromatograms of isolates: m) ZA37, m) QDD1, n) QDD2

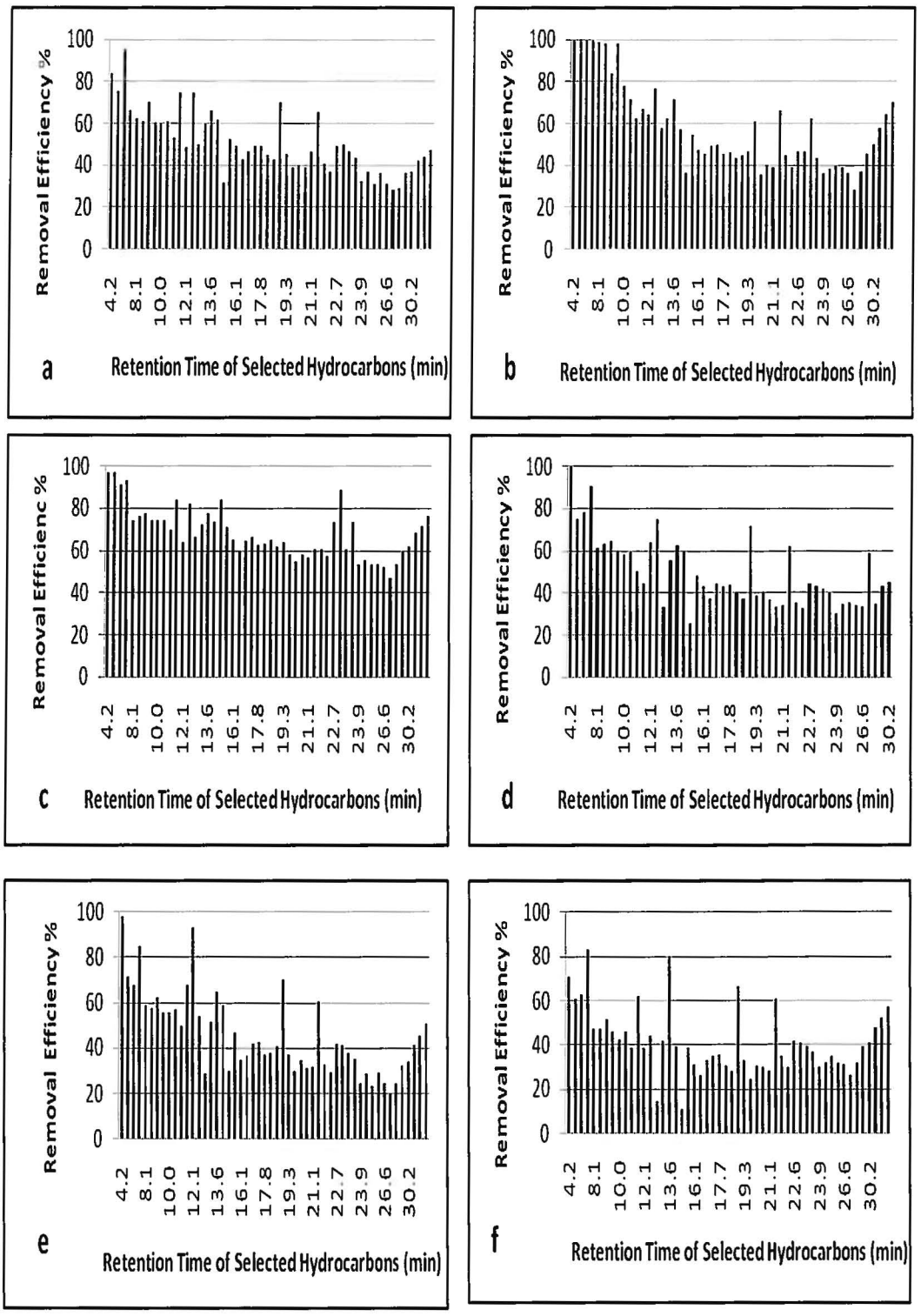

Figure 7: Removal Efficiency (RE) of selected hydrocarbons on 5% Diesel (indicated) by their retention time in their corresponding GC chromatograms of isolates: a) ZA2, b) ZA8, c) ZA9, d) ZA18, e) ZA36, f) QDD1

Section 3: Investigation of the bacterial activities at several nutritional conditions

Considering the results obtained in Section 1 and Section 2 with respect to our isolates, as well as those reported in the literature review, it is obviously clear that the biological activity of each strain is totally different from the others. This is not only related to their growth and tolerance to toxicity, but also to their ability to degrade or convert the diesel hydrocarbons. It is expected that some of the isolates belong to the same bacterial species. However, the ability to grow in a specific medium, depending on the metabolism, may change in nature by adaptation of the cells to the micro-environmental conditions. Random mutations affecting genes involved in the metabolism as well as those engaged in the biological activities may occur due to the exposure of the microorganisms to chemical mutagens, UV light, and such other factors. Consequently, it is possible that the nutritional requirements of each isolate may affect the corresponding biological activity exhibited in degrading diesel hydrocarbons. In fact, potentialities of the strains to grow on diesel as the sole carbon source may be improved by the use of adequate media composition, as already described in the literature (Coulon, et al., 2005).

In this section, we investigate the role of several nutritional parameters in the biological activity of the preselected isolates.

3.1. Study of the effect of nitrogen source on the growth of the selected strains

In order to investigate the effect of the composition of the nitrogen source on the growth and biological activity of the selected strains, two different nitrogen

sources were used. Ammonium Nitrate (NH_4NO_3) was initially the nitrogen source used at 1.4 g/l in the isolation program. It is named MSM1, corresponding to C/N ratio of 60/1 (based on rough estimation of 75% (750 g/l) carbon content as in the raw diesel sample). Ammonium Chloride (NH_4Cl) was used at 1.05 g/l in the medium named MSM2, corresponding to C/N ratio of 80/1. This may be considered actually, as a comparison between two conditions, which were the most described in the literature (Coulon, et al., 2005). In this study, one representative from each strain group was selected, except the strains ZA8, ZA9 and QDD1 from the same interesting group. The strain ZA19, which rapidly enters into the death phase, was not used. Results are shown in Figure 8.

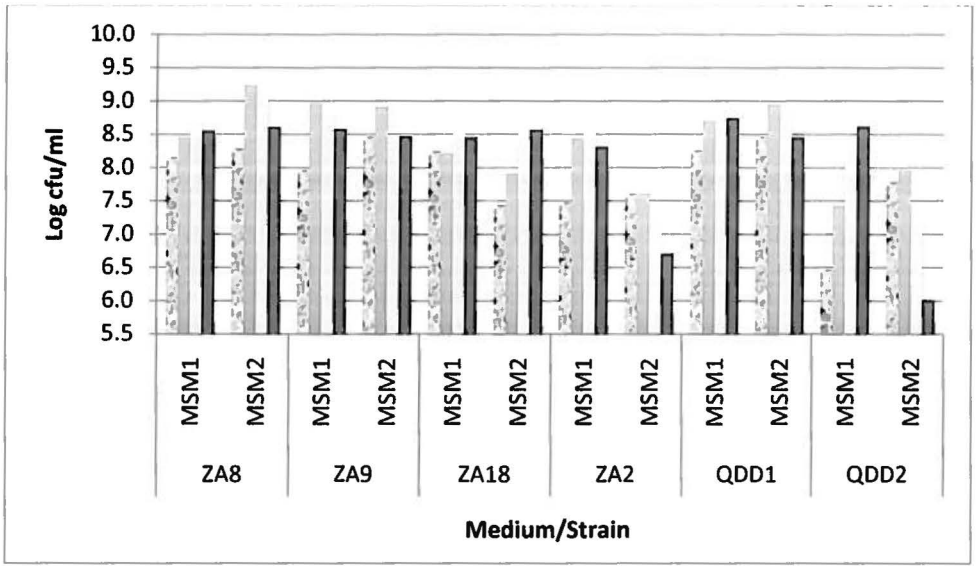

Figure 8: Comparison of growth of selected isolates on the two media MSM1 & MSM2 after: □ 3 days, ■1 week, □ 10 days, ■ 2 weeks.

3.2. Study of the effect of C/N ratios with different nitrogen sources on growth of the selected isolates

In order to study the effect of the C/N ratio on growth of the different isolates using hydrocarbons from diesel, different C/N ratios were ensured in both media, MSM1 (Ammonium Nitrate) and MSM2 (Ammonium Chloride). This investigation was performed with the most sensitive strains (ZA2, ZA8 and QDD1) to nitrogen source composition. The results obtained after 7 days of incubation are shown in Figure 9 and Figure 10.

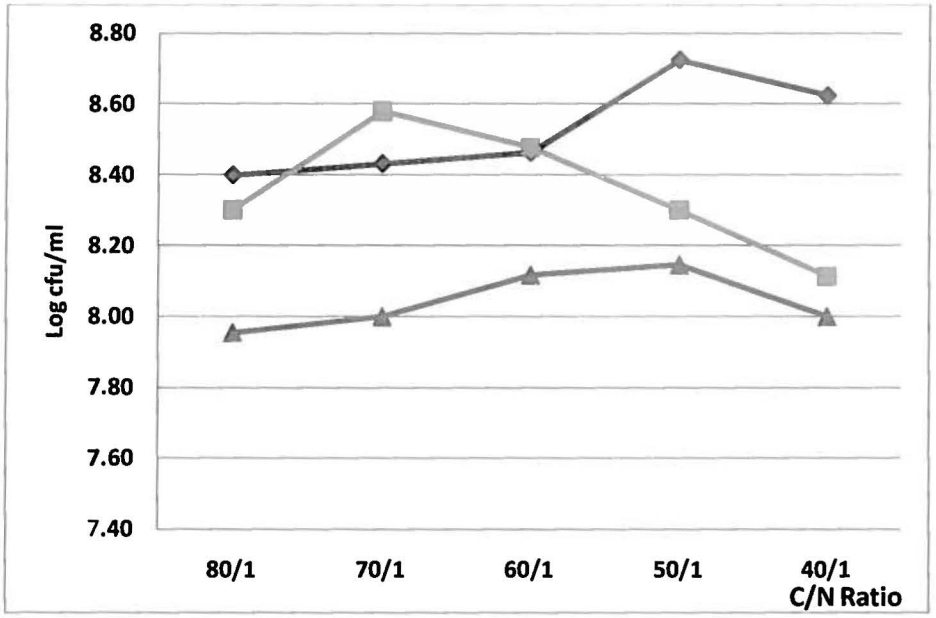

Figure 9: Effects of Ammonium Nitrate C/N Ratios on growth of selected isolates: QDD1, ZA8, ZA2

