QATAR UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

HYDROCARBON DEGRADING CANDIDATE BACTERIA ISOLATED FROM QATAR POLLUTED SOIL AND MOLECULAR IDENTIFICATION OF KEY ENZYMES CODING GENES

BY

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ABSTRACT

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Title: Hydrocarbon Degrading Candidate Bacteria Isolated from Qatar Polluted Soil and Molecular Identification of Key Enzymes Coding Genes

Supervisor of Thesis: Prof. Samir Jaoua.

Sixteen bacterial strains were isolated form contaminated soils and identified as *Bacillus subtilis, Bacillus circulans, Burkholderia cepacia, Pseudomonas aeruginosa, Micrococcus luteus, Raoultella ornithinolytica* and *Staphylococcus capitis*. The hydrocarbon degradation potentialities of these strains revealed that they can be considered as good degraders of diesel, toluene and xylene reaching high biomasses in diesel as sole carbon source and high efficiencies of degradation of different molecular weight hydrocarbons. These hydrocarbon catabolism potentialities were confirmed by the identification and sequencing of seven key genes encoding haloalkane dehalogenase, hydrocarbon binding protein, alkane 1-monooxygenase, alkane hydroxylase, naphthalene dihydrodiol dehydrogenase, 1,2-Dihydroxynaphthalene dioxygenase, toluene monooxygenase large α subunit and xylanase. These findings demonstrate the importance of both the strains and gene bank since having very high values and can be exploited for many applications including the enhancement of the genes expression in order to create improved hydrocarbon degrading strains to become one of the best environmentally friendly solution in bioremediation, competing with chemical and physical methods and allowing to achieve highest remediation efficiencies.

DEDICATION

This thesis is dedicated to my parents, Ehab Saleh and Hoda Al-Sharkawy.

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CHAPTER 1: INTRODUCTION

Hydrocarbons are one of the most utilized sources of energy by mankind. The recent anthropogenic activities resulted in many accidental environmental pollutions such as oil leakage and spills through the process of exploration, extraction, and transportation of hydrocarbons (Albokari et al., 2015a). Many of these environmental harms are area of interest to scientists and surrounding communities to find a solution to remediate the toxic effects and degrade the highly toxic compounds. Oil spills can cause massive destructions to ecosystems such in water and soil. Soil pollution can cause complete destruction of the soil microbial community as well as destroying plant crops and can cause huge losses (Das & Chandran, 2010). Methods of their clean- ups can be physical, chemical or biological (Li et al., 2020). Bacterial communities are found to have the ability of degrading and remediating many pollutants such as heavy metals, polychlorinated biphenyls (PCBs), and hydrocarbons despite the harsh conditions (Al Disi, 2013; Ruiz et al., 2015; Solís-González & Loza-Tavera, 2019). Bioremediation is believed to be a cost-effective process of cleaning up pollutants in an a non-invasive method that will not be exposing the environment to further destruction as in other chemical and physical methods (Das & Chandran, 2010). Despite the ability of remediation of many polluting compounds, hydrocarbons are known to be toxic to most of the microorganisms. Thus, it is challenging to find bacterial species that are tolerating the toxic environment in a contaminated site and efficiently hydrocarbon degrading as well (Disi & Ali, 2013).

It has been found that the biological compounds called surface-active agents (SAAs) have the high ability to biodegrade crude oil organic compounds. These SAAs are enzymes produced by specific bacterial strains called “hydrocarbon-degrading bacteria” (Mohanram, et al., 2016).

The present work has been designed primarily to set up efficient tools to address the environment contamination due to hydrocarbon wastes.

This M. Sc. Thesis project aims to isolate, identify and explore hydrocarbon degrading bacterial strains, evaluate their hydrocarbon degradation potentialities and investigate the genes encoding key enzymes in hydrocarbon catabolism.

- ***Hypotheses:***

1- The Hydrocarbon degrading bacterial strains explored in this study have different hydrocarbon degradation activities

2- Key genes encoding the hydrocarbon degrading enzymes might be evidenced and explored

3- The candidate strains key genes, encoding the hydrocarbon degrading enzymes, might have sequence polymorphism among them and allow the prediction of differences among these enzymes

CHAPTER 2: LITERATURE REVIEW

Almost all ecosystems are susceptible to oil pollution. Oil spills have a massive disturbance effect that might deteriorate an ecosystem completely. These effects were seen in devastating oil spill accidents such as gulf war oil spill in 1991 and Deepwater horizon in the Gulf of Mexico in 2010 and many others (Jernelöv, 2010). Such oil spills have many ways of clean up, including chemical destruction of the organic compounds through surfactants or dispersants as an example, or mechanical by physical removal of bulk majority of the spill such as pressure washing, or the latest discovered method, biological treatment by biological agents such as bacteria or fungi (Dewling and McCarthy, 1980; USEPA, 1999). The biological agents are preferred in many cases because of their ability to biodegrade those harmful compounds without having harmful side effects to the environment as the chemical or physical methods that might be harmful to the environment as well as the biota and the whole ecosystem (Li et al., 2020).

Oils are hydrocarbons that are chemical organic compounds known for their combustible property and comprise the main source of the energy worldwide in the meantime. Those chemicals possess toxicity to human and the environment. While the exploration and extraction of oil has been a frequently occurring activity, the problem of oil spills are being faced. Besides the oil spills on land, the spills in water reach shorelines and cause environmental and ecological problems (Li et al., 2020), as well as economic issues, as what was seen in the Deepwater horizon oil spill in Gulf of Mexico (Kostka, et al., 2011).

Methods of oil spill treatment are firstly, chemical through surfactants that are classified in 3 types: dispersants, bio-based surfactants, and chemical herders. Biological methods are based on the biodegradation which is done either by bio-emulsifiers, that work on the dispersal of oil slicks to smaller droplets and that help in the process of biodegradation by bacteria, or

by biosurfactants that increase the solubility and bioavailability of the oil to the bacterial populations that are performing the biodegradation process (figure 1) (Doshi et al., 2018).

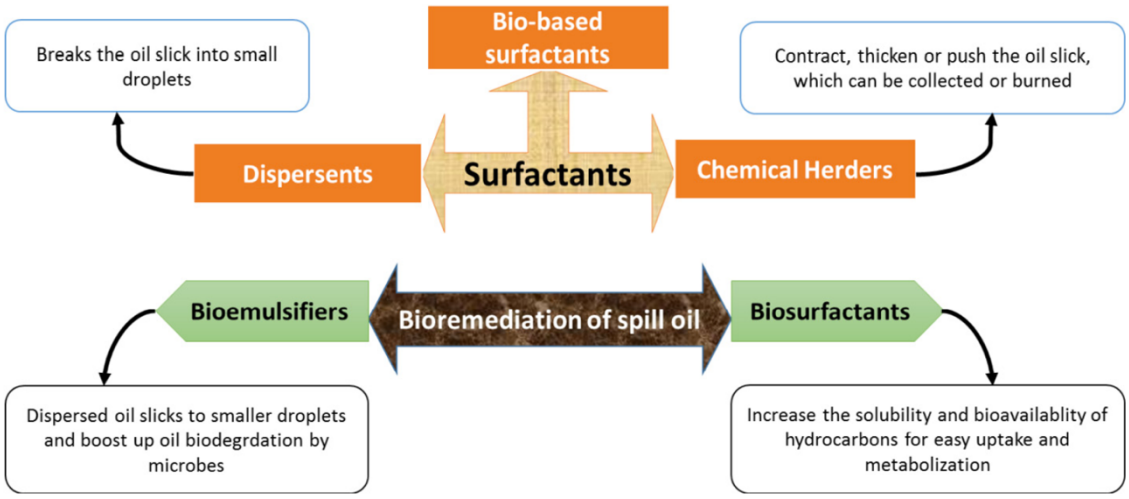


Figure 1: Classification of Surfactants for Oil Spill Treatment (Doshi et al., 2018).

Many chemical solutions have been used to clean oil spills, but their toxicity is still questionable and unavoidable (Fingas, 2013). Thus, hydrocarbon-degrading bacteria have been found to be the solution as reported in many studies (Obi, Atagana, & Adeleke, 2016). These bacteria have this potential activity through secretion of extracellular enzymes that breaks down the hydrocarbons and facilitate the process of degradation and bioremediate the site from hydrocarbon pollutants. Bioremediation is defined as the process of complete mineralization of oil to CO_2, H_2O, inorganic compounds, and cellular constituents (Albokari et al., 2015a). Bioremediation has been an active topic of research for around 30 years, and increased after the Deepwater horizon disaster, it was seen that bacterial communities have been stimulated and their activity was observed and noticed. Many studies isolated numerous bacterial strains from this spill and identified their species and their hydrocarbon degrading activities (Gutierrez

et al., 2013). Bioremediation became one of the main methods of hydrocarbon remediation because of its' advantages (Obi et. al., 2016). Technologies started to improve this field of study and experimentation showed that adjusting oxygen concentration, and addition of fertilizers containing nitrogen and phosphorus produces improved results of hydrocarbon degradation (Atlas, 2007).

2.1 Bioremediation using microbes

The process of decontamination or transformation of contaminants to less toxic substances in the environment using microbial species or enzymes is called bioremediation (Kaur & Balomajumder, 2020). Several species of bacteria, fungi, algae, as well as plants have been found to have this capability (Dua et al., 2002). Bioremediation is chosen over the other methods of detoxification using chemicals because it is usually found to be less disturbing to the ecosystem as well as cost-effective (Li et al., 2020). Bioremediation processes have to be optimized in terms of the environmental factors such as pH and temperature (Owabor et al., 2011). Also, it was found that the concentration of the contaminants affects the efficiency of bioremediation. All of these parameters are very important, however, the most vital is the microbial concentration and the biomass volume that is present to perform the bioremediation process (Kaur & Balomajumder, 2020). Usually the biomass of the microbes is not enough to perform efficient bioremediation unaided, thus addition of nutrients and oxygen is done to enhance the efficiency of the process (Li et al., 2020). Other studies such as of Ibrar & Zhang (2020) suggested artificially making a consortium of hydrocarbon-degrading bacterial species in order to enhance the efficiency of HCs and PAHs degrading.

2.2 Degradation of hydrocarbons by microbes

Hydrocarbons are hydrophobic compounds. This means that they are non-soluble thus not available for biodegradation process by microbes. However, hydrocarbon-degrading species overcome this by their natural ability to survive in contaminated areas by producing extracellular enzymes that work as bio-surfactants and makes the hydrocarbons bioavailable for bacterial degradation (Ibrar & Zhang, 2020). Bacterial communities are found to have the ability of degrading and remediating many pollutants such as heavy metals, polychlorinated biphenyls (PCBs), and hydrocarbons despite the harsh conditions (Al Disi, 2013; Ruiz et al., 2015; Solís-González & Loza-Tavera, 2019). Bioremediation is believed to be a cost-effective process of cleaning up pollutants in an a non-invasive method that will not be exposing the environment to further destruction as in other chemical and physical methods (Das & Chandran, 2010). Despite the ability of remediation of many polluting compounds, hydrocarbons are known to be toxic to most of the microorganisms. Thus, it is challenging to find bacterial species that are tolerating the toxic environment in a contaminated site and efficiently hydrocarbon degrading as well (Disi & Ali, 2013). Despite many bacterial species were isolated from hydrocarbon contaminated environments it is not logical that a single specie would be able to degrade all types of hydrocarbons, and this is the reason that many microbes require further experimentation and investigation (Ruiz, Brown, et al., 2021).

Among many bacterial species found to be inhabiting oil sludge and contaminated site samples in the middle east region, it was found that *Bacillus* species were the most common ones. A study done in Saudi Arabia investigated the microbial communities present in oil samples from the biggest Saudi oil company Saudi ARAMCO using PCR techniques. In this study, they identified bacterial strains were mostly belonging to the following genera *Bacillus, Spingobacteria, Flavobacteria, Clostridia, and Gammaproteobacteria.* As previously mentioned, it was found that the most common genus between all samples either from crude

oil or oil sludge was *Bacillus* species(Albokari et al., 2015a). These novel results identify that there are *Bacillus* candidate strains in the region that are promising hydrocarbon degrading.

The widely studied and most novel biosurfactant is called surfactin (figure 2), which is produced mainly by *Bacillus subtilis* (Kecskeméti et al., 2018). Surfactin gained this popularity because of its prosperities of having very efficient SAAs as well as having antimicrobial capabilities (Ibrar & Zhang, 2020).

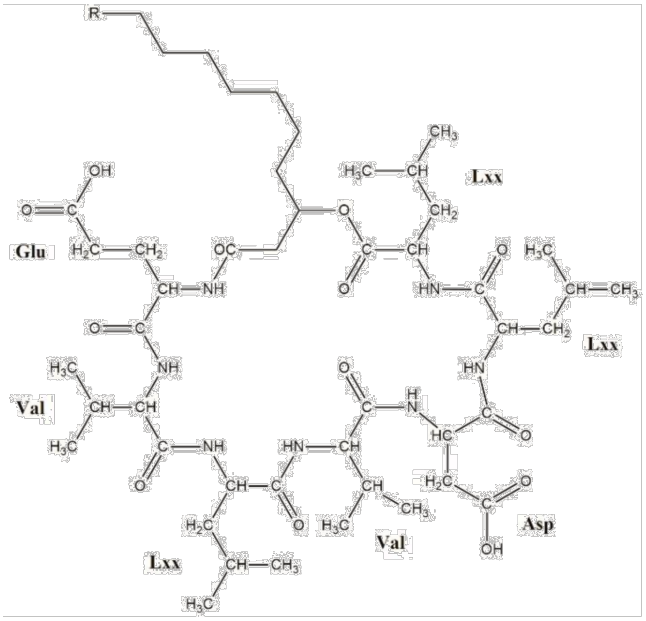


Figure 2: Chemical structure of Surfactin (Kecskeméti et al., 2018).

Scientists have studied Alkane Hydroxylases producing gene alkB for decades and linked it with surfactant production in bacteria (Olivera et al., 2009).

Alkanes as the most common hydrocarbons used in industry, have gained scientific attention for decades. These compounds are found to be degraded using bacteria producing Alkane Hydroxylases (AHs) enzymes (Figure 3). The degradation of alkanes by AHs results into their degradation into alcohols that can be utilized by bacteria in the catabolic reactions after their oxidation into fatty acids (Ayala & Torres, 2004; Nie et al., 2015).

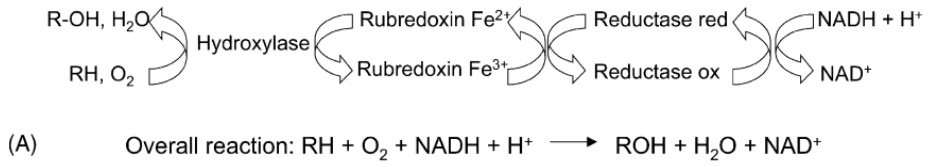


Figure 3: Alkane hydroxylase catalyzes oxidation reaction of alcohol (Ayala & Torres, 2004).

The alkane hydroxylase is produced by many bacterial species including *Acinetobacter calcoaceticus Acinetobacter* spp., *Burkholderia Cepacia*, *Pseudomonas putida*, *Pseudomonas oleovorans*, and *Rhodococcus* spp., that in total covers the range between C5 (5 carbons chain) up to C24 (24 carbons chain) (Ayala & Torres, 2004). This range covers oil range starting from petroleum, to jet fuel and kerosene, to more heavy oils such as diesel and motor oil (Collins, 2007).

Microorganisms tend to produce enzymes depending on the hydrocarbon available and the present substrate. *alk* gene encodes an enzyme that has metallic center called diiron cluster which plays as the active site that activates di-oxygen in the oxidation reaction (Table 1) (Ayala & Torres, 2004).

Table 1: The producing bacterial species of AH, paraffinic substrate, and its' active site (Ayala & Torres, 2004).

Enzyme	**Microorganism**	**Paraffinic substrate**	**Active site**
Alkane hydroxylase	*Acinetobacter calcoaceticus Acinetobacter* spp., *Burkholderia Cepacia*, *Pseudomonas putida*, *Pseudomonas oleovorans*, and *Rhodococcus* spp.	C5–C24 alkanes	Diiron cluster

2.3 Identification of hydrocarbon degrading of strains

Studies tend to isolate potential degrading bacteria and identify their biodegradation ability through incubation for a period of time and measuring the degradation rate to verify that these strains are suitable for bioremediation processes (Obi et al., 2016).

The metabolism of these bacterial species varies, and these varieties affect their biodegradation capacity. Some bacteria have been found to be exclusively hydrocarbon degraders such as species from the genera *Alcanivorax, Cycloclasticus, Oleispira, Oleiphilus, and Thalassolituus.* These bacterial species occupy different habitats including marine, soil, freshwater, oil fields and sediments. Some of these bacterial species have been discovered and known for several years such as *Alcanivorax borkumensis* whose genome has been studied in detail. The observation of this species found that it survives very well in alkanes. By the genome study, it was discovered that this is achieved by an exopolysaccharide that is encoded by the bacterial strain and works as a bio-surfactant that increase the surface area of interaction between water and hydrocarbon and increase its solubility (Brooijmans et al., 2009).

A study conducted in China found a bacterial strain from the genus *Rhodococcus* was able to degrade DEHP which is a very common PVC product. By culturing in DEHP, it was observed that the strain was able to degrade 96.4% of the hydrocarbon compound in 7 days only and without any nutrient additives.

The degradation potentials of bacterial species depend on the environmental factors and the surrounding chemicals and physical states (Das & Chandran, 2010; Gouma et al., 2014). It was found that the degradation potentials of petroleum products differ from one environment to another. As mentioned in figure 4, it was found that it reaches the maximum degradation rate in freshwater environment, soil environment, and marine environment at 20-30°C, 30-40°C, and 15-20°C, respectively. these factors affect the chemical composition of the

hydrocarbon and thus ease the mission of hydrocarbon-degrading bacteria. However, in the case of optimal temperature of hydrocarbon degradation rates, the optimal temperature of bacterial growth has to be considered as well. Based on that, we conclude that a bacterial specie that is found to degrade a specific hydrocarbon could be effective in one environment but not the other.

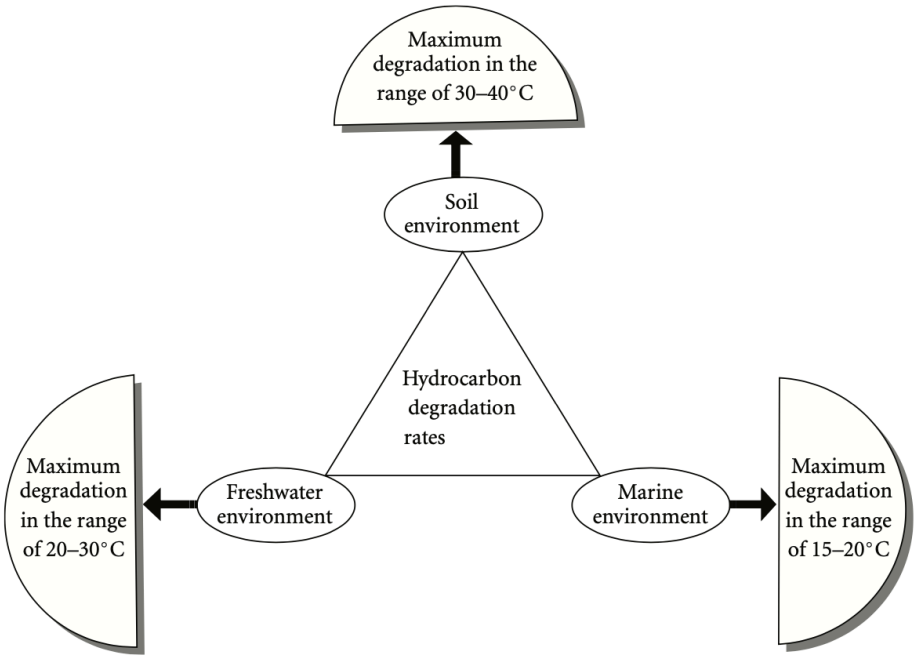


Figure 4: Hydrocarbon degredation rates and ranges based on the surrounding medium (Das & Chandran, 2010)

Despite, it was found that some bacterial species such as *Pseudomonas frederiksbergensis* can degrade hydrocarbons in relatively low temperatures that can reach as low as 4°C in some cases and its degradation abilities were tested and its degradation profiles were investigated and showing unique results (Ruiz et al., 2015; Ruiz, Radwan, et al., 2021). It is found that degradation profiling can be detected by Gas chromatography mass spectrometry to find the characteristics of the degradation potentials of bacterial species. GC-MS is found to be effective as well as in cases investigating metagenomics. It is believed that

collaborations between bacterial species in a culture would be highly efficient in degrading hydrocarbons compared to single isolate degradation. In addition to that, metagenomic analysis would include the unculturable bacterial species (Ruiz, Brown, et al., 2021). Current studies depend on the isolation of single species and investigation of its hydrocarbon degrading potentials alone, however it might be a better idea to form a microbial community of many hydrocarbon degrading isolates in-situ and investigate their efficiency.

2.4 Genes encoding the enzymes responsible for hydrocarbon degradation

Hydrocarbon degrading bacteria grow in harsh environments containing high concentrations of oil. These circumstances give them the ability to produce surface-active agents (SAAs) that breakdown hydrocarbons and provide the bacterial communities with carbon source from these oils. There are 2 types of SAAs that are biosurfactants (BSs) and bioemulsifiers (BEs) that vary in molecular weight. BSs have low molecular weights while BEs have high molecular weights (Mohanram et al., 2016). Some of the best-known BSs and BEs are listed in the table 2 below:

Table 2: Well-known Biosurfactants (BSs) and Bioemulsifiers (BEs) and the bacterial species that produces them

	Bacterial strain	**BSs/BEs type**
BSs	*Pseudomonas aeruginosa*	Rhamnolipids
	Bacillus subtilis	Surfactin
BEs	*Acinetobacter venetianus* recombination - activating gene 1 (RAG1)	Emulsan
	Acinetobacter radioresistens	alasan

A *Pseudomonas putida* isolate found in a Russian oil refinery was found to be naphthalene degrading through salicylate and gentisate secretion. These were secreted through the expression of genes in a non-conjugative plasmid called IncP-7. 2 operons were evolved in the process, the well-known nah-1 operon and "salicylate-gentisate pathway (sgp) operon" that comprises 6 opening frames sgp-A, sgp-I, sgp-K, sgp-G, sgp-H, and sgp-B. This was discovered by mapping the transcription start site by specific primers including nahC, nahE, and nagI. Then, predicted similarity of identified gene products and amino acid sequences using BLAST searches, revealed that 7 of the 13 plasmids have ORFs responsible for the degradation activity (Izmalkova et al., 2013).

Several species are found to be potential hydrocarbon-degrading species were isolated from the Arabian Gulf region and investigated belonging to various genera including *Pseudomonas, Bacillus, Staphylococcus,* and *Burkholderia* and found to have promising potentialities (Albokari et al., 2015a).

