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INVESTIGATION OF THE NUTRITIONAL REQUIREMENTS AND CORRESPONDING CODING GENES OF HYDROCARBON-DEGRADING BACTERIAL STRAINS FOR THE PRODUCTION OF BIOMASS USEFUL IN BIOREMEDIATION OF PETROLEUM POLLUTION

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THESIS APPROVAL

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الملخص

يعد التلوث الناجم عن النفط ومشتقاته أحد أهم مصادر التدهور البيئي في الدول الصناعية. وتصنف المركبات الهيدروكربونية على أنها ملوثات عضوية سامة ومسرطنة وتقاوم التحلل. تهدف جهود المعالجة البيولوجية إلى حصر وتخفيف درجة التلوث؛ سعياً لمنع المزيد من الأضرار البيئية وحماية كل أشكال الحياة من التعرض للمواد الضارة. يهدف هذا المشروع البحثي إلى دراسة دور مكونات الوسط المستخدم لزراعة البكتيريا في النمو والنشاط البيولوجي لثلاث عينات بكتيرية قادرة على تحليل المواد الهيدروكربونية عزلت محلياً من سلالة *Pseudomonas aeruginosa*. بالإضافة إلى إجراء دراسة تفصيلية حول دور مكونات البيئة الغذائية للخلايا البكتيرية من خلال التحكم بثلاث عوامل: الكربون، النيتروجين، والفوسفور. كما يهدف المشروع للقيام بمسح جيني بحثاً عن الجينات المسؤولة عن تحليل المواد الهيدروكربونية بين العينات الخاضعة للدراسة.

كشفت نتائج البحث بوضوح الدور الهام للعناصر الغذائية مثل الكربون، والنيتروجين، والفوسفور وتأثيرها على نمو الخلايا البكتيرية وعلى عمليات هدم وتحليل المركبات الهيدروكربونية. بعض العينات أظهرت قدرة على تحمل تراكيز عالية من الديزل (المصدر الوحيد لعنصر الكربون) عند زراعتها في وسط دون آخر. لوحظ انخفاض النمو البكتيري في الأسبوع الثاني من الزراعة عن استخدام تراكيز عالية من الديزل. بينما كان تأثير النيتروجين إيجابياً وهاماً على نمو الخلايا، في حين بدا أن الفوسفور يؤثر بشكل أقل. وعلى الرغم من كون العينات البكتيرية الثلاث تنتمي لنفس السلالة التصنيفية، إلا أنها تجاوبت بشكل مختلف مع ظروف النمو المستخدمة. وجدنا أن بعض العينات أكثر حساسية لتغير نسب العوامل الغذائية (الكربون: النيتروجين: الفوسفور) من البعض الآخر. كما أثبت تحليل طبقة الديزل باستخدام تقنية Gas Chromatography قدرة العينات البكتيرية الخاضعة للدراسة على تحليل المواد الهيدروكربونية في مختلف الأوساط الغذائية. من جهة أخرى، تبين من الكشف الجزيئي أن العينة QDD1 تمتلك الجين *alkB1* المسؤول عن إنتاج إنزيم alkane hydroxylase والذي يساهم في هدم المركبات الألكينية. وقد لوحظ اختلاف المكونات الجينية لهذه العينة بالإضافة لاختلاف سلوكها البيولوجي وحساسيتها للبيئة الغذائية المحيطة.

تعرف منطقة الخليج العربي في السجلات العلمية كإحدى أكثر المناطق تلوثاً بالنفط ومشتقاته حول العالم. بالعودة إلى التاريخ، نجد أن منطقة الخليج قد تعرضت لأحد أكبر أحداث التسرب النفطي وأشدها تدميراً على الصعيد البيئي. أضف إلى ذلك الظروف الطبيعية الصعبة التي تتعرض لها المنطقة والضغوطات البشرية الهائلة التي تشهدها. كل ذلك يجعل النظام البيئي في منطقة الخليج هشاً ومعرضاً لمزيد من التدهور. هذا المشروع البحثي يحاول استخدام تكنولوجيا " المعالجة الميكروبية " من أجل إيجاد حلول للتلوث الناجم عن صناعتي الغاز والنفط في المنطقة. وبالتوافق مع التنمية البيئية المستدامة لدولة قطر، يعد هذا البحث الأول من نوعه بحيث يدرس ميكروبات البيئة المحلية ومدى أهليتها لتحليل الملوثات البترولية.

Abstract

Petroleum-derived contamination events constitute one of the most dominant sources of environmental deterioration in the industrialized countries. Hydrocarbon compounds are recognized as toxic and carcinogenic organic pollutants and environmentally persistent. Bioremediation efforts aim to confine, restrain and mitigate the magnitude of contamination, in order to prevent additional decline of the environment and to protect all life forms from exposure to hazardous materials. The aim of this project is to study the role of medium components on the growth and biological activity of three locally isolated hydrocarbon degrading *Pseudomonas aeruginosa* strains. Furthermore, a factorial investigation of the strains' potentiality to degrade diesel hydrocarbons by manipulating the nutritional microenvironment has been conducted. Then, stains are screened for genes encoding for hydrocarbon degradation activity.

Results clearly showed the major role of nutritional elements such as carbon (supplied solely as diesel), nitrogen, and phosphorous play on the growth and metabolism of hydrocarbon degrading strains. Some strains were found tolerant to higher diesel concentrations under certain growth condition, but not in another. Inhibition by elevated carbon (diesel) concentration by the second week of culturing was noted. Nitrogen was significantly affecting cells growth under all experimental conditions and to a lesser extent was phosphorous. The strains' response to altered

growth conditions varied substantially; although they were from the same taxonomical group. Some strains were found more sensitive to changing C/N/P ratios than others. The analysis of diesel removal by GC confirmed the capability of our strains to degrade hydrocarbons under different growth conditions. Molecular investigation of our strains demonstrated that one *Pseudomonas aeruginosa* strain (QDD1) possessed the *alkB1* gene encoding for alkane hydroxylase enzyme participating in the catabolic pathway of n-alkanes. The other strains were found to lack this particular gene. The genetic makeup of strain QDD1 was distinguished to be different from other strains as well as its biodegradation potential and its sensitivity to nutritional manipulations.

The Arabian Gulf including Qatar is recognized as one of the most oil contaminated areas worldwide. With the experience of the worlds' largest and most devastating oil incidents in history and with the natural and anthropogenic stresses; the Gulf environment is highly vulnerable and susceptible to further deterioration. This research project attempts to use the technology of microbial bioremediation in order to solve the environmental pollution resulting from the oil and gas industry. It will be the best local environmental-friendly solution and the most favorable for maintaining Qatar's environment sustainability. To the best of our knowledge, the work of this project is the first in the state, which investigates the naturally existing microbes in remediating petroleum pollution. Qatar's extreme weather conditions and the special characteristics of local soils make this project of interest to implement local microbes in local polluted areas.

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1. Introduction

Through decades, petroleum and its derivatives have been intensively utilized. Oil exploration, transportation, refining and usage have cost the environment expensive prices. The Arabian Gulf countries possess one of the largest worldwide oil and gas reservoirs. Qatar peninsula is placed along the western coast of the Arabian Gulf. The Gulf waters are considered exceptional for their enclosed nature. Several natural environmental stresses have been addressed impacting the quality of marine and coastal life forms of the Gulf. The shallow Gulf waters suffer naturally from low hydrodynamic energy, high salinities, low exchange rates, high temperatures, and high evaporation rates (Khan and Al-Ajmi, 1998; Sheppard *et al.,* 2010). Such peculiar environmental status promotes pollutants trapping and particulates deposition. In addition, the Gulf's history of oil liberation disastrous events and the continuous intensive anthropogenic activities make research attempts for remediation solutions an urgent need.

Bioremediation technology offers environmental friendly alternative where pollutants are being removed from contaminated sites by biological means, particularly microorganisms. Bioremediation is grapping scientists' attentions due to its promising approach of treating nature with natural tools and exploiting the processes that are occurring naturally. Indeed, microorganisms are being isolated from petroleum-polluted environments for their potentials to degrade hydrocarbons.

Qatar and the Gulf region are distinguished by an extreme weather and special soil characteristics. Microbial communities may provide under stress conditions, very special and interesting biological degradation routes, due to their adaptations to extreme temperatures, desiccation and salinity. These specific potentialities of the local microbial strains would be beneficial for the implementation of the remediation of the polluted areas using biotechnological tools, which are bioprocesses, based on the use of microorganisms, i.e. balanced microecosystems consisting of very different kinds of species. In fact, several microorganisms are reported as good candidates for bioremediation, but their implementation at the Qatari conditions may not be possible with the highest efficiency.

The research team in our lab has successfully isolated, purified, and conserved a database of hydrocarbon degrading strains collected from highly contaminated sites all over the state of Qatar. A well-designed screening program has been adapted in order to isolate the best growing bacterial strains with highest biological activity and tolerance to hydrocarbon toxicity. Interestingly, isolates have exhibited high dynamicity in growth as well as biodegradative activity upon the exposure to various culturing media. It was evidenced that the composition and the concentration of nitrogen sources affected a lot the biological activities of the isolates towards low or high molecular weight hydrocarbons. A shifting of the activities is easily achieved by the composition of the medium (Al Disi *et al*, 2015).

Here comes the motive behind the present research work. It was of great interest to us to study the role of the medium components on the growth and the degradation of diesel hydrocarbons using three *Pseudomonas aeruginosa* strains reported to be most effective among our laboratory culture collection. The second objective was to investigate the role of carbon, nitrogen, and phosphorous sources and their interactions on the biological activity of each isolate. A three-factor experimental design was adapted for this purpose. From another perspective, the molecular investigation of genes encoding hydrocarbon degrading activity formulated our third aim in this study.

The present research effort is, to our knowledge, the first in the state to screen and investigate the nutritional microenvironment of locally isolated bacterial strains as candidates to bioremediation implementation in Qatar.

2. Literature Review

2.1 Petroleum Pollution: a critical environmental issue

Since the beginning of petroleum industry, the demands for petroleum or its derivative products are increasing worldwide. Early after the dawn of the 20th century, petroleum has been distinguished as a potential environmental hazard (Albers, 2007). Petroleum-derived contamination events constitute one of the most dominant sources of environmental deterioration in the industrialized countries. Assessments of oil compounds have found them toxic to various living beings including humans plus environmentally persistent. Hydrocarbon compounds are recognized as carcinogens and neurotoxic organic pollutants (Das and Chandran, 2011).