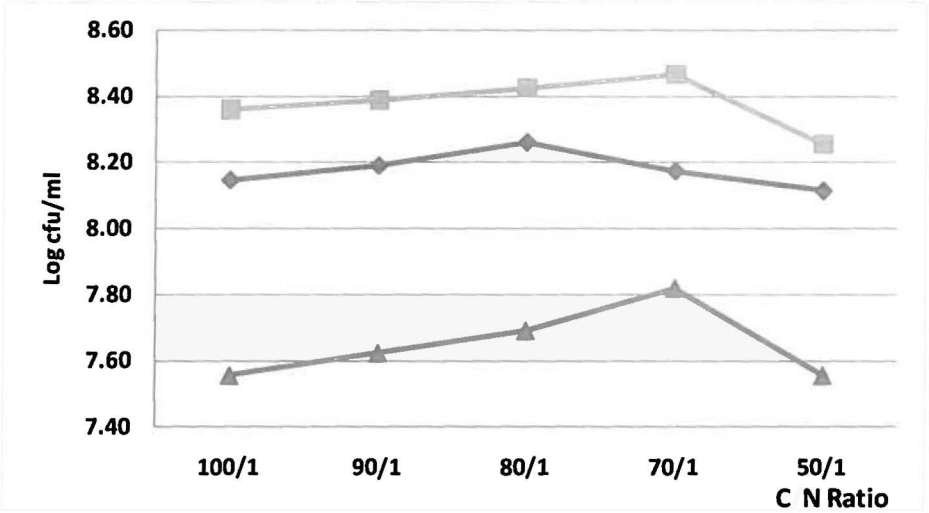

Figure 10: Effects of Ammonium Chloride C/N Ratios on growth of selected isolates: QDD1, ZA8, ZA2

From Figure 9, it is clear that each strain has an optimal C/N at 10% (v/v) diesel, corresponding to 75 g/l hydrocarbons:

- With strain ZA8, the optimal C/N is equal to 70/1, corresponding to 3.5 g/l Ammonium Nitrate.
- With strain QDD1, the optimal C/N is equal to 60/1, corresponding to 4 g/l Ammonium Nitrate.
- With strain ZA2, the optimal C/N is equal to 50/1, corresponding to 3 g/l Ammonium Nitrate.

3.3. Study of the effect of amino acids supplemental on growth of the selected strains

Since the inoculum of every strain is prepared in the LB medium, which contains all the growth factors, including amino acids and peptides among others, for high and fast growth of bacteria, we added to MSM1 and MSM2, 1ml of sterile LB

medium into the final volume of 20ml. It is then expected that the modified MSM media contains 0.5 g/l tryptone and 0.25 g/l yeast extract, both serving as amino acid sources. The C/N ratios were 80/1 with Ammonium Chloride and 60/1 with Ammonium Nitrate. In fact, we preferred, in this study, to diversify all the strains and not only those pre-selected for the nitrogen source study. Indeed, ZA9 was used as one of the best strains in our collection. ZA8, ZA18 and ZA20 were used to enrich the study as strains having potentialities in degradation of diesel hydrocarbons, but which may be improved. It was previously shown (Table 3) that ZA8 and ZA9 seemed not to be so highly sensitive to toxicity. On the contrary, strains ZA18 and ZA20 were more sensitive to hydrocarbon toxicity. This step is also considered for investigations included in the screening program. Growth of the selected isolates was followed after periods of 3 days, 1 week and 2 weeks. The results of growth of the selected strains in the media are shown in Figure 11.

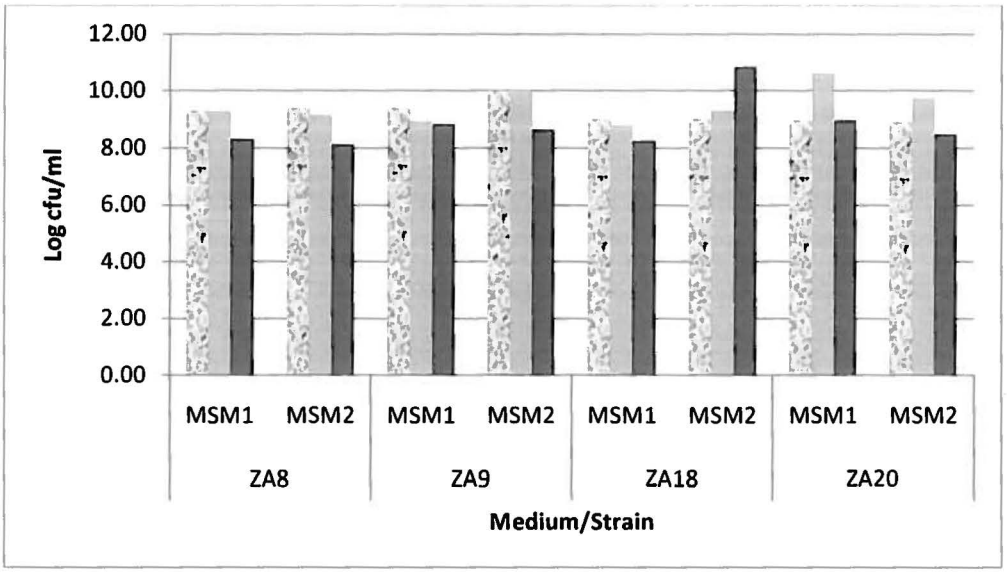

Figure 11: Effects of Amino Acid source (LB medium) on growth of selected isolates in MSM1 & MSM2 after: □ 3 days, □ 1 week, ■ and 2 weeks of incubation.

3.4. Role of yeast extract as amino-acid source for hydrocarbon-degrading bacteria

In microbiology, yeast extract is mainly considered as an amino acid source for microorganisms. It may also be used as carbon and nitrogen sources. Different concentrations of yeast extract were added into the final volume of 20 ml MSM1. Cell counts of the pre-selected strains (ZA2, ZA8 and QDD1) were recorded after one week of incubation. The results are shown in Figure 12.

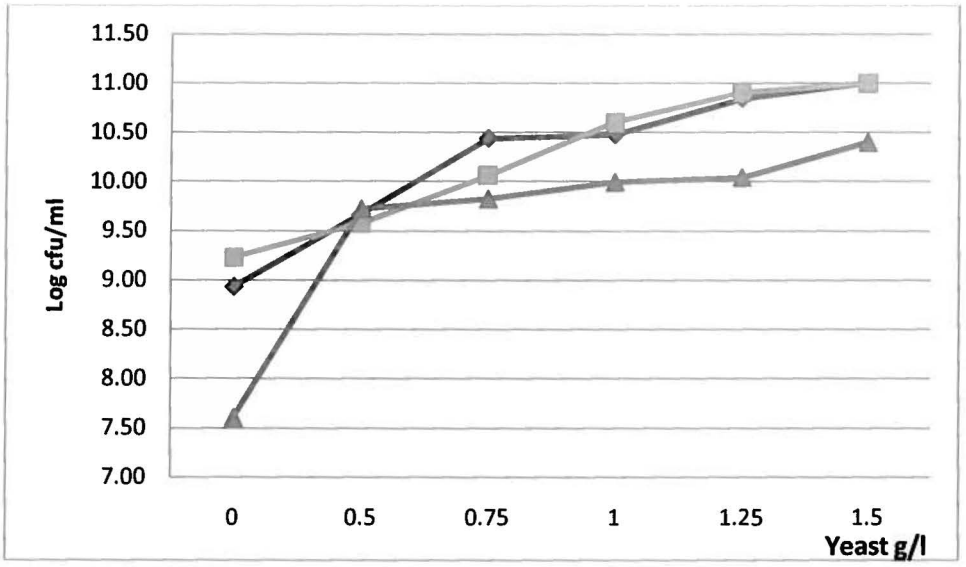

Figure 12: Biomass production of selected isolates, QDD1, ZA8, ZA2, after 2 weeks incubation, on 10% diesel, 4 g/l Ammonium Chloride and different yeast extract concentrations.

3.5. Establishment of growth Curves of the isolated strains

In the screening program, it is sometimes suitable to select strains according to their growth rate in a rich medium so as to predict if the optimization of the cultural conditions and of the media may lead to improvement of biomass production. Here, LB medium was selected to determine the growth rates of the

pre-selected strains ZA2, ZA8 and QDD1. Cell counts (Figure 13) and Optical density at 600nm (Figure 14) were measured at regular intervals for 4 days.

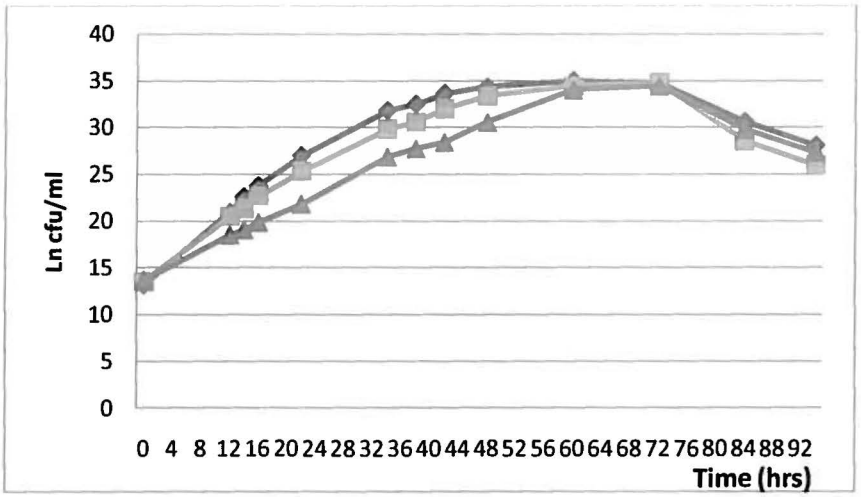

Figure 13: Growth Curves of the pre-selected isolates QDD1, ZA8, ZA2, in LB medium. Growth was determined by cfu in the culture broths.