The above-mentioned species will be further explored in this study. Moreover, it was found that the literature is lacking in the term of investigation of key genes encoding the important pathways that encodes the enzymes such as the previously mentioned responsible for the hydrocarbon-degrading potentials of the microbial species. Thus, this study will focus on investigation of hydrocarbon degradation potentialities of bacterial species at both levels, chemical composition and degradation of hydrocarbons and genetic determinants as well by exploration of the presence of corresponding key genes.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials:

3.1.1 Bacterial strains isolated in the current study

	Strain	Isolation location
1	**QDD1**	Polluted soil with weathered hydrocarbons (Dukhan)
2	**QBC3**	Locally isolated from the Qatari feed market
3	**QMSM10**	Locally isolated strains from industrial contaminated water
4	**BG8**	
5	**Afnan 5**	Locally isolated from oily soil far from the sea line
6	**Afnan 24**	Locally isolated from oily soil from an automotive workshop
7	**Afnan 27**	
8	**Afnan 33**	Locally isolated from oily soil from a dump site
9	**2.2.1**	Locally isolated strains from hydrocarbon contaminated soil sample (Umm Slal)
10	**2.2.2**	
11	**2.2.3**	
12	**2.2.4**	
13	**2.2.5**	
14	**2.2.6**	
15	**3.1.1**	
16	**3.1.2**	

3.1.2 Media preparation

Different media were used in the current study. The composition of each medium is given as follows:

LB (Luria Bertani):

10 g/L peptone (Acumedia, Heywood, UK), 5 g/L Yeast extract (HIMEDIA, Mumbai, India) and 5 g/L NaCl (BDH, England), all components are dissolved in 1 L of dH_2O.

MSM1 medium (Attar et al., 2017):

4.0 g/L NH_4NO_3 (VWR BDH Chemicals, Germany), 2.0 g/L Na_2HPO_4 (VWR BDH Chemicals, Leuven, Belgium), 0.53 g/L KH_2PO_4 (VWR BDH PROLABO Chemicals, Leuven, Belgium), 0.17 g/L K_2SO_4 (VWR BDH Chemicals, Spain), 0.10 g/L $MgSO_4 \bullet 7H_2O$ (LABOSI, Paris, France) and 1ml trace element solution previously prepared as follows: 0.1g/100mL EDTA (BDH Chemicals Ltd, Poole, England), 0.042g/100mL $ZnSO_4$ (BDH Chemicals Ltd, Poole, England), 0.178g/100mL $MnSO_4$ (Sigma-Aldrich, Germany), 0.05 g/100mL H_3BO_3 (Breckland, Norfolk, U.K.), 0.1 g/100mL NiCl (Fulka-Garantie, Switzerland).

All media were sterilized at 121ºC, 15 psi for 15 minutes and equally poured 90 mm X 15 mm Petri dishes (20 mL each).

3.1.3 Solutions and buffers

Hydrocarbons:

- Diesel (ρ = 0.750 kg/L), provided by QP oil refinery Unit (Mesaieed, Qatar).
- Toluene (Riedel-de Haen [Honeywell], Germany).
- Xylene (Riedel-de Haen [Honeywell], Germany).

DNA extraction:

- B I: 1% SDS, 1mM EDTA, 20 mM Sodium-acetate, 40 mM Tris-acetate pH 7.8
- B II: 250 mM NaCl, 100 mM EDTA , 100 mM Tris-HCl pH 8.0

- Buffer A: 50 mM Glucose, 10 mM EDTA-Na2, 25 mM Tris-HCl, pH 8.0.
- Buffer AL: buffer A + 4 mg / ml lysozyme.
- Buffer B: 1 % SDS , 0.2 N NaOH, freshly prepared.
- Buffer C: Glacial Acetic acid,11.5 ml; Potassium acetate 5 M, 60 ml ; H2O, 28.5 ml
- TER: 10 µg/ml RNAse in 10 mM Tris pH 8.0, 1 mM EDTA,
- TE: 10 mM Tris pH 6.8; 1 mM EDTA
- Glycerol 30%
- TE(10X) : 10 mM EDTA-Na2, 100 mM Tris-HCl, pH 8.0
- Phenol: Chloroform: Isoamyl alcohol (25:24:1)
- NaCl: 5 Mole/L.

PCR:

- Emerald Amp MasterMix (2x Premix) ((Takara, Kusatsu, Japan).
- Molecular dH_2O (Takara, Kusatsu, Japan).
- Taq DNA Polymerase, DreamTaq (Thermo Scientific, Lithuania)
- Gel Electrophoresis:
- Loading buffer (6X): 0.25% Bromophenol blue, 40% sucrose (Thermo Scientific, Lithuania).
- 100 bp DNA Ladder (Bio Labs New England).
- Molecular dH_2O (Takara, Kusatsu, Japan).
- TAE buffer (0.5X) containing 0.4 mM tris-acetate and 0.01 mM EDTA-Na_2.

3.1.4 Kits

MinElute PCR Purification Kit (Qiagen, Germany) containing:

- Buffer PB: guanidine hydrochloride and isopropanol
- Buffer EB: 10 mM Tris-Cl, pH 8.5

- pH indicator I
- MinElute column and 2mL collection tubes

3.1.5 Equipment and machines

- Autoclave (P SELECTA, Spain)
- Centrifuge (ThermoSCIENTIFIC, Germany)
- Electrophoresis (Mupid, Japan)
- Fridges (-80°C,-20°C,4°C) (SANYO, Japan)
- Incubator (BINDER, Germany)
- Laminar air flow (LABCONCO, USA)
- Oven (Friedberg, Germany)
- PCR Machine (appliedbiosystems, Germany)
- Shaking incubator (SHEL LAB, USA)
- Spectrophotometer (Jenway, UK)
- Ultraviolet light transilluminator (Cole-Parmer, USA).

3.1.6 Primers for PCR

All primers used in this study are listed below in table 3. The expected fragment sizes are mentioned. We focused on the following genes encoding important enzymes of the pathways of the degradation of hydrocarbon using primers.

AlkB1 encoding Alkane hydroxylase

AlkB encoding Alkane hydroxylase

tmoA encoding Toluene monooxygenase large α subunit (tmoA)

nahC 1,2-Dihydroxynaphthalene oxygenase

nahB encoding Cis-Naphalene dihydrodiol dehydrogenase

xylE1: (*xylE1)*Catecholextradiol dioxygenases

Table 3: List of primers needed to explore the presence of genes encoding important enzymes of the pathways of the degradation of hydrocarbon.

Primer pair	Primer	Sequence	Gene / Enzyme	Size (kbp)	Reference
1.	B6-F.	5'CAATCAACAAGTCGTTTC3'	Naphalene dihydro diol	0.773	Ferrero*et al.*,2002
	B778-R.	5'ACTTGCGACCGAGCG 3'			
2.	C118-F.	5'GAGAAGGACCGTTTCTATC3'	(*nah C)*1,2-Dihydro xynaphthalene oxygenase	0.697	Ferrero*et al.*,2002
	C814-R.	5'CACCTCGCCAGCCGGG3'			
3.	E207-F.	5'CGCYACGTTGACCTGGG3'	(*nahE)*2-Hydroxybenzalpyrovate aldolase	0.620	Ferrero*et al.*,2002
	E826-R.	5'CCGAAAAGTCGCCACGC3'			
4.	ALK-1F.	5'CATAATAAAGGGCATCACCGT3'	(*alkB)* Alkane hydroxylase	0.185	Kohnoet al., 2002
	ALK-1R.	5'GATTTCATTCTCGAAACTCCAAC3'			
5.	ALK-2F.	5'GAGACAAATCGTCTAAAACGTAA3'	(*alkMa*) & (*alkMb*) Alhkane hydroxylase	0.271	Kohnoet al., 2002
	ALK-2R.	5'TTGTTATTATTCCAACTATGCTC3'			
6.	ALK-3F.	5'TTGTTATTATTCCAACTATGCTC3'	(*alkB1*) Alkane hydroxylase	0.330	Kohno et al., 2002
	ALK-3R.	5'CCGTAGTGCTCGACGTAGTT 3'			
7.	TBMD-F.	5'GCCTGACCATGGATGC(C/G)TACTGG3'	(*tbmD)*Toluenebenzene monooxygenase large subunit	0.640	Hendrickx.*et al.,*2006
	TBMD-R.	5'CGCCAGAACCACTTGTC(A/G)(A/G)TCCA3'			

8.	AlkB1-E.	5’GGAATTCATGTTTGAAAATTTCTCT3’	(*alkB*)Alkane hydroxylase	1.149	This study
	AlkB1-X.	5’GTCTAGATCAGGAAGCTGCCGG3’			
9.	AlkB1-F.	5’ATGTTTGAAAATTTCTCTCCCAG 3’	*(alkB)* Alkane hydroxylase	1.149	This study
	AlkB1-R.	5’TCAGGAAGCTGCCGGCCGC3’			
10.	TMOA-F.	5’CGAAACCGGCTT(C / T) A CCAA(C/T)ATG3 ‘	(*tmoA*) Toluene monooxygenase largeα subunit	0.505	Hendrickx. *et al.,*2006
	TMOA-R.	5’ACCGGGATATTT(C/T)TCTTC(C/G)AGCCA3’			
11.	TOD C1-F.	5’CAGTGCCGCCA(C/T) CGTGG (C/T)ATG3’	(*todC1)* Aromatic dioxygenases (TOD pathway) large subunit	0. 51	Hendrickx. *et al.,*2006
	TOD C1-R.	5’GCCACTTCCATG(C/T)CC(A/G)CCCCA3’			
12	BED C1-F.	5’TCGTCGTCAGACACTACGTA3’	(*bed C1)* Benzene dioxygenase *alpha subunit*	0.358	Wang *et al.,* 2008
	BED C1-R.	5’AATCTGATGCTTGCCATCATGG3’			
13	XYL A- F.	5’CCAGGTGGAATTTTCAGTGGTTGG3’	(*xylA*) Xylene monoxygenases (TOLp pathway)	0.291	Hendrichx *et al.,* 2006
	XYL A- R.	5’AATTAACTCGAAGCGCCCACCCCA3’			
14.	XYL E1-F.	5’CCGCCGACCTGATC(A/T) (C/G)CATG3’	(*xylA*) Xylene monoxygenases (TOLp pathway)	0.242	Hendrichx *et al.,* 2006
	XYL E1-R.	5’TCAGGTCAGCACGGTCAGGA3’			
15.	ALK B-F	5’AAYACNGCNCAYGARCTNGGNCAYAA3’	*alkB* Alkane hydroxylase	0.550	(Kloos et al., 2006; Smith et al., 2013)
	ALK B-R	5’GCRTGRTGRTCNGARTGNCGYTG3’			

3.2 Methods:

3.2.1 Isolation of hydrocarbon degrading bacterial strains from petroleum polluted samples

In addition to 8 Strains selected for this work, we proposed to increase the number by isolating additional strains from Qatari hydrocarbon polluted soil.

The Hydrocarbon degrading strains were isolated using Enrichment cultures as a first step. 2g from polluted samples were suspended in 25mL (LB 0.4% glucose) as Enrichment medium for 7 days at 30°C. Then, 2mL were transferred from this LB enrichment culture to 25 mL [MSM1 + 0.4% glucose] medium containing 1mL diesel for one week. This culture condition is very important for the adaptation of bacteria to hydrocarbon as the sole carbon source and selective for bacteria able to use crude oil and diesel components as sole carbon source. Then, 2 successive cultures of 7 days each were performed by the inoculation of 1mL reculture into a fresh 25mL MSM1 containing 0.4% glucose and 1ml Crude oil/diesel. Then, 100µl from MSM-diesel liquid culture were plated on MSM1 solid medium and 100 µl of oil diesel are plated on this solid medium surface. In addition, and in parallel, 100µl from MSM-diesel liquid culture were spread on LB solid medium.

Cell's biomass after 1 week and 2 weeks of incubation correlate the ability of the isolates to use the diesel as a sole source of carbon.

The purification of each isolate is carried out by repeated streaking of individual colonies 3 consecutive times. Isolated stains were preserved in 30% glycerol at -80°C.

3.2.2 Identification of the bacterial strains

Based on the protein profiles the newly isolated strains have been identified using matrix assisted laser desorption ionization-time of flight mass spectrometry MALDI-TOF analysis and dendrograms have been constructed showing the relationships and similarities between the selected strains (Nacef et al., 2017).

3.2.3 Protein deposition on MALDI Biotarget plate

The MALDI biotarget plate (48-sample spots) was used from Bruker Daltonics / Germany. 1 µL of the sample was pipetted onto the plate. After drying at room temperature, the spot was overlaid with 1 µL of a-cyanohydroxycinnamic acid (CHCA) matrix to allow the crystallization of proteins. The MALDI biotarget plate was then installed in the MALDI-TOF MS instrument (Bruker Daltonics / Germany) and the sample spots were labelled in MALDI Biotyper Real Time Classification to initiate the analysis (Samad et al., 2020; Shao et al., 2012).

3.2.4 Mass Spectra Acquisition

Bruker Flex Control software was used to acquire mass spectra of proteins. The software was run using linear and positive mode at 60 Hz laser frequency and intensity of 35%. The acceleration and source voltages were set as 20 and 18.7 kV, respectively. From different areas of the sample spot, 240 laser shots in 40-shot steps for each spectrum were obtained and analyzed using default settings. The mass range was set from 0 – 10,000 Da (Samad et al., 2020; Shao et al., 2012).

3.2.5 Mass Spectra processing

The mass spectra were acquired using Flex analysis software. The raw peak spectra were processed through baseline subtraction, smoothing and then peak detection using default algorithms in Flex Analysis 3.4. The profiles were then visualized in stack mode for better comparison of mass spectra acquired from different samples (Samad et al., 2020; Shao et al., 2012).

3.2.6 Comparison of Hydrocarbon degrading potentials of the studied strains based on biomass production

The selected strains from MSMI are cultured in LB medium at 30°C for 48 h. For each culture the (OD) is to be determinied at 600 nm. 20 mL MSM1 were inoculated

with each isolate supplemented with diesel (1 mL or 1.5 mL) at initial OD of 0.15 at 600nm. The preculture is prepared through 2 days growth on LB solid. Then, in order to obtain a more concentration cells suspension, a loop of cells were transferred in 1 mL MSM medium in Eppendorf tube from 48 hours culture. This suspension was then diluted 20x by transferring 50 µL to cuvette containing 950 µL MSM1 and measured absorbance (Abs.) using spectrophotometer. Then, the volume needed to be transferred to the 50 mL falcon tube was based on the OD of the bacterial suspension using the following formula below. For the preparation of the mixture of the 16 strains, 16 cells suspensions were prepared as above individually in 16 tubes. Their OD were measured and adjusted to the same OD with MSM1. Then, equal volumes were mixed in the one sterile tube. OD was measured again and then, the volume needed to be transferred to the 50 mL falcon tube was based on the OD of the bacterial suspension using the following formula below, to start the culture, as above, at initial OD of 0.15 at 600nm.

$$OD = Abs. \times 20\ (Dilution\ factor)$$

$$V = \frac{20\ mL\ (V_f) \times\ 0.15}{OD}$$

The growth of each culture was checked by plating 100 µL cells from the MSM1 culture on LB (CFU determination). The control is a non-inoculated MSM1 supplied with 1 mL (or 1.5 mL) diesel. All tubes were wrapped in aluminum foil, incubated in dark, tilted at 45° angle in a shaking incubator at 300 rpm at 30°C for 2 weeks.

3.2.7 Determination of CFU of the bacterial culture supplemented with diesel as sole carbon source

The growth of each strain was checked by plating 100 µL cells from the MSM1 culture after a number of serial dilutions on LB to determine CFU. The following equation was used:

$$\boldsymbol{CFU/mL = \frac{no.of\ colonies \times\ dilution\ factor}{0.100\ mL}}$$

3.2.8 Analysis of Diesel Degradation by Gas Chromatography (GC-FID)

The method of this experiment was previously described by Attar et al., (2017). The extraction of the diesel layer was carried out using a micropipette. The diesel layer was transferred into a sterile Eppendorf tube, and the Eppendorf tube containing the diesel was centrifuged for 1 min at 13,000 rpm mainly to separate any remaining liquid medium. Then the layers of pure diesel were transferred to a new sterile Eppendorf tube to be used for GC analysis. The diesel contaminants removal is identified by the reduction of the area under the hydrocarbon peaks in the chromatograms when compared to that of the control.

The removal efficiency (RE) is calculated, based on the reduction in the peak area of selected hydrocarbons from the chromatogram of diesel inoculated with cells in compare with control culture, and was expressed as the following: RE (%), whereas is the total area of the peak in each sample, Ac is the total area of the peak in the control sample, and RE (%) is the efficiency of removal (Al Disi et al., 2017).

After the incubation periods GC- FID is used to analyze the degrading capacity of each isolate during 2 weeks of incubation using a Perkin Elmer Clarus 680 Gas Chromatography – Flame Ionization Detector (GC-FID) under the following conditions:

Table 4: GC-FID machine specifications and analysis conditions

Machine	**PerkinElmar Carlus 680 Gas Chromatography**
Injection volume	1 µl
Oven Temp.	50°C hold for 5 min, to 250°C with rate 8°C/min, hold for 40 min
Injector temp.	300°C
Carrier gas	Helium

Flow of carrier gas	1ml/min
Split ratio	1:50
Column	PerkinElmar Elite-1, L: 60m, ID: 0.25, DF: 0.25
Detector	FID
Detector Temp.	200°C
Software	TotalChrom Navigator

3.2.9 Investigation and comparison of hydrocarbon degradation potentialities of the tested strains on solid media

This experiment was designed to test the ability of the selected 16 strains to utilize 3 different hydrocarbons as their sole carbon source.

MSM1 plates were prepared each containing 25 mL of media. The experimental design included 3 hydrocarbons to be tested which are diesel, xylene, and toluene. Each hydrocarbon was poured into the medium before it is poured into the plate to ensure homogenous distribution of hydrocarbon throughout the plates.

A similar experimental design was performed by Martínez-Ávila et al., (2021). Different concentrations of hydrocarbons were tested including 100 ppm, 200 ppm, 500 ppm, 1000 ppm, 3000 ppm. LB media plates were used as positive control. Plates were divided into a grid of 4x4 to include all 16 strains in each plate as shown in the figure below:

Figure 5: Petri dish showing the grid used for distribution of bacterial strains inoculation

The concentrations of each hydrocarbon in each 25 mL media plates were calculated as follows:

	MSM1	**LB (+ve control)**	**MSM1 + Diesel**	**MSM1 + Toluene**	**MSM1 + Xylene**
100 ppm	0 µL	0 µL	3 µL	3 µL	3 µL
200 ppm	0 µL	0 µL	6 µL	6 µL	6 µL
500 ppm	0 µL	0 µL	15 µL	15 µL	15 µL
1000 ppm	0 µL	0 µL	30 µL	30 µL	30 µL
3000 ppm	0 µL	0 µL	90 µL	90 µL	90 µL

Bacterial inoculation was done by transferring a single colony using a sterile toothpick to the grid shown in the figure above. All plates were incubated at 30°C for 48 hours.

3.2.10 Genomic DNA Extraction

Genomic DNA from the 16 chosen strains were extracted. The following method was used:

The DNA of the strains were be isolated from cells plated on LB solid. The cells were harvested and suspended in 1 mL water, then mixed and centrifuged for 3 min at 12000 rpm. The cell pellet was re-suspended by vigorous shaking in 200 µl Lysis Buffer-I. 66 µl of 5M NaCl was added and mixed well to remove proteins and cell debris. The mixture was centrifuged at 12000 rpm for 10 min at 4 °C. The supernatant was transferred to a new tube, and an equal volume solution of (Phenol, chloroform and isoamyl alcohol 25: 24: 1) was added and mixed by gently inverting the tube 50 times at least until a milky solution is formed. After centrifugation, the upper phase was transferred to a new tube and the DNA was precipitated by adding 2 volumes of absolute ethanol and incubated overnight at -20°C. The pellet (DNA) was washed with 70% ethanol and dried. The DNA was dissolved in TER (50 µl) and incubated at 37 C for 1h before analyzing the DNA by electrophoresis.

3.2.11 Polymerase Chain Reaction (PCR)

In all carried *PCR* reactions performed in this study, the total volume of each one was of 25 µl and contains 6 µl H2O, 12.5 µl Master mix, 2.5 µl of each primer 13.5 µM, 0.5 µl Taq DNA polymerase enzyme [1U], 1 µl template DNA.

The thermocycler program

- 94°C for 4 minutes, followed by 35 cycles of:
- 94°C for 45 seconds, then annealing for 45 seconds, at annealing temperature of primer (depending on the primers used), and polymerization at 72°C for 1 minute.
- An additional extension step at 72°C for 7 minutes was carried out.
- 5 µl of each reaction is run in 1% agarose gel
  25V/cm for 10 minutes followed by 50 Volts/ cm for 50 minutes and visualized using ultraviolet transilluminator.

3.2.12 Gel Electrophoresis:

DNA (fragment size)	% Agarose

DNA Extraction- mini gels	0.8% Agarose gel
PCR amplified products- mini gels	1% Agarose gel

Agarose gel of 0.8 or 1% was prepared with 0.5% TAE buffer. The gel was stained using 0.5 µg/ml Ethidium bromide. 0.5% TAE buffer was used as tank buffer as well.

Ladder was prepared by mixing 2 µL of ladder, 3 µL of H_2O and 1 µL of loading dye. Samples were prepared by mixing 5 µL of DNA sample and 1 µL of loading dye. Gels were run on 25 V/cm for 10 min and then 50V/cm for 60 min.

3.2.13 DNA sequencing

PCR amplification products corresponding the corresponding bands to the investigated gene encoding the enzyme anticipated responsible of hydrocarbon degradation, were sequenced using Sanger method using Genetic analyzer 3500.

3.2.14 Investigation of the nature of the genes amplified by PCR using NCBI database

The Alignment Search Tool (BLAST) was used to look for similar sequences in the NCBI gene bank (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

3.2.15 Statistical Analysis

Principle component analysis (PCA) was performed as a statistical tool for comparison of multi-variate data. PCA helps to categorize similar spectra together resulting in the formation of groups (of closely related spectra) that are dissimilar to each other. The PCA results can be visualized in 2D or 3D graphs depending upon the variability in the data set. To investigate the hierarchical relationships among the spectra, dendrogram clustering was performed.

Both PCA and Dendrogram analysis were done within the MALDI-TOF MS instrument using Biotyper RTC 3 software. All the mass spectra were pre-processed through baseline subtraction and smoothing. The processed spectra were then analyzed using PCA and Dendrogram tools of the software using standard operating procedure (Samad et al., 2020; Shao et al., 2012).

CHAPTER 4: ISOLATION OF BACTERIA DEGRADING HYDROCARBONS FROM CONTAMINATED SITES IN QATAR

Introduction

In this MSc project, hydrocarbon degrading bacteria were explored. The first part was to isolate local hydrocarbon degrading bacteria to start investigating their potentialities. The bacterial strains were isolated from soil that is sampled from a hydrocarbon contaminated site in Qatar and were isolated to undergo further investigation assuming that since it has been living in a hydrocarbon contaminated site, thus it has the ability of utilizing the hydrocarbon and use it as a source of carbon. The project was already designed to contain 8 previously isolated strains, but in order to enhance the results with more diversity, it was important to isolate more strains. The strains were isolated from hydrocarbon contaminated soil from Umm Slal municipality in Qatar. The soil was sampled and brought to the lab, undergo enrichment culture using glucose, then selective culture by providing diesel as the sole carbon source (as mentioned in 3.2.1), then plating and isolation of bacterial colonies that are morphologically different in shape as shown in figure 6 below. The isolated bacterial strains were then identified and classified based on their protein profiles using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

4.1 Isolation of Hydrocarbon Degrading Bacterial Strains

In this project we focused on including a variety of bacterial strains from different genera to experiment the extent of their hydrocarbon degradation potentialities. As mentioned in 3.2.1, the bacteria were isolated from hydrocarbon contaminated soil samples brought from Umm Slal municipality in Qatar. It was expected that the bacteria would be exhausted form the harsh environmental conditions

from direct sunlight and UV-radiation, to high temperature, in addition to high salinity as well. Thus, it was firstly cultured in an enrichment culture using 0.4% glucose, then transferring into another media containing 0.4% glucose and 5% diesel, and then selective media with 5% diesel only to ensure all the bacterial strains that survive are only hydrocarbon degrading. Plating was done from the bacterial cultures (figure 6).