2.1.1 Petroleum Components

Petroleum is a naturally forming substance that results from decaying living material, which has been covered by a succession of layers over time. Due to pressure and heat action, these organic residues are converted into oil (petroleum). Then, the formulated petroleum either travels upward to the earth surface, or gets trapped in reservoirs (Kingston, 2002). Petroleum is a complex mixture of numerous hydrocarbons and non-hydrocarbons where the latter is less abundant (Wilson and LeBlanc, 2000). Hydrocarbons can be divided into four classes: (1) saturates, (2) aromatics, (3) asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and (4) resins

(pyridines, quinolines, carbazoles, sulfoxides, and amides). Whilst non-hydrocarbons are comprised of: (1) sulfurs, (2) nitrogens, (3) oxygens, and (4) metallics (Leahy and Colwell, 1990).

2.1.2 Causes of Petroleum Pollution

Environmental contamination with petroleum occurs naturally through seepage in the ocean floor, or anthropogenically during extraction, refining or processing, distribution, and consumption. Although, anthropogenic releases of petroleum products into the environment are of particular concern, global estimates propose that natural seepage accounts for about 47% of crude oil currently entering the marine environment (Kvenvolden and Cooper, 2003). Nonetheless, environmental effects of the natural seeping phenomenon seem to be limited because of the slow but fixed rate of liberation that allows adaptation of the surrounding ecosystems (Burgherr, 2006). Catastrophic oil spills are probably the most visible source of petroleum-derived pollution. Accidents may occur as a result of transportation of crude oil among countries either via ocean tankers or land pipelines. Extraction well blowouts or storage facility explosions may also cause oil to spill (Burgherr *et al.,* 2004). Other causes of petroleum pollution could be due to leakages of underground fuel tanks, improper disposal of wastes and other oil handling operations (Gray, 2004).

2.1.3 Fate of Petroleum Pollution in the Environment

When crude petroleum is introduced into the environment, several physicochemical and biological processes tend to act on and alter the released compounds including evaporation, dissolution, photochemical and biological degradation (Wang *et al.,* 1999). As oil spreads on the sea, a few millimeters slick layer forms all over the surface. With the wind and current influence; light, volatile oil components typically evaporate adding to air pollution. Another portion of oil constituents is subjected to photolysis by UV light, in which compounds that are more toxic may result. Moreover, winds, waves, currents and turbulence at the sea surface facilitate the distribution and fragmentation of the slick layer and further promote processes as dissolution, biodegradation, and sedimentation (Kingston, 2002). It is also possible that oil compounds sink to the seabed either due to high molecular weight or due to incorporation with other particulates like clay or sand (Wilson and LeBlanc, 2000).

After the evaporation, dissolution, and biological degradation of the relatively light fractions of the oil, the remaining heavy and highly viscous compounds like asphaltenes form oil aggregates of variable sizes and shapes (Gray, 2004). The latter are perceived to have longer existence in the environment due to their low bioavailability and thus slow degradation.

2.1.4 Environmental Impact of Petroleum Pollution

2.1.4.a Pollution Impact on Wild Life

The size of petroleum-derived contamination event is rarely connected to the resulting harm on wild life. The time/ season of the event, the type of liberated oil, weather conditions and the nature of affected environment are rather more critical in this issue (Kingston, 2002). Exposed wild life such as seabirds, marine mammals, turtles, and fish are physically coated by oil. When fur and feather get oiled; buoyancy is imbalanced and swimming and flying abilities are reduced as a result. Thermoregulation is also affected leading to body temperature fluctuation. Oiled organisms may eventually die (Wilson and LeBlanc, 2000; Kingston, 2002).

Wild life organisms are also threatened by the toxicity of oil compounds. Wild sensitive species tend to either inhale or ingest oil components. When the soil is contaminated with oil hydrocarbons, local systems are severely affected as pollutants accumulate in animals and plants' tissues (Alvarez and Vogel, 1991). Acute toxicity is mostly observed at early stages when oil concentrations in the surroundings are still high. However, long-term effects are harder to measure, where organisms are chronically exposed. Contributors as the additive/synergistic/antagonistic effects and the diversity/complexity of ecosystems usually complicate the situation (Wilson and LeBlanc, 2000). One of the most detected long-term effects on wild life communities

was the alteration of macrofaunal composition due to loss of some species (Huz *et al.,* 2005).

2.1.4.b Pollution Impact on Habitats

The oil slick spread on the sea surface decreases light penetration into beneath strata by up to 90% reduction. Consequently, photosynthesis processes are hindered, thus; food availability for grazers is highly diminished (Wilson and LeBlanc, 2000). Habitats such as; seagrass beds, coral reefs and mangrove forests are shown to be particularly vulnerable (Mendoza-Cantú *et al.*, 2011). Because coral reefs and mangrove forests are considered as nurseries for diverse communities of organisms, exposing such habitats to toxic oil components extends the risks to wide spectrum of life forms. On the other hand, replacing a mangrove tree can take decades because mangrove forests are established very slowly. It is worth mentioning that for such sensitive habitats, large scale cleaning operations cause physical disturbance and may result in greater damage (Linden and Husain, 2002).

2.1.4.c Pollution Impact on Human Health

Human exposure routes to toxic oil residues can be various: inhaling evaporated compounds (volatile organic compounds, VOC's), being in contact with contaminated soils or water, drinking, swimming in affected areas, or ingesting oiled seafood. In general, hydrocarbons vary in terms of hydrophobicity; which determines their susceptibility to degradation. High molecular weight hydrocarbons are usually more

viscous, more hydrophobic, less bio-available (Gray, 2004). It is well known that accumulation in the environment is inversely related to susceptibility to metabolization (i.e.; the less bioavailable the compound, the higher the tendency to accumulate in the environment) (Wilson and LeBlanc, 2000). Throughout the food chain, humans tend to receive the highest concentration of oil residues when consuming seafood.

As mentioned earlier, evaporation can release huge portions of toxic volatile oil compounds to the atmosphere. VOC's combine with NO_x in presence of sunlight reaction to create O_3 in what is recognized as photochemical smog (Baird and Cann, 2008). Ground level O_3 is classified as a secondary air pollutant, thus; contributing to decreased air quality.

Workers involved in cleanup operations are exposed to direct health hazards of oil components. Records of acute exposure to volatile organic compounds (VOC's) shown neurological symptoms, such as headache, nausea, dizziness, and sleepiness. Other symptoms like breathing difficulties, vomiting, and abdominal pain are also common (Bosch, 2003). According to International Agency for Research on Cancer (IARC), VOC's are classified as Group 1 agents that proved to be human carcinogens. Based on evidences from animal studies, oil substances such as toluene and ethylbenzene are considered possibly carcinogenic to humans. An increase in DNA damage was also observed in some cleanup workers (Trigo *et al.,* 2007).

2.1.5 Petroleum Pollution in the Arabian Gulf

The Arabian Gulf is recognized as one of the most oil contaminated areas worldwide (Khan and Al-Ajmi, 1998). It has experienced probably the world's largest and most devastating oil incidents in history. Historically, there have been two main disastrous oil spillages in the Gulf waters: the Nowruz oil well blowout in 1983, which released about two million barrels and the Gulf war oil spills and fires during the 1991. The latter have released between 6 and 8 million barrels of crude oil into the Gulf waters (Massoud *et al.,* 1998; Khan and Al-Ajmi, 1998).

Of the world multi-national water basins, the Gulf is relatively exceptional for its enclosed nature. The shallow Gulf waters are located in hyper-arid region with very peculiar natural stresses, such as; low hydrodynamic energy, high salinities, low exchange rates, high temperatures, and high evaporation rates (Khan and Al-Ajmi, 1998; Sheppard *et al.,* 2010). The Gulf's distinct physical features create the favorable conditions for trapping pollutants and depositing particulates (Healey and Harada, 1991). Adding to the natural environmental stresses, the Gulf is intensively exposed to many anthropogenic activities. The rapid developmental construction projects and the substantial coastline alterations stand beside the international heavily oil traffic in the region (Figure 1) and the national oil activities, including exploration, exploitation, drilling, storage, and export (Massoud *et al.,* 1998). Collectively, the Gulf environment is highly vulnerable and many of its habitats are critically endangered.

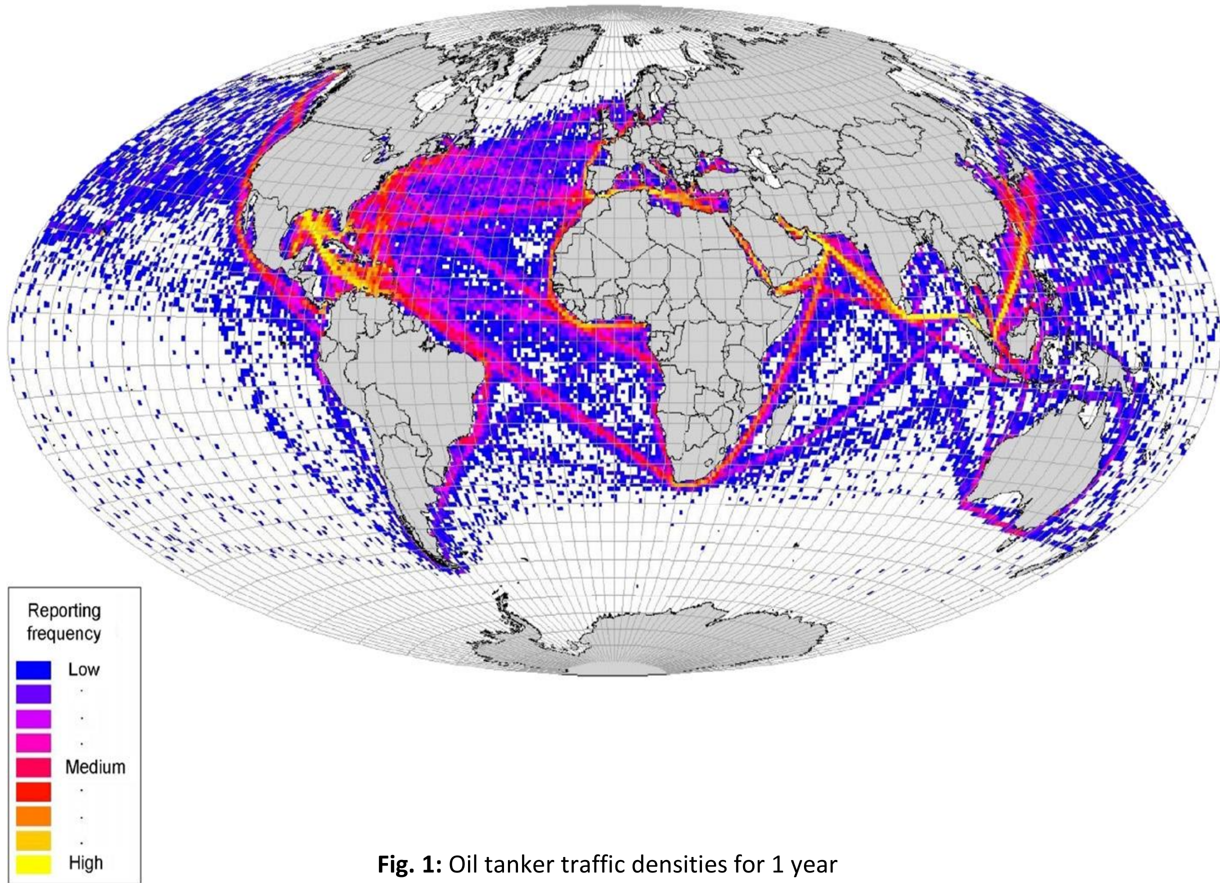

Fig. 1: Oil tanker traffic densities for 1 year

Source: (Vanem *et al.,* 2008)