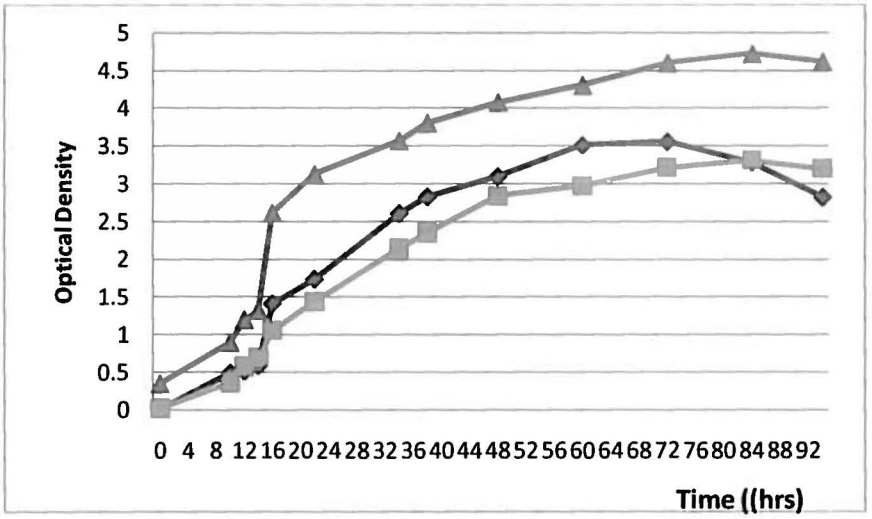

Figure 14: Growth Curves of the pre-selected isolates QDD1, ZA8, ZA2, in LB medium. Growth was determined by measuring optical density at 600 nm of the culture broths.

Calculation of approximate growth rates of the studied strains was performed according to results of Figure 13:

- ZA8: $\mu = 0.54\ h^{-1}$
- ZA2: $\mu = 0.37\ h^{-1}$
- QDD1: $\mu = 0.64\ h^{-1}$

The growth rate of ZA2 is the lowest, which means that even in a rich medium, there is a possibility to distinguish strains.

3.6. Effect of nitrogen source, C/N ratios and amino acid supplemental on the biological activity of the isolates

As illustrated above, it was clearly observed that the growth of strains is affected by different nutrition conditions; hence, a further study was carried out on selected strains to assess the impact of changing conditions on diesel degradation efficiency.

Figure 15 - **Error! Reference source not found.** present hydrocarbon removal efficiencies for isolates ZA2, ZA8 and QDD1 with different nutrition conditions.

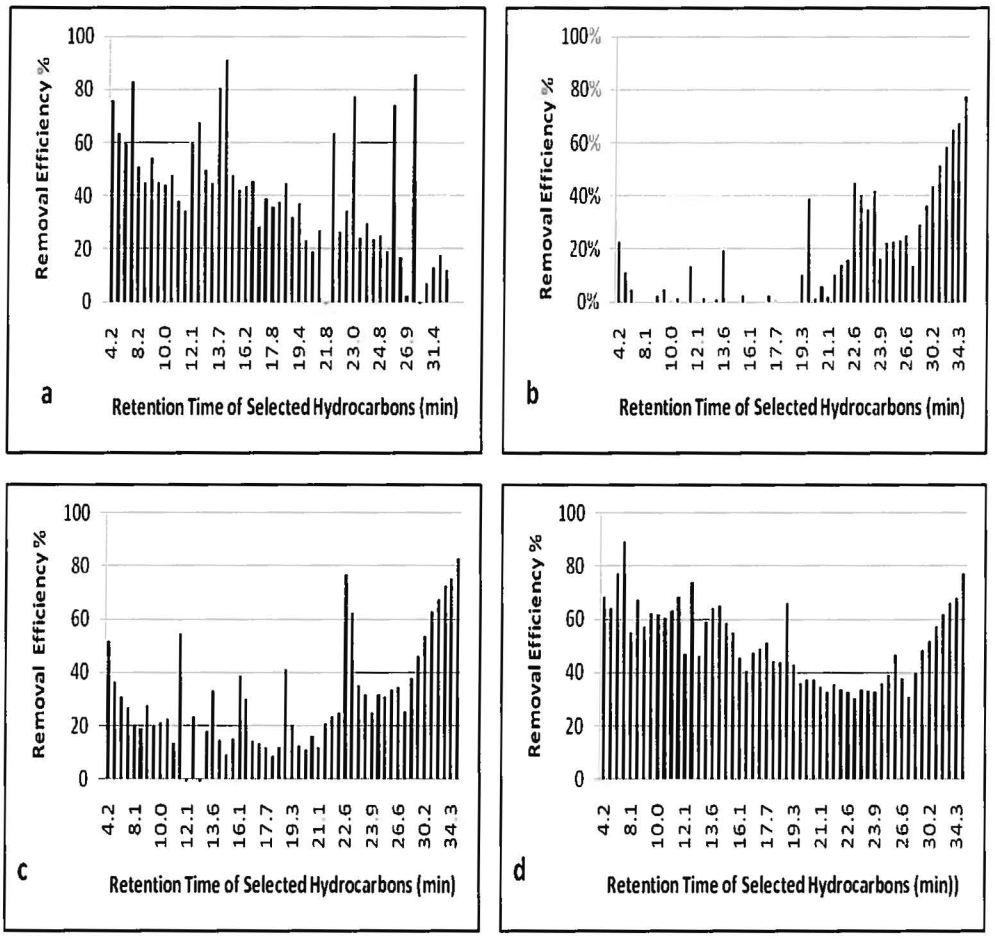

Figure 15: Hydrocarbon Removal Efficiency for QDD1 with Different nutrient conditions:

a) MSM1 Nitrogen Source; NH_4NO_3 (4 g/l), corresponding to C/N ratio equal to 60/1
b) MSM 1 condition (4) Nitrogen Source NH_4NO_3 (5 g/l) , C/N optimized to 50/1
c) MSM 2 condition (3) Nitrogen Source NH_4Cl (4.5 g/l) C/N optimized to 70/1
d) MSM2 Nitrogen Source is NH_4Cl (4 g/l), C/N is 80/1, with Yeast extract 1.5 g/l

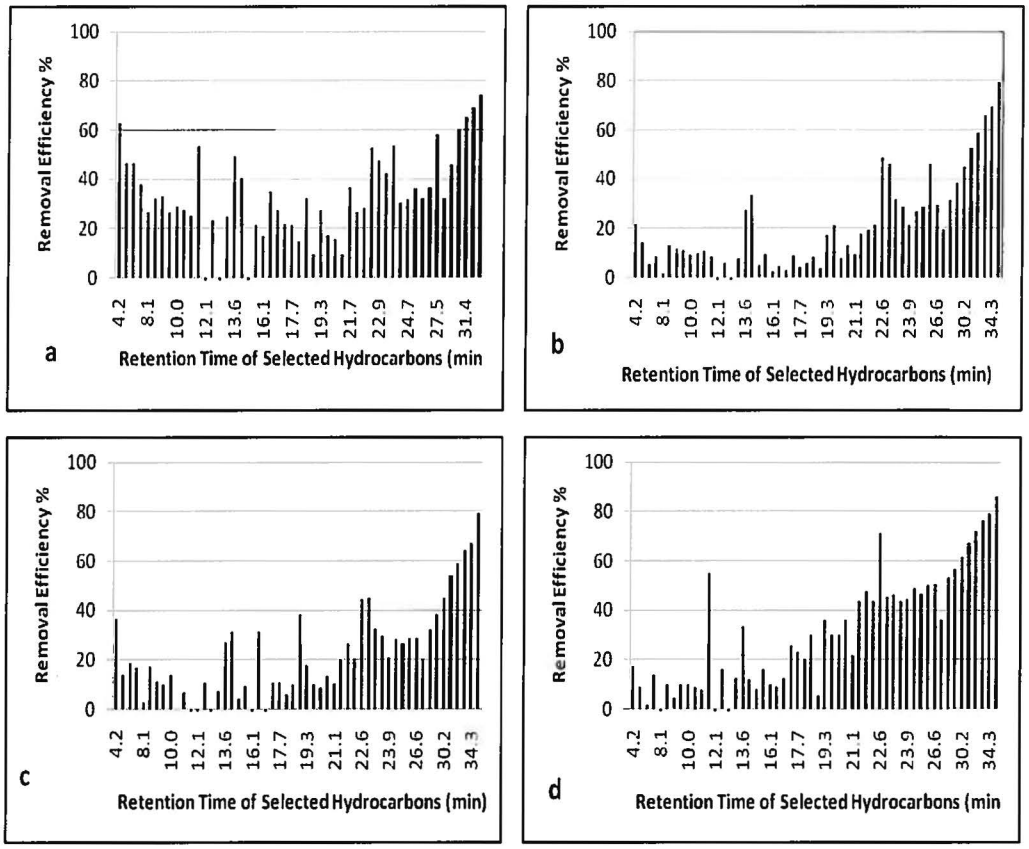

Figure 16: Removal Efficiency (RE) of selected hydrocarbons on 10% Diesel (indicated) by their retention time in their corresponding GC chromatograms of isolate ZA8 with Different nutrient conditions

a) MSM1 Nitrogen Source; NH_4NO_3 4 g/l, corresponding to C/ N ration equal to 60/1.
b) MSM1 condition (2); Nitrogen NH_4NO_3 (3.5 g/l), C/N ratio optimized to 70:1.
c) MSM2 condition (4) Nitrogen Source NH_4Cl (4.5 g/l), C/N ratio optimized to 70:1.
d) MSM2 Nitrogen Source is NH_4Cl (4 g/l), C/N ratio is 80/1, with Yeast extract 1.5 g/l.