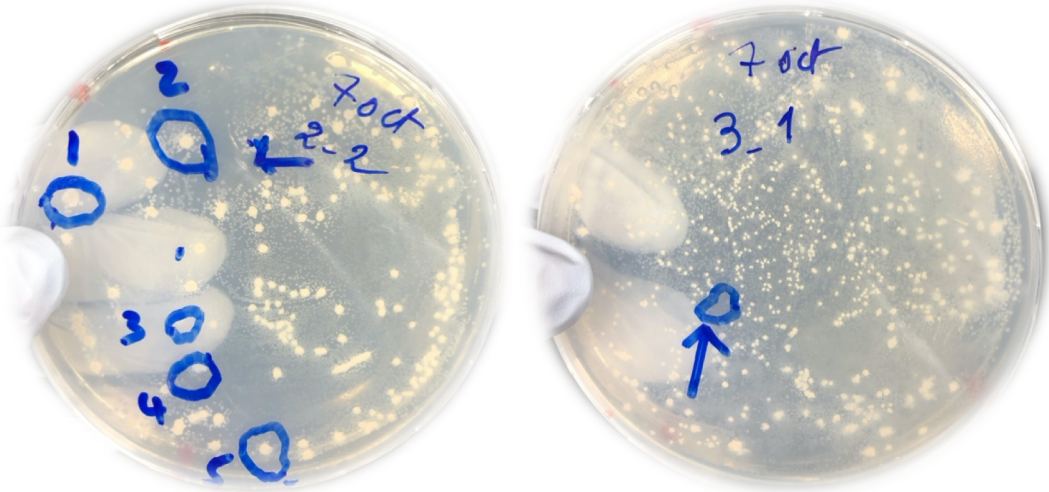


Figure 6: Isolation of hydrocarbon-degrading bacterial strains

The isolation was successful, and we could isolate 8 more strains (in addition to the 8 we previously had) and they were coded as shown in the table below:

Table 5: Strain coding/numbering of the isolated strains from hydrocarbon contaminated soil in Qatar

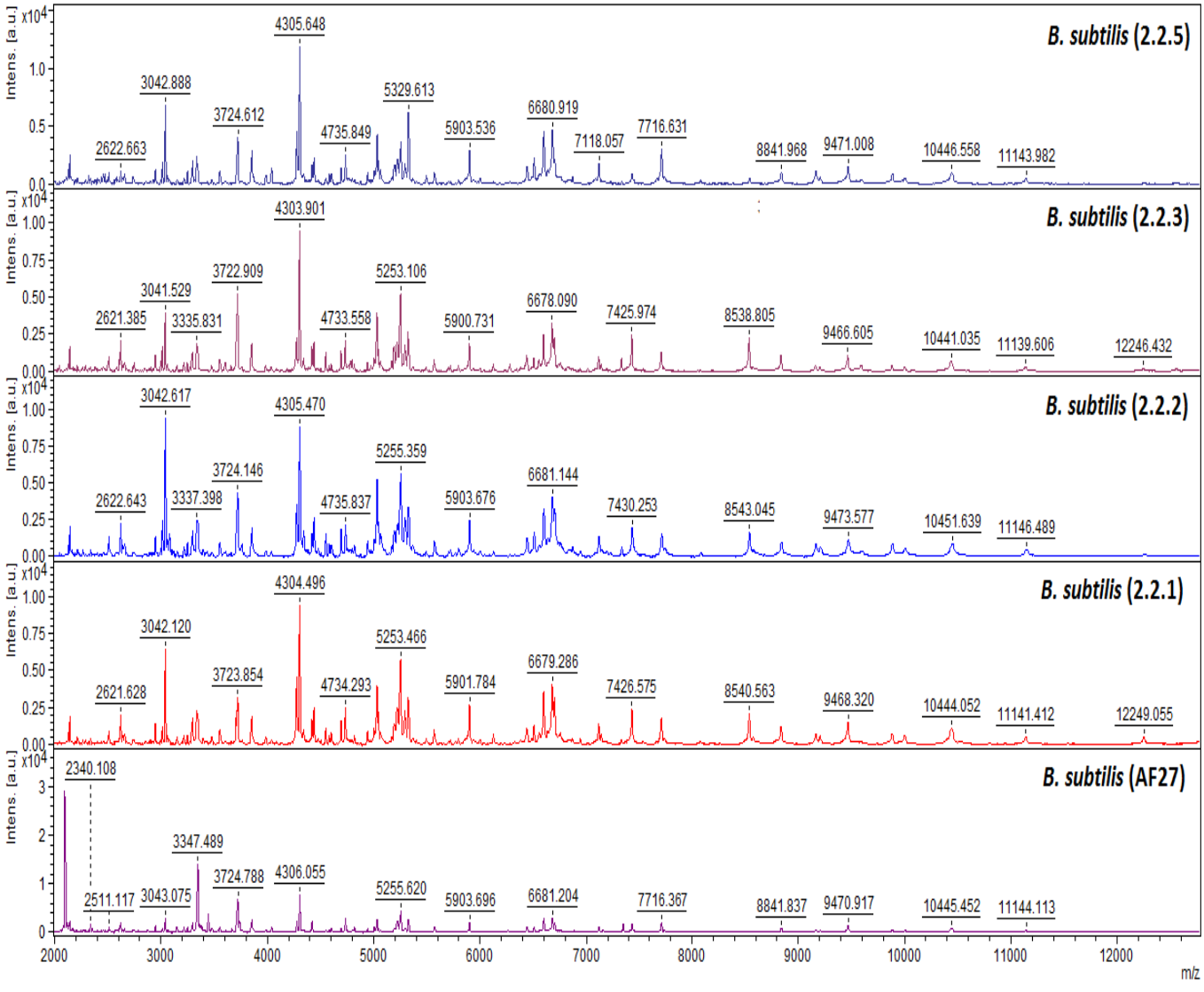
	Strain
1	2.2.1
2	2.2.2
3	2.2.3
4	2.2.4
5	2.2.5
6	2.2.6
7	3.1.1
8	3.1.2

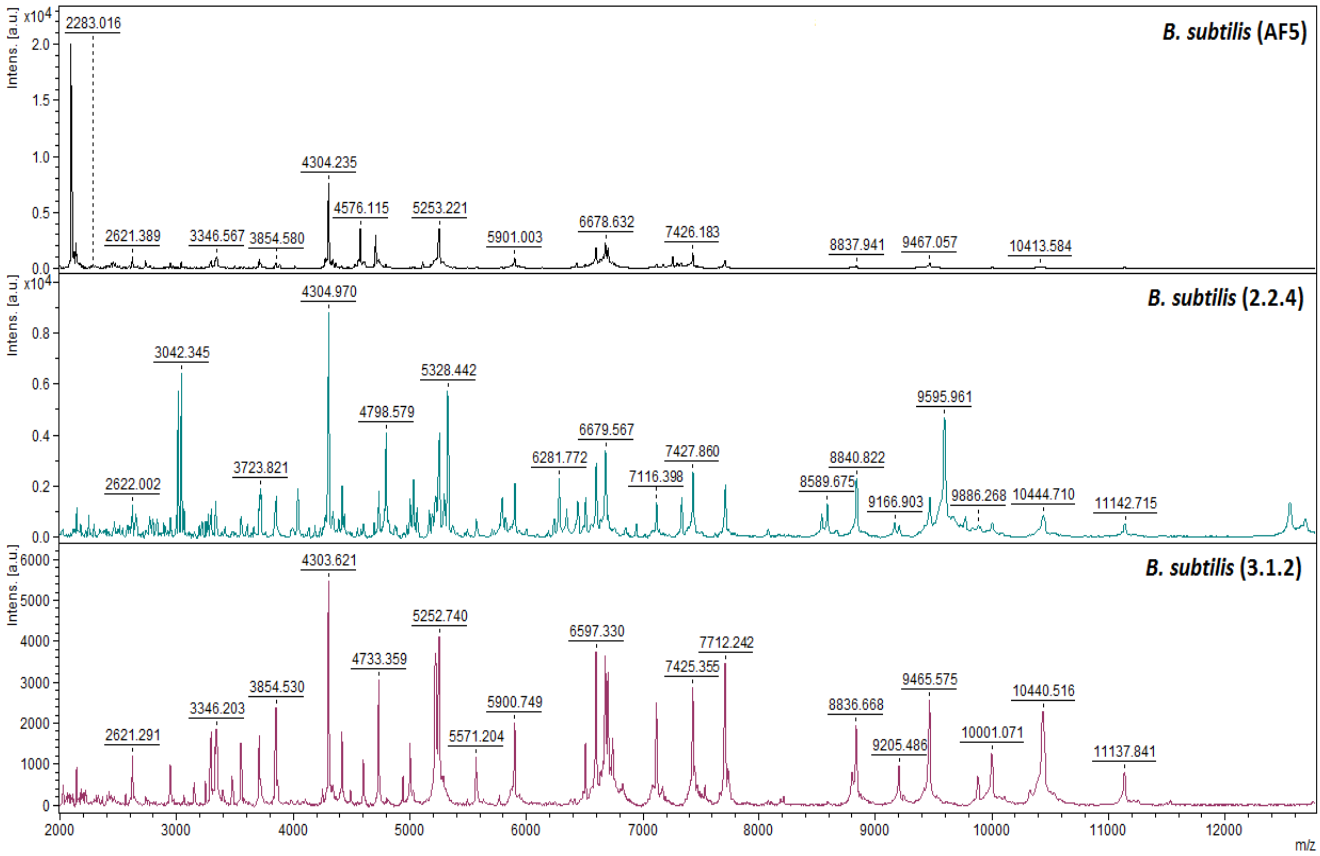
4.2 Identification of Bacterial Strains

After successful isolation of additional 8 bacterial strains, 16 potential hydrocarbon-degrading bacterial strains were selected for this current work. The following step was to identify them to establish their taxonomy. All bacterial strains (previously and newly isolated ones) were identified and confirmed by protein profiling using Bruker MALDI Biotyper as mentioned in Figure 7.

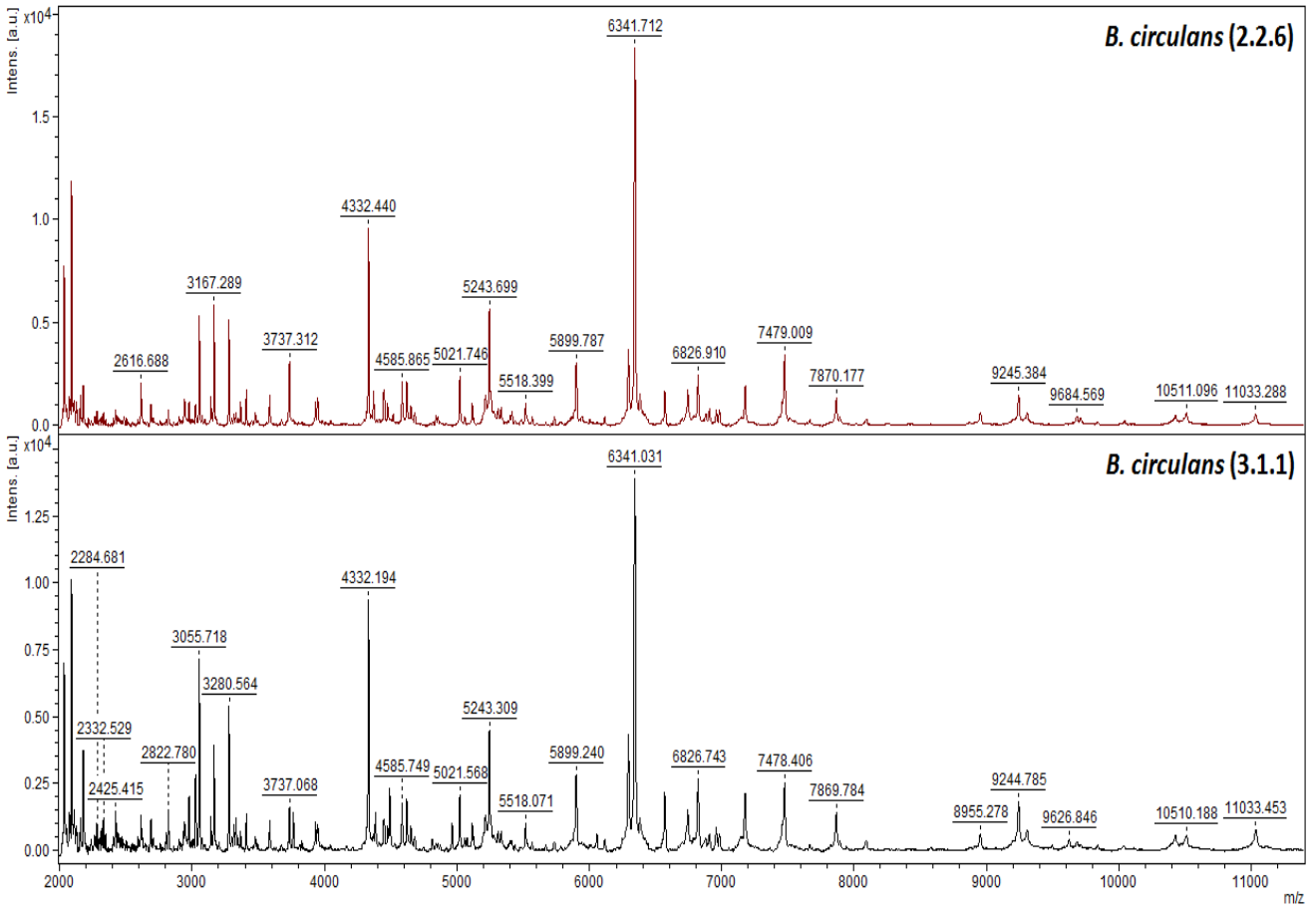
Figure 7: Protein profiles of the identified strains analyzed by MALDI-TOF.

Strains #5 (2.2.5); #3 (2.2.3); #2 (2.2.2); #1 (2.2.1); #15 (AF27); #13 (AF5); #4 (2.2.4); #8 (3.1.2); of *Bacillus subtilis*

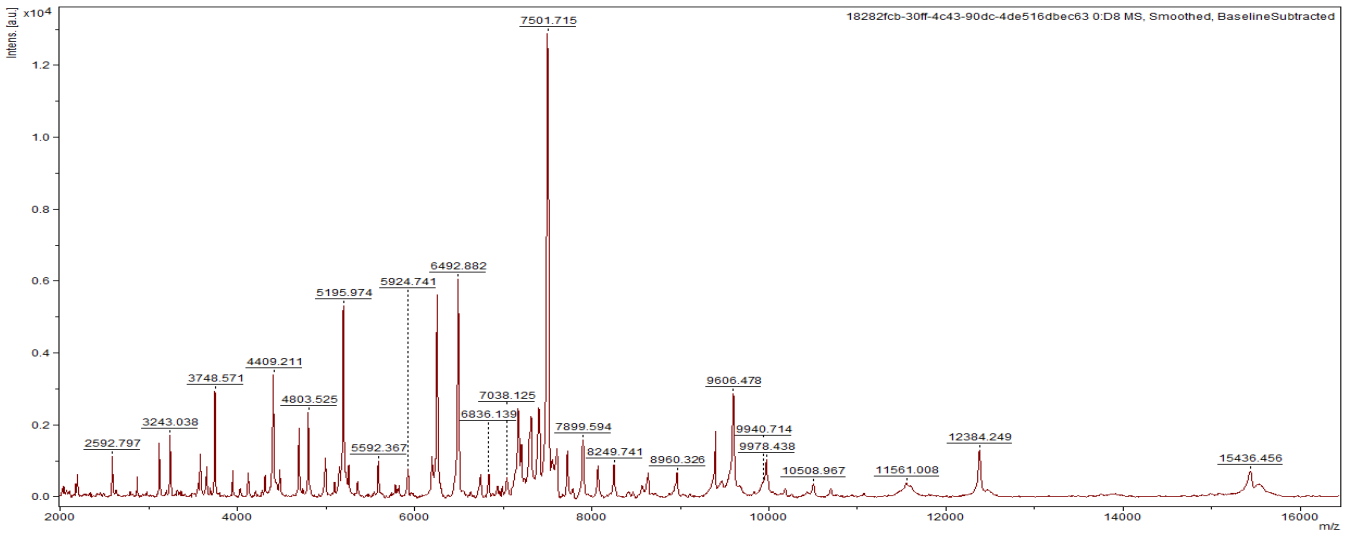




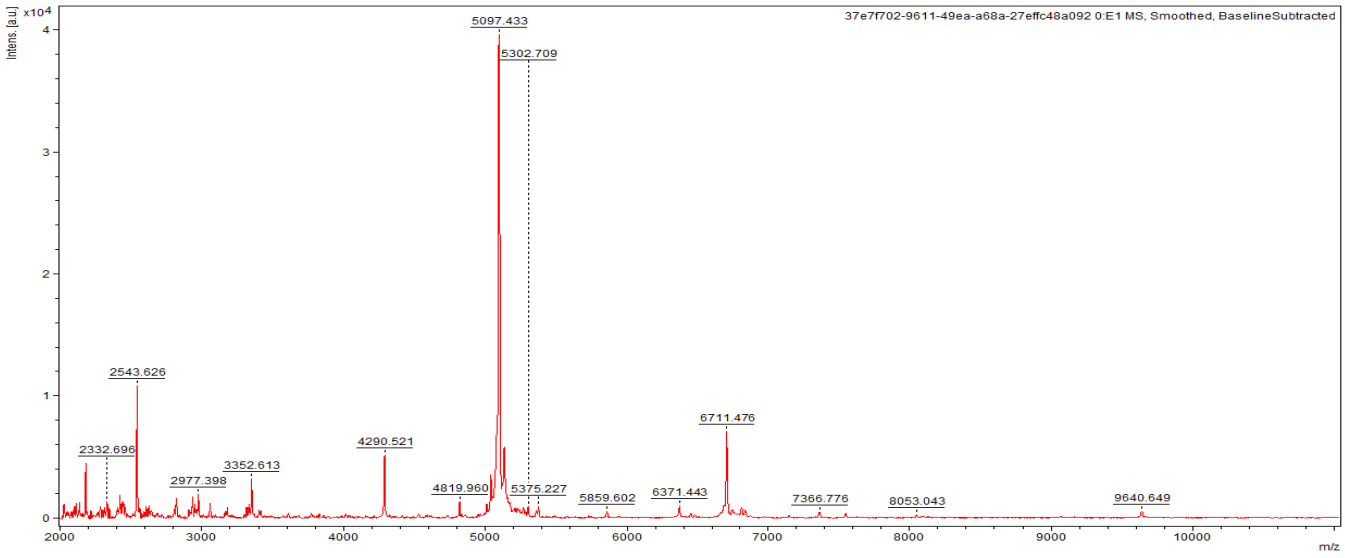
Strains # 6 #2.2.6, 7# 3.1.1-of *Bacillus circulans*



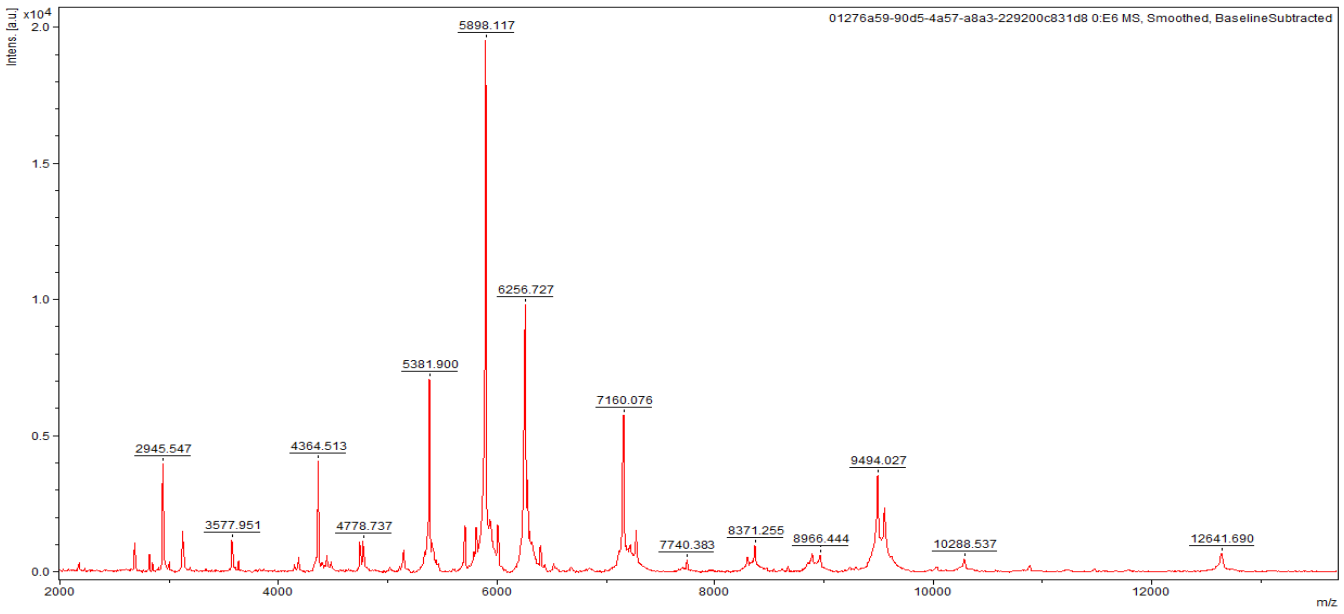
Strain #11- QBC3 *Burkholderia cepacian*



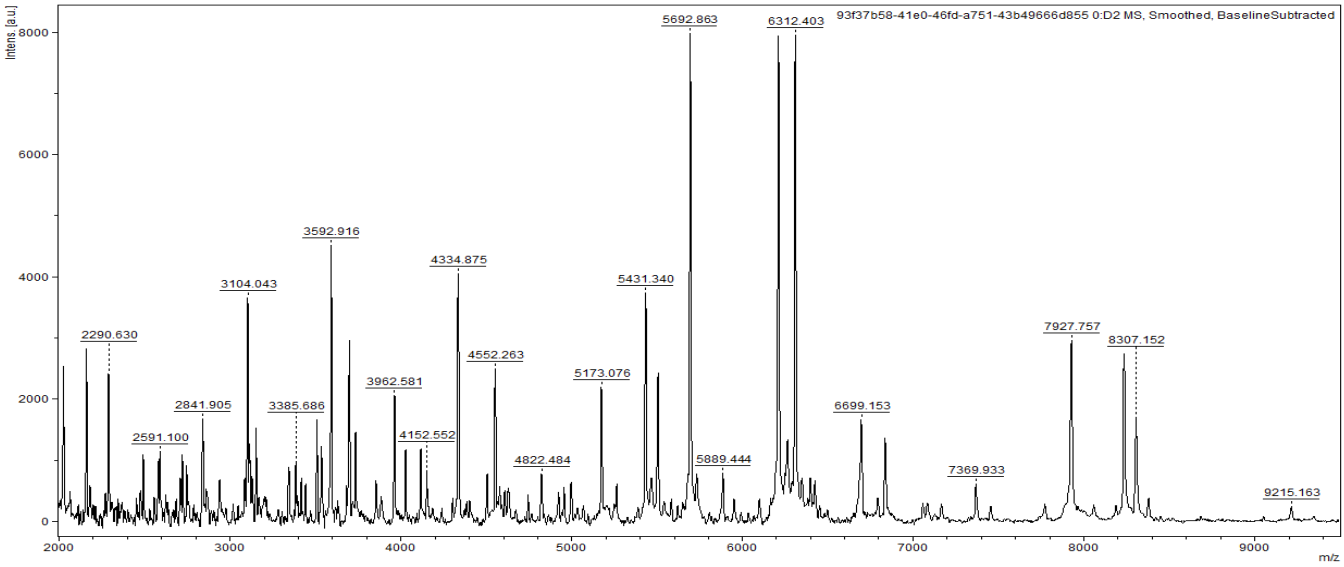
Strain #12- QMSM10 *Staphylococcus capitis*