2.2 Remediation Technologies

Emerging from the fact that almost 47% of the crude oil leaching into the marine environment is caused by natural seepage, the question to be raised is how could nature cleanup itself? As noted earlier, soon after the contamination event, a number of physical, chemical, and biological processes start acting on the oil components. These processes change the properties and behavior of the oil. Having the existing slow and

fixed rate of oil liberation, nature seems to be handling the situation. However, continuous anthropogenic activities and oil spill accidents discharge massive quantities of oil into the environment to the extent that human interference becomes crucial. Human attempts to accelerate what occurs naturally by the implementation of technology using several techniques. Remediation efforts aim to confine or restrain the magnitude of contamination, in order to prevent additional decline of the environment and to protect all life forms from exposure to hazardous materials (Pavel and Gavrilescu, 2008).

2.2.1 Mechanical Containment

Containment of the polluted area via physical means is perceived as the primary defense line in response mechanisms. Following petroleum pollution, booms are inserted in the contaminated water area to border and insulate the oil slick, or to prevent its passage to vulnerable areas. Then, skimmers collect the oil at the top of the slick confined within the boom. Skimmed oil is either stored in tanks on nearby vessels or on the shore. However, bad weather conditions can decrease the effectiveness of booms and skimmers (University of Delaware, 2004). In the case of land contamination events, physical containment can be achieved by capping the soil with clean material, making physical boundaries surrounding the contaminated area. The scenario is more complicated in the attempts to contain contaminated ground water (Pavel and Gavrilescu, 2008).

2.2.2 Physical Methods

Wiping contaminated shorelines with sorbent materials is a common physical response to petroleum pollution. Some oil hydrocarbons show tendency to bind to solids (Sayed and Zayed, 2006). Sorbents are used to recover oil from polluted sites through the mechanism of *ab*sorption, or *ad*sorption, or both. They can be organic or inorganic, natural or synthetic. A study examined the adsorption capacity of different kinds of materials: sludge from petroleum refineries and thermal power stations, garlic and onion peel used in oil uptake. Results showed significantly higher adsorption capacity of the sludge compared to the garlic and onion peels (Sayed and Zayed, 2006).

2.2.3 Chemical Methods

Chemicals are often used along with mechanical and biological techniques to overcome oil pollution. Among many, dispersants or emulsifiers are synthetic detergent-like chemicals proved to reduce environmental impact when sprayed onto petroleum polluted sites (Lessard and Demarco, 2000). Like detergents, dispersants are designed with two ends; lipophilic and hydrophilic. When applied onto the oil slick, they attempt to bridge the gap between the oil and water molecules. Dispersant molecules rearrange themselves so that the lipophilic end is oriented towards the oil and the hydrophilic tail towards the external water phase. This action decreases the interfacial surface tension and helps dispersing the oil in the water body as tiny droplets. The formation of droplets also increases the oil surface area, thus increasing the exposure to natural physical,

chemical, and biological degradation processes (Lessard and Demarco, 2000; University of Delaware, 2004). Figure 2 illustrates the mechanism of dispersants in facilitating the removal of hydrophobic hydrocarbons from water environment.

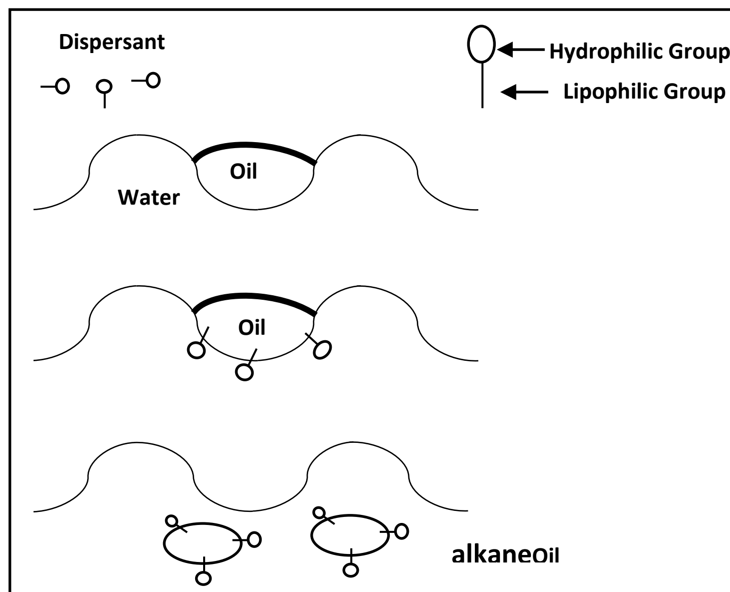

Fig. 2: Dispersants mechanism

Source: Modified from: Lessard and Demarco, 2000

2.3 Bioremediation Technologies

In nature, some hydrocarbons were found readily assimilable by variant classes of microorganisms. Consequently, scientists proposed the opportunity of sustaining and accelerating these naturally occurring processes for the sake of removal and degradation of several environmental pollutants encompassing the products of petroleum industry (Medina-Bellver *et al.,* 2005). More recently, scientific community started recognizing the term 'bioremediation' as the use of living organisms, particularly

microorganisms, to transform, degrade, and mineralize pollutants and reclaim environmental quality (Gray, 2004). Nevertheless, ensuring the success of microbial bioremediation requires many factors to be enhanced and/or provided. The first essential requirement is the presence of candidate microorganisms with the appropriate metabolic capabilities. When this is guaranteed; the establishment and the maintenance of the abiotic conditions that favor the optimal rates of growth and biodegradation comes next (Das and Chandran, 2011). In bioremediation, bacteria are typically exploited, yet the usage of fungi, algae and plants have also been recorded (Leung, 2004).

Bioremediation of hydrocarbons can involve the 'bio-transformation' of molecules into less or non-hazardous ones by altering their chemical structures and thus properties. Microorganisms are also capable of breaking down hydrocarbons into smaller and simpler sub-units or inorganic molecules in what is known as 'biodegradation'. When degradation is complete to final inorganic constituents as CO_2 and H_2O, the process is named 'mineralization' (Leung, 2004). Among the available remediation technologies, bioremediation is perceived as relatively cost-effective and non-invasive (April *et al.,* 2000).

2.3.1 The Interaction between Microorganisms and Hydrocarbons

Microbial degradation of hydrocarbons is a complicated process influenced by many elements. In fact, a major inquiry in the biodegradation of hydrocarbons is how

the microbes actually contact the substrates. In application, many challenges encounter the interaction between microorganisms and hydrocarbons. Broadly speaking, hydrocarbons are ordered in terms of their susceptibility to microbial attack from the most susceptible as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes, while high molecular weight aromatics like poly aromatic hydrocarbons (PAHs) being greatly recalcitrant or exhibiting extremely low rates of biodegradation (Leahy and Colwell, 1990; Ulrici, 2000; Atlas and Bragg, 2009). According to Monod Law, the rates of uptake and mineralization of many organic compounds by microbial populations in the aquatic environment are proportional to the concentrations of the compounds. This relation stands true for low molecular weight and highly soluble oil components such as toluene. However, concentration dependent degradation seems not to apply for high molecular weight hydrocarbons with low solubility (Boethling and Alexander, 1979).

2.3.1.a Toxicity

A basic requirement for microorganisms to catabolize hydrocarbons is the absence of toxicity. Some oil compounds, due to their chemical and physical properties, perform as inhibitors to microorganisms. However, even toxic hydrocarbons can be biodegraded under favorable conditions (Leahy and Colwell, 1990). Generally, the toxic effect of majority of hydrocarbons on microorganisms was attributed to accumulation in the phospholipid bilayer of the cell membrane altering its fluidity (Sikkema *et al*., 1995; Heipieper and Martínez, 2010).

2.3.1.b Bioavailability (Surfactants)

A major limiting factor encountering microbial degradation is the bioavailability of pollutant molecules with the appropriate chemical and physical conditions in order to be degraded or catabolized. Most petroleum hydrocarbons display hydrophobicity and low water solubility, hence; they need to be solubilized or emulsified prior to being attacked by microbial cells. To break this oil/water barrier, microorganisms have shown the ability to produce surface acting agents 'biosurfactants' that reduce the surface tension between individual microbial cells and the surrounding oil-contaminated microenvironment (De´Ziel *et al*., 1996).

Biosurfactants are amphiphilic compounds that possess both hydrophilic (water-loving) and lipophilic (fat-loving) moieties. Research studies have shown high interest in biosurfactants, particularly due to their low toxicity, high biodegradability, and their role in enhancing microbial degradation by reducing the surface tension between active microbes and hydrophobic substrates (Zhang and Xiang, 2010).

Scientists have identified and characterized numerous and various categories of biosurfactants produced by microorganisms. However, biosurfactants can be broadly classified into two major groups: low molecular weight biosurfactants comprised of glycolipids and lipopeptides and high molecular weight biosurfactants made up of polymers and lipopetides (Satpute *et al.*, 2010). Several microorganisms have been reported to produce surfactants including *Bacillus, Pseudomonas, Acinetobacter,*

Achromobacter, Arthrobacter, Brevibacterium, Corynebacterium, Candida, and *Rhodotorula,* among others (Cameotra and Makkar, 1998). According to Hisatsuka *et al.*, the rhamnolipids of *Pseudomonas aeruginosa* are among the heavily reviewed and well-studied biosurfactants.

2.3.1.c Biodegradability

As mentioned earlier in this text, hydrocarbons are different in terms of susceptibility to microbial degradation. Some oil fractions seems to be recalcitrant or less biodegradable. In this matter, microorganisms have developed naturally various mechanisms to overcome recalcitrance of substrates in order to proliferate and grow. Here, two metabolic pathways will be touched briefly: 'co-metabolism or co-oxidation' and 'commensalism'.