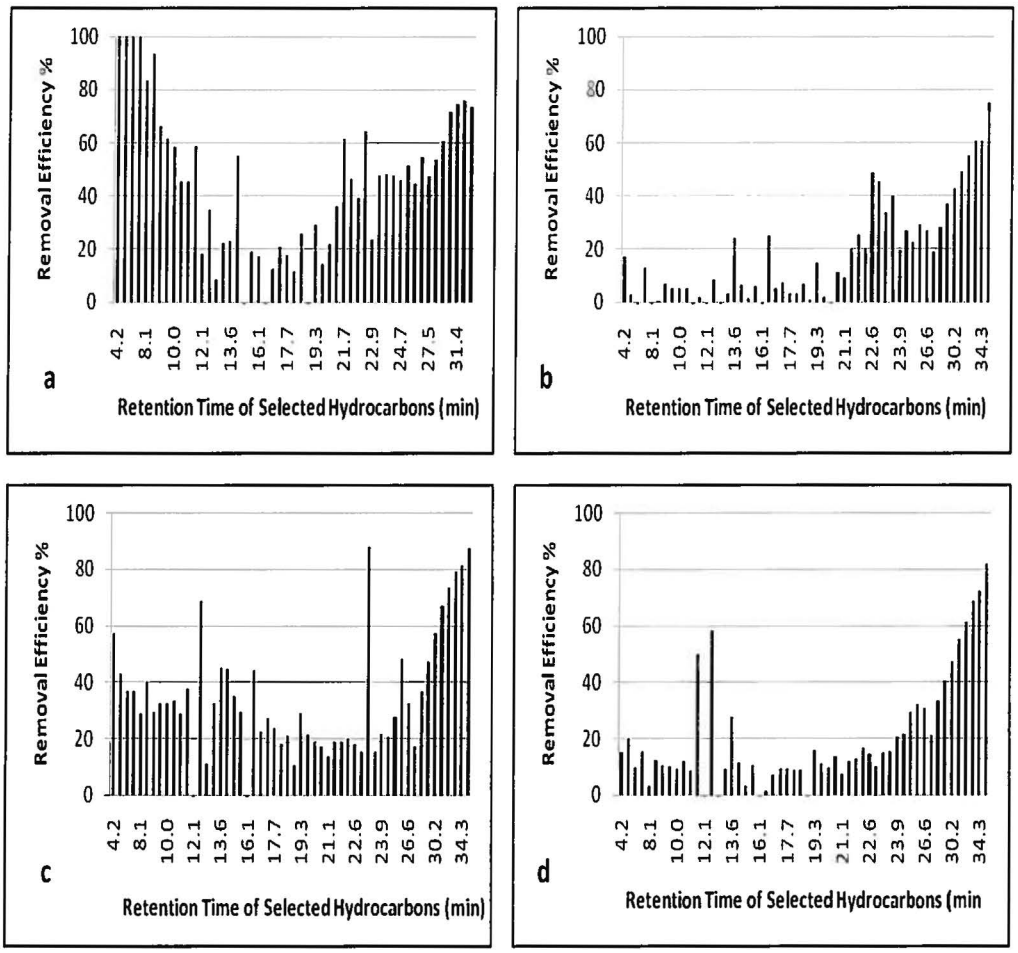

Figure 17: Removal Efficiency (RE) of selected hydrocarbons on 10% Diesel (indicated) by their retention time in their corresponding GC chromatograms of isolate ZA2 with Different nutrient conditions

a) MSM1 Nitrogen Source; NH_4NO_3 (4 g/l), corresponding to C/N equal to 60/1.
b) MSM1 condition (4); NH_4NO_3 (5 g/l) optimizing C/N ratio optimized to 50/1.
c) MSM2 condition (4); NH_4Cl (5 g/l) C/N ratio optimized to 70/1.
d) MSM2 Nitrogen Source NH4Cl (4 g/l), C/N is 80/1, with yeast extract 1. 5 g/l.

3.7. Investigation of toxicity by diesel

It was observed in several previous studies, that biodegradation rates are affected by diesel oil concentration. It may be considered that at high diesel concentration,

hydrocarbons used as substrates are also at higher concentration for the growth of bacteria. The fast growth of bacteria may promote the biodegradation of the diesel components. Very high concentrations of hydrocarbons may also cause inhibition to biodegradation by several compounds or oxygen limitations (Lou, et al., 2012).

The investigation of the growth of the selected isolates by increasing the diesel concentration from 5% to 50% was performed. In order to compare the growth and biological activities of the three selected strains for further application in hydrocarbons degradation, increased diesel concentrations corresponding to C/N ratio of 60/1 in the MSM1 medium were used. According to our previous results, it was clear that this comparison may be more informative with incubation periods of 1 week and 2 weeks. Indeed, with time, the removal of hydrocarbons with several strains may continue, but much slower than with the others. Interactions between hydrocarbons as well as their reactivity need strains with rapid growth and fast bioremediation.

The Results of Figure 18, obtained after one week of incubation, clearly show that the growth of the three strains increased with diesel concentration, up to 40% (v/v). The strain ZA8 exhibited the best biomass and growth. At 50% (v/v) diesel, the growth of all the strains decreased. Here, many reasons may be highlighted, especially:

- Toxicity with several hydrocarbons reaching critical concentrations;
- Inappropriate aeration of the medium;
    - Oxygen, an essential parameter for growth of aerobic microorganisms as substrate and as final acceptor of electrons;
    - Oxidation of the substrates mainly, hydrocarbons;

- High nitrogen concentration, employed to maintain the C/N ratios.

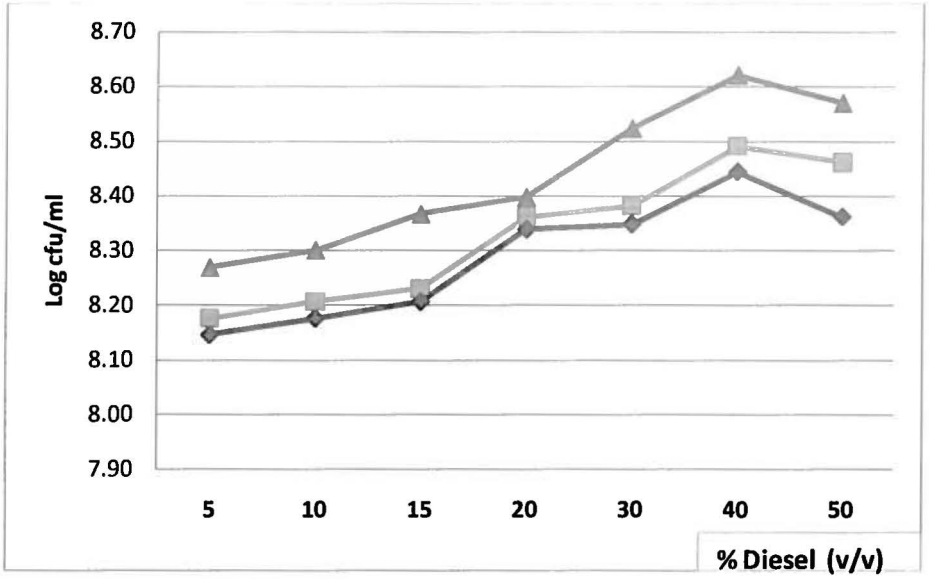

Figure 18: Growth of selected isolates QDD1, ZA8, ZA9, at increasing diesel concentrations in MSM1 medium after 1 week of incubation, C: N ratio is maintained at 60:1 with all diesel concentrations.

Moreover, ZA8, which had the best growth after 1 week of incubation, may have the ability to adapt to the substrate in the medium (diesel) better than the other strains. This might reduce the time of the short lag phase before entering the exponential phase.

Results obtained after 2 weeks incubation (Figure 19), clearly show that the biomass production by each isolate was almost the same in the range of 10 to 30% diesel.

Growth of QDD1 and ZA9 was slightly increased at 40% diesel, to decrease dramatically at 50%. First, this study could show that the three strains are normally different. Second, the explanation of these results is related to several potential phenomena, such as co-metabolism, balance between growth due high substrate concentration and their toxicity…

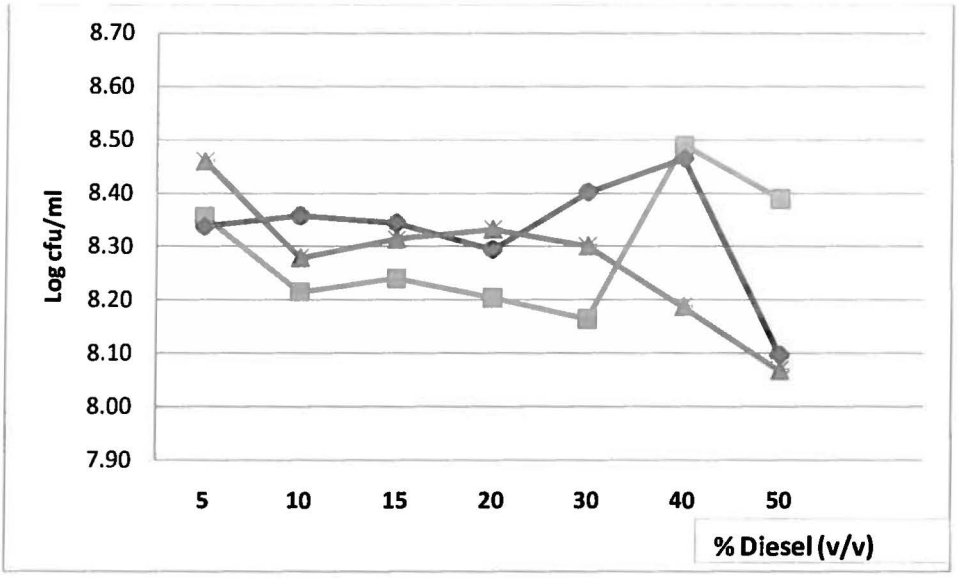

Figure 19: Growth of selected isolates QDD1, ZA8, ZA9, at increasing diesel concentrations in MSM1 medium after 1 week of incubation, C/N ratio is maintained at 60/1 with all diesel concentrations.

The GC analysis was carried out in the diesel fraction and the results of the removal of the selected hydrocarbons are shown in Figure 20- Figure 22.