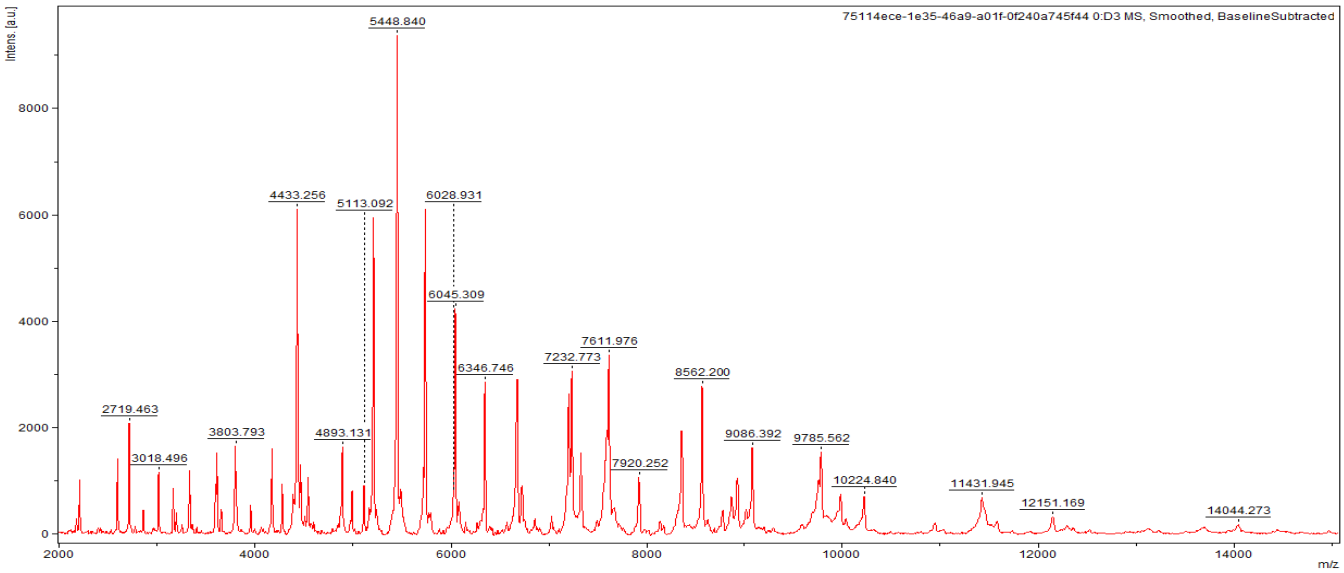
Strain #14 - AF24 *Raoultella ornithinolytica*



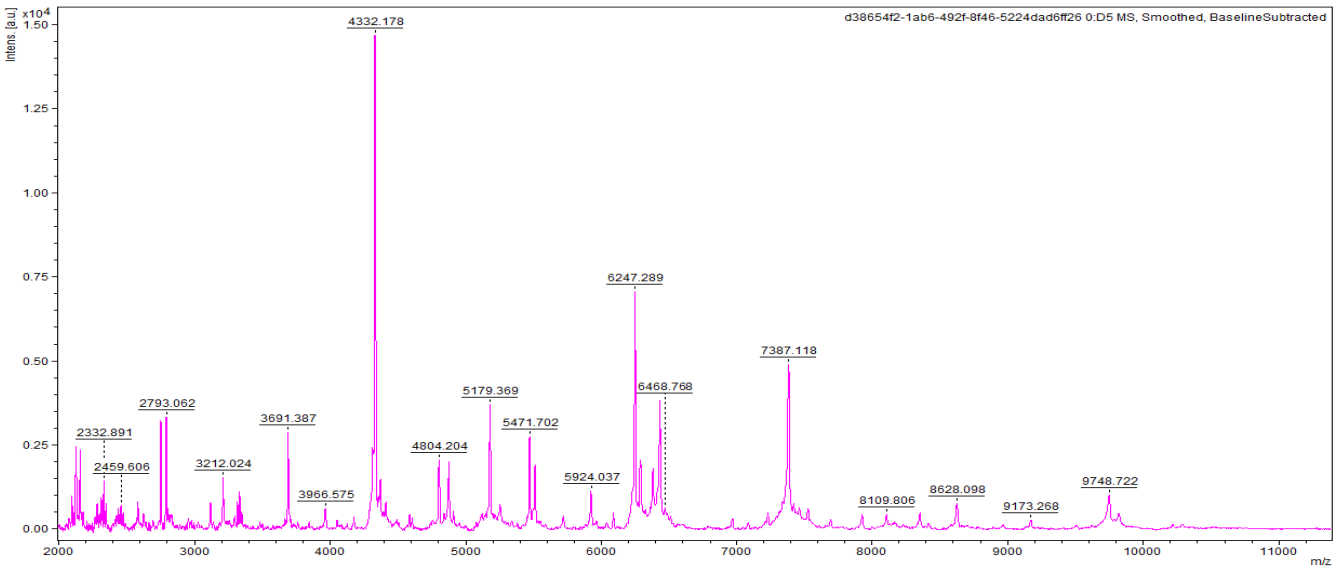
Strain #16- *Bacillus cereus*



Strain #9 - QDD1 - *Pseudomonas aeruginosa*



Strain #10- BG8 *Micrococcus luteus*



Many of the strains were identified as *Bacillus subtilis* which is a known hydrocarbon degrader found in the middle east region (Albokari et al., 2015b) as well as other species showed in table (6) below:

Table 6: Identification and taxonomy classification of the isolated strains (Identified using MALDI-TOF analysis).

	Strain	Identification result	**MALDI Scores**
1	2.2.1	*Bacillus subtilis*	2.19
2	2.2.2	*Bacillus subtilis*	2.12
3	2.2.3	*Bacillus subtilis*	2.21
4	2.2.4	*Bacillus subtilis*	2.11
5	2.2.5	*Bacillus subtilis*	2.17
6	2.2.6	*Bacillus circulans*	2.00
7	3.1.1	*Bacillus circulans*	1.96
8	3.1.2	*Bacillus subtilis*	2.11
9	QDD1	*Pseudomonas aeruginosa*	2.33
10	BG8	*Micrococcus luteus*	1.98
11	QBC3	*Burkholderia cepacia*	2.44
12	QMSM10	*Staphylococcus capitis*	2.20
13	Afnan 5	*Bacillus subtilis*	1.91
14	Afnan 24	*Raoultella ornithinolytica*	1.74
15	Afnan 27	*Bacillus subtilis*	2.15
16	Afnan 33	*Bacillus cereus*	1.84

After the identification of species, it was noticeable that many of them (11) are from the same genera *Bacillus* and half of them (8) were all *Bacillus subtilis.* Therefore, it was important to us to understand if they are genetically identical, or they might be only similar. Thus, dendrograms were constructed to clarify the difference level between the similar isolates. As shown in the dendrograms below (figure 8), there is a difference in the hierarchical relationship between the different strains isolated.

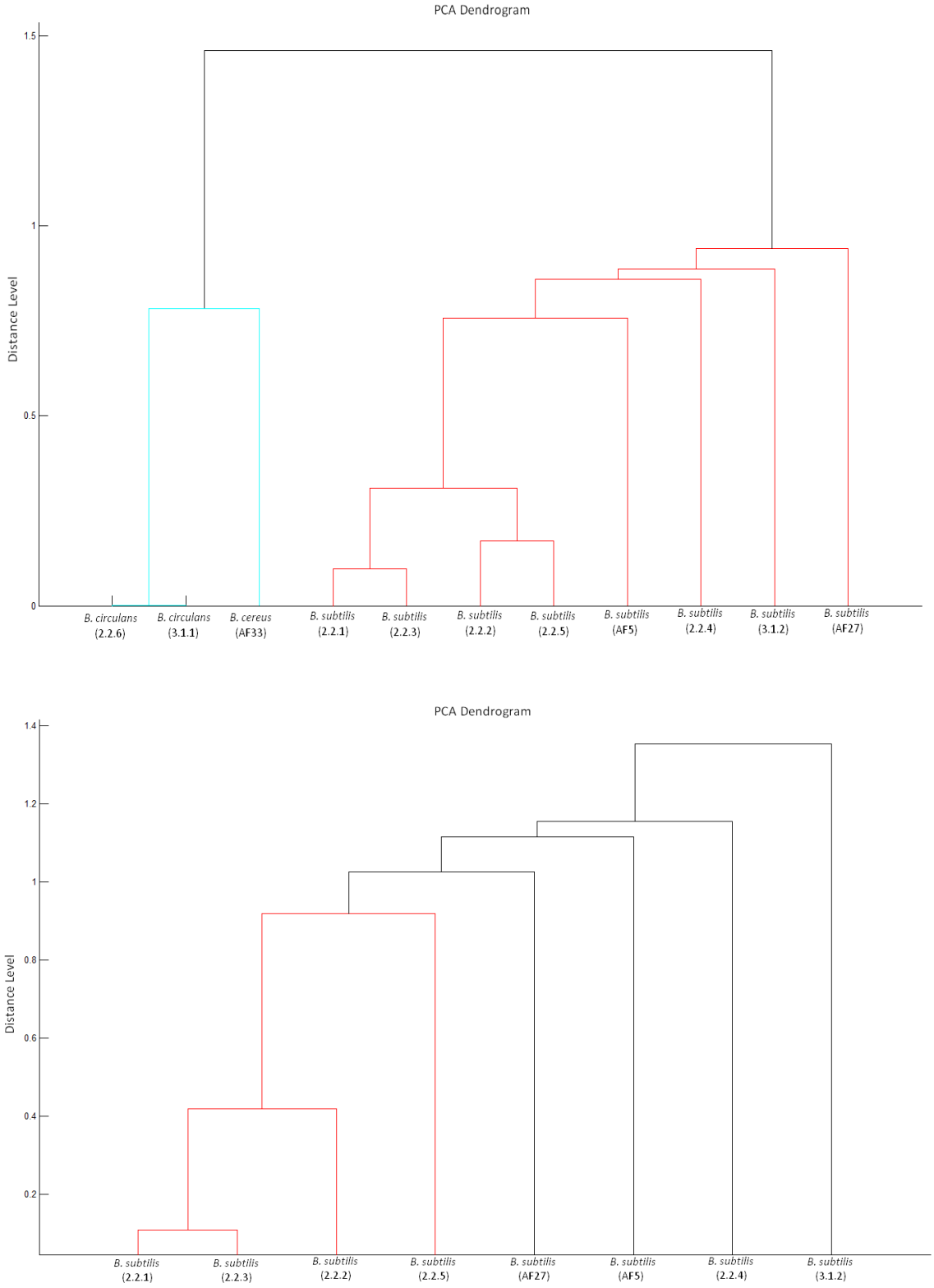


Figure 8: Dendrogram clustering of the 16 bacterial strains demonstrating the differences among the same species (Details in Appendix)

To demonstrate the results of the dendrograms, it was important to analyze the protein profiles of each of the strains to confirm there are genetic differences between the different strains that lead to a difference in their protein content and overall protein profiles. As shown in Figure 7, protein profiles were analyzed by MALDI-TOF and are showing the differences in protein profiles between the 16 isolates. It was found that even between the strains of the same species, there are differences in their protein profiles as demonstrated in *Bacillus subtilis* and *Bacillus circulans* strains. This makes it important to study each one of them as a different strain since they are not all producing the same proteins. This potentially means that some might be better or worse hydrocarbon-degrading than the others since they do not all produce the same proteins.

Discussion

The successful isolation of bacterial strains despite the harsh environmental conditions of high temperature and salinity and the presence of hydrocarbons that are known to be toxic to microbial species confirms that it was only possible because of the ability of these strains to degrade the hydrocarbons and utilize it as its' sole carbon source. The enrichment and selective culture protocol previously described in 3.2.1 allowed the isolation of bacterial strains that are tolerating to high hydrocarbon concentrations and toxicity levels only (Al-Kaabi et al., 2018). In order to have an acceptable variety of strains in this project to investigate the hydrocarbon degradation potentialities it was important to have additional isolates, and thus this was the aim of this part of the project. The isolation process was successful in isolating 8 additional strains to the 8 we already have. Many of the isolated strains have been under investigation and has been already reported to have hydrocarbon activity in previous literature (Table 7).

Table 7: Identified strains previously reported in literature.

Strains Isolated in this work	**Genus and species**	**Reference**
2.2.1, 2.2.2, 2.2.3, 2.2.4, 2.2.5, 3.1.2, Afnan 5, Afnan 27	*Bacillus subtilis*	(Albokari et al., 2015b; Zhang et al., 2014)
2.2.6, 3.1.1	*Bacillus circulans*	(AlKaabi et al., 2020; Hlordzi et al., 2020; Yu et al., 2013)
QDD1	*Pseudomonas aeruginosa*	(Attar et al., 2017; Disi & Ali, 2013; Martin-Sanchez et al., 2018)
BG8	*Micrococcus luteus*	(Abdollahinejad et al., 2020; de Vasconcellos et al., 2009; Imron et al., 2020)
QBC3	*Burkholderia cepacia*	(Ayala & Torres, 2004; Das & Chandran, 2010; Dua et al., 2002; Sarkar et al., 2017; Zeidan et al., 2019; Zhang et al., 2014)
QMSM10	*Staphylococcus capitis*	(Hentati et al., 2021; Vaishnavi et al., 2021)
Afnan 24	*Raoultella ornithinolytica*	(Imron et al., 2020; Morales-Guzmán et al., 2017; Obi et al., 2016)
Afnan 33	*Bacillus cereus*	(Al Disi et al., 2017; Albokari et al., 2015b; Al-Kaabi et al., 2018; Alsayegh et al., 2021; Disi & Ali, 2013)

Subsequently, it was important to identify the hierarchical relationship between the isolates. This was demonstrated by the dendrograms shown in figure 8 that demonstrates that the different bacterial strains have some differences in their genetic data, and this was further investigated and verified by studying their protein profiles using MALDI-TOF-MS. MALDI-TOF method was chosen for this analysis because it is a rapid and reliable method of identification of species and analysis of protein profiles. It was discovered that the dominancy of the bacterial community goes to *Bacillus subtilis*. This is an expected finding because *B. subtilis* is a well-known Surfactin producer (Kecskeméti et al., 2018) as well as a common specie found in the region (Albokari et al., 2015b). The protein profiles of the strains to identify if there is any difference between them or if they are identical. This step was important because

it allowed us to understand that there are differences between the strains in terms of protein production. For instance, the difference in peaks is noticeable between the 2 *Bacillus circulans* strains 2.2.6 and 3.1.1. the number of peaks between the mass of 2000-3000 m/z is significantly different which gives us an indication that there is a difference in the proteins produced by the 2 strains. Therefore, they are not identical. In addition to that, it was essential to compare the protein profiles between the 8 *Bacillus subtilis* strains. Similarly, to what was found with *B. circulans* strains, there are difference in their protein profiles. Some of the differences were minor such as with 2.2.2 and 2.2.5, however others had significantly different peaks such as Afnan5, Afnan27, and 2.2.4.

Conclusion

In this 1st chapter, 16 hydrocarbon degrading candidate strains were successfully isolated from highly oil contaminated soil. The identification of these strains revealed that all of the locally isolated strains belong to genera and species that were shown to have hydrocarbon degradation potentials as reported in literature (references mentioned in the table 7), which confirms our hypothesis. MALDI-TOF analysis was chosen to study the strains protein profiles and the hierarchical relationship between the isolates. It was verified that several bacterial isolates are closely related but not identical. The identification of the species demonstrated that *Bacillus subtilis* dominates the bacterial community. The MALDI-TOF-MS analysis identified that the isolates have differences in their protein profiles, which makes it necessary to include all of them in the following experiments due to their unique protein profiles.

CHAPTER 5: INVESTIGATION AND QUALITATIVE COMPARISON OF HYDROCARBON DEGRADATION POTENTIALITIES OF THE INVESTIGATED STRAINS

Introduction

The field of bioremediation using microbes is getting additional attention in the recent decades due to the increasing amounts of oil pollution due to the exploration, extraction, and transportation of fuel (Albokari et al., 2015b). It is known that hydrocarbon degradation process is complex and includes the presence of many compounds with variety of molecular weights, thus it was found that a better scenario is to form a complex or microbe community or consortia to have more degradation capabilities and achieve a higher bioremediation efficiency (Morales-Guzmán et al., 2017). Many bacterial species were found to have hydrocarbon degradation potentialities (Al Disi et al., 2017; Al-Kaabi et al., 2018; Attar et al., 2017; Brooijmans et al., 2009; Gouma et al., 2014; Morales-Guzmán et al., 2017). Many studies focus on the study of biosurfactant producing bacteria (Ibrar & Zhang, 2020; Morales-Guzmán et al., 2017; Olivera et al., 2009). On the one hand, our isolates after identification were found to be mainly *Bacillus spp.* that were previously discovered and reported in Saudi Arabia by (Albokari et al., 2015b). In addition to *Pseudomonas aeruginosa* that was previously isolated from Qatar and was proven to have hydrocarbon degrading potentialities *(Al Disi et al., 2017)*. On the other hand, *Raoultella ornithinolytica* was isolated in this study and it was not previously reported to be studied or found in the region, thus it was interesting to include this diversity of species and compare their hydrocarbon degradation potentialities against each other, as well as in consortia.

5.1 Comparison of The Survival and Growth Abilities of The Tested Strains in Hydrocarbon Presence Based on the determination of the CFU (Colony Forming Units)

The investigation of strains survival and growth potentialities was carried out by the determination of the cell biomass (cfu/ml) after 14 days of incubation in the presence of hydrocarbons. Bacterial strains were inoculated with an initial Optical Density (OD) of 0.15 as mentioned in section 3.2.6. The cfu/ml was determined using the equation mentioned in section 3.2.7. All of the bacterial strains had the ability to tolerate and survive in the presence of diesel and utilize it as their sole carbon source. Some of the bacterial strains had higher CFU than the others as shown in the table below:

Table 8: Determination and statistical analysis of cell biomass (10^6 cfu/ml) of the isolated strains after 2 weeks incubation in 7.5% Diesel medium.

Strains	**Growth (cfu/ml) x 10^6**			**Average growth (cfu/ml) x 10^6**
	I	II	III	
Control	0	0	0	**0** +/- 0.00
QDD1	50	48	47	**48.00** +/- 1.55
QBC3	33	32	34	**33.00** +/- 0.82
QMSM10	33	30	35	**32.57** +/- 2.26
BG8	37	34	36	**35.97** +/- 1.69
Afnan24	41	44	39	**41.00** +/- 2.61
Afnan27	33	32	39	**34.43** +/- 4.06
Afnan33	26	24	26	**25.10** +/- 1.32
2.2.1	29	21	28	**26.03** +/- 4.10
2.2.2	29	23	30	**27.27** +/- 3.78
2.2.3	16	19	19	**17.93** +/- 1.33
2.2.4	25	24	30	**26.43** +/- 2.86
2.2.5	39	38	36	**37.40** +/- 1.51
3.1.1	16	18	18	**17.23** +/- 1.50
Mixture	60	63	64	**61.93** +/- 2.06

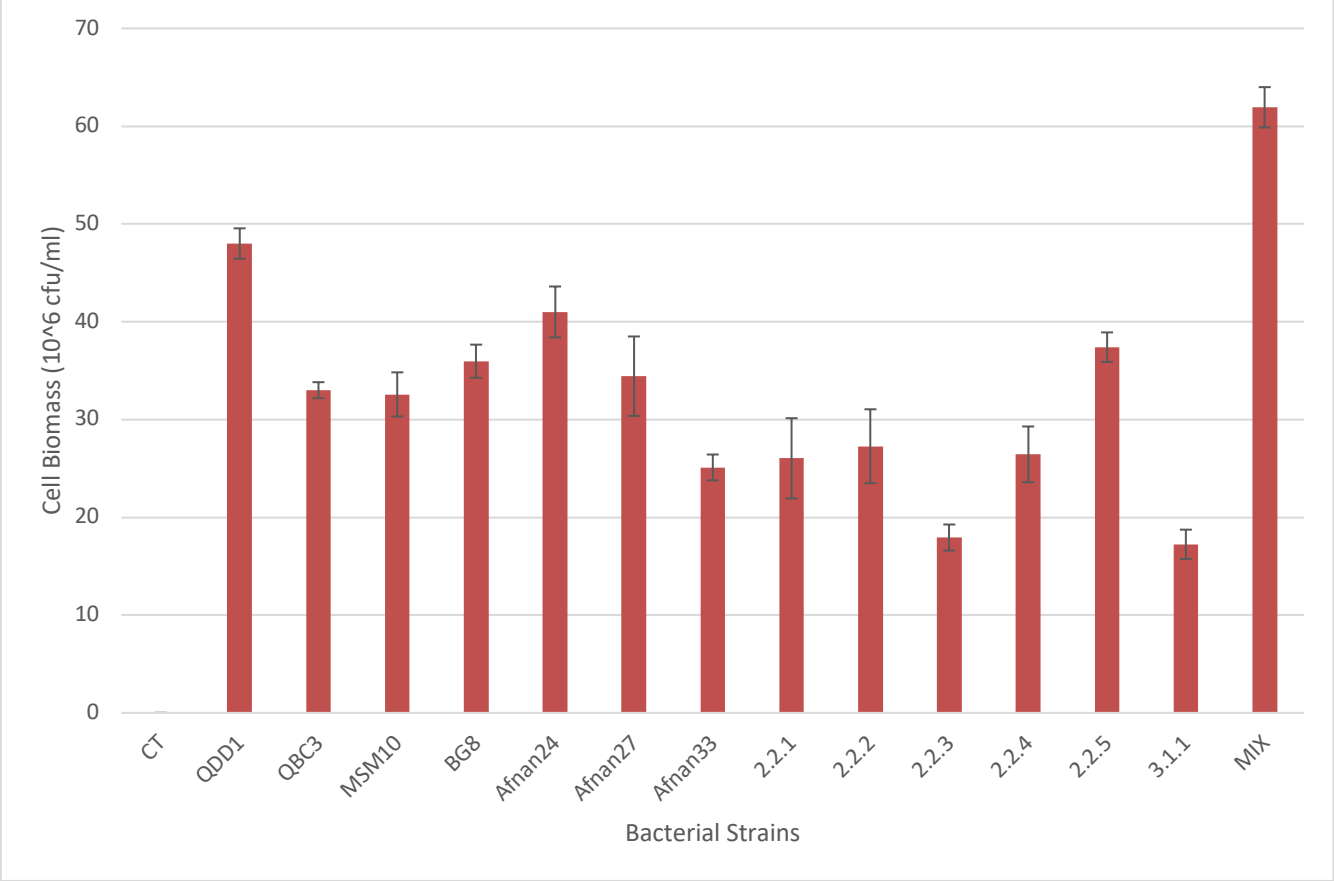


Figure 9: Comparison of the cell biomass (10^6 cfu/ml) of the isolated strains after 2 weeks growth in 7.5% Diesel MSM medium. [CT=Control (without bacterial inoculation); MIX= consortuim of all bacterial isolates].