In some hydrocarbon microbial degradation cases, scientists have noticed the disability of the microorganism to degrade the certain hydrocarbon as sole carbon and energy source, unless another co-substrate is added to the medium. This phenomenon was described as 'co-metabolism or co-oxidation' and defined as: any oxidation process of substances such that the yielded energy of the oxidation will not be used to support microbial growth (Horvath, 1972). Several methods could be used to detect the occurrence of co-metabolism. Monitoring the oxygen consumption levels by the microorganism upon the oxidation of a co-substrate, while failing to support growth. In addition, demonstrating substrate disappearance or intermediate product accumulation

can also be an indicator of co-metabolism. Further, enzyme induction could act as a clue (Walker and Harris, 1970).

As in co-metabolism, microbial degradation need an inducer substrate to catabolize certain hydrocarbon, many microbial isolates have demonstrated the requirement of the presence of another microorganism in order to attack specific hydrocarbons. In commensalism, the co-existence of one organism is obligatory for the other, since both participate in the same catabolic pathway of the same hydrocarbon. In other words, both are complementary to each other in terms of the catabolic enzymatic machinery (Beam and Perry, 1974). In natural situations, microorganisms seem to favor mixed growth like in biofilms, flocs, or granules under conditions of nutrient and waste gradient, in which growth is better maintained (Christensen *et al.,* 2002).

2.3.2 Factors Affecting Microbial Degradation

Microbial degradation of petroleum hydrocarbons is complex and many factors influence the rate and effectiveness of degradation. Among many, few factors are to be tackled. Firstly, the chemical composition of the crude oil fraction or the single hydrocarbon being catabolized. Typically, *n*-alkanes and light aromatics contribute to highest biodegradation rates compared to high molecular weight poly aromatics. However, the case might be inverted when signs of co-oxidation are evident. Studies recorded high oxidation rate of resins and asphaltenes when mixed culture of bacteria

were grown on crude oil (Bertrand *et al*., 1983). Therefore, co-oxidation of non-growth substrates was detected in the presence of growth supporting hydrocarbons.

The physical state of petroleum hydrocarbons also determines the susceptibility to biodegradation. The differences between water and soil oil contamination are attributed to movement and distribution patterns of hydrocarbons in the surroundings. Oil in the marine environment spreads horizontally forming the slick, whilst in soil it infiltrates vertically throughout the soil depth (Bossert and Bartha, 1984).

The relation between hydrocarbon concentration and the uptake and mineralization by cells has been discussed earlier. This correlation stands true only with small and relatively soluble hydrocarbons, while the concentration of high molecular weight and immiscible hydrocarbons seems not proportional to growth and activity of microbes (Button and Robertson, 1986).

Another important limiting factor is temperature affecting the structure of hydrocarbon degrading microbial communities and the physical and chemical properties of oil composition (Foght *et al.,* 1996). At low temperatures, elevated viscosity of the oil moiety is observed. As a result, bioavailability of oil fractions to microbial cells is substantially diminished. In addition, volatile small alkanes tend to become more soluble and impose toxicity to viable microorganisms. Further, catabolic enzymatic activity reduces at low temperatures, hence; reducing microbial biodegradation rates (Atlas and Bartha, 1972).

Microbial catabolism of hydrocarbons is believed to be oxidation/reduction reactions. Molecular oxygen is crucial demand in the first steps of any hydrocarbon catabolic pathway (Figure 3). Oxygen availability is considered an indicator of the rate and effectiveness of biodegradation. Only low rates of anaerobic catabolism is detected and mostly of incomplete degradation (Das and Chandran, 2011).

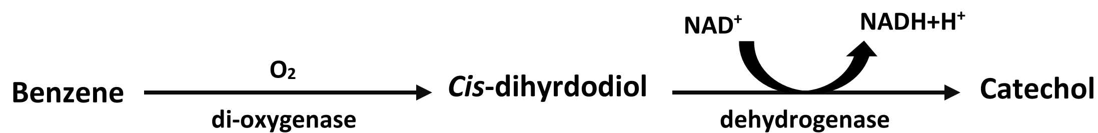

Fig. 3: Part of the cxidative catabolic pathway of Benzene.

Source: Modified from: Juhasz and Naidu, 2000

In order for microorganisms to degrade hydrocarbons as the sole carbon source, other nutritional requirements should be supplied. Inorganic nitrogen and phosphorous sources have been attributed to enhanced growth and biodegradation activity of cells (Atlas and Bartha, 1973). Consequently, carbon: nitrogen: phosphorous ratios are substantially important for the optimization of microbial biodegradation (Jasmine and Mukherji, 2014). Scientists have reported that excess of nutrients might also inhibit the microbial hydrocarbon degradation as well as their shortage (Chaillan *et al.,* 2006).

2.3.3 Types of Bioremediation Technologies

Various biological approaches are verified to detoxify oil components and reverse environmental impacts. There are the two main methodologies of

bioremediation: (a) bioaugmentation, in which known, well-characterized non-native oil-degrading microorganisms are added to the contaminated site supporting the existing microbial population and enhancing the rate and/or extent of oil biodegradation (Leung, 2004). In bioaugmentation also named 'seeding', the provided microorganisms are mostly isolated previously by enrichment cultures from contaminated locations (Riser-Roberts, 1998). Nevertheless, the seeded microorganisms must meet several conditions: (1) they have to survive in the new destination and compete with indigenous. (2) They need to possess elevated rates of enzymatic activity and biodegradation of broad hydrocarbon spectrum. (3) Genetic stability is also required. (4) The absence of pathogenicity and toxicity must be ensured (Gray, 2004). On the other hand, there exist some arguments on the application of non-native microorganisms into an environment. The unknown fate of such micro-degraders in the environment after accomplishing their bioremediation job is one argument for example, among others.

The second bioremediation approach is (b) biostimulation, in which the growth of indigenous oil degraders is stimulated by the addition of nutrients or other growth-limiting substrates. The enhancement of the capability of native hydrocarbon biodegraders upon feeding with nutrient solutions encompassing the major nutritional requirements of nitrogen, phosphorous and sulfur is evident (Riser-Roberts, 1998). Commercial fertilizers (nutrients to be inoculated) have calculated ratios of nitrogen and phosphorous for instance. The scientific rationale behind this calculation is the amount

of N and P needed to mineralize a certain amount of hydrocarbons into carbon dioxide and water (Gray, 2004).

Bioremediation can also be divided as in-situ (treatment at the contamination site), and ex-situ (contaminants are retrieved out of the contaminated location and processed elsewhere). Ex-situ is further split into solid phase and slurry phase bioremediation (Pavel and Gavrilescu, 2008).

Several systems are designed for the application of microbial bioremediation. For example, the most well-known ex-situ, slurry phase bioremediation systems are the 'bioreactors'. In this method, contaminated water or soil is transferred to huge vessels where conditions are highly controlled and favorable for the growth and activity of microorganism populations (Gray, 2004; Pavel and Gavrilescu, 2008). Over the years, scientists along with engineers have developed huge number of various treatment designs in order to achieve high pollutant removal.

Another ex-situ bioremediation technique is the Landfarming. Contaminated soil is excavated and spread on a thin surface. Growth of native microorganisms is promoted by tilling the soil and providing the proper nutrients and minerals. To deliver adequate aeration to the soil, water is sprinkled. This will also minimize the dust created while tilling the soil (Rubinos *et al.,* 2007). However, landfarming has some disadvantages, like: (1) difficulty to achieve remediation more than 95% (2) the need for large areas (3) and generation of dust and vapor decreasing air quality (Pavel and Gavrilescu, 2008).

Further, bioventing is an in-situ remediation technology that uses indigenous microorganisms to biodegrade oil constituents adsorbed to soils. Microorganisms are enhanced by inducing air (or oxygen) flow into the soil via injection wells and, if necessary, by adding nutrients (Norris *et al.,* 1994). Bioventing is claimed to be not suitable for treating soils with shallow groundwater tables. It also capable of tackling the unsaturated zone of the soil; other methods are needed to treat the saturated zone (Pavel and Gavrilescu, 2008).

2.4 Investigation of Genes Encoding Hydrocarbons Degradation

For comprehensively understanding the phenotypic profiles of hydrocarbon degrading microorganisms and overcoming the limitation in microbiological culturing methods, molecular techniques based on the genotypes have been developed. Scientists have been oriented for years towards analyzing the enzymatic catabolic pathways of various crude oil hydrocarbon components. The catabolic pathways of petroleum compounds like aliphatic *n*-alkanes and simple polycyclic aromatic hydrocarbons (PAHs) have been fully elucidated (Aoki *et al.,* 1996; Goyal and Zylstra, 1996; Ferrero *et al*., 2002; Kloos *et al.,* 2006). Molecular approaches comprise PCR amplification of particular genes encoding for catabolic enzymes using oligonucleotide primers within chromosomal or plasmid isolated DNA. In addition, hybridization techniques with oligonucleotide probes have been utilized for more specific analysis of the PCR amplicons (Sei *et al.,* 1999).

Several bacterial isolates have received particular attention in terms of their biodegradation potential. Hence, their hydrocarbon degrading genes have been well studied and used as reference, such as *Pseudomonas putida* NCIB 9816, *Pseudomonas putida* G7, *Pseudomonas aeruginosa* PaK1, and *Acinetobacter calcoaceticus* NCIB 8250, among others (Denome *et al*., 1993; Takizawa *et al.,* 1999; Hamann *et al.*, 1999; Kohno *et al.,* 2002).

Research studies of the functional catabolic genes among hydrocarbon degrading bacterial strains have reported that they can be plasmid-borne or of chromosomal loci (Chakrabarty, 1973; Takizawa *et al.,* 1999). Bacterial plasmids carrying aliphatic and aromatic degrading genes have been characterized. A plasmid can possess the whole set of functional genes in a catabolic pathway or only few degradative steps (Obayori and Salam, 2010). Of the major known plasmids are; TOL plasmid carrying catabolic genes of toluene or xylene, SAL plasmid assigning the biodegradation of salicylate (Chakrabarty *et al.,* 1978). Other plasmids are between the most reported for naphthalene degrading genes such as; NAH7, pDTG1, and pUC18 (Dunn, 1973; Kurkela *et al.,* 1988; and Denome *et al.,* 1993, respectively).