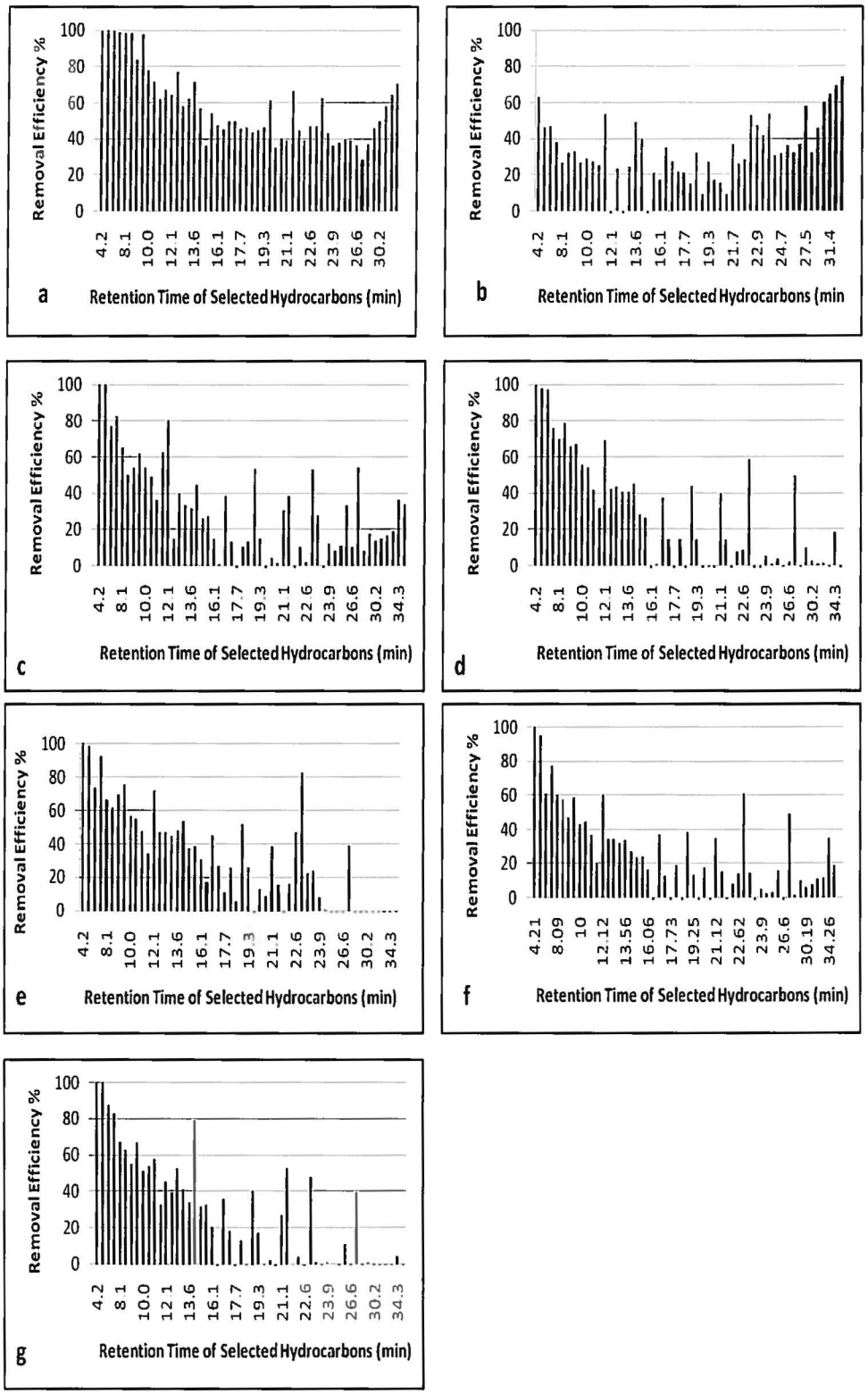

Figure 20: Removal Efficiency of ZA8 with increased diesel concentrations on MSM1, a) 5%, b) 10%, c) 15, d) 20%, e) 30%, f), 40% g) 50% diesel (v/v).

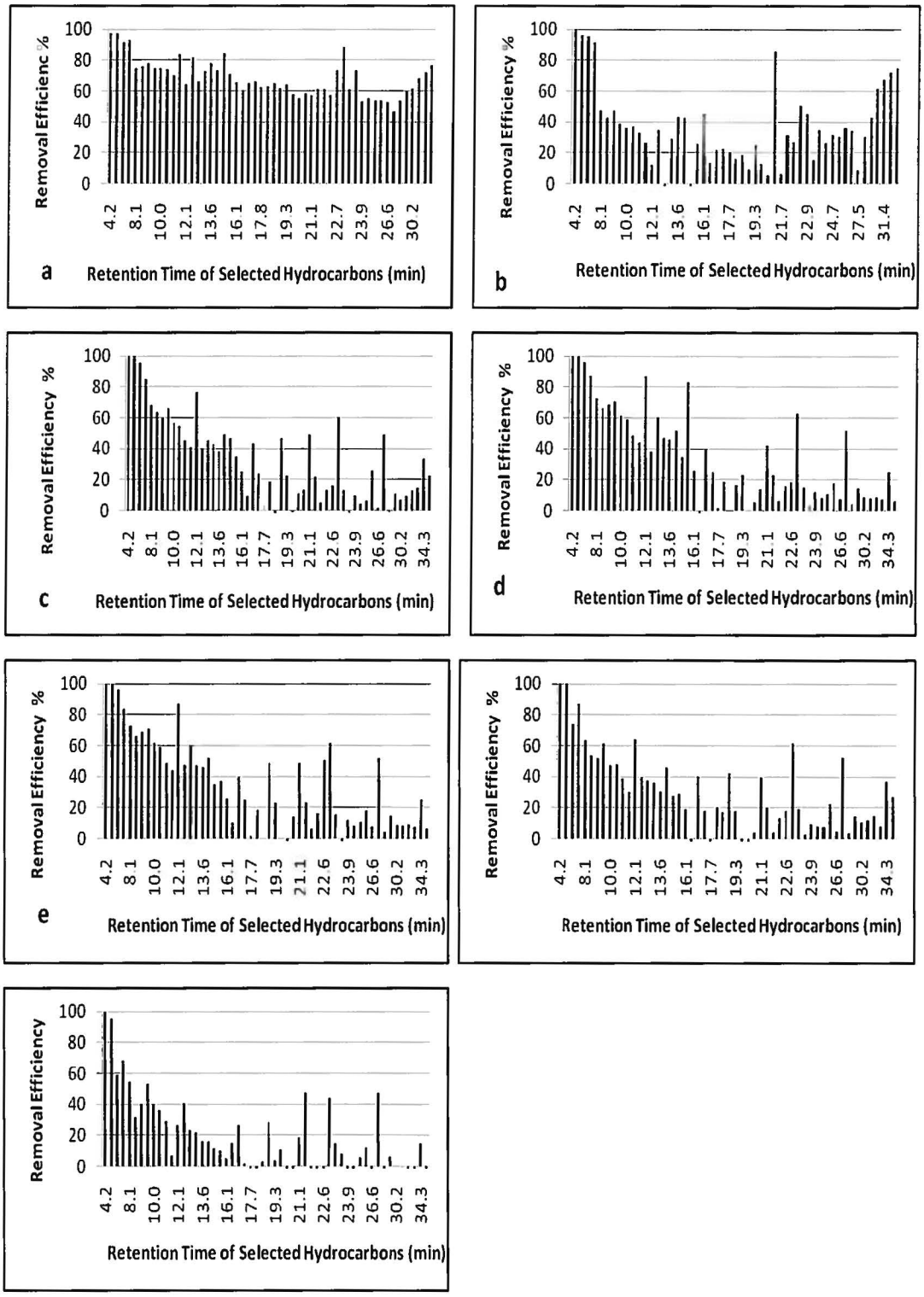

Figure 21: Removal Efficiency of ZA9 with increased diesel concentrations on MSM1, a) 5%, b) 10%, c) 15, d) 20%, e) 30%, f), 40% g) 50% diesel (v/v).

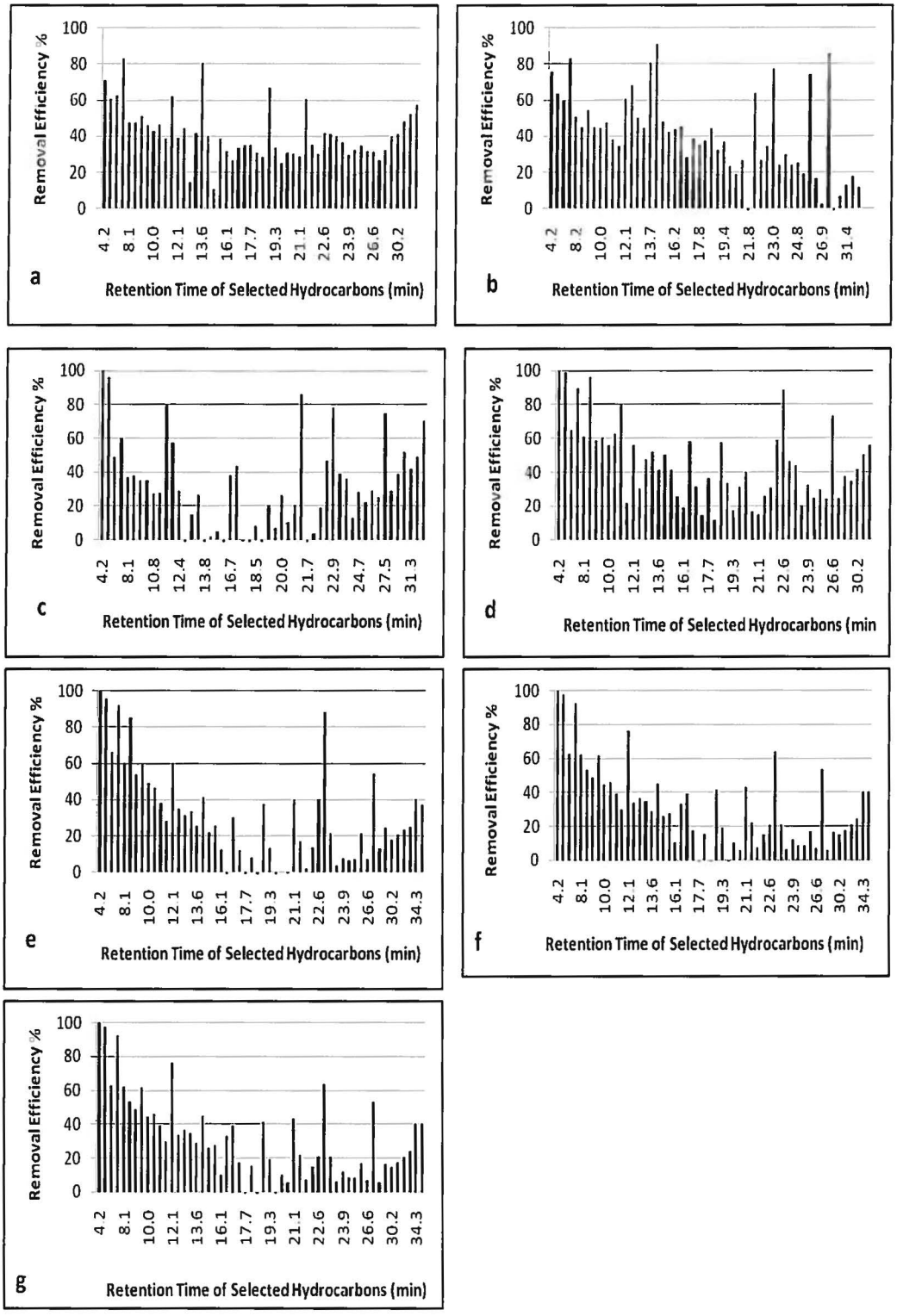

Figure 22: Removal Efficiency of QDD1 with increased diesel concentrations on MSM1, a) 5%, b) 10%, c) 15, d) 20%, e) 30%, f), 40% g) 50% diesel (v/v).