Based on the cell biomass analysis shown above in figure 9, it was observed that QDD1, Afnan24, and strain 2.2.5 had the highest cell biomass among all of the others strains. QDD1 has showed the highest biomass. The mixture of the 16 strains (QDD1, QBC3, QMSM10, BG8, Afnan5, Afnan24, Afnan27, Afnan33, 2.2.1, 2.2.2, 2.2.3, 2.2.4, 2.2.5, 2.2.6, 3.1.1, and 3.1.2) showed the highest biomass after 2 weeks of growth in the presence of diesel as sole carbon source. This might be well explained by the synergistic effects between the strains and their co-degrative potentials making low molecular weight molecules more available to bacterial cells.

These results demonstrate very well the degradation potentials of the strains that keep growing in the presence of hydrocarbon sole carbon source.

5.2 Qualitative Analysis of Diesel Degradation by Gas Chromatography (GC-FID)

In this part of the study, the investigation of the diesel degradation potentials was explored after incubation with the bacterial strains as explained in the previous sections. The exploration of the diesel degradation was carried out by GC-FID MS as described previously in 3.2.8. The removal efficiency (RE%) is calculated using the following equation (Disi & Ali, 2013):

$$\boldsymbol{RE\% = 100 - \left(A_s \times \frac{100}{A_C}\right)}$$

As = the total area of the peak in each sample

Ac = the total area of the peak in the control

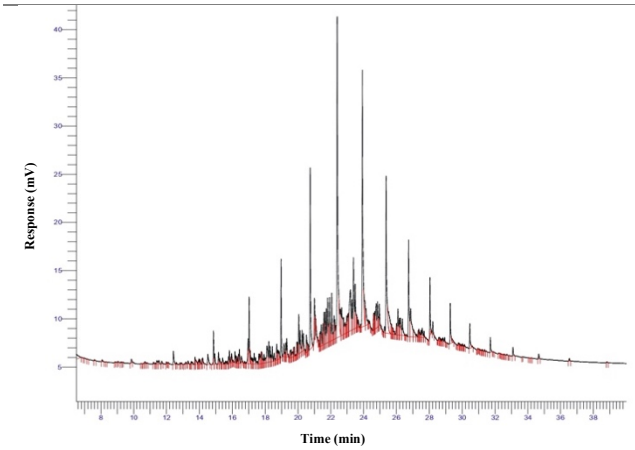
RE% = hydrocarbon degradation/removal efficiency

The shaking conditions were very important and affect a lot the degradation potentials of the strains. In fact, the purpose is to ensure that the diesel is mixed all the time with the cells to increase the contact surfaces between the cells and the hydrocarbons that are not miscible in the MSM medium.

The first experiment was done to confirm the ability if the strains to degrade diesel and to measure the removal efficiency when supplemented with 5% diesel in MSM. The GC-FID MS analysis was done measuring low, medium, and high molecular weight (MW) diesel. Uninoculated sample was used as a control for this experiment containing sterile MSM1 media and diesel incubated with the samples under the same conditions.

It was shown that all the species were hydrocarbon degrading. Removal efficiencies of all species are shown in table 9 below. Generally, the highest removal efficiencies were found in the high molecular weight (HMW) hydrocarbons. The medium and low molecular weight hydrocarbons (LMW and MMW) were showing

relatively lower RE which is reasonable giving that the HMW hydrocarbons when degraded they can be converted into MMW or LMW. The decrease in the LMW hydrocarbons is an indicator that some of the diesel was completely degraded and removed.



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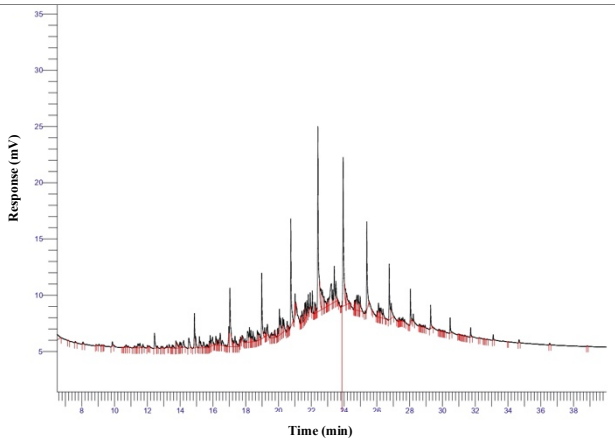

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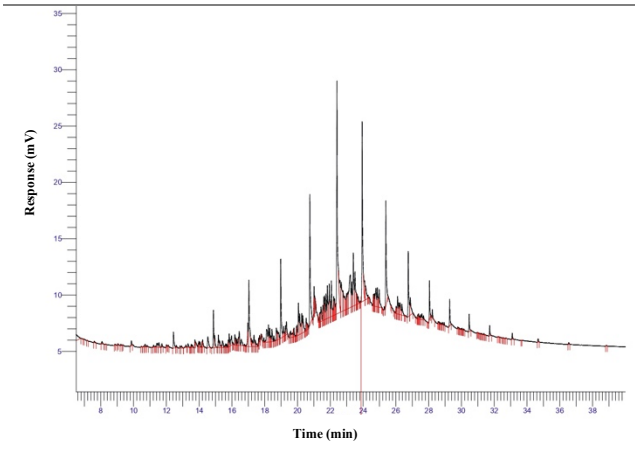

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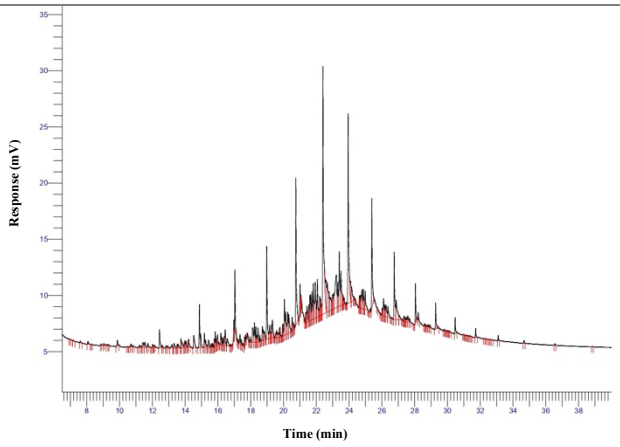

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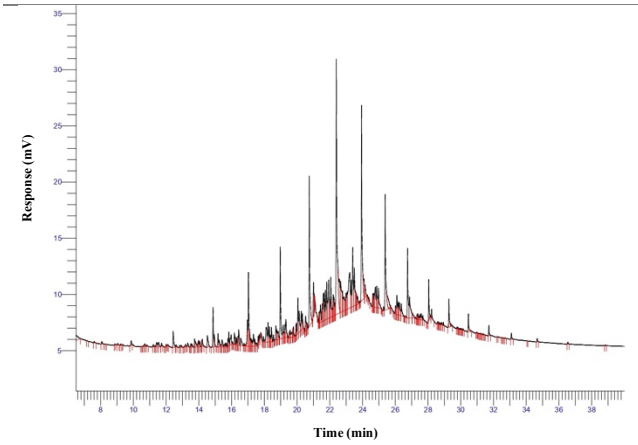
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Time (min)

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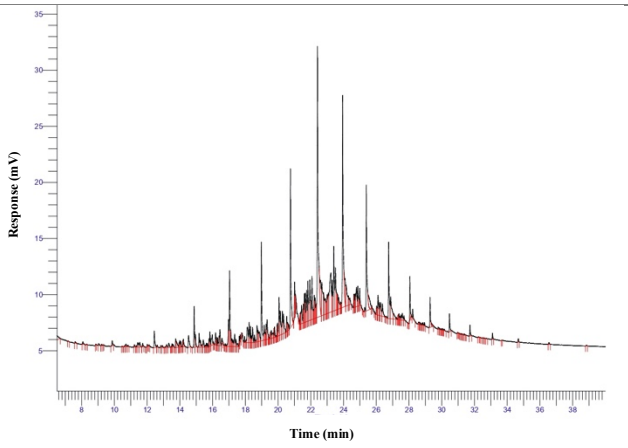
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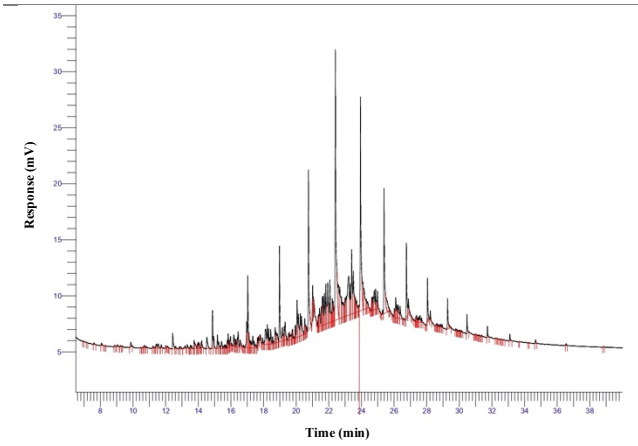
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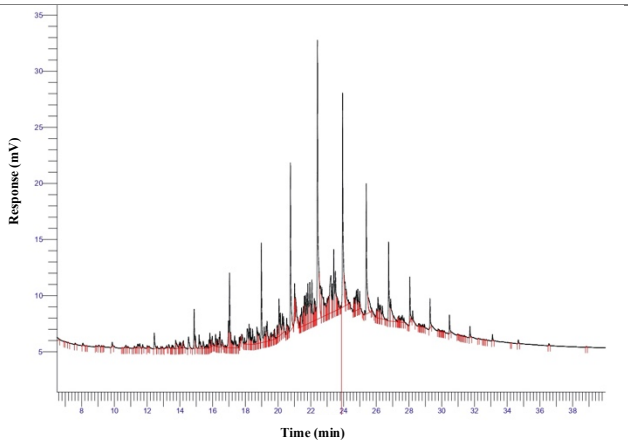
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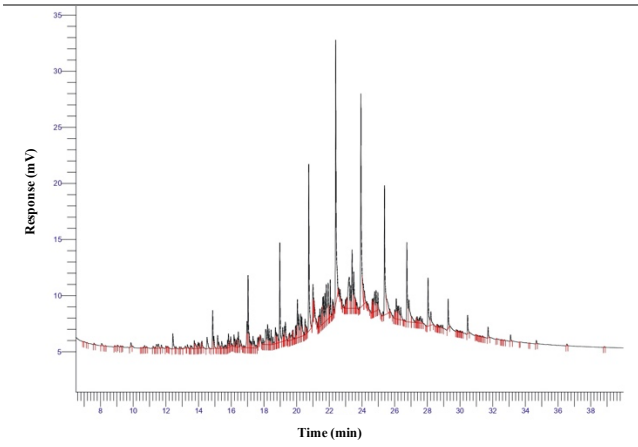
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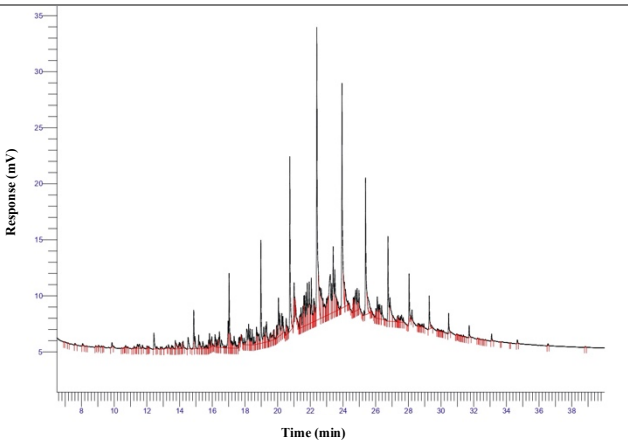
Response (mV)
Time (min)

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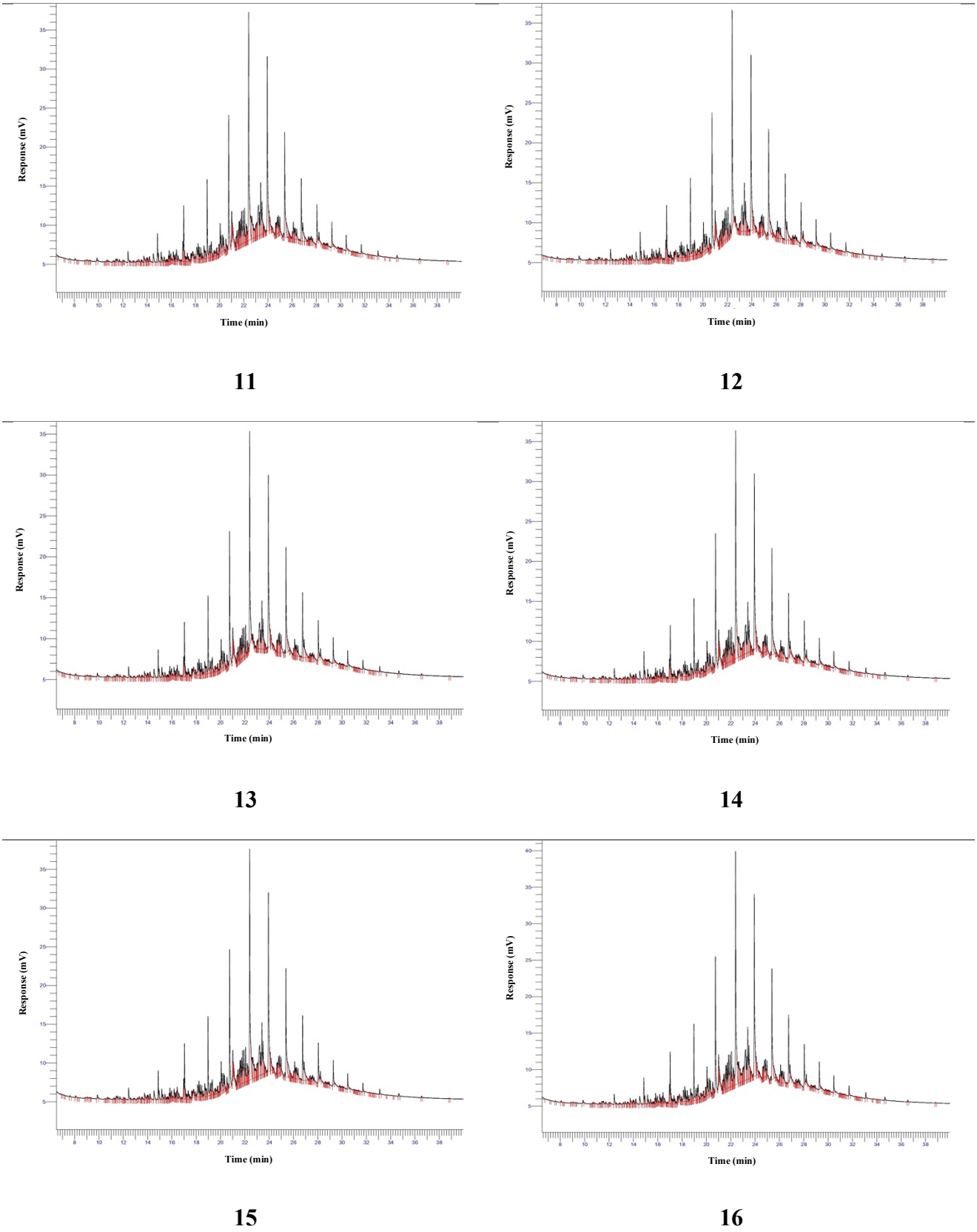

Figure 10: GC-FID Chromatograms demonstrating the peaks of absorbance vs retention time of Diesel compounds separated based on size through mass spectrophotometry [1: Control (without bacterial inoculation); 2:QBC3; 3:QMSM10;

4:BG8; 5:Afnan5; 6:Afnan24; 7:Afnan27; 8:Afnan33; 9:2.2.1; 10:2.2.2; 11:2.2.3; 12:2.2.4; 13:2.2.5; 14:2.2.6; 15:3.1.1; 16:3.1.2.

Table 9: Removal efficiency (RE) of bacterial isolates after 3 weeks of incubation in MSM media supplemented with 5% diesel. (LMW is low molecular weight; MMW is Medium molecular weight; HMW is high molecular weight) (LMW; nC_9-nC_{16}), (MMW; nC_{17}-nC_{20}) and (HMW; nC_{21}-nC_{25}).

Removal efficiency (%)	RE of LMW (%)	RE of MMW (%)	RE of HMW (%)
QBC3	47.4%	56.9%	52.5%
QMSM10	28.6%	47.7%	48.4%
BG8	24.9%	43.6%	54.9%
Afnan5	20.5%	38.5%	45.2%
Afnan24	12.2%	34.7%	43.9%
Afnan27	14.7%	42.9%	42.9%
Afnan33	17.3%	34.5%	39.0%
2.2.1	29.2%	29.3%	42.1%
2.2.2	14.9%	28.4%	37.1%
2.2.3	5.7%	20.3%	31.6%
2.2.4	20.8%	22.3%	28.5%
2.2.5	22.9%	24.6%	33.8%
2.2.6	13.8%	31.5%	24.3%
3.1.1	5.8%	21.7%	32.8%
3.1.2	2.2%	22.7%	12.7%

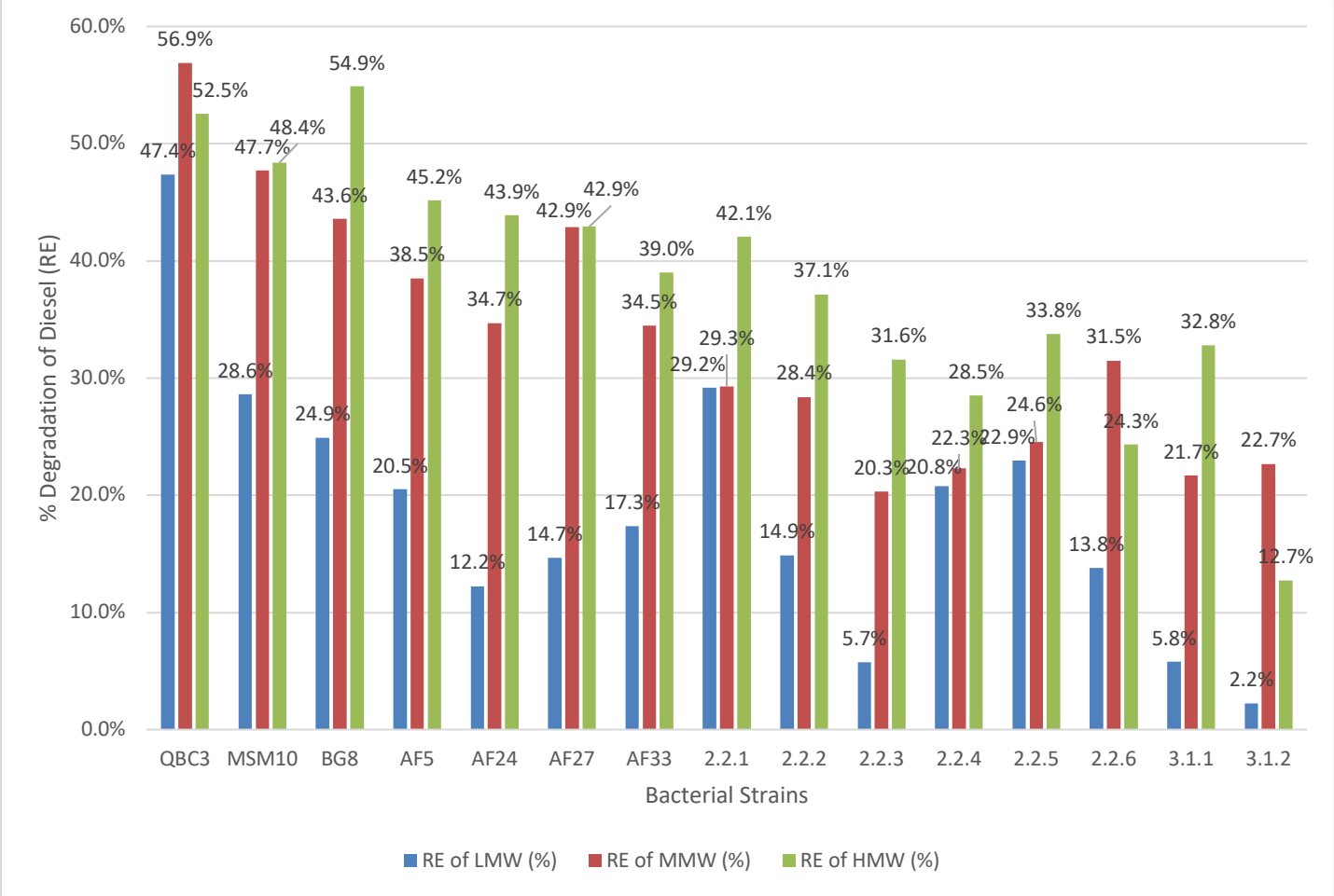

Figure 11: Removal Efficiency (RE) of 5% diesel supplemented to hydrocarbon degrading bacteria in MSM1 media after incubation period of 3 weeks analyzed by GC-FID MS.

As shown in figure 11 above, the Removal Efficiency (RE) of the 5% diesel supplemented to hydrocarbon degrading bacterial strains in MSM1 media after an incubation period of 3 weeks differs from one strain to another. Some strains showed very high efficiency in degradation such as QBC3 (*Burkholderia cepacia*), QMSM10, and BG8 that degraded high molecular weight (HMW) hydrocarbons with 52.5%, 48.4%, and 54.9%, respectively, while others were not as efficient degraders to diesel such as 2.2.4, 2.2.6, and 3.1.2 that had RE of 28.5%, 24.3%, and 12.7%, respectively.

5.3 Investigation of the Potentialities of the Tested Strains to Degrade Different Aromatic Hydrocarbons

The ability of the 16 strains to grow on different concentrations of hydrocarbons as their sole carbon source was already investigated and confirmed as mentioned in the previous sections. In this part of the project, the 16 selected strains were tested for their hydrocarbon utilization potentialities utilizing aromatic hydrocarbons (Toluene and Xylene) and the extent of their growth was observed based on their colony size. The method used was similar to the described by (Martínez-Ávila et al., 2021) with some adjustments. Media were prepared regularly as described in 3.1.2, and before pouring a specific amount of different hydrocarbon was inoculated (Table 10) and shaken into the media in order to ensure homogenous distribution of hydrocarbon over the whole plate grid where the 16 strains were inoculated. The 16 strains were tooth picked into the media by picking up a single colony from freshly grown culture. A negative control was assigned for the experiment using MSM1 plates that had no hydrocarbon inoculation.

Table 10: Experimental design showing the concentrations of hydrocarbons inoculated in MSM1 media.