2.4.1 Genes Encoding Alkanes Degradation

Among the oil fractions, alkanes (saturates) are perceived the most biodegradable. Scientific estimates have reported that upon the Exxon Valdez oil spill into the Alaskan waters, 50% of the liberated oil was biodegradable (Kohno *et al.,* 2002).

It is then crucial to understand the genetic involvement in the catabolic pathways of alkanes. Intensive studies of bacterial aerobic degradation of alkanes have revealed that alkane hydroxylases (AHs) are the principal enzymes catalyzing the first step in the pathway (Kohno *et al.,* 2002; Kloos *et al.,* 2006; Nie *et al.*, 2014). AHs are of three known types; the integral membrane alkane hydroxylase (monooxygenase) that relies on rubredoxins as electrons transfer system. This AH is encoded by a gene called *alkB* and considered the most common among bacteria. Generally, *alkB*-hydroxylases target *n*-alkanes between C10 and C16 (Nie *et al.,* 2014). The second type of AHs is cytochrome P450 dependent, which targets the short or medium length chains of *n*-alkanes. A third group of AHs utilizes NADH for electron shuttling. The *CYP52* gene encodes for the latter two classes of AHs (Kloos *et al.,* 2006). Figure 4 illustrates partial catabolic pathway of *n*-alkanes and the role of alkane monooxygenases in initiating the first oxidative step.

Fig. 4: Partial aerobic pathway for the degradation of *n*-alkanes by terminal oxidation. Initial activation of the alkane molecule requires O2 as a reactant. AH, alkane hydroxylase; AD, alcohol dehydrogenase; ALD, aldehyde dehydrogenase.

Source: Modified from: Rojo, 2009

2.4.2 Genes Encoding Aromatics Degradation

The mechanisms involved in the degradation of aromatic compounds such as; benzene, toluene, and xylene and simple polycyclic aromatic compounds like; naphthalene, phenanthrene, and anthracene have been the subject of extensive research. The genes encoding several aromatic compounds are found to be clustered in 'operons' mostly borne on 'plasmids' (Aoki *et al.,* 1996).

In the degradation of BTEX (benzene, toluene, ethylbenzene and xylene), several genes have been detected and amplified. For instance, the *bedC1* gene was identified to encode for alpha subunit of benzene dioxygenase (Wang *et al.,* 2008). In addition, genes for toluene and xylene monoxygenases such as *tmoA* and *xylA,* respectively, were also discovered. However, the *todC1* gene was attributed to the direct dioxygenase attack of benzene, toluene, and ethylbenzene (Hendrickx, *et al*., 2006).

For polycyclic aromatic hydrocarbons, the naphthalene catabolic pathway for *Pseudomonas putida* strains G7 and NCIB 9816 has been deeply investigated (Takizawa *et al.,* 1999; Hamann *et al.*, 1999). However, very few publications examined PAH degrading genes in non-pesudomonads strains (Goyal and Zylstra, 1996; Hamann *et al.*, 1999; Andreoni *et al*., 2000). In the catabolic pathway of naphthalene, the functional genes encoding naphthalene degradation were clustered in the *nah* operon (Takizawa *et al.,* 1999). Other genes such as; *ndo* for naphthalene dioxygenase, *dox* for

dibenzothiopene oxidation, and *pah* for polycyclic aromatic hydrocarbon degradation have been identified as well (Kurkela *et al.,* 1988; and Denome *et al.,* 1993).

Generally, most of the aromatic compounds including the PAHs are catabolized into mutual intermediates. The first degradative step is catalyzed by dioxygenases, where the aromatic ring is transformed into *cis*-dihydrodiol intermediates (Hamann *et al.*, 1999; Takizawa *et al.,* 1999; Brezna *et al*., 2003). Cleavage of the aromatic ring follows via multiple enzymatic steps. Heavy research effort has been dedicated for amplifying and cloning fragments of genes encoding for enzymes or enzymes' subunits participating in aromatics degradation. Figure 5 provides a schematic presentation of the enzymatic catabolic pathway of BTEX. Whilst, figure 6 demonstrates the upper pathway for naphthalene catabolism by *Pseudomonas* strains.

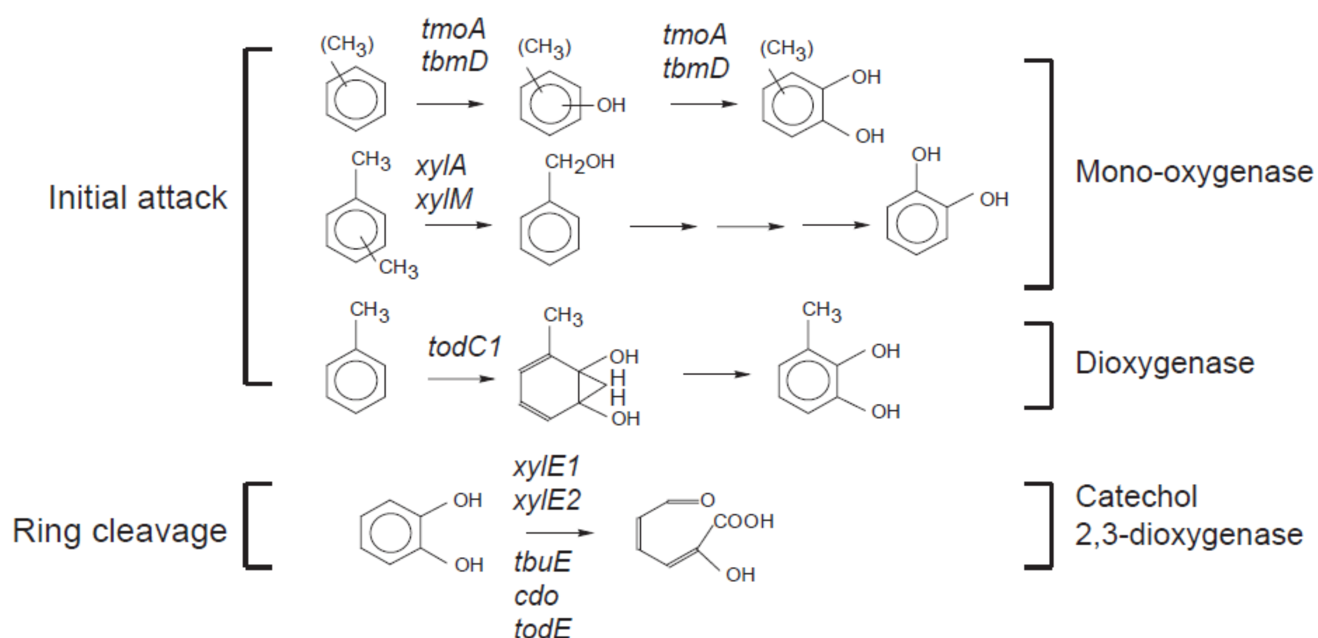

Fig. 5: schematic presentation of the enzymatic catabolic pathway of BTEX.

Source: Wang *et al.,* 2008

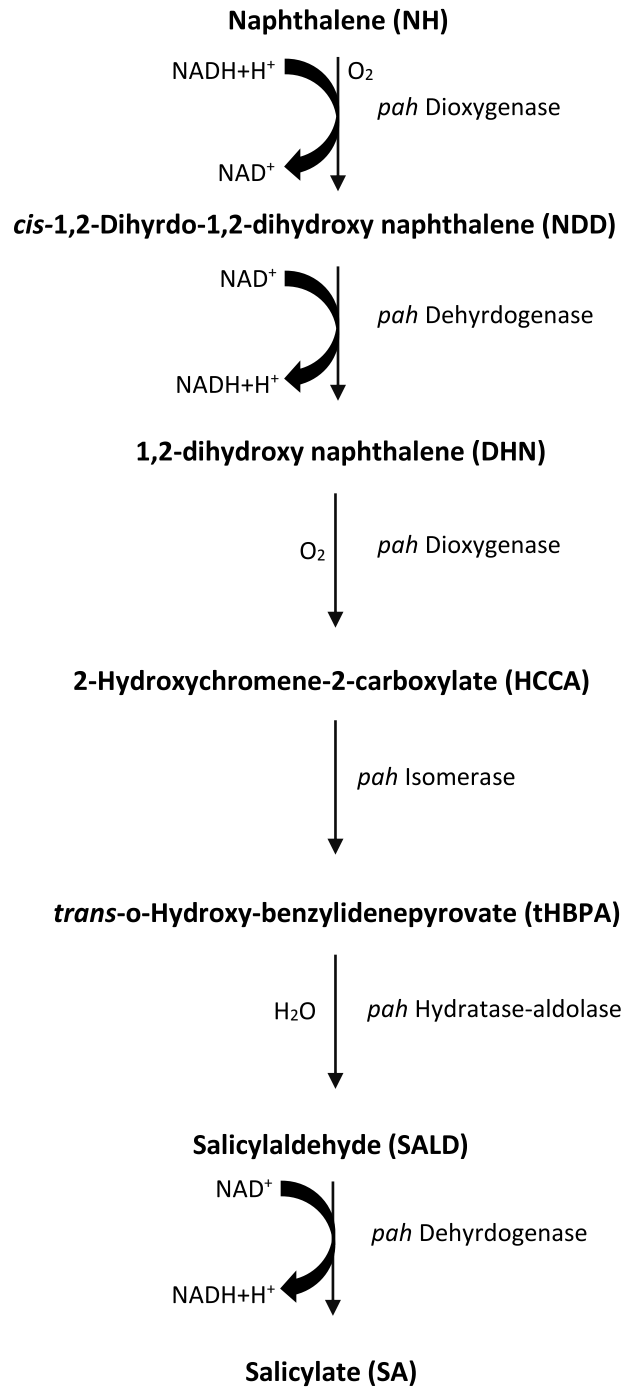

Fig. 6: Upper pathway for naphthalene catabolism by *Pseudomonas* strains.

Source: Modified from: Takizawa *et al.,* 1999

3. Materials and Methods

3.1 Bacterial Strains

Three strains of *Pseudomonas aeruginosa* coded QDD1, QDD8, and QDD9 were utilized in this study. These strains were previously isolated in our lab from highly contaminated soil sites in the state of Qatar (Jaoua and Al Thani, unpublished (QDD1); Al Disi *et al.,* 2015 (QDD8 and QDD9). Their identification was performed using molecular techniques by sequencing the 16S RNA. Table 1 lists the original locations of strains isolated in the lab. For this study, selected strains were initially recovered from the established Qatari Hydrocarbon Degrading Strains Bank preserved on glycerol, at -80 ^{o}C by streaking on solid Luria-Bertani (LB) medium. These strains were routinely streaked ahead of each experiment to obtain viable, fresh, and pure cells.