These results show that the best efficiency was obtained with 5% diesel. Then, several fluctuations were observed from one concentration to another. These results were repeated two times and the same conclusions drawn.

Section 4: Molecular identification of the pre-selected isolates.

Molecular Ribotyping of the selected strains

The molecular ribotyping was carried out by amplifying by PCR and sequencing a part of 16S ribosomal DNA using the two primers RibS73 and RibS74 and Ion torrent sequencing technology.

Primer sequences used in molecular ribotyping:

Primers	Sequence(5'->3')
RibS73sp	**AGAGTTTGATCCTGGCTCAG**
RibS74sp	**AAGGAGGTGATCCAGCCGCA**

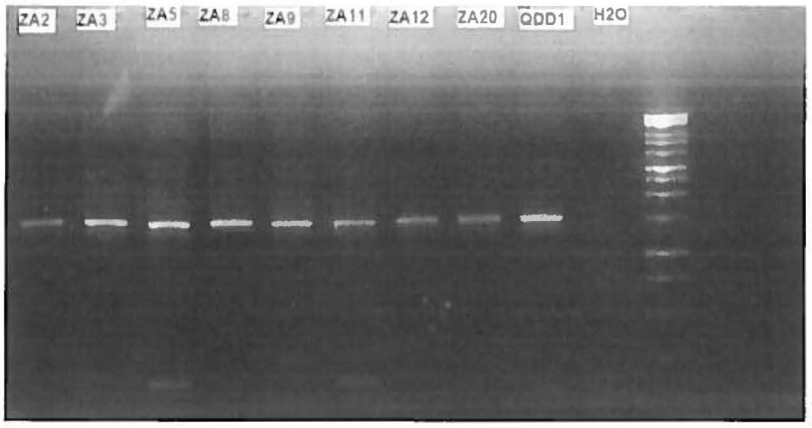

Figure 23 : PCR amplification of the 16S ribosomal DNA fragment

M: DNA Ladder: 250bp, 500 bp, 750 bp, 1kb, 1.5kb, 2 kb, 2.5 kb. 3 kb, 4 kb, 5 kb, 6 kb, 7 kb, 8 kb, 10kb.

The DNA ladder includes fragments ranging from 0.5-10.0 kilobases (kb). The 1.5 kb fragment has increased intensity to serve as a reference band.

For all the studied strains, a fragment of an expected size of 1.5 kb was amplified by PCR.

These fragments were purified from the gel and subjected to DNA sequencing.

Then, the obtained sequences were analyzed and compared to published 16s ribosomal DNA sequences of known bacteria, using BLAST software [http://www.ncbi.nlm.nih.gov/]

Figure 24 demonstrates sample blast done for ZA9

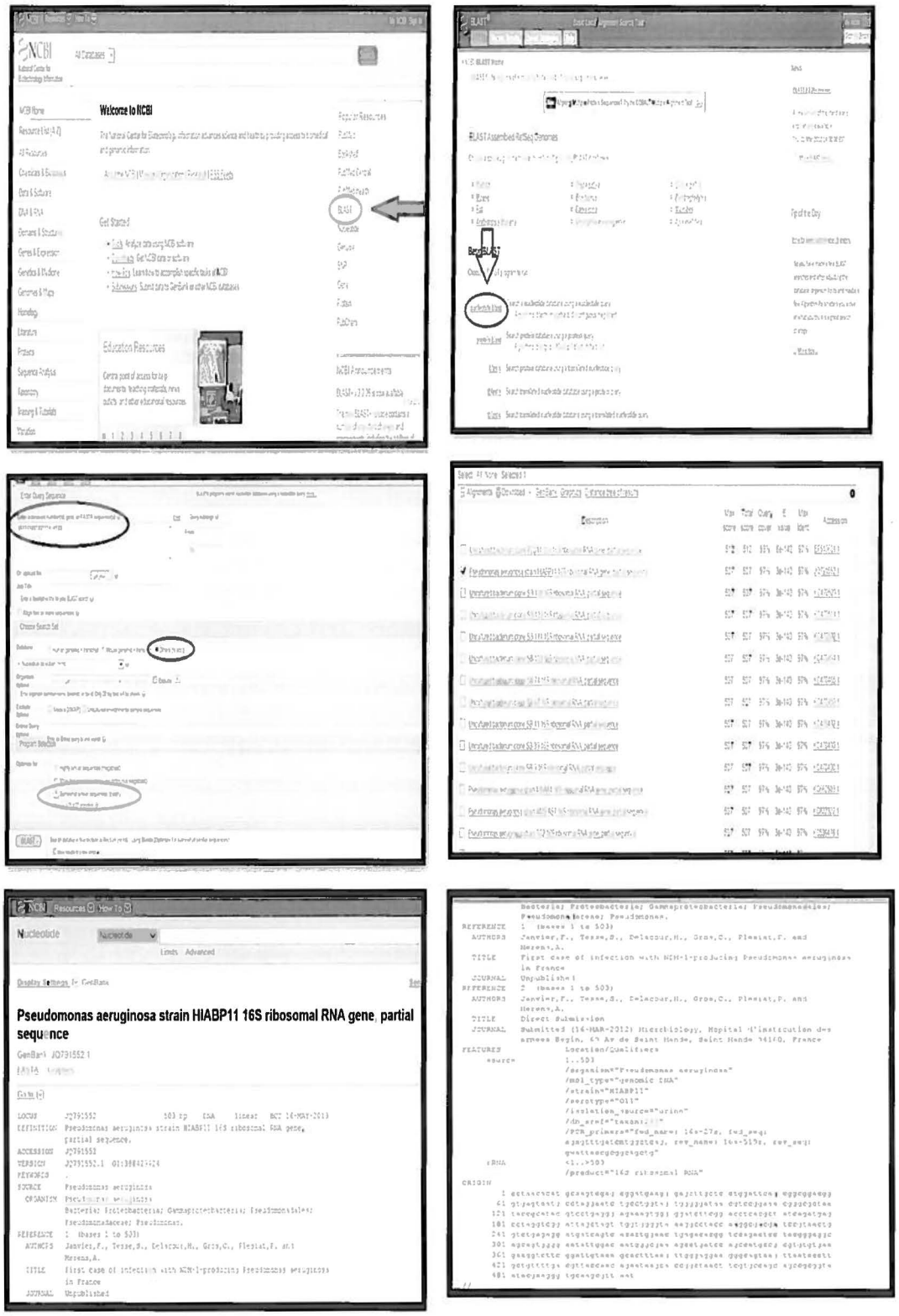

Figure 24: Screenshots demonstration of Sample blast done for ZA9- *Pseudomonas aeruginosa*

For each 16S rDNA sequence, the most closely related sequence and the sequence of the most closely related cultured bacterial strain were retrieved from the Gene Bank database using the Blast server at NCBI.

Table 4 : Results of taxonomic classification of selected isolates

Code	Sequence	Strain
ZA2	ACCCGTATGCGGCCTCTTTACCATGCAAGTCGAACGATGATGCTGGTGCT TGCACTGGTGGATTAGTGGCGAACGGGTGAGAAACACGTGAGTAACCTG CCCCTGACTTCGGGATAAGCCCGGGAAATGGTCTATCCGGTTGCTTCTGCT CTGACGCCTCG	*Arthrobacter sp.*
ZA3	GCCTTGGCTCCTTGGTTCGACTTCCCCCAGTCATGAATCACAAAGTGGCA GCGCCCTCCCGAAGGTTAAGCTACTAGAAAAAGGAGGGGACCCACTCCC ATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGGGG CATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGTT TACTCCTATCCGGACTATGAT	*Citrobacter sp.*
ZA5	GCCAATGGCTTCTTGTTCGACTTCACCCCAGTCATGAATCACAAAGTGGT AAGCGCCCTCCCGAAGGTTAAGCTACCTACTACTTTTGCAACCCACTCCC ATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGGC ATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCA GACTCCAATCCGGACTACGACATACTTTATGAGGTCCGCTTGCTCTCGCG AGGTCGCTTCTCTTTGTATATGCCATTGTAGCACGTGTGTAGCCCTGGTCG TAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACT GGCAGTCTCCTTTGAGTTCCCGGCCTAACCGCTGGCAACAAAGGATAAGG GTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACG ACAGCCATGCAGCACCTGTCTCACAGTTCCCGAAGGCACCAAGGCATCTC TGCCAAGTTCTGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATC GAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTG AGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCTATTTAACGCGTTAGC TCCGGAAGCCACGCCTCAAAGGGCACAACCTCCAAATAGACATCGTTTAC GGCGTGGACTACCAGGGTATCTAAATCCTGTTTGCTCCCCACGCTTTCGCA CCTGAGCGTCAGTCTTCGTCCAGGGGGGCCGCCTTCGCCCACCGGTATCC CTCCAAGATCTCTACGCATTTTCACCGCTACACCTGGGAAATCTACCCCCC CTTCTACCGAAGACTTCAAGGCCTGCAAGTTTCGAAATGCAGATCCCCAA GGTTGAAGCCCGGGGAATTCACAATCCCGAACTCTGGAACAGAACCCGG CTCCTGGCCGTAGCTCACGC	*Citrobacter amalonaticus*
ZA8	GATTTGGCCGGGAAGGCTACACATGCAGTCGAGCGGATGAAGGGGAGCTT GCTCTCTAGGATTCAGCGGCGGACGGGTGAGCTAATGCCTAGGAATCTGC CTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTC CTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCT AGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCC GTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCC AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAG CCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGC ACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGT TACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA CGAAGGGGGCAATCGTTAATCGGAATTACTGGGGTGGAAAGCGCGCGTAC GTGGTTCATCAAT	*Pseudomonas aeruginosa*
ZA9	CGAAAGGGCCCGGGGAATGGAAAGGGAGCTTGCTTCCTGGATTCAGCGGC GGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTC CGGAAACGGGCGCTAATACCGCATACTCCTGAGGGAGAAATTGCGGGTTC TTCTTCCTCTCTCTATCT	*Pseudomonas aeruginosa*
QDD1	GTCCSGAAGSCCKGGAWKGGAAGGKAKCTTGCTCCCTGGATTTCAGCGGC GGACGGGTGASCWATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTC CGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGA TCTTCGGAYCTCACGCTATCWRATGAGCCTAGGTCGGATTAGCTAGTTGG TGGGGTAAAGGCCTACCMAGGCKACRATCCGTAACTGGKCT	*Pseudomonas aeruginosa*
ZA11	TGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTCGCTGACGAGTGG CGGACGGGTGAKTAATGTCTGGGAAACTGCCTGATGGAGGGRGATAACT ACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGG GACCTTCGGGCCTCATGCCATCAKATGTGCCCAGATGGGATTAGCTTGTT GGTGGGGTAACGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGG ATGACCAGCCACWCTGGAACTGAYACACKGTCCAKACTCCTACGGGAGG CAGCAGTGGGGAATATTGCACAATGGGYGCAAGCCTGATGYA	*Enterobacter helveticus*