	MSM1 (-ve control)	**LB (+ve control)**	**MSM1 + Toluene**	**MSM1 + Xylene**
100 ppm	0 µL	0 µL	3 µL	3 µL
200 ppm	0 µL	0 µL	6 µL	6 µL
500 ppm	0 µL	0 µL	15 µL	15 µL
1000 ppm	0 µL	0 µL	30 µL	30 µL
3000 ppm	0 µL	0 µL	90 µL	90 µL

After the verification and measurement of the potentialities of the selected strains in degradation of the polyaromatic hydrocarbon (PAH) diesel, it was found important to investigate their potentialities in degradation of other hydrocarbons. This

experiment was designed to test the rationality of the presence of genes encoding enzymes that are responsible for toluene and xylene degradation.

Table 11: Growth of bacterial colonies utilizing toluene as their sole carbon source. (-) indicates no growth, (+) indicates minimum growth, (++) indicates meduim growth, (+++) indicates maximum growth.

	Strain	MSM	LB	T-100	T-200	T-500	T-1000	T-3000
1	QDD1	-	+++	-	-	-	-	-
2	QBC3	-	+++	-	-	-	-	-
3	QMSM10	-	+++	-	-	-	-	-
4	BG8	-	+++	-	-	-	-	-
5	Afnan5	-	+++	++	+	+	+	+
6	Afnan24	-	+++	++	++	++	++	+++
7	Afnan27	-	+++	+	+	+	+	+
8	Afnan33	-	+++	++	++	++	++	++
9	2.2.1	-	+++	+	++	+	+	+
10	2.2.2	-	+++	+	+	+	+	+
11	2.2.3	-	+++	+	+	++	++	+++
12	2.2.4	-	+++	+	+	+	+	+
13	2.2.5	-	+++	+	+	+	+	+
14	2.2.6	-	+++	+	+	+	+	+
15	3.1.1	-	+++	+	+	+	+	+
16	3.1.2	-	+++	+	+	+	+	+

Table 12: Growth of bacterial colonies utilizing xylene as their sole carbon source. (-) indicates no growth, (+) indicates minimum growth, (++) indicates medium growth, (+++) indicates maximum growth.

	Strain	MSM	LB	X-100	X-200	X-500	X-1000	X-3000
1	QDD1	-	+++	-	-	-	-	-
2	QBC3	-	+++	-	-	-	-	-
3	QMSM10	-	+++	-	-	-	-	-
4	BG8	-	+++	-	-	-	-	-
5	Afnan5	-	+++	+	+	+	+	+
6	Afnan24	-	+++	+	+	+	+	+
7	Afnan27	-	+++	+	+	+	+	+
8	Afnan33	-	+++	++	++	++	++	++
9	2.2.1	-	+++	+	+	+	+	+
10	2.2.2	-	+++	+	+	+	+	+
11	2.2.3	-	+++	-	-	-	-	-
12	2.2.4	-	+++	-	++	++	++	++
13	2.2.5	-	+++	-	++	++	+++	+
14	2.2.6	-	+++	-	-	-	-	-
15	3.1.1	-	+++	-	-	-	-	-
16	3.1.2	-	+++	-	-	-	-	-

Most of the bacterial strains have shown positive results when inoculated in toluene and xylene (tables 11 and 12). Despite the fact that in both toluene and xylene, the strains from 1 to 4 were not indicating visible growth. However, it was confirmed that many of the strains are degrading toluene and xylene. As shown in table 11, strains

5-16 were all showing growth in media containing toluene as their sole carbon source. Similarly, as shown in table 12, the strains 1, 2, 3, 4, 11, 14, 15, and 16 were not showing growth on the media, however the strains 5, 6, 7, 8, 9, 10, 12, and 13 were all showing growth utilizing xylene as their only carbon source.

Discussion

In this chapter, the potentialities of survival, growth, and degradation of hydrocarbons of bacterial isolates were investigated. The findings from the first experiment through the statistical analysis of cell biomass (cfu/ml) demonstrated that all the bacterial isolates had the ability to grow and survive for 14 days utilizing diesel as their only carbon source. After the confirmation of the survival abilities of the bacterial strains, it was observed that some of the strains had higher cell biomass than others and this was hypothetically explained by the fact that they have better degradation abilities since they have higher cfu. In addition, a bacterial consortium was made in order to examine the symbiotic relationship of the bacterial strains in an ecosystem. The mixture of the 16 strains (QDD1, QBC3, QMSM10, BG8, Afnan5, Afnan24, Afnan27, Afnan33, 2.2.1, 2.2.2, 2.2.3, 2.2.4, 2.2.5, 2.2.6, 3.1.1, and 3.1.2) showed the highest biomass after 2 weeks of growth in the presence of diesel as sole carbon source. This might be well explained by the synergistic effects between the strains and their co-degrative potentials making low molecular weight molecules more available to bacterial cells.

The diesel degradation analysis done by GC-FID MS revealed the hydrocarbon removal efficiencies by the bacterial strains. Between all the strains it was found that the following strains were having the highest %RE in HMW diesel: QBC3, QMSM10, and BG8 with efficiencies of 52.5%, 48.4%, and 54.9% after 21 days of incubation in 5% diesel medium. Similarly, MMW had comparable degradation ratios to the HMW,

and the same 3 strains obtained the highest degradation efficiencies with MMW as what have been observed with HMW hydrocarbons. However, it was observed that the LMW hydrocarbons as an overall was lesser in %RE compared with MMW and HMW. Generally, it is known that the HMW are usually degraded into MMW and LMW and by this observation it is concluded that the HMW and MMW were degraded into LMW in this case, thus the reduced degradation ratios in the LMW hydrocarbons. Similar results were obtained by (Wang et al., 2020), where they obtained HMW-PAHs removal of 52.2% in comparison to LMW-PAHs of 29.3% only.

Investigating the degradation potentialities of the selected strains for other aromatic hydrocarbons presented in toluene and xylene. It was found that many of the strains did have the ability of surviving in toluene and xylene, which can be only possible if they are utilizing the aromatic hydrocarbons to obtain carbon that is essential for their growth. In addition to that, it was observed that there was difference in colony sizes which was correlated with the hydrocarbon concentration. The difference in colony size was visible with some strains showing positive correlation between colony size and toluene concentration such as with the strainsAfnan24 and 2.2.3.

These results corroborated with those of the following chapter where we demonstrate that the strain AF33, 2.6 and 3.11 have the gene encoding Toluene monooxygenase for the degradation of Toluene and on the other hand strains 2.1. 2.2. 2.3, 2.4 and 2.5 harbor the xylanase coding gene responsible of xylene degradation.

These results demonstrate that the bacterial strains used and isolated in this work do not only tolerate the toxicity of the hydrocarbons but utilize the toluene as their source of carbon which has been previously reported in literature (Chao & Hsu, 2004; Feng et al., 2021; Kesavan et al., 2021; Ruiz, Radwan, et al., 2021).

Conclusion

The investigation of degradation potentials of the tested strains demonstrated that some bacterial isolates might be successful hydrocarbon degraders matching the efficiencies to a bacterial consortium. Based on the preliminary results obtained through the investigation of hydrocarbon-degradation potentialities of the tested strains, it is now confirmed that the bacterial strains have potentialities in the degradation of other aromatic hydrocarbon compounds determined by growth abilities in minimal media containing toluene and xylene as the only sources of carbon. Therefore, the investigation of the presence of genes encoding important enzymes responsible of the degradation of hydrocarbon compounds was found to be fundamental as has been carried out in the following section.

CHAPTER 6: EXPLORATION OF KEY GENES ENCODING IMPORTANT HYDROCARBON DEGRADING ENZYMES

Introduction

The exploration of the availability of genes that are encoding the important enzymes that are responsible for the degradation of hydrocarbons were investigated in this part of the project. After investigating the degradation potentialities and abilities by the tested strains demonstrated in chapter 5, it was important to go deeper into studying the genes that encode the synthesis of the enzymes degrading hydrocarbons. The main focus of the study was to explore the genes encoding diesel hydrocarbon degrading enzymes. However, after finding that many of the strains are able to use toluene and xylene as carbon sources as presented in section 5.3, it was important to take into consideration that the bacterial isolates might have the genes that give them the ability of degradation of different aromatic hydrocarbons. It was found that most of the available literature focusing on the study of hydrocarbon-degrading bacteria focus on the degradation potentials and the enzymatic structure and chemical composition, however the area of molecular studies is lacking. In is part of the project, the objective was to identify the presence of key genes that are encoding the synthesis of enzymes that have hydrocarbon degradation abilities. In order to do that, we selected several pairs of primers that were designed for the exploration of the presence of the mentioned genes encoding important enzymes responsible of the degradation of hydrocarbons. Some of these primers were reported in literature and others are new for this study (Table 13).

6.1 Exploration of the presence of the key genes encoding hydrocarbon degrading enzymes

In this part of the project, the key genes encoding the most important enzymes of the pathways of the degradation of hydrocarbons, were explored by PCR using specific pairs of primers (Table 13).

Table 13: List of Primer pairs used in this study and corresponding gene/enzyme and expected size of the PCR amplified fragments.

Primer pair	Primers	Gene / Enzyme	Size (kb)
1.	B6-F.	Naphalene dihydro diol	0.773
	B778-R.		
2.	C118-F.	(*nah C)*1,2-Dihydro xynaphthalene oxygenase	0.697
	C814-R.		
3.	E207-F.	(*nahE)*2-Hydroxybenzalpyrovate aldolase	0.620
	E826-R.		
4.	ALK-1F.	(*alkB)*Alkane hydroxylase	0.185
	ALK-1R.		
5.	ALK-2F.	(*alkMa*) & (*alkMb*)Alkane hydroxylase	0.271
	ALK-2R.		
6.	ALK-3F.	(*alkB1*) Alkane hydroxylase	0.330
	ALK-3R.		
7.	TBMD-F.	(*tbmD)* Toluene benzene monooxygenase large subunit	0.640
	TBMD-R.		
8.	AlkB1-E.	(*alkB*)Alkane hydroxylase	1.149

	AlkB1-X.		
9.	AlkB1-F.	*(alkB)* Alkane hydroxylase	1.149
	AlkB1-R.		
10.	TMOA-F.	(*tmoA*) Toluene monooxygenase large α subunit	0.505
	TMOA-R.		
11.	TOD C1-F.	(*todC1)* Aromatic dioxygenases (TOD pathway)	0. 510
	TOD C1-R.	large subunit	
12	BED C1-F.	(*bed C1)* Benzene dioxygenase *alpha subunit*	0.358
	BED C1-R.		
13	XYL A- F.	(*xylA*)Xylene monoxygenases (TOLp pathway)	0.291
	XYL A- R.		
14.	XYL E1-F.	(*xylA*) Xylene monoxygenases (TOLp pathway)	0.242
	XYL E1-R.		
15.	ALK B-F.	*(alkB)* Alkane hydroxylase	0.550
	ALK B-R.		

In this study, the PCR cycles were optimized by changing the amplification conditions and particularly by optimizing the annealing temperatures and timing for many pairs of primers (Data not shown).

6.1.1 Investigation of the presence of the genes encoding alkane hydroxylases

Due to the importance of alkane hydroxylases in hydrocarbon degradation, several pairs of primers were used to look for this important family of genes that are found in different hydrocarbon degrading bacteria (Table 14).

Table 14: List of Primer pairs used in the exploration of the alkane hydroxylase genes

Primer pair	Gene	Expected size
ALK-1F.	(*alkB)* Alkane hydroxylase	0.185
ALK-1R.		
ALK-2F.	(*alkMa*) & (*alkMb*) Alhkane hydroxylase	0.271
ALK-2R.		
ALK-3F.	(*alkB1*) Alkane hydroxylase	0.330
ALK-3R.		
AlkB1-E.	(*alkB*) Alkane hydroxylase	1.149
AlkB1-X.		
AlkB1-F.	*(alkB)* Alkane hydroxylase	1.149
AlkB1-R.		
ALK B-F	*alkB* Alkane hydroxylase	0.550
ALK B-R		

1. Exploration of (alkB) Alkane hydroxylase coding gene

The prediction of the presence of (*alkB)* Alkane hydroxylase coding gene was carried out using the Primer pair 4 (ALK-1F. & ALK-1R) and as templates the DNA of the 16 strains explored in this study.

The PCR products were electrophoresed in an agarose gel (Figure 12)

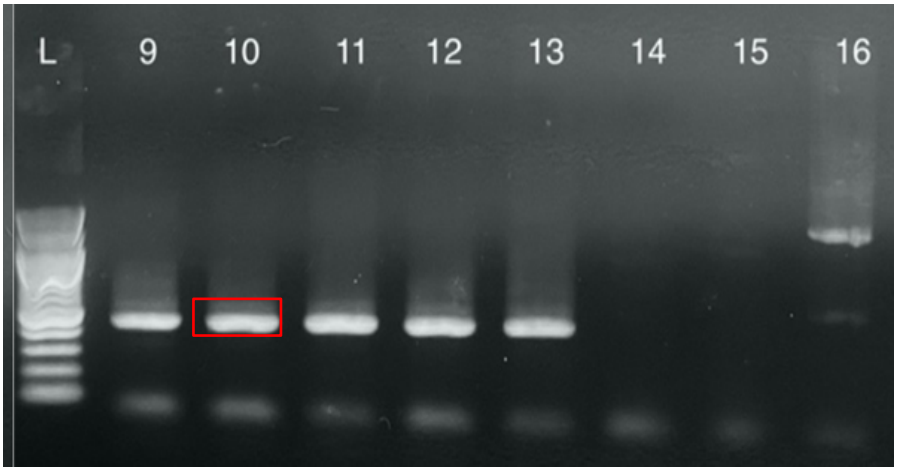

Figure 12: PCR fragment amplification using Primer pair 4: (ALK-1F. & ALK-1R). Lanes; L, 100-bp DNA ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1517 bp); 9, 2.2.1; 10, 2.2.2; 11, 2.2.3; 12, 2.2.4; 13, 2.2.5; 14, 2.2.6; 15, 3.1.1; 16, 3.1.2.

It was found that none of the 16 strains gave the amplification of the expected size of 185 bp . However, a very strong amplification of a fragment of 500bp was noticed for the strains 2.2.1; 2.2.2; 2.2.3; 2.2.4 and 2.2.5. This amplified fragment is not within the expected size of 185 bp. This might be explained by the fact that the primers were designed primarily for *Pseudomonas putida* (Smits et al., 1999) genes. Therefore, the gene sequence polymorphism might affect the annealing of the primers and give fragments of different sizes. In fact, all these strains that gave amplification are *Bacillus subtilis* but not *Pseudomonas* strains. Therefore, the fragment of 500bp of the strain 2.2.1 was sequenced.

5'TAATGGATGACTGCTGAGAATAGGCAGATGAAGATGTGCGAGCGCCAAGGA
CCGCCAGACGATGATCGGCATCCTAAGCTAAAAAAATAAATGCCGGGCTCACCTTT
ATACGTTGACATAGGTGCGGAGGTTAAGCTCAGGAAATGCACGCGCACCGGGAATG
GCAGGAAGATAACGCGCCCTCAGGTTTCGTGAGCATAAAGGGAAGCATGCTGATCC
AAGCTTTGCCCGTTTATATTGTATCTAATTCCTAAAACGGACGGTACCAAAGCGCGA
AGGATAGAAACATCAACAGGCCAGTGAGCAAATAAGACATCATTCCCAGGTTTTGT
CTCATGATCCAGATTCCTTTCAGGTACAGAAATGTTTTTTTTGCTGAAAGTGTTCCGT
CAACGTATATGCTCCTTTCGTTTGGAGTTCGAAAAAAAAAAAAAACGCCTCAGTGGGG
ATTGAAAGCCAGAGAGCTTAACCGGTTTATGCGTATTATTCCAGATTCTGCTGCGGT
GGCGGGAGATTACTACACAACGATGATGTTTTTTTATGAGGGGGGG3'

Figure 13: Sequence of the 500 bp PCR fragment of the strain 2.2.1 using the primer ALK-1F

The alignment of this sequence revealed similarities with the genome of *Bacillus subtilis s*train SRCM102756 particularly the 2 genes encoding the Alkanesulfonate monooxygenase and the Haloalkane dehalogenase

-Alkanesulfonate monooxygenase: Protein id:QHM17555.
MEILWFIPTHGDARYLGSESDGRTADHLYFKQVAQAADRLGYTG
VLLPTGRSCEDPWLTASALAGETKDLKFLVAVRPGLMQPSLAARMTSTLDRISDGRLL
INVVAGGDPYELAGDGLFISHDERYEATDEFLTVWRRLLQGETVSYEGKHIKVENSNL
LFPLQQEPHPPIYFGGSSQAGIEAAAKHTDVYLTWGEPPEQVKEKIERVKKQAAKEGR
SVRFGIRLHVIARETEQEAWEAAERLISHLDDDTIAKAQAALSRYDSSGQQRMAVLHQ
GDRTKLEISPNLWAGIGLVRGGAGTALVGDPQTIADRIAEYQALGIESFIFSGYPHLE
EAYYFAELVFPLLPFENDRTRKLQNKRGEAVGNTYFVKEKNA

-Haloalkane dehalogenase: Protein id:QHM20145.1
MKSAWMEKTYTIDGCAFHTQHRKGSSGVTIVFEAGYGTSSETWK
PLMADIDDEFGIFTYDRAGIGKSGQSRAKRTADQQVKELESLLKAADVKPPYLAVSHS
YGAVITGLWACKNKYDIIGMVLLDPALGDCASFTFIPEEMHKSHTRKMMLEGTHAEFS
KSLQELKKRQVHLGNMPLLVLSSGERTEKFAAEQEWQNLHSSILSLSNQSGWIQAKNS
SHNIHHDEPHIVHLAIYDVWCAACQQAAPLYQAVN

Figure 14: Sequences of *Bacillus subtilis* Proteins encoded by genes showing similarities with the 500bp of the strain 2.2.1

These 2 enzymes might have an important role in the degradation of hydrocarbon alkanes.

2. *Exploration of Alkane hydroxylase alkM coding genes*

No amplification was found using the DNA template of any of the tested strains. This primer pair 5 (ALK-2F. & ALK-2R) is corresponding to the genes (*alkMa*) & (*alkMb*) that are responsible for production of Alkane hydroxylase alkM (Tani et al., 2001). The results demonstrated above show that none of the tested strains has this gene.

3. *Exploration of Alkane hydroxylase alkb1 coding genes*

Using the Primer pair 6 (ALK-3F. & ALK-3R) no amplification with the expected size of 330 bp was obtained. However, a very strong amplification was shown in a different size of around 600 bp (Figure 15). Although this amplified fragment is not within the expected size of 330 bp, it was decided to sequence it (Figure 16)

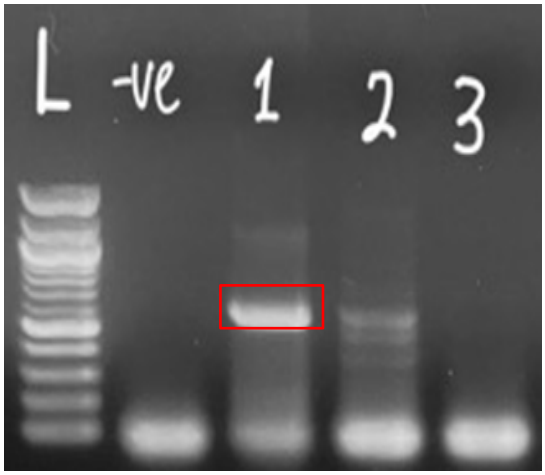

Figure 15: PCR fragment amplification using Primer pair 6: (ALK-3F. & ALK-3R). Lanes; L, 100-bp DNA ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1517 bp); -Ve: negative control; 1, QDD1; 2, QBC3; 3, MSM10

GATTCCTCCGGGAGTACTTCTTCTGCGCCTATCGCCCAATCCTCTAACCGTTC
TGCCCAATGCCACCAACACCACCTTCTCCACCCCCGGGCCCTGTAGGCCGATCGCCC
TGCGCGCAAGGGGCTGCCGCTGTGCACCGGGCCAACCAAATGATCTGGTGGTACCT
GTTGACCCTCTCTTCCTGGGTCGGTTTTCGCCTGGCCTTTCCGCTGGGTGGGAGCTAT
CTTCTTCCTCTGCCCATCCGTTATGGCCTTCACCCTGGTGGAAAATCTCCACTAACAA
AAACCCCTACGGG.

Figure 16: Sequence of the 600 bp PCR fragment of the strain QDD1 using the primer ALK3-F

The alignment of this sequence revealed similarities with the genome of *Pseudomonas aeruginosa s*train PA0750, hydrocarbon binding protein (Figure 17).

MAKHAPQLPIEVDSETGVWTTDALPMLYVPRHFFVNNHMGIEEV

LGADAYAEILYKAGYKSAWHWCEKEAECHGLEGVAVFEHYMKRLSQRGWGLFEIERIN

LEEGTAEVRLRHSAFVYVYGKVNRKVDYMFTGWFAGAMDQILAARGSSLRTVAEQVYS

GAEDGHEDGLFVVKPL

Figure 17: Sequences of *Pseudomonas aeruginosa s*train PA0750 hydrocarbon binding protein: Protein id:QAA05717 encoded by a gene showing similarities with the 600bp of the strain QDD1

4. Exploration of Alkane monooxygenase alkB1 coding genes

Using the Primer pair 9 (ALK-B1F. & ALK-B1R), only the strain QDD1 gave an amplification of an expected size of 1140 bp (Figure 18).

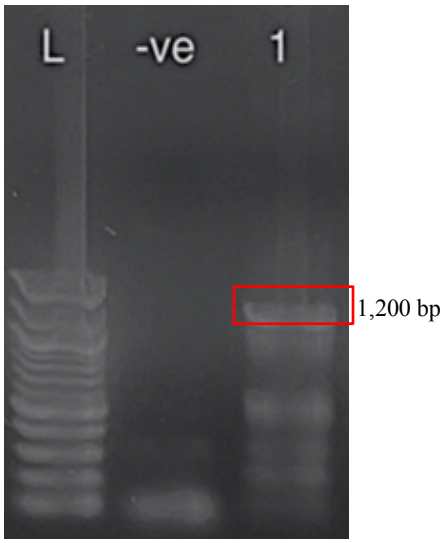

Figure 18: PCR fragment amplification using Primer pair 9: (AlkB1-F. & AlkB1-R).

This allows to predict that this strain has the gene (*alkB1*) that encodes the synthesis of the enzyme Alkane hydroxylase that is responsible for the metabolism of alkanes (Canosa et al., 2000; Ruettinger et al., 1977; J. Shanklin et al., 1997; John Shanklin & Whittle, 2003).