For DNA isolation, cell suspensions were prepared as follows: pre-cultures of 5 ml LB were prepared using fresh, pure isolated colony for 4 hours. After that, overnight (ON) cultures (same volume of LB) were inoculated by 100 µl from the pre-cultures.

3.2 Culture Media

3.2.1 Luria-Bertani Medium (LB)

Ready mix of LB agar and LB broth were used to prepare both liquid and solid LB media.

Table 1: List and origin of the coded 39 isolates and media used for isolation (Al Disi *et al.,* 2015) including the strains used in this study (in bold).

Sample	Origin	MSM Solid + Crude Oil	MSM Liquid + Crude Oil	MSM Liquid + Diesel	MM Liquid + Crude Oil
S1	Auto-workshop		QDD11, QDD19	QDD7	
S2	Auto-workshop			QDD1, QDD10	
S3	Auto-workshop		**QDD8, QDD9,** QDD36, QDD37	QDD6	QDD3, QDD12, QDD15, QDD18
S4	Auto-workshop	QDD4, QDD5, QDD16, QDD17	QDD2, QDD13, QDD14, QDD20		
S5	Soil (in depth)			QDD26, QDD27, QDD28, QDD32, QDD34	
S6	Beach Soil	QDD25	QDD22	QDD33	
S7	Surface Soil		QDD21, QDD23, QDD24	QDD29, QDD30, **QDD1**, QDD2	
S8	Beach Soil			QDD31	
S9	Surface Soil			QDD35	

3.2.2 Minimal Salt Medium (MSM)

The composition of MSM medium was as follows (g/l): NH_4NO_3 (4.0), Na_2HPO_4 (2.0), KH_2PO_4 (0.53), K_2SO_4 (0.17), $MgSO_4.7H_2O$ (0.10), and 1ml of trace-element solution (g/100 ml): EDTA (0.1), $ZnSO_4$ (0.042), $MnSO_4$ (0.178), H_3BO_3 (0.05), NiCl (0.1). pH was adjusted to 7.2. (Al Disi *et al.*, 2015). Two different nitrogen sources were used: NH_4NO_3

and NH_4Cl. When adding 4 g of ammonium nitrate to 1 L of the medium, the resulting 'nitrogen' concentration was 1.4 g/l, corresponding to a C/N ratio of 60/1. The latter was called MSM1, however; mixing 4 g of ammonium chloride in 1 L of the medium gives 1.05 g/l 'nitrogen' concentration. This medium was named MSM2 and the corresponding C/N ratio was 80/1.

3.2.3 Factorial Experiment Media

Emerging from the original MSM1 components, a factorial experimental design was applied to study the influence of the main constituents of the media on growth of the three strains and their biological activities in terms of hydrocarbon degradation. Three main parameters were chosen for the study: Nitrogen source (X1), Phosphorous source (X2), and Diesel concentration as carbon source (X3). Each factor was investigated at two levels; low and high. The nitrogen source concentration as [NH_4NO_3] varied between: 2g/l and 8g/l. Phosphorous source concentration as [Na_2HPO_4, KH_2PO_4] varied between: 1g/l and 0.265g/l - 4g/l and 1.06g/l respectively. Diesel concentration as carbon source varied between: 5% (v/v) and 20% (v/v). Building on that, (2^3 = 8) eight different growth conditions resulted. The remaining components of MSM1 medium remained the same. The chosen model was *Linear additive model for a Completely Randomized Design (CRD)* and represented by:

$$Y_{ij} = \mu + C + N + P + C*N + C*P + N*P + C*N*P + \varepsilon_{ij}$$

Where Y_{ij} is any growth observation reported in terms of the Colony Forming Units (CFU) in cells/ml. μ is the grand mean of all recorded observations. C is the single factor effect of carbon concentration on growth. N is the single factor effect of nitrogen concentration on growth. P is the single factor effect of phosphorous concentration on growth. While (C*N), (C*P), and (N*P) are the first order interaction between two factors. The second order interaction is represented as (C*N*P). Finally, ε_{ij} represents the experimental error.

This formulated equation explains the sources of variance that can be observed in the growth results when conducting statistical analysis. A low response is coded as (-1) and a high response as (+1) (Tajber *et al*., 2009). Table 2 shows the composition of the various eight conditions in a symbolic demonstration; where (+1) refers to maximum concentration and (-1) refers to minimum concentration.

Table 2: Factorial Experiment Model: three factors involved Carbon, Nitrogen, and Phosphorous where each has two levels (High: +1 and Low: -1).

Factor / Experiment	N-Source	P-Source	C-Source
1	-1	-1	+1
2	-1	+1	+1
3	-1	+1	-1
4	-1	-1	-1
5	+1	+1	-1
6	+1	-1	-1
7	+1	-1	+1
8	+1	+1	+1

3.3 Solutions and Buffers

A description of all solutions and buffers used in this study is noted here:

- Lysis Buffer I (40 mM Tris-acetate pH 7.8, 20 mM Sodium-acetate, 1 M EDTA, 1% SDS)
- Lysis Buffer II (100 mM Tris-HCl pH 8.0, 100 mM EDTA, 250 mM NaCl)
- Solution A (25 mM Tris-HCl, 50 mM Glucose, 10 mM EDTA-Na_2, pH 8.0)

- Solution B (0.2 % NaOH, 1 % SDS) freshly prepared
- Solution C (60 ml of Potassium acetate 5 M, 11.5 ml of Acetic acid glacial, 28.5 ml H_2O)
- TER Buffer (10 mM Tris pH 8.0, 1 mM EDTA, 10 µg/ml Rnase)
- TE Buffer (10 mM Tris pH 6.8, 1 mM EDTA)
- CTAB Solution (0.8 M NaCl, 0.5% hexadecyltrimethylammonium bromide)
- Phenol: Chloroform: Isoamyl alcohol (25:24:1)
- Chloroform: Isoamyl alcohol (24:1)
- NaCl (5 M)
- Ethanol (70% and 100%)
- Agarose gel (0.7%, 1%, and 1.2%)
- TAE Buffer 0.5X (Tris-acetate, EDTA)
- Ethidium bromide solution: 10 mg/ml in water. Final concentration in agarose gel is 0.6 µg/ml

All culture media, solutions and buffers were autoclaved at 121°C for 20 min.

3.4 Screening of *Pseudomonas aeruginosa* Strains on MSM Medium

Pseudomonas strains were grown on liquid MSM1 and MSM2 culture media of chemical composition mentioned earlier. In 50 ml falcon tubes, strains were subjected to 5% (v/v) and 10% (v/v) diesel as the sole carbon source. Inoculum was

prepared from a single isolated colony of a fresh LB streak in sterile MSM suspension. Final volume of growth reaction was 20 ml. Culture tubes were kept at 30°C in rotating shaker 300 rpm. Results were retrieved after a week of incubation. The cells growth was assessed by counting the Colony Forming Units (CFU) using the serial dilution technique.

3.5 Gas Chromatographic Analysis (GC) of Diesel Hydrocarbons

Strains' capability to degrade diesel hydrocarbons was examined through Gas Chromatographic Analysis (GC). As GC fractionates all hydrocarbons present in the diesel matrix in terms of their molecular weight (MW), large spectrum of low MW and high MW hydrocarbons are illustrated in chromatograms as peaks. The area under the peak represents the quantitative estimate of each hydrocarbon in the examined diesel layer. Diesel fraction from control samples (lacks inoculum) was analyzed to select representative peaks over the whole hydrocarbon spectrum. Chromatograms of the biological samples were analyzed likewise the control with respect to the same selected retention times. Hydrocarbon biodegradation was estimated via the percentage of Removal Efficiency (RE) or Biodegradation Efficiency (BE); using the following formula: $\%RE = 100 - (A_s*100/A_{ac})$ (Michaud *et al.*, 2004).Where A_s is the total area under the peak of the biological sample, whilst A_{ac} is the total area under the same peak in the abiotic control.

3.6 Factorial Investigation of Strains' Potentiality to Degrade Diesel Hydrocarbons

Each *Pseudomonas aeruginosa* strain was cultured at eight different growth conditions with three main changing variables (Nitrogen, Phosphorous, and Carbon). Inoculum was prepared similarly to that of MSM experiment. Cells were inoculated in a 20 ml growth reaction containing the final concentration of each factor either maximum or minimum as described in the Factorial Experiment Media section (3.2.3). Growth results as well as hydrocarbon degradation estimates were obtained in the same way of MSM experiment and on a weekly basis. Incubation temperature was 30 ^{o}C, in 300 rpm rotating shaker. Each growth condition was accompanied with a negative control sample (lacks inoculum). All assays were carried out in triplicate runs: biological samples are repeated three times, thus; three CFU counts and three chromatograms analyzed per sample.

3.7 Statistical Analysis

Triplicate growth results were entered into Minitab statistical software. Analysis of Variance (ANOVA) was performed at a significance level $\alpha = 0.05$. ANOVA facilitates testing the significance of differences among two or more means (populations). Decisions are made based on the p – value, which evaluates the evidence against null hypothesis H_o. If p – value is less than the level of significance α, we reject H_o, and the differences between the means are significant. Calculation of the Least Significant

Difference (LSD) using the equation: LSD = t_α sqrt (2MSe/r), allows examining which particular combination of means were the source of significant variance. t_α represents the theoretical *t* - value at a significance level 0.05 and degrees of freedom of the error. MSe is the means square of the error. r is the replication, which is in our study r = 3.

3.8 Extraction of Genomic DNA

For the preparation of genomic DNA from our *Pseudomonas aeruginosa* strains, two different protocols have been retrieved from literature and adjusted for the purpose of this study.

3.8.1 DNA Isolation Method – I

This method is used to extract DNA from *Pseudomonas* species. It was adapted from the Chen and Kuo (1993) publication and modified as follows:

- 1.5 ml of saturated culture was centrifuged for 3 minutes at 12000 rpm.
- After discarding the supernatant, the cell pellet is re-suspended in 200 µl Lysis Buffer I
- To remove proteins and cell debris, 66 µl of 5M NaCl was added and mixed well
- 300 µl from the phenol: chloroform: isoamyl alcohol (25: 24: 1) mix, was added and then centrifuged for 10 minutes, at 12000 rpm and 4 ^{o}C.
- Clear supernatant was transferred to new tube and DNA was

precipitated by adding two volumes of 100% ethanol, incubated at -20 ^{o}C for at least 1 hour.

- The supernatant was decanted upon centrifugation for 10 minutes.
- DNA pellet was washed twice with 500 µl of 70% ethanol
- After drying the pellet at room temperature, it was re-suspended in 50 µl of TER solution.
- Incubation at 37 ^{o}C for 1 hour prior to storage.