Code	Sequence	Strain
ZA18	GYWRRRRRSGSSRGGCCCWAAMAMATGCAAGTCGAACGGTAACAGGAAG CYAGCTTGCTGCTTTGCTGACGAGTGGCGGACGGSTKAKTAATGTCTGGG AAACTGCCCGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGC ATAATGTCGCAAGACCAAAKAGGGGGACCTTCGGGCCTCTTGCCATCGGA TGTGCCCWGATGGGATTAKCTTGTTGGTGAGGTAACGGCTCACCAAGGCG ACGATCCCTAGCTGGTCTGAKARGATGACCASCCWCA	*Citrobacter amalonaticus*
ZA19	ATGCARGTTCGAMCGGTAMCAGGAAGCAGCTTGCTGCTTTGCTGACGAGT GGCGGACGGGTGWGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAA CTACTGGAAACGGTAGCTAATACCGCATAACGTCKCAAGAYCAAAGTGGG GGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGT AGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGASAGG ATGACCAGCCACACTGGAWCTGAGACACGGTCCAGACTCCTACGGGAGG CASCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC GCGTGTATGAAGAAGGCCTTCGGKTTGTAA	*Cronobacter muytjensii*
ZA20	ATATGCCCCCCTTGTTTCGACTTACCCCAATCTTCTGTCCCACCTTAGGCTG CTGGCTCCAAAAAGGTTACCCCACGGTCCATAGACGTTACAAAATCACGT GGGGGGACGGGCGGGGTGTACAAGGCCCGGCAACGTATTCACCGCGCGA TGCTGATCCGATATTACTAGACAATCCAGCTTCAGTAGGGGAGTTGCGTCC TACAATCCGAACTGAAAACGGTTTTATGAGATTAGCTCCACCTCCCGGCA CTGCATCTCTTTGCACCGTCCATTGTATCACGTGTGTAGCCCAGGTCATAA GGTGCATGATAATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCTCCAGC ACTCACCTT	*Bacillus cereus*
QDD2	GSYAAWKMGGSSRGGCCTWAMMAMMATGCAAGTCGAGCGGTAGCACAG AGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTYTWKTAATGTCTGGG AAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGC ATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGA TGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCG ACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGAC ACGGTCCAGACTCCTACGGGAGGCAGCAKTGGGGAATATTGCACAATGGG CGCAAGCCTGATGCAGCCATGCCGCGTGTRTGAAGAAGGCCTTCGGGTTG TAAAGYACTTTCAGCGGGGAKGAAGGCGATAAGGTTAATAACCTTGTCGA TTGACGTTACCCKCATAARAAGCACCGGCTAACTY	*Klebsiella pneumoniae*

Discussion

1. Isolation of Hydrocarbon degrading bacteria

The Isolation and Screening program was designed to obtain the most interesting strains that are capable of degrading hydrocarbon compounds.

a) The nine samples were collected from recently polluted areas as well as areas polluted for very long time to ensure adaptation of bacteria to pollution potential of hydrocarbons, and to different compositions of the hydrocarbons.

b) After the enrichment in LB media, adaptation step of microorganisms to oil/diesel as sole carbon sources was repeated 2 folds to enrich the MSM and MM media only with microorganisms able to grow using oil and/or diesel components as sole carbon source.

At first steps of the Isolation program, 39 purified bacterial isolates were recorded out of hundreds isolates, which were lost in the phase of purification, due to loosing hydrocarbon degrading activity, known to be exhibited by plasmidic genes easily lost at several growth conditions.

We can conclude that diversification of samples as well as media and hydrocarbons led to a potential large variety of isolates with biological activities which may be different.

2. Screening program for the isolated strains

2.1. Screening of the Isolates on MSM-Oil solid Medium

The Spread plate technique was used to spread LB-cultures on MSM agar medium supplied with Crude oil/Diesel as sole carbon source. Finally, the best diesel and/or petroleum degrading bacteria were purified on solid MSM agar plates containing by spray-plate technique petroleum or diesel respectively, as the sole carbon and energy source. As a result, only 23 were able to form clear zones on the coated solid MSM medium and were selected for further screening programs.

2.2. Screening of bacterial isolates based on biomass production on 10%

A high pressure selection criterion was applied using 10% of Raw Diesel. Consequently, only strains that can tolerate high hydrocarbon concentrations would be selected. The selection criteria was enhanced by including the Gas Chromatograph analysis combing diesel biodegradation efficiency for each strain with biomass production, in order to obtain wide biological varieties isolated collection of strains.

By the end of the screening program the isolated strains were categorized according to their biological activities to strains that attack low molecular weight carbon compounds and others that are more effective with high molecular weight carbon compounds, while others are efficient with both low and high molecular weight carbon compounds.

11 isolates producing cells counts above 10^7 cfu/ml, were selected for further screening programs. These results may reflect the expected variability within the ability of our isolates to degrade diesel hydrocarbons.

2.3. Screening of bacterial isolates based on biomass production on 5% diesel

The 11 isolates were incubated using 5% diesel as sole carbon source. Based on the obtained results presented in Table 3 , our 11 isolates may be classified into 5 classes:

Based on the obtained results presented in Table 3 , our 11 isolates may be classified into 5 classes:

- **Class 1:** The strains QDD1, ZA8, ZA9, and ZA36 produced higher biomass with 10% than with 5%; they should degrade several hydrocarbons, but not all. The final production depends on the relative concentrations of the assimilated hydrocarbons. The growth of the strains seems not inhibited by diesel components.
- **Class 2:** The biomass production of the strains ZA2 and ZA20 is similar at 5% and 10%. Here, a combined effect of hydrocarbons concentration and toxicity may occur.
- **Class 3:** The strains ZA5, ZA10, and ZA18 produced biomass with higher yield at 5% than with 10%. Their growth should be partially inhibited by high diesel concentration; they also seem sensitive to toxicity effect of diesel.
- **Class 4:** The strain ZA19 is able to degrade a fraction of diesel hydrocarbons, with fast entering in the death phase.
- **Class 5:** The strain QDD2 is able to grow on few hydrocarbons without a significant inhibition of growth due to diesel components.

It is clear, from these results, that using 5% diesel; better interpretation could be drawn concerning the hydrocarbon-biodegradability by the pre-selected strains. Indeed, the 5% (v/v) diesel corresponds to almost 37.5 g/l hydrocarbons.

Considering the distribution of hydrocarbon compositions in diesel, it is acceptable that some of them may be at low concentrations. If the isolate is able to use a wide range of hydrocarbons, a diauxic growth may occur, which refers to a growth in media containing more than one substrate, one of which is easier to metabolize than the others. First, the bacterium will metabolize all the easier substrates and grow at a higher speed. Eventually, after all has been consumed, the bacterium will begin the process of expressing the genes to metabolize the others. For these reasons, diauxic growth occurs in multiple phases.

The first phase is the fast-growth phase, since the bacterium is consuming (in the case of the above example) exclusively easier substrates and is therefore capable of rapid growth. The second phase is a lag phase where the genes used in metabolism are expressed and observable cell growth stops. This is followed by another growth phase which is slower than the first. The final stage is the saturation phase. If the isolate is not able to use a variety of hydrocarbons, its growth should stop with the exhaustion of the useful substrates and it enters the stationary phase of growth or even the death phase.

2.4. Screening of the strains based on their ability to degrade Diesel hydrocarbons

The results of Gas Chromatography (GC) analysis (Figure 4 - Figure 7) clearly show high variability within our isolated strains at the cultural conditions. The combination of the results obtained with 5% and 10% diesel was useful to classify the strains depending on their potential of using hydrocarbons from diesel, as well as sensitivity to their toxicity.

It is obviously clear that each isolate exhibited a specific profile of degradation of diesel hydrocarbons. Several ones were effective in degradation of low MW hydrocarbons, while others were more effective on high MW hydrocarbons. ZA12 was shown with relatively low activity but able to degrade almost all categories of hydrocarbons.

Here, the results are not enough to correlate the produced biomass by the ability of each strain to degrade the pre-selected area. Indeed, the GC analysis provides us with an idea about the category of the hydrocarbons that may be degraded by each isolate. This is a qualitative study, because, the GC analysis does not provide us with the concentration of each hydrocarbon, so to calculate the quantity of hydrocarbons removed by each strain.

This study confirms the variability of biological activity within our collection of strains.

3. Investigation on biological activities of the isolated strains

3.1. Study of the effect of nitrogen source on growth of the selected strains

Several studies were done on the effect of nutrients, two MSM mediums were used, MSM1 & MSM2; both media have similar chemical composition except for Nitrogen source, in MSM1 we used Ammonium Nitrate as Nitrogen source corresponding to 1.4 g/l, while in MSM2 we used Ammonium Chloride corresponding to 1.05 g/l.

As it was very clear; by using same concentration of diesel 10%, consequently, each medium represent different Carbon/ Nitrogen ratio.

Furthermore, the biological activity for selective strain and Gas Chromatography results were examined in different nutrition conditions. Including adding the yeast extract as source of Amino Acids.