Therefore, the fragment of 1149-bp was sequenced (Figure 19):

GAATAAGGCATTCATACACGGCTGCAGGCGGCATTCTGCTGGCCGCCGTGTGCTACGCGG
GGTTCAAGGTCGAGCATGTGCGCGGCCACCATGTGCATGTGTCTACGCCGGAGGACGCTT
CGTCGGCGCGTTTCGGCCAGTCGGTCTACCAGTTCCTGCCGCATGCCTACAAGTACAACTT
CCTCAACGCCTGGCGCCTTGAAGCGGTGCGGCTGCGCAAGAAGGGCCTGCCGGTGTTCGG
CTGGCAGAACGAACTGATCTGGTGGTACCTGCTGAGCCTGGCGTTGCTGGTCGGTTTCGGT
TGGGCGTTCGGCTGGCTGGGGATGGTTTTCTTCCTTGGCCAAGCGTTCGTCGCGGTGACCC
TGCTGGAGATCATCAACTACGTCGAGCACTACGGCCTGCATCGGCGAAAGGGCGAGGACG
GGCGCTACGAGCGGACCAACCATACCCACTCCTGGAACAGCAACTTCGTCTTCACCAACC
TGGTCCTGTTCCATCTGCAACGCCACTAGAACCCACCCCAGACCATGTCGGGGGGGGGGG
GAAAAAACATTCCGGTTGATCGGCGTGGGGGGGGGTTTTCTATCTCTCTTTACCATCATCG
AATTATAAG

Figure 19: Sequence of the 1149 bp PCR fragment of the strain QDD1 using the primer ALKB1-F

Then, using Blast alignment software, the following figure shows the closed similar sequence (Figure 20).

```
Query  20   GGCTGCAGGCGGCATTCTGCTGGCCGCCGTGTGCTACGCGGGGTTCAAGGTCGAGCATGT  79
            ||||||  ||||||||  ||||||||||||||||||||||||||||||||||||||||||
Sbjct  41   GGCTGCCGGCGGCATCCTGCTGGCCGCCGTGTGCTACGCGGGGTTCAAGGTCGAGCATGT  100

Query  80   GCGCGGCCACCATGTGCATGTGTCTACGCCGGAGGACGCTTCGTCGGCGCGTTTCGGCCA  139
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  101  GCGCGGCCACCATGTGCATGTGTCTACGCCGGAGGACGCTTCGTCGGCGCGTTTCGGCCA  160

Query  140  GTCGGTCTACCAGTTCCTGCCGCATGCCTACAAGTACAACTTCCTCAACGCCTGGCGCCT  199
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161  GTCGGTCTACCAGTTCCTGCCGCATGCCTACAAGTACAACTTCCTCAACGCCTGGCGCCT  220

Query  200  TGAAGCGGTGCGGCTGCGCAAGAAGGGCCTGCCGGTGTTCGGCTGGCAGAACGAACTGAT  259
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  221  TGAAGCGGTGCGGCTGCGCAAGAAGGGCCTGCCGGTGTTCGGCTGGCAGAACGAACTGAT  280

Query  260  CTGGTGGTACCTGCTGAGCCTGGCGTTGCTGGTCGGTTTCGGTTGGGCGTTCGGCTGGCT  319
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  281  CTGGTGGTACCTGCTGAGCCTGGCGTTGCTGGTCGGTTTCGGTTGGGCGTTCGGCTGGCT  340

Query  320  GGGGATGGTTTTCTTCCTTGGCCAAGCGTTCGTCGCGGTGACCCTGCTGGAGATCATCAA  379
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  341  GGGGATGGTTTTCTTCCTTGGCCAAGCGTTCGTCGCGGTGACCCTGCTGGAGATCATCAA  400

Query  380  CTACGTCGAGCACTACGGCCTGCATCGGCGAAAGGGCGAGGACGGGCGCTACGAGCGGAC  439
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  401  CTACGTCGAGCACTACGGCCTGCATCGGCGAAAGGGCGAGGACGGGCGCTACGAGCGGAC  460

Query  440  CAACCATACCCACTCCTGGAACAGCAACTTCGTCTTCACCAACCTGGTCCTGTTCCATCT  499
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  461  CAACCATACCCACTCCTGGAACAGCAACTTCGTCTTCACCAACCTGGTCCTGTTCCATCT  520

Query  500  GCAACGCCACT  510
            ||||||  ||||
Sbjct  521  GCAACGGCACT  531
```

Figure 20: Blast alignment of the sequence of the 1149 bp PCR fragment of the strain QDD1 using the primer pair ALKB1-F

These results confirm the presence in the strain **QDD1** of *Pseudomonas aeruginosa* of the gene *alkB1* encoding alkane 1-monooxygenase

However, it was noticed that there are 3 mutations that were double checked and confirmed in the sequencing picks. These results show that this QDD1 gene has at least 3 mutations in its ORF sequence compared to the published ones.

5. *Exploration of Alkane hydroxylase alkB coding genes using the primer pair15*

Using the Primer pair 15 (ALK-BF. & ALK-BR) with an expected fragment size of 550 bp the 16 strains results are shown in Figure 23.

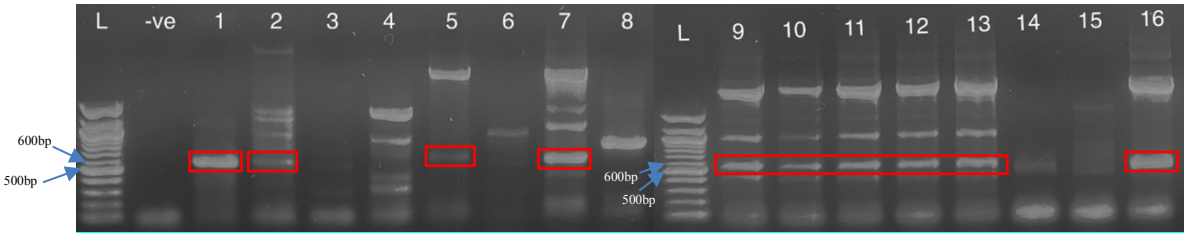

Figure 21: PCR fragment amplification using Primer pair 15: (ALK B-F. & ALK B-R)
Lanes; L, 100-bp DNA ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1517 bp); -Ve, negative control; 1, QDD1; 2, QBC3; 3,MSM10; 4, BG8; 5, Afnan5; 6, Afnan24; 7, Afnan27; 8, Afnan33; 9, 2.2.1; 10, 2.2.2; 11, 2.2.3; 12, 2.2.4; 13, 2.2.5; 14, 2.2.6; 15, 3.1.1; 16,3.1.2.

The PCR results showed amplification with expected size of 550 bp with QDD1, QBC3, Afnan5, Afnan27, 2.2.1, 2.2.2, 2.2.3, 2.2.4, 2.2.5, and 3.1.2. This results allow the prediction of the presence in these strains of the gene (*alkB*) that encodes the production of the enzyme Alkane hydroxylase responsible for the metabolism of alkanes (Canosa et al., 2000; Ruettinger et al., 1977; J. Shanklin et al., 1997; John Shanklin & Whittle, 2003).

6.1.2 Exploration of Naphthalene dihydrodiol dehydrogenase *nahB* coding genes using the primer pair 1

In order to predict the presence of the gene encoding the Cis- Naphthalene dihydrodiol dehydrogenase (*nahB*), the pair (B6-F. & B778-R) was used in PCR amplification (Figure 23).

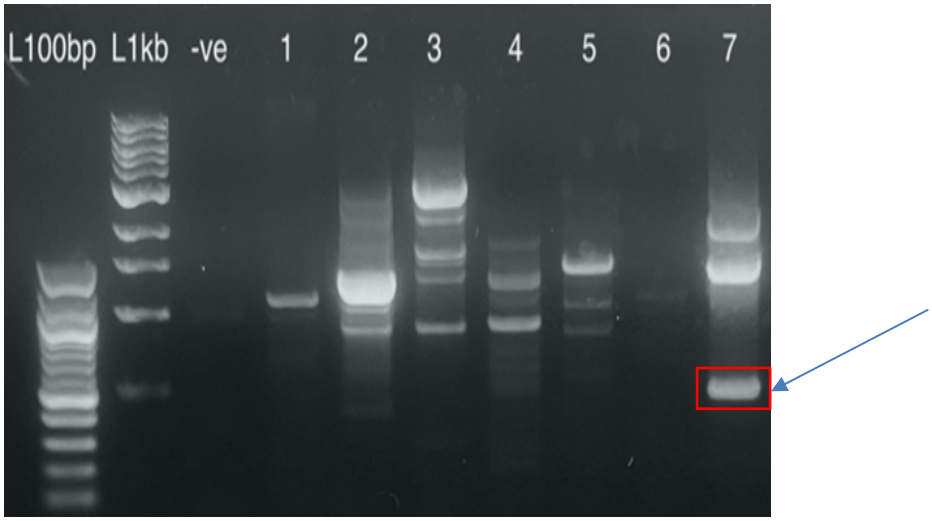

Figure 22: PCR fragment amplification using Primer pair 1: (B6-F. & B778-R). Lanes; L, 100-bp DNA ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1517 bp); , 1kb- DNA ladder (0.5 kb, 1kb, 1.5kb, 2kb, 3kb, 4kb, 5kb, 6kb, 8kb, 10-kb); -Ve, negative control; 1, QDD1; 1, QDD1; 2, QBC3; 3, MSM10; 4, BG8; 5, Afnan5; 6, Afnan24; 7, Afnan27.

As shown in figure 22, only the strain Afnan27 gave a strong amplification of a fragment of expected size of 700 bp. The other strains showed fragments of different sizes. This amplification obtained with the strain Afnan27 might allow to predict the presence of the gene *nahB* in the strain Afnan27.

The other amplified fragments might be explained by partial similarity with this gene (Denome et al., 1993).

6.1.3 Exploration of the 1,2-Dihydroxynaphthalene oxygenase *nah C* coding genes using the primer pair 2

In order to predict the presence of the gene encoding the (*nah C)*1,2-Dihydro xynaphthalene oxygenase, the pair (C118-F. & C814-R) was used for PCR amplification (Figure 24).

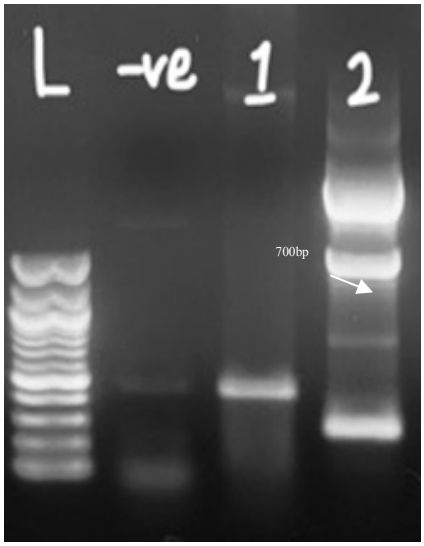

Figure 23: PCR fragments amplification using Primer pair 2: (C118-F. & C814-R), Lanes; L, 100-bp DNA ladder; -Ve, negative control; 1, QDD1; 2, QBC3

As shown in figure 23, only the strain QDD1 gave an amplification of a fragment of the expected size of 690 bp. The other strains showed fragments of different sizes (results not shown). This amplification might predict the presence of the gene *nah C* in the strain QDD1. This gene (*nah C)* encodes the synthesis of 1,2-Dihydroxynaphthalene oxygenase, which degrades naphthalene (Patel & Barnsley, 1980). In order to confirm the nature of the gene, this PCR fragment of was sequenced (Figure 26).

5'AGCTCTGGGTCAAAAGCTTATTGATGCCGGTTACAAGATCCGCATCTGCGACAAAGTTG
AGGCTCAGGAGCGTATGGTGTTGGGTCTGATGAAGACAGA AGATCCG GGC GGCAACCC
GACCGAGATATTCTGGGGCCCCCGGATCGACATGAGCAACCCGTTCCATCCCGGTCGCCC
CCTGCACGGAAAGTTTGTGACCGGTGACCAAGGCTTGGGCCATTGCATCGTTCGCCAAAC
CGACGTCGCAGA AGCTCATAAGTTTTATAGCCTGCTGGGCTTCCGTGGG GACGTCGCCG
GATTCCGTTGCCCAACGGCATGACT GCCG AACTGTCGTTCATGCATTGAACGCCCGT GAT
CACTCCATTGCGTTTGGTGCCATGCCCGCTGCCAAGCGA CTCAATCACT TGATGCTTGA5'
Figure 24: Sequence of the 690 bp PCR fragment of the strain QDD1 using the primer pair C118F

The obtained sequence was subjected to blast alignment search (Figure 25) that showed 100% similarity with the gene *nahC* encoding1,2-dihydroxynaphthalene dioxygenase of *Pseudomonas putida.*

Full lenghth
1,2-dihydroxynaphthalene dioxygenase
***Pseudomonas putida* plasmid NPL-41 nahC gene**
Sequence ID: Y14173.1Length: 2285Number of Matches: 1

```
Query  1     AGCTCTGGGTCAAAAGCTTATTGATGCCGGTTACAAGATCCGCATCTGCGACAAAGTTGA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1061  AGCTCTGGGTCAAAAGCTTATTGATGCCGGTTACAAGATCCGCATCTGCGACAAAGTTGA  1120

Query  61    GGCTCAGGAGCGTATGGTGTTGGGTCTGATGAAGACAGAAGATCCGGGCGGCAACCCGAC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1121  GGCTCAGGAGCGTATGGTGTTGGGTCTGATGAAGACAGAAGATCCGGGCGGCAACCCGAC  1180

Query  121   CGAGATATTCTGGGGCCCCCGGATCGACATGAGCAACCCGTTCCATCCCGGTCGCCCCCT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1181  CGAGATATTCTGGGGCCCCCGGATCGACATGAGCAACCCGTTCCATCCCGGTCGCCCCCT  1240

Query  181   GCACGGAAAGTTTGTGACCGGTGACCAAGGCTTGGGCCATTGCATCGTTCGCCAAACCGA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1241  GCACGGAAAGTTTGTGACCGGTGACCAAGGCTTGGGCCATTGCATCGTTCGCCAAACCGA  1300

Query  241   CGTCGCAGAAGCTCATAAGTTTTATAGCCTGCTGGGCTTCCGTGGGGACGTCGAATACCG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1301  CGTCGCAGAAGCTCATAAGTTTTATAGCCTGCTGGGCTTCCGTGGGGACGTCGAATACCG  1360

Query  301   GATTCCGTTGCCCAACGGCATGACTGCCGAACTGTCGTTCATGCATTGCAACGCCCGTGA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1361  GATTCCGTTGCCCAACGGCATGACTGCCGAACTGTCGTTCATGCATTGCAACGCCCGTGA  1420

Query  361   TCACTCCATTGCGTTTGGTGCCATGCCCGCTGCCAAGCGACTCAATCACTTGATGCTTGA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1421  TCACTCCATTGCGTTTGGTGCCATGCCCGCTGCCAAGCGACTCAATCACTTGATGCTTGA  1480
```

Figure 25: Blast alignment of the sequence of the 690 bp PCR fragment of the strain QDD1 using the primer pair C118F-C118R

These results confirm the presence in the strain QDD1 of *Pseudomonas* of the gene *nahC* encoding the 1,2-dihydroxynaphthalene dioxygenase of *Pseudomonas putida*.

6.1.4 Investigation of the presence of the gene *nah E* encoding the 2-Hydroxybenzalpyruvate aldolase

In order to predict the presence of the gene *nahE* encoding the 2-Hydroxybenzalpyruvate aldolase, the pair (E207-F. & E826-R) was used in PCR amplification (Figure 26).

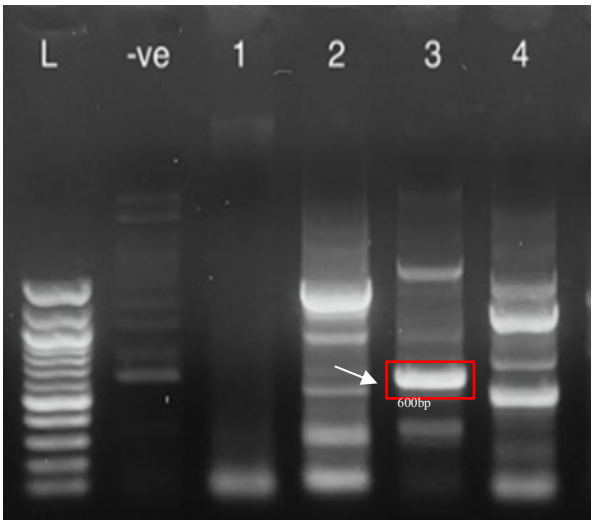

Figure 26: PCR fragments amplification using Primer pair 3: (E207-F. & E826-R), Lanes; L, 100-bp DNA ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1517 bp); 1, QDD1; 2, QBC3; 3, MSM10; 4, BG8.

As shown in figure 26, only the strain MSM10 (lane 3) gave an amplification of a fragment of expected size of 620 bp. All the other strains gave fragments of different sizes or did not give amplification. The fragment was sequenced and did not show similarity with any gene encoding this enzyme.

6.1.5 Investigation of the presence of the gene *tbmD* encoding the Toluene benzene monooxygenase

No amplification was found from the DNA templates for any of the tested strains Figure 27. This primer pair 7 (TBMD-F. & TBMD-R) is corresponding to the gene (*tbmD)* which is responsible for the production of the enzyme Toluene benzene monooxygenase *large subunit* that degrades toluene and benzene (Johnson & Olsen, 1995). The results demonstrated above show that none of the tested strains contain the target gene.

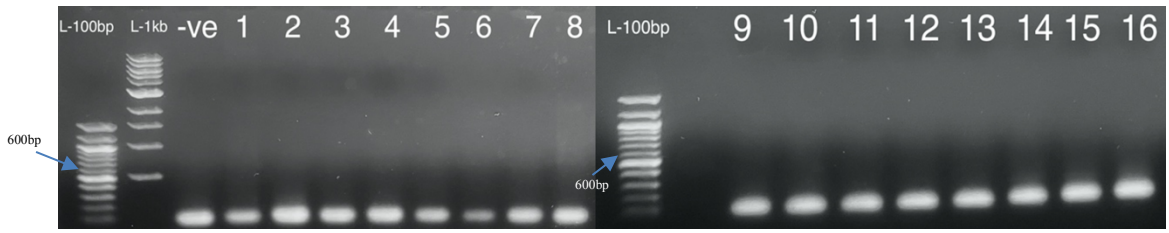

Figure 27: PCR fragment amplification using Primer pair 7: (TBMD-F. & TBMD-R). Lanes; L, 100-bp DNA ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1517 bp); , 1kb- DNA ladder (0.5 kb, 1kb, 1.5kb, 2kb, 3kb, 4kb, 5kb, 6kb, 8kb, 10-kb); -Ve, negative control; 1, QDD1; 1, QDD1; 2, QBC3; 3, MSM10; 4, BG8; 5, Afnan5; 6, Afnan24; 7, Afnan27. 8, Afnan33; 9, 2.2.1; 10, 2.2.2; 11, 2.2.3; 12, 2.2.4; 13, 2.2.5; 14, 2.2.6; 15, 3.1.1; 16, 3.1.2.

6.1.6 Investigation of the presence of the gene *tmoA* encoding the Toluene monooxygenase large α subunit

PCR amplification using Primer pair 10 (TMOA-F & TMOA-R) with expected fragment size 505 bp showed positive amplification results with the strains Afnan33, 2.2.6, and 3.1.1 (Figure 28). This demonstrates that these strains have the gene (*tmoA*) that encodes the Toluene monooxygenase large α subunit. This enzyme is responsible of the toluene degradation (Acheson et al., 2014, 2017; L. J. Bailey et al., 2008; Lucas J. Bailey et al., 2012; Lucas J. Bailey & Fox, 2009; Elsen et al., 2009; Tao et al., 2004; Whited & Gibson, 1991; Yen et al., 1991).

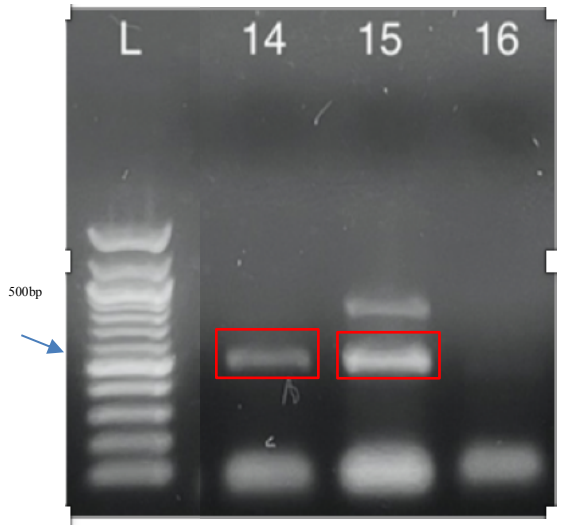

Figure 28: PCR fragment amplification using Primer pair 10: (TMOA-F& TMOA-R). Lanes; L, 100-bp DNA ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1517 bp); 16, -Ve, Negative control; 1, 14, 2.2.6; 15, 3.1.1.

6.1.7 Investigation of the presence of the gene todC1 encoding the Aromatic dioxygenases

When using the pair of primers (TOD C1-F & TOD C1-R), no amplification was evidenced with the DNA templates of any of the tested strains. This primer pair 11 (TOD C1-F & TOD C1-R) is corresponding to the gene (todC1) that is responsible of the synthesis of the enzyme Aromatic dioxygenases (TOD pathway) large subunit (Mosqueda & Ramos, 2000). These results show that none of the tested strains harbor the target gene.

6.1.8 Investigation of the presence of the genes encoding the Xylene monoxygenases

The target gene is (*xylA*) encoding Xylene monoxygenases (Shaw & Harayama, 1992; Suzuki et al., 1991). When using the pair 13 of primers (XYL A-F & XYL A-R), no amplification was evidenced with the DNA templates of any of the tested strains.

These results show these primers did not allow to evidence this gene in all the tested strains.

Targeting the same gene, another pair#14 of primers (XYL E1-F. & XYL E1-R.) was used with expected size of 242 bp showed positive results (Figure 29) with 2.2.1, 2.2.2, 2.2.3 2.2.4, and 2.1.5. These results allow to predict that these strains harbor the gene (*xylA*) encoding Xylene monoxygenases that is responsible of the degradation of xylene.

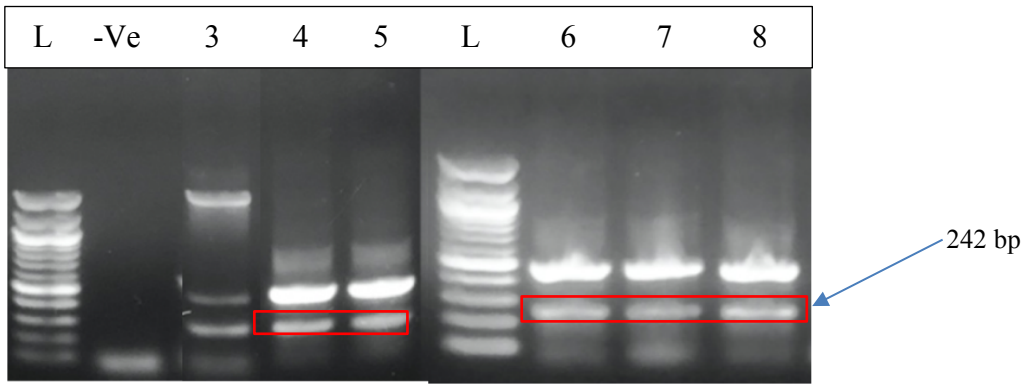

Figure 29: PCR fragment amplification using Primer pair 14: (XYL E1-F. & XYL E1-R). Lanes; L, 100-bp DNA ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1517 bp); -Ve, negative control; 3, MSM10; 4, 2.2.1; 5, 2.2.2; 6, 2.2.3; 7, 2.2.4; 8, 2.2.5.

In order to confirm the nature of the gene, this PCR fragment was sequenced (Figure 30).