3.8.2 DNA Isolation Method – II

This genomic DNA extraction protocol was obtained and adjusted from Cavalcaa *et. al*., (2000). Extraction was performed as follows:

- 1.5 ml of cell culture was centrifuged for 10 minutes at 14000 rpm and 5°C
- Supernatant was discarded while the cell pellet was re-suspended in 200 µl of Lysis Buffer II
- 200 µl of SDS and proteinase K mixture was added in a final concentration of (0.5 % SDS and 100 µg/ml proteinase K).
- Cells were incubated for 1 hour at 37 ^{o}C
- Precipitation was done by adding 400 µl of hexadecyltrimethylammonium bromide (CTAB) solution containing (0.8M NaCl and 0.5% CTAB)
- In a water bath, mixture was incubated for 20 minutes at 65 ^{o}C

- An equal volume was added from chloroform: isoamyl alcohol (24: 1)
- Clear supernatant was transferred to a new tube after centrifugation for 10 minutes at 12000 rpm and 5 ^{o}C
- RNase was of final concentration (10 µg/ml) was added and DNA solution was incubated for 15 minutes at 37 ^{o}C
- DNA was precipitated by adding 0.6 volume of isopropanol, and incubated overnight (ON) at -20 ^{o}C
- Precipitated DNA was centrifuged the day after for 10 minutes at a maximum speed.
- DNA pellet was washed with 500µl of 70 % ethanol, centrifuged for 20 minutes at a maximum speed, and left to dry
- Re-suspension of DNA pellet was done in 50 µl of TE buffer, pH 6.8 prior to storage.

Genomic DNA was run on 0.7 % agarose gel electrophoresis, 100 Volts/ cm and visualized using UV documentation system.

3.9 Extraction of Plasmid DNA

The plasmid DNA of our strains was extracted using the following protocol:

- 1.5 ml of cell culture was centrifuged for 5 minutes at 14000 rpm
- The cell pellet was suspended in 100 µl of solution A
- 200 µl of solution B was added and mixed by inverting the tube 5 times

- While working in ice, 100 µl of solution C was added and mixed
- Prior to centrifugation for 5 minutes at 14000 rpm, the mixture was incubated in ice for 5 minutes
- Clear supernatant was transferred and equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added
- Then, centrifugation for 5 minutes at 14000 rpm was done and the supernatant was moved to new clean tube
- Two volumes of 100 % ethanol was added for each one volume of DNA
- Slowly, the DNA was mixed and incubated at room temperature (RT) for 2 minutes
- After centrifugation for 5 minutes at 14000 rpm, the DNA pellet was washed with 70 % ethanol and left to dry
- The DNA pellet was re-suspended in 50 µl TER buffer and incubated for 45 minutes at 37 ^{o}C prior to storage

Plasmid DNA was run on 0.7 % agarose gel electrophoresis, 10 Volts/ cm and visualized using UV documentation system.

3.10 Primers Selection

The set of primers used in this study to detect hydrocarbon-degrading genes among our *Pseudomonas aeruginosa* strains were selected from previous research studies. The strategy of this selection was based on testing our strains' genetic potentials

and diversity over a broad spectrum of hydrocarbons. As elaborated earlier in the reviewed literature, several genes or gene fragments encoding for alkane hydroxylases have been characterized. To explore the ability of our strains to degrade n-alkanes; primers ALK1-F/ ALK1-R, ALK2-F/ ALK2-R, ALK3-F/ ALK3-R, and ALKB1-F/ALKB1-R have been selected (Kohno, *et al*., 2002; Kloos, *et al.,* 2006). Further, in order to screen our strains biodegradation capability of BTEX; the following primers were chosen: TMOA-F/ TMOA-R, TBMD-F/ TBMD-R, XYLA-F/ XYLA-R, XYLE1-F/ XYLE1-R, TODC1-F/ TODC1-R, and BEDC1-F/ BEDC1-R (Hendrickx, *et al*., 2006; Wang *et al.,* 2008). For molecular screening on PAHs, oligonucleotide primers of genes encoding for dehydrogenase, oxygenase, and aldolase in the upper catabolic pathway of naphthalene have been selected; B6-F/ B778- R, C118-F/ C814-R, and E207-F/ E826-R (Ferrero, *et al*., 2002). Table 3 lists the primers set used in this study along with the corresponding sequence, and the amplicon expected size.

Table 3: List of primers used in this study for molecular screening of hydrocarbon degrading genes

No.	Name	Sequence	Corresponding Gene	Size (bp)	Reference
1	B6-F B778-R	5’ CAATCAACAAGTCGTTTC 3’ 5’ ACTTGCGACCGAGCG 3’	Cis- Naphthalene dihydrodiol dehydrogenase *(nahB)*	773	Ferrero *et al.*, 2002
2	C118-F C814-R	5’ GAGAAGGACCGTTTCTATC 3’ 5’ CACCTCGCCAGCCGGG 3’	1,2-Dihydroxynaphthalene oxygenase (*nahC)*	697	Ferrero *et al.*, 2002
3	E207-F E826-R	5’ CGCYACGTTGACCTGGG 3’ 5’ CCGAAAAGTCGCCACGC 3’	2-Hydroxybenzalpyrovate aldolase (*nahE)*	620	Ferrero *et al.*, 2002
4	ALK1-F ALK1-R	5’ CATAATAAAGGGCATCACCGT 3’ 5’ GATTTCATTCTCGAAACTCCAAAC 3’	Alkane hydroxylase (*alkB)*	185	Kohno *et al.*, 2002
5	ALK2-F ALK2-R	5’ GAGACAAATCGTCTAAAACGTAA 3’ 5’ TTGTTATTATTCCAACTATGCTC 3’	Alkane hydroxylase (*alkMa)* & (*al*kMb)	271	Kohno *et al.*, 2002
6	ALK3-F ALK3-R	5’ TCGAGCACATCCGCGGCCACCA 3’ 5’ CCGTAGTGCTCGACGTAGTT 3’	Alkane hydroxylase *(alkB1)*	330	Kohno *et al.*, 2002

Table 3: Cont.

7	TBMD-F TBMD-R	5′ GCCTGACCATGGATGC(C/G)TACTGG 3′ 5′ CGCCAGAACCACTTGTC(A/G)(A/G)TCCA 3′	Toluene/ benzene monoxygenase large subunit (*tbmD*)	640	Hendrickx *et al.*, 2006
8	TMOA-F TMOA-R	5′ CGAAACCGGCTT(C/T)ACCAA(C/T)ATG 3′ 5′ ACCGGGATATTT(C/T)TCTTC(C/G)AGCCA 3′	Toluene monooxygenase large α subunit (*tmoA*)	505	Hendrickx *et al.*, 2006
9	XYLA-F XYLA-R	5′ CCAGGTGGAATTTTCAGTGGTTGG 3′ 5′ AATTAACTCGAAGCGCCCACCCCA 3′	Xylene monoxygenases (TOL pathway) (*xylA*)	291	Hendrickx *et al.*, 2006
10	XYLE1-F XYLE1-R	5′ CCGCCGACCTGATC(A/T)(C/G)CATG 3′ 5′ TCAGGTCA(G/T)CACGGTCA(G/T)GA 3′	Catechol extradiol dioxygenases (*xylE1*)	242	Hendrickx *et al.*, 2006
11	TODC1-F TODC1-R	5′ CAGTGCCGCCA(C/T)CGTGG(C/T)ATG 3′ 5′ GCCACTTCCATG(C/T)CC(A/G)CCCCA 3′	Aromatic dioxygenases large subunit (TOD pathway) (*todC1*)	510	Hendrickx *et al.*, 2006
12	BEDC1-F BEDC1-R	5′ TCGTCGTCAGACACTACGTA 3′ 5′ AATCTGATGCTTGCCATCATGG 3′	Alpha subunit of benzene dioxygenase (*bedC1*)	358	Wang *et al.*, 2008
13	ALKB1-F ALKB1-R	5′ AAYACNGCNCAYGARCTNGGNCAYAA 3′ 5′ GCRTGRTGRTCNGARTGNCGYTG 3′	Alkane monooxygenase (*alkB)*	550	Kloos *et al.,* 2006

3.11 Detection of hydrocarbon degrading genes by PCR

Extracted genomic DNA from pure strains was used as template for the amplification of various hydrocarbon degrading genes using the primer set in section (3.9). PCR reaction was performed in total volume of 25 µl, with 1.3 µM forward and reverse primers, 0.2 mM dNTP mix, 1µl of Taq DNA polymerase corresponding to 0.5-1 Unit (QIAGEN), 1X incubation buffer containing 1.5 mM $MgCl_2$, 10-20 ng of DNA was added, and the water volume was adjusted accordingly.

The PCR program was set as follows: first cycle: denaturation at 94 ^oC for 3 minutes, 35 cycles: denaturation at 94 ^oC for 45 seconds, various annealing temperatures used according to each primer (45, 50, 52, 54, and 56 ^oC) for 45 seconds, polymerization at 72 ^oC for 1 minute, and elongation step for 7 minutes at 72 ^oC. PCR products were run on 1% or 1.2% agarose gel electrophoresis, stained with 0.6 µg/ml ethidium bromide under 50 or 100 Volts/ cm and visualized using UV documentation system.

4.1 Screening of *Pseudomonas aeruginosa* Strains on MSM Medium

The degradation of hydrocarbons by bacteria is known to be a complex process. Many factors play a fundamental role in influencing the rate and effectiveness of hydrocarbon degradation. Among the formerly discussed factors (section 2.3.2) distinguished to hinder the degradation of hydrocarbons are nutrients availability and concentration of hydrocarbons present in the microenvironment of microbial cells (Button and Robertson, 1986; Coulon *et al.,* 2005; Jasmine and Mukherji, 2014).

This study was conducted in order to characterize the nutritional profile as well as the biodegradation potential among three selected *Pseudomonas aeruginosa* strains, which were previously isolated from contaminated sites in Qatar (Al Disi *et al*., 2015). Our *P. aeruginosa* strains (QDD1, QDD8, and QDD9) have been screened over two minimal salt media with different nitrogen sources and C/N ratios. MSM1 contains ammonium nitrate (NH_4NO_3) as a nitrogen source at a concentration of 1.4 g of nitrogen/l with the corresponding C/N ratio of 60/1. MSM2 contains ammonium chloride (NH_4Cl) as a nitrogen source at a concentration of 1.05 g of nitrogen/l with the corresponding C/N ratio of 80/1. These two media are the most cited in literature (Coulon *et al.,* 2005). In fact, this can be considered as a comparative study of two parameters; the type of inorganic nitrogen source and the different C/N ratios. The variability of nitrogen in source type and concentration was the core of this study, due to its major role in hydrocarbon degradation among other nutritional requirements (Coulon *et al.,* 2005).

In addition, strains were subjected at the same time to two diesel concentrations as the sole carbon source, 5% (v/v) and 10% (v/v). Studies have shown a proportional relation between hydrocarbons concentration and the uptake by microbial cells as well as growth. However, this might not be the situation when cells encounter high molecular weight and immiscible hydrocarbons (Boethling and Alexander, 1979). Consequently, four different growth conditions resulted: MSM1 5% diesel, MSM1 10% diesel, MSM2 5% diesel, and MSM2 10% diesel.

Figure 7 shows the growth results of *P. aeruginosa* strains in the different growth conditions over a week period obtained as CFU counts. Growth results are represented as the average 10^7 cells/ml.

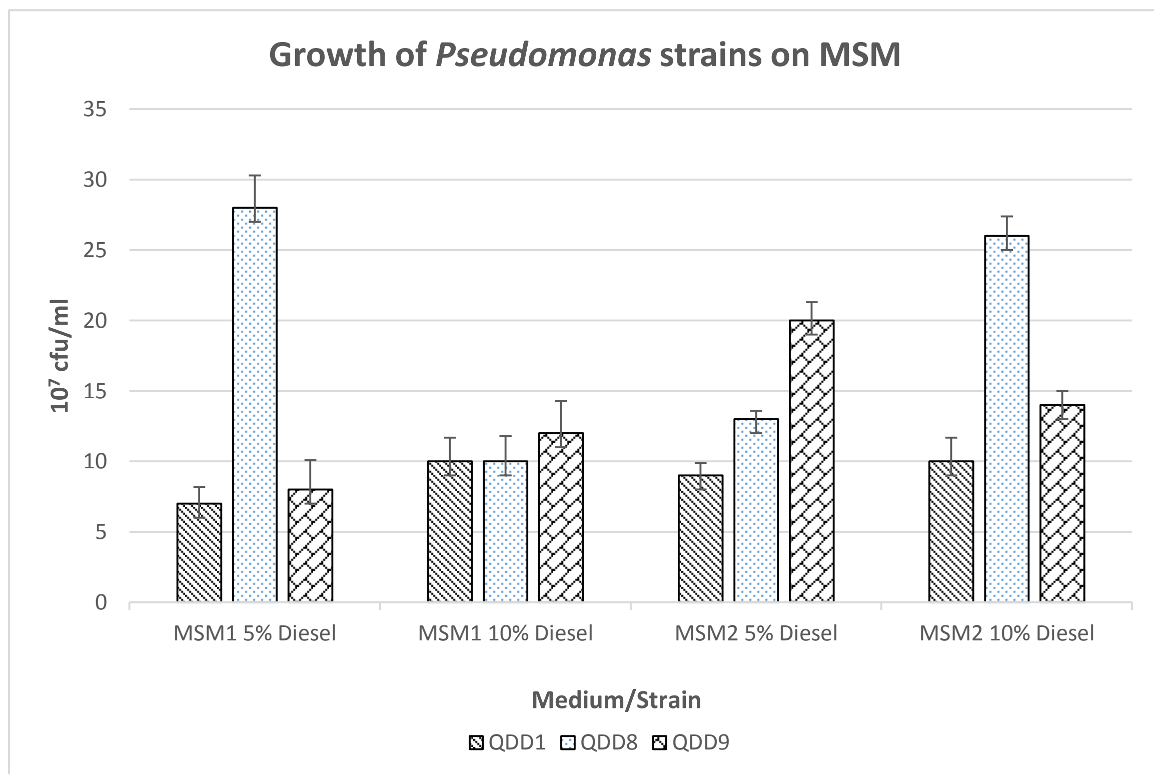

Fig. 7: Screening of *Pseudomonas aeruginosa* strains on two media; MSM1 and MSM2 under 5% and 10% diesel over a week period (average of triplicate with the standard error).

The results shown in Figure 8 demonstrate how the components of culturing medium are crucial for growth and tolerance to toxicity. Some strains may be tolerant to higher diesel concentrations in a medium, but not in another. For instance, the growth of strain QDD8 increased in MSM2 medium, when diesel concentration was elevated, from 13 to 26 10^7 cells/ml. However, when diesel concentration was increased in MSM1 from 5% to 10%, the growth of QDD8 has decreased from 28 to 10 10^7 cells/ml. Similar phenomenon was observed with strain QDD9, yet growth was inhibited under higher diesel concentrations in MSM2 rather than MSM1.

For strain QDD8, the optimal growth was detected with 5% diesel and C/N ratio of 60/1 of ammonium nitrate (MSM1). Whilst for strain QDD9, the optimal growth was observed with 5% diesel and C/N ratio of 80/1 of ammonium chloride (MSM2). On the other hand, strain QDD1 maintained similar growth under the two examined nitrogen sources and C/N ratios, while its growth was generally enhanced when diesel concentration increased in both media.

High variability within the same group of isolates is noticeable. Even that our strains are all *Pseudomonas aeruginosa* isolates, they had different types of activity toward various growth conditions.

Inhibition of microbial growth under high diesel concentrations has been reported in many bioremediation studies. In fact, diesel at certain concentrations could be toxic to microorganisms via the solvent impacts that can alter the plasma membrane (Shukor *et*

al., 2009; Heipieper and Martínez, 2010). Since our strains have tolerated high diesel concentrations of 10% (v/v), they can be considered interesting candidates for bioremediation application.

However, our strains' evaluation in tolerating the growth under high diesel concentrations and various nitrogen sources is still limited until we analyze their capabilities in degrading diesel hydrocarbons. The removal of low MW and high MW hydrocarbons was monitored using GC analysis. A sample of the diesel layer from each culture was analyzed and the percentage of removal efficiency (RE) of the most representative hydrocarbons was calculated from the obtained chromatograms. Results are illustrated in Figures 8, 9, and 10 for strains QDD1, QDD8, and QDD9 respectively. Each figure shows the strain behavior under the four growth conditions plotted as RE (%) versus the retention time (in minutes) of the selected peaks. Certainly, the calculation of RE considered the abiotic control of each condition (lacking inoculum) as a reference for both the initial area under the hydrocarbon peak and the retention time by which peaks have been selected.

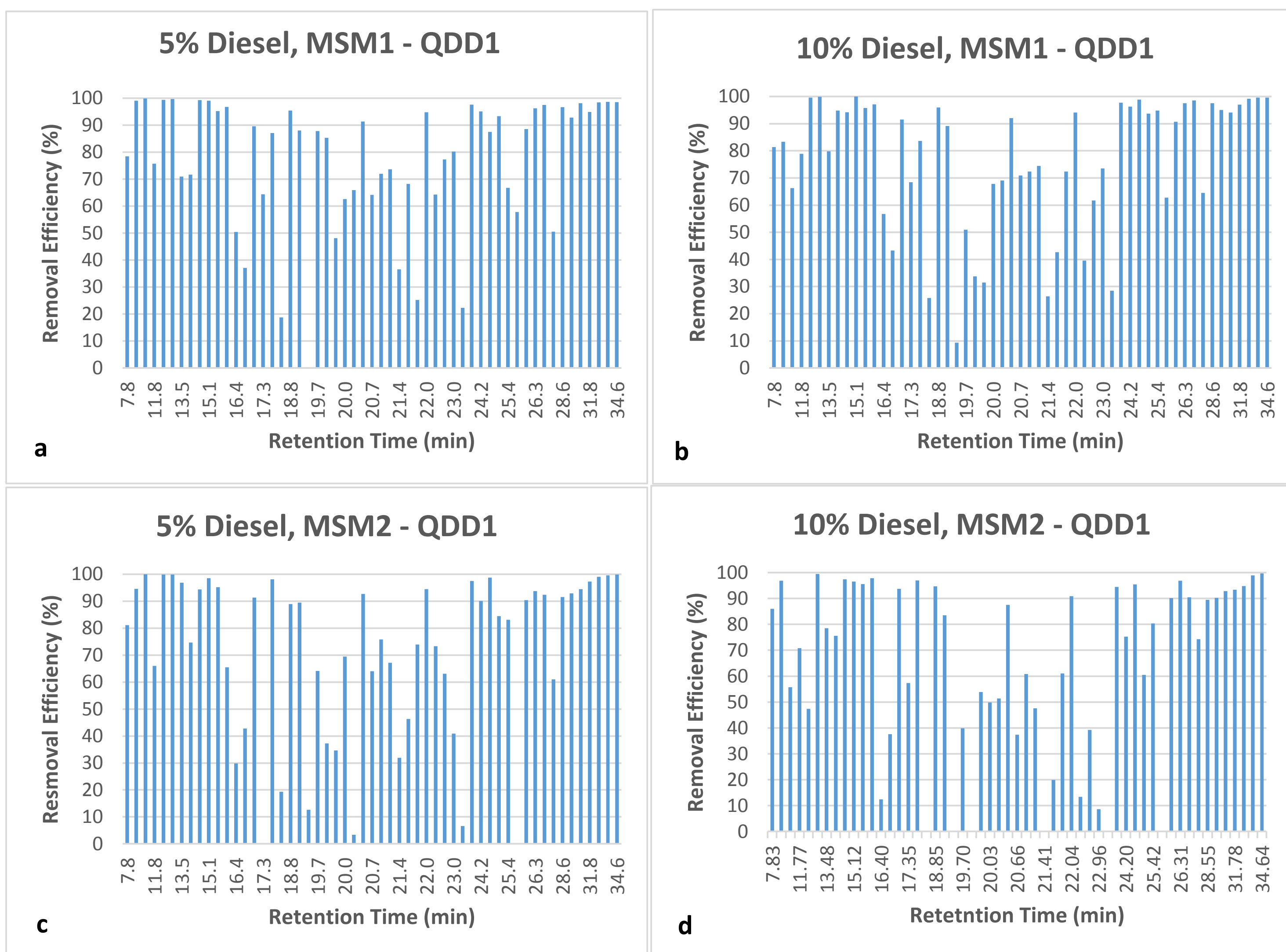

Fig. 8: Percentage of Removal Efficiency (% RE) of selected hydrocarbons by their retention time in the corresponding GC chromatograms over one week period for strain QDD1 under four different growth conditions. (a) MSM1 medium and 5% diesel, (b) MSM1 medium and 10% diesel, (c) MSM2 medium and 5% diesel, (d) MSM2 medium and 10% diesel