Since it was shown in sections 1 and 2 that there was diauxic growth, we followed the growth after periods of 3 days, 1 week, 10 days and 2 weeks. From the observation, it is obviously clear that slight variations were noticed in most of the strains, especially when compared after 2 weeks of incubation, except for strains ZA2 and QDD2 which grow better with Ammonium Nitrate as the nitrogen source (MSM1) than with Ammonium Chloride (MSM2). These differences are higher with incubation periods exceeding 1 week.

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. Mostly, this requires synthesis of amino-acids by the cell. Since, in the used MSM media, there are no supplemental amino-acids; cells synthesize the amino-acids from two different nitrogen sources, which may not have the same efficiency and rate. The addition of amino-acids to the media may increase the rate of growth, depending on the nitrogen source.

Hypothesis 2: In the two media, there is the same carbon source concentration (10% diesel corresponding to almost 75g/l hydrocarbon concentration) but different nitrogen concentrations supplied by the different nitrogen sources. The C/N ratios are of 60/1 in MSM1 and 80/1 in MSM2. The study of the effect of C/N ratio on the growth is necessary.

These two hypotheses are further studied.

3.2. Study of the effect of C/N ratios with different nitrogen sources on growth of the selected isolates

From Figure 10, it may be concluded that the growth of the three strains (ZA2, ZA8, and QDD1) was less sensitive to the C/N ratio in the Ammonium Chloride (as the nitrogen source) sample than in that of Ammonium Nitrate. C/N ratios of 70/1 to 80/1 may be adequate for all strains.

3.3. Study of the effect of amino acids supplemental on growth of the selected strains

Comparing these results with those obtained without supplemented amino acids, we can conclude that with amino acid supplemental, the growth of all strains was improved and may be considered similar. Fluctuations were exhibited, with the highest biomass being reached after 1 week of incubation. This result is expected since the MSM media are missing the amino acids that all the strains, that need to synthesize, hence affecting their growth rate and final biomass yields.

Considering these finding, it is not possible to conclude on the effect of the nitrogen source on the growth. Indeed, similar results were obtained with both sources and amino acids.

Interestingly, all the strains did not enter into a highly pronounced death phase. It may be therefore concluded that adding several concentrations of amino acids in the screening medium may be of interest.

3.4. Role of yeast extract as amino-acid source for hydrocarbon-degrading bacteria

It is obviously clear that yeast extract improved greatly, the growth of all the strains as recorded after 2 weeks of incubation. This is compared to results of Figure 8 Yeast extract concentration of between 0.5 g/l to 1.5 g/l is enough to provide the necessary amino-acids. With higher concentrations, yeast extract may also act as a carbon and nitrogen source.

Consequently, it may be considered that yeast extract supplemental is not a parameter to be included in the screening.

3.5. Effect of nitrogen source, C/N ratios and amino acid supplemental on the biological activity of the isolates

It is obvious from the figures 15-17 that ZA2 and ZA8 were less responsive than QDD1 to the changes in nutrition conditions/nitrogen sources, slight shifts to higher molecular weights and increased efficiency obtained by adding the Amino Acids was observed.

In conclusion, 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. Moreover, the biodegradation efficiency also increased but to different extents for each of the selected strains through addition of amino acids.

3.6. Establishment of growth Curves of the isolated strains

The results of Figure 13 clearly show that growth rates of the three strains were not too different in the LB medium. From Figure 14, it may be concluded that

proportionality between cell counts and optical densities varies from one strain to another.

The growth rate of ZA2 is the lowest, which means that even in a rich medium, there is the possibility to distinguish strains. However, we do not consider that the determination of the growth curves in rich media is an appropriate parameter for the screening of the strains according to growth rates. Similarly, the determination of the optical density of the broths is not a useful parameter in distinguishing between the growth ability of the different strains. It may be a criterion for differentiation of the isolated strains.

4. Investigation of toxicity by diesel

Tolerance to high concentrations is an important factor in our selection criteria. The adaptation of each strain and its tolerance to diesel toxicity could be improved by optimizing the nutrition requirements.

The investigation of the growth and biological activities of the selected isolates were carried out by increasing the diesel concentration from 5% to 50% while maintaining the MSM1 C/ N ratio at 60/1.

In spite of the growth increased up to 40% diesel, best efficiency was obtained with 5% diesel, while some variations were recorded from one concentration to another.

To interpret these findings, it is necessary to reconsider the phenomenon of diauxic growth. Indeed, by changing diesel concentration, it was shown that growth was much improved, implying that several hydrocarbons were used as substrates and partially converted into biomass. With the selected peaks in the GC chromatograms, it is not obvious and easy to determine the correlation between the

removal of hydrocarbons and growth. Using the selected peaks is not enough to quantify the fraction of hydrocarbons converted into biomass.

Moreover, depending on each hydrocarbon concentration, the microbial cell should adapt its metabolisms. Here, it is also possible to talk about co-metabolism, well known in hydrocarbon-degrading bacteria. This special metabolism of co-oxidation of substrates is dependent on the presence of the substrate and the co-substrate.

5. Identification of the selected strains:

Following the steps described in section 4, we carried out our identification program for the most interspersing strains according to the work of this research, list of strains where identified and reported in the literature as hydrocarbon degrading strains is illustrated in **Error! Reference source not found.**

Table 5: List of identified diesel degrading strains and references for the same species reported in literature as diesel / hydrocarbon strains

Isolate	Identity	References in Literature
1 (ZA2)	*Arthrobacter sp.*	(Cipinyte, et al., 2011) (Dussan & Numpaque, 2012)
2 (ZA3)	*Citrobacter sp.*	(Erdogan, et al., 2011) (Singh & Lin, 2008)
2 (ZA5, ZA18)	*Citrobacter amalonaticus*	(Erdogan, et al., 2011)
3 (ZA8, ZA9, & QDD1)	*Pseudomonas aeruginosa*	(Chaeruna, et al., 2004) (Obuekwe, et al., 2009) (Zhanga, et al., 2010) (Erdogan, et al., 2011) (Luo, et al., 2012) (Hassanshahian, et al., 2012)
1 (ZA11)	*Enterobacter helveticus*	Not reported
1 (ZA19)	*Cronobacter muytjensii*	Not reported
1 (ZA20)	*Bacillus cereus*	(Al-Sharidah, et al., 2000) (Nwaogu, et al., 2008) (Obuekwe, et al., 2009) (Zhanga, et al., 2010) (Jyothi, et al., 2012)
1(QDD2)	*Klebsiella pneumoniae*	(Nwaogu, et al., 2008) (Obuekwe, et al., 2009) (Erdogan, et al., 2011)

We can conclude that within the identified strains, we have 3 strains of *Pseudomonas aeruginosa,* which are the most effective strains. This result is expected due to the fact that this bacterium is the most involved in bioremediation, especially of hydrocarbons. There are also several strains of *Arthrobacter* and

Citrobacter. These species are also reported in literature for their biological activities for bioremediation of hydrocarbons.

Bacillus and *Klebsiella* species are also reported in literature for their hydrocarbon degrading activity. 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 bacterial species of *Enterobacter helveticus* or *Cronobacter muytjensii*, are involved in degradation of hydrocarbon.

Conclusion

Hydrocarbon compounds, as products of the oil industry and related activities, are considered serious environmental pollutants. Bioremediation is one of the most rapidly growing areas of environmental biotechnology, by microorganisms that have specific metabolic capacities for cleaning up pollutants. The application of bioremediation is distinguished by its low costs and simple implementation with safer environmental impacts and public acceptability.

It is evident from this study that, hydrocarbon-degrading organisms are abundant in the Qatari environment and they can be isolated from hydrocarbon polluted sites. An isolation and screening program has been designed to isolate interesting hydrocarbon degrading strains and in this case, the combination of growth parameters and biological activities resulted in the isolation of 39 bacterial strains. Indeed, we showed that a direct isolation of strains using one medium, as usually reported in literature, may be not enough to build a collection of strains exhibiting variability of biological activities. For the isolation and screening, we diversified media composition, nitrogen source composition, C/N ratios,.. and we applied high pressure selection with high diesel concentration. Moreover, we combined the screening program based on growth capability of the isolates to their biological activities in removing hydrocarbons from diesel. We showed that all our isolated strains behave differently. By this program, we reduced the risk to discard interesting strains from the collection.

Further studies were conducted on several selected bacterial strains which exhibited variations in growth dynamics, biological activities and tolerance to diesel toxicity.

Biological activities shifted from low MW to High MW hydrocarbons and vice versa by using different nitrogen sources, C/N ratio and amino-acid supplemental. These findings ware never reported in literature. This is important due to the fact that according to the composition of hydrocarbons (pollutants), the conditions of the bioremediation as well as the used strain may change. It is just to analyze the pollution parameters to select the strain and the conditions of its implementation. We evidenced high tolerance of several strains up to 50% diesel and removal of more than 70% of most hydrocarbons in 40 g/l culture media in optimized conditions.

In the last part of this work, 12 isolates were identified using molecular ribotyping techniques. Amongst them, 3 are *Pseudomonas aeruginosa*. Occurrence of this bacterium with polluted area is more than expected, especially with hydrocarbons. It seems according to our screening program, that these strains are different in term of biological activity and growth. Several other species identified within our isolates are also reported in literature. There are also several strains of *Arthrobacter* and *Citrobacter* in our collection. These species are reported in literature for their biological activities for bioremediation of hydrocarbons.

Bacillus and *Klebsiella* species are reported in literature for their hydrocarbon degrading activity. 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 bacterial species of *Enterobacter helveticus* or *Cronobacter muytjensii*, involved in degradation of hydrocarbon. This finding may also be interesting to investigate.

It is highly recommended that further studies be conducted to determine and optimize the best condition for biomass production, combined with best degradation efficiency. It is in the research team's interest and intention to carry this study forward.

Indeed, in further work, we propose to start by the investigation of:

- the rate of growth and hydrocarbon degradation during longer incubation, for better selection of the isolates.
- the physical and chemical composition of media, allowing the highest biological activities of the selected strains.
- the possibility of selecting strains tolerating higher temperature, salinity and drought, according to Qatari weather.
- the possibility to improve of the selected strains through random mutagenesis, in term of tolerance and biological activity
- the relationship between surfactant production and biological activity
- the molecular mechanisms of hydrocarbon degradation, including the cloning of the genes involved in the biological activities of the selected strains.

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