5'GGCAAAATTGGACTGATGGG GGCGGTATAGTAAACGCTGTCAATGGGTCTGGCGGGAA T TACAGTGTTAATTGGTCTAATACCGGAAATT TTGTTGTTGGTAAAGGTTGGACTACAGG TTCGCCATTT3'

Figure 30: Sequence of the 242 bp PCR fragment of the strain 2.2.2 of *B. subtilis* using the primer pair XYLE1

The obtained sequence was subjected to blast alignment search (Figure 31) that showed 100% similarity with the gene *akk1* xylanase of *Bacillus subtilis*. KJ540928.1

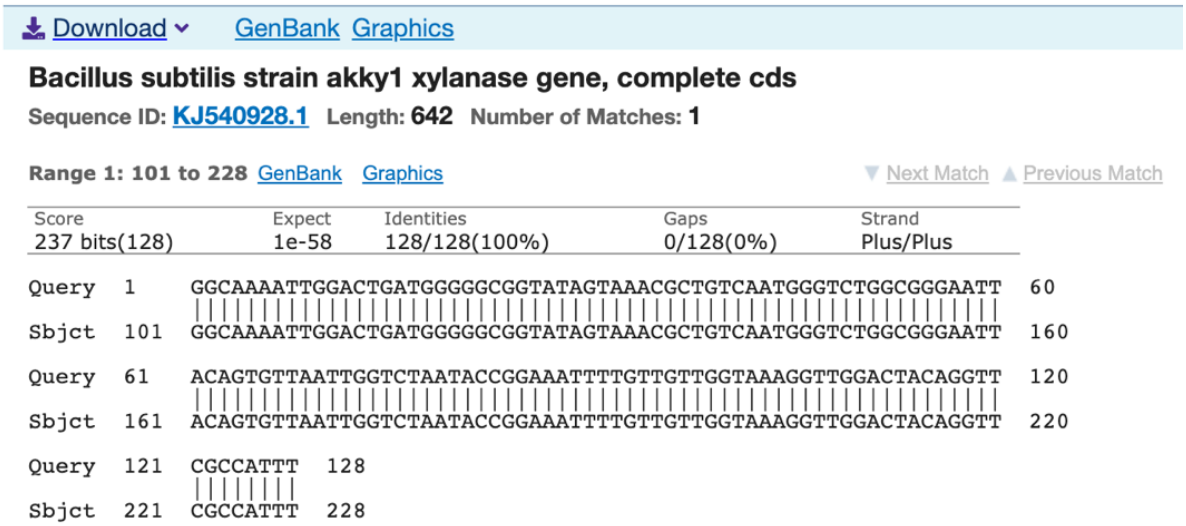

Figure 31: Blast alignment of the sequence of the 242 bp PCR fragment of the strain 2.2.2 of *B. subtilis* using the primer XYLE1-F

These results confirm the presence in the strain 2.2.2 of *B. subtilis* of the gene *akk1* encoding the xylanase of *Bacillus subtilis.*

Discussion and conclusion

In this chapter, the hydrocarbon degrading enzyme coding gene content was explored among the 16 selected strains that are able to grow on diesel as sole carbon source. The adopted method, based on PCR amplification using conserved primers belonging to key genes encoding these enzymes, was successful for several strains but not for all explored ones. The sequencing of several PCR fragments, allowed to confirm the specific amplification of the majority of the fragments and the determination of the nature of the corresponding genes and enzymes and the degree of similarities.

The gene content of the explored strain is presented in Table 15 where one can see all genes and corresponding enzymes synthesized by the explored strains. These results demonstrate that the explored strains in this study harbor very important genes that encode key enzymes of hydrocarbon catabolism. Seven main genes were identified

in the explored strains encoding the haloalkane dehalogenase, hydrocarbon binding protein, alkane 1-monooxygenase, alkane hydroxylase, naphthalene dihydrodiol dehydrogenase, 1,2-Dihydroxynaphthalene dioxygenase, toluene monooxygenase large α subunit and xylanase.

Table 15: Hydrocarbon degrading enzyme coding genes content of the strains explored in this study

Number	Primers / Strain	ALK-1F. & ALK-1R	ALK-2F. & ALK-2R	ALK 3-F& ALK 3-R	ALK-B1F. & ALK-B1R	ALK-BF. & ALK-BR	B6-F. & B778-R	C118-F. & C814-R	TMOA-F & TMOA-R	XYL E1-F. & XYL E1-R
1	QDD1	-	-	*hbp*	*alk-B1*	*alkB*	-	*nahC*	-	-
2	QBC3	-	-	-	-	*alkB*	-	-	-	-
3	QMSM3	-	-	-	-	-	-	-	-	-
4	BG8	-	-	-	-	-	-	-	-	-
5	Afnan 5	-	-	-	-	*alkB*	-	-	-	-
6	Afnan 24	-	-	-	-	-	-	-	-	-
7	Afnan 27	-	-	-	-	*alkB*	*nahB*	-	-	-
8	Afnan 33	-	-	-	-	-	-	-	-	-
9	2.2.1	*had*	-	-	-	*alkB*	-	-	-	*akk1*
10	2.2.2	*had*	-	-	-	*alkB*	-	-	-	*akk1*
11	2.2.3	*had*	-	-	-	*alkB*	-	-	-	*akk1*
12	2.2.4	*had*	-	-	-	*alkB*	-	-	-	*akk1*
13	2.2.5	*had*	-	-	-	*alkB*	-	-	-	*akk1*
14	2.2.6	-	-	-	-	-	-	-	*tmo*	-
15	3.1.1	-	-	-	-	-	-	-	*tmo*	-
16	3.1.2	-	-	-	-	*alkB*	-	-	-	-

Abbreviation:
Genes and encoded enzymes:
[*had*]: Haloalkane dehalogenase
[*hbp*]: Hydrocarbon binding protein
[*alk-B1*]: Alkane 1-monooxygenase
[*alkB*]: Alkane hydroxylase
[*nahB*]: Naphthalene dihydrodiol dehydrogenase
[*nahC*]: 1,2-Dihydroxynaphthalene dioxygenase
[*tmo*]: Toluene monooxygenase large α subunit
[*akk1*]: Xylanase of *B. subtilis*

The obtained results confirm as well the results of the previous chapter in term of correlation between gene content and degradation potentials. In fact, these results corroborate with those of the previous chapter where we demonstrate that the strain AF33, 2.6 and 3.11 having the gene encoding Toluene monooxygenase for the degradation of Toluene, degrade toluene and on the other hand strains 2.1. 2.2. 2.3, 2.4 and 2.5 harboring xylanase coding gene responsible of xylene degradation, do degrade xylene since using it as sole carbon source.

These strains and most importantly the gene bank that are identified have a very important value since can be investigated further to improve their expression, but they can be expressed in other hydrocarbon degrading strain to improve their degradative potentials. Moreover, these genes can be transferred to the same strain to obtain a super powerful strain for the same application in bioremediation.

CHAPTER 7: CONCLUSION AND FUTURE PERSPECTIVES

In this MSc thesis, the hydrocarbon degradation potentialities of several locally isolated strains was investigated. The candidate strains QDD1, QBC3, QMSM10, BG8, Afnan5, Afnan24, Afnan27, Afnan33, 2.2.1, 2.2.2, 2.2.3, 2.2.4, 2.2.5, 2.2.6, 3.1.1, and 3.1.2 were isolated from Qatari hydrocarbon contaminated soil and their hydrocarbon degradation potentialities were explored against diesel, toluene, and xylene side by side with molecular investigation of the presence of key genes encoding important pathways enzymes of degradation of hydrocarbons.

MALDI-TOF MS analysis identified these isolated strains. The 16 bacterial strains were identified as *Bacillus subtilis, Bacillus circulans, Burkholderia cepacia, Pseudomonas aeruginosa, Micrococcus luteus, Raoultella ornithinolytica* and *Staphylococcus capitis*. *Bacillus subtilis* was found to be a common strain isolated from the hydrocarbon contaminated site and it dominates the bacterial community. These finding were encouraging for further investigation; thus, protein profiling analysis was carried, and it revealed that they have unique protein profiles, and it was proven through hierarchical relationships that the isolated strains were closely related but not identical which was important for the molecular investigation of the genes encoding the hydrocarbon degrading enzymes.

The degradation potentialities of the isolates against diesel was conducted and the results revealed that the bacterial isolates were successful hydrocarbon degraders and able to reach very high biomasses by using diesel as sole carbon source. A consortium of 16 strains could reach the highest biomass in MSM diesel medium proving the synergistic effects among the strains allowing them to cooperate in diesel degradation. Moreover, we demonstrated that the 3 weeks-diesel Removal Efficiency (RE) of a 5% diesel supplemented MSM medium differs from one strain to another.

Some strains showed very high efficiency in degradation such as QBC3 (*Burkholderia cepacia*), QMSM10, and BG8 that degraded high molecular weight (HMW) hydrocarbons with 52.5%, 48.4%, and 54.9%, respectively.

Moreover, in addition to the previously explored potentialities against diesel, the exploration of the degradation potentialities against other aromatic hydrocarbons revealed the ability of many of the tested strains to degrade toluene and xylene.

These obtained findings demonstrate that all of the bacterial isolates have hydrocarbon catabolism potentialities. This conclusion was confirmed by the evidence of the key genes encoding the enzymes responsible of the degradation of these hydrocarbons. Seven main genes were identified and sequenced, in the explored strains, encoding haloalkane dehalogenase, hydrocarbon binding protein, alkane 1-monooxygenase, alkane hydroxylase, naphthalene dihydrodiol dehydrogenase, 1,2-Dihydroxynaphthalene dioxygenase, toluene monooxygenase large α subunit and xylanase

The obtained results demonstrate that the explored strains in this study harbor very important genes that encode key enzymes of hydrocarbon catabolism. These strains and most importantly the gene bank that are identified have a very important value since can be investigated further to improve their expression but also, they can be expressed in other hydrocarbon degrading strain to improve their degradative potentials. Moreover, these genes can be transferred to a same strain to obtain a super powerful strain for the same application in bioremediation.

Additional research should focus on the enhancement of the expression of these genes in order to create improved hydrocarbon degrading strains to be the environmentally-friendly solution in bioremediation, competing with chemical and physical methods and achieve highest remediation efficiencies.

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APPENDIX A: MALDI-TOF MS Specie Identification Analysis

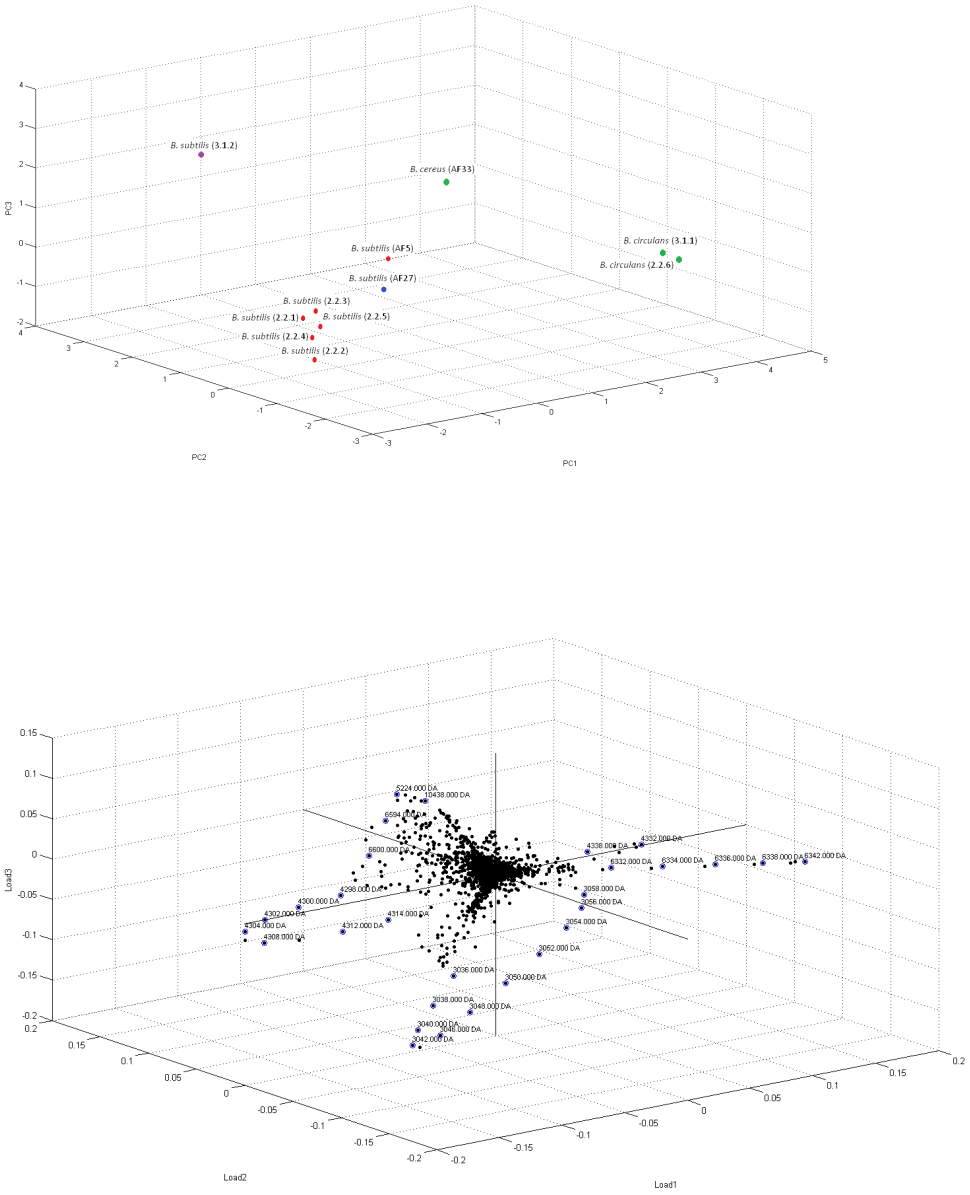


Figure 32: PCA results for all *Bacillus* strains

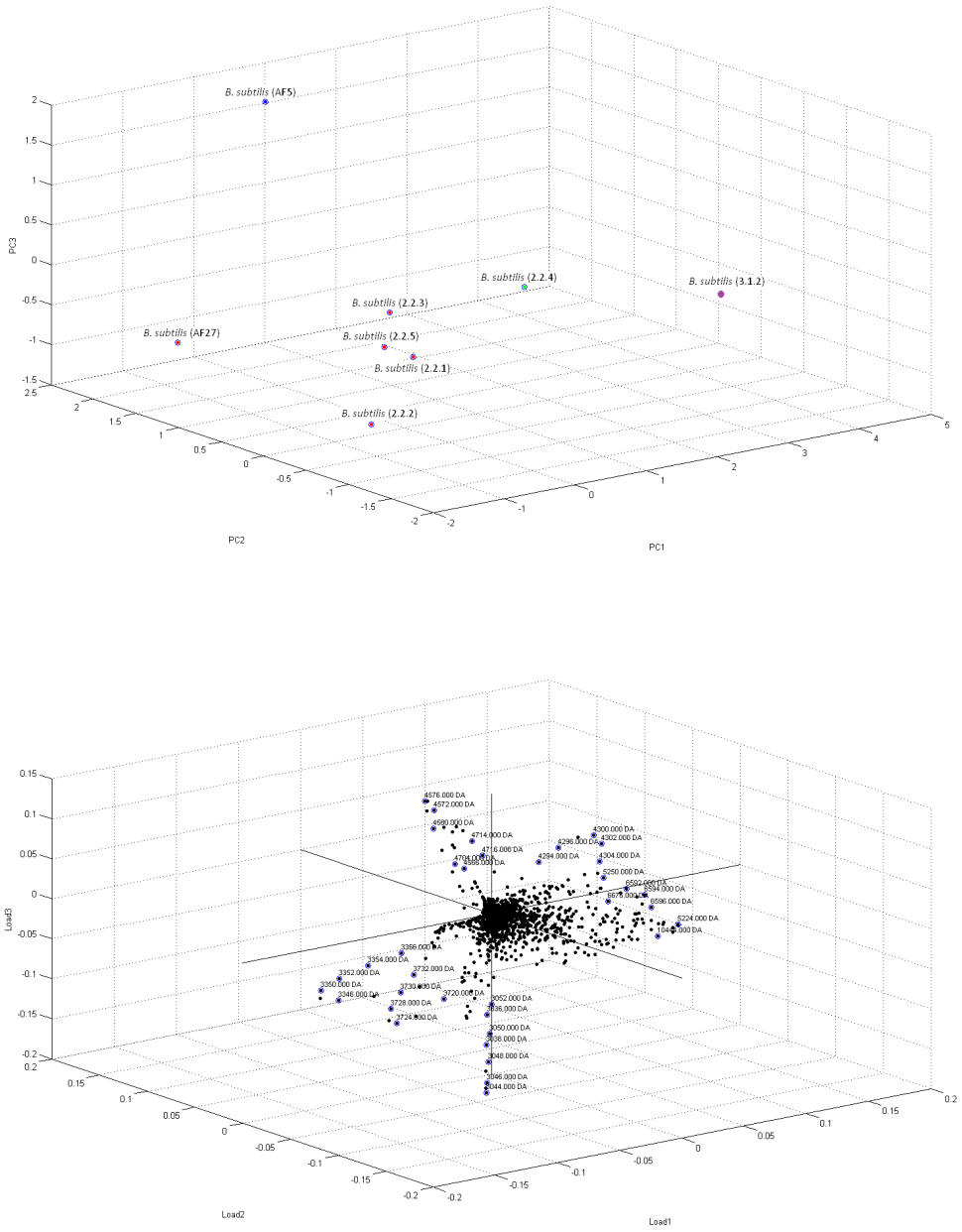
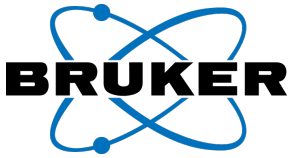


Figure 33: PCA results for only *Bacillus subtilis* strain

Bruker MALDI Biotyper Identification Results



Run Info:

Run Identifier:	201102-1605-001013760
Comment:	
Operator:	Admin@FLEX-PC
Run Creation Date/Time:	2020-11-02T16:13:21.235
Number of Tests:	34
Type:	Standard
BTS-QC:	not present
BTS-QC Position:	
Instrument ID:	269944.00397
Server Version:	4.1.80 (PYTH) 102 2017-08-226_04-55-52

Result Overview

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
A1 (+) (B)	Haneen (Standard)	Bacillus subtilis	1.76	**No Organism Identification Possible**	1.68
A2 (-) (B)	Haneen (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
A3 (+) (A)	2.2.1 (Standard)	Bacillus subtilis	1.97	Bacillus subtilis	1.96
A4 (+++) (A)	2.2.1 (Standard)	Bacillus subtilis	2.19	Bacillus subtilis	2.16
A5 (+++) (A)	2.2.2 (Standard)	Bacillus subtilis	2.12	Bacillus subtilis	2.12
A6 (+) (A)	2.2.2 (Standard)	Bacillus subtilis	1.97	Bacillus subtilis	1.96
A7 (+++) (A)	2.2.3 (Standard)	Bacillus subtilis	2.21	Bacillus subtilis	2.18

Result overview table--continued on next page

Result overview table--continued from previous page

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
A8 (+++) (A)	2.2.3 (Standard)	Bacillus subtilis	2.19	Bacillus subtilis	2.15
B1 (+) (A)	2.2.6 (Standard)	Bacillus circulans	1.76	**No Organism Identification Possible**	1.37
B2 (+++) (A)	2.2.6 (Standard)	Bacillus circulans	2.00	**No Organism Identification Possible**	1.34
B3 (+++) (A)	2.2.5 (Standard)	Bacillus subtilis	2.17	Bacillus subtilis	2.14
B4 (-) (A)	2.2.5 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
B5 (+++) (A)	2.2.4 (Standard)	Bacillus subtilis	2.11	Bacillus subtilis	2.08
B6 (-) (A)	2.2.4 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
B7 (+) (B)	3.1.1 (Standard)	Bacillus circulans	1.96	**No Organism Identification Possible**	1.49
B8 (+) (B)	3.1.1 (Standard)	Bacillus circulans	1.82	**No Organism Identification Possible**	1.36
C1 (+++) (A)	3.1.2 (Standard)	Bacillus subtilis	2.11	Bacillus subtilis	2.10
C2 (+) (A)	3.1.2 (Standard)	Bacillus subtilis	1.95	Bacillus subtilis	1.88
C3 (+) (B)	AF5 (Standard)	Bacillus subtilis	1.91	Bacillus subtilis	1.81
C4 (-) (B)	AF5 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
C5 (-) (C)	AF24 (Standard)	**No Organism Identification Possible**	1.57	**No Organism Identification Possible**	1.55
C6 (-) (C)	AF24 (Standard)	**No Organism Identification Possible**	1.67	**No Organism Identification Possible**	1.60
C7 (+++) (A)	AF27 (Standard)	Bacillus subtilis	2.15	Bacillus subtilis	2.12

Result overview table--continued on next page

Result overview table--continued from previous page					
Sample Name	**Sample ID**	**Organism (best match)**	**Score Value**	**Organism (second-best match)**	**Score Value**
C8 (+) (A)	AF27 (Standard)	Bacillus subtilis	1.99	Bacillus subtilis	1.85
D1 (-) (B)	AF33 (Standard)	**No Organism Identification Possible**	1.40	**No Organism Identification Possible**	1.29
D2 (+) (B)	AF33 (Standard)	Bacillus cereus	1.84	Bacillus cereus	1.77
D3 (+++) (A)	QDD1 (Standard)	Pseudomonas aeruginosa	2.33	Pseudomonas aeruginosa	2.12
D4 (+++) (A)	QDD1 (Standard)	Pseudomonas aeruginosa	2.32	Pseudomonas aeruginosa	2.22
D5 (+) (B)	BG8 (Standard)	Micrococcus luteus	1.98	Micrococcus luteus	1.96
D6 (+) (B)	BG8 (Standard)	Micrococcus luteus	1.95	Micrococcus luteus	1.88
D7 (+++) (A)	QBC3 (Standard)	Burkholderia cepacia	2.40	Burkholderia cepacia	2.13
D8 (+++) (A)	QBC3 (Standard)	Burkholderia cepacia	2.44	Burkholderia cepacia	2.21
E1 (+++) (A)	MSM10 (Standard)	Staphylococcus capitis	2.20	Staphylococcus capitis	2.20
E2 (+++) (A)	MSM10 (Standard)	Staphylococcus capitis	2.06	Staphylococcus capitis	1.95

APPENDIX B: GC-FID MS Raw Data

Table 16: GC-FID MS Raw data analysis

	Low	Medium	High	Low-CT	Medium-CT	High-CT
CT	918760	151260	2840	0	0	0
QBC3	483587	65201	1348	-435173.31	-86058.7	-1492.08
MSM10	656004	79099	1466	-262756.89	-72160.76	-1374.4
BG8	689904	85339	1282	-228856.12	-65920.44	-1558.81
AF5	730411	93027	1558	-188349.3	-58232.71	-1282.47
AF24	806413	98799	1594	-112347.17	-52461.25	-1246.01
AF27	784093	86416	1622	-134667.43	-64843.98	-1218.51
AF33	759470	99151	1733	-159290.38	-52109.28	-1107.16
2.2.1	650800	106997	1646	-267960.01	-44262.66	-1194.4
2.2.2	782037	108354	1785	-136723.32	-42905.8	-1054.98
2.2.3	866139	120511	1944	-52621.71	-30748.57	-896.78
2.2.4	728074	117536	2031	-190686.54	-33723.9	-809.44
2.2.5	707930	114100	1882	-210830.97	-37159.39	-958.66
2.2.6	792056	103660	2150	-126704.76	-47599.43	-690.69
3.1.1	865500	118482	1909	-53260.71	-32777.65	-930.91
3.1.2	898096	116976	2479	-20664.21	-34283.57	-361.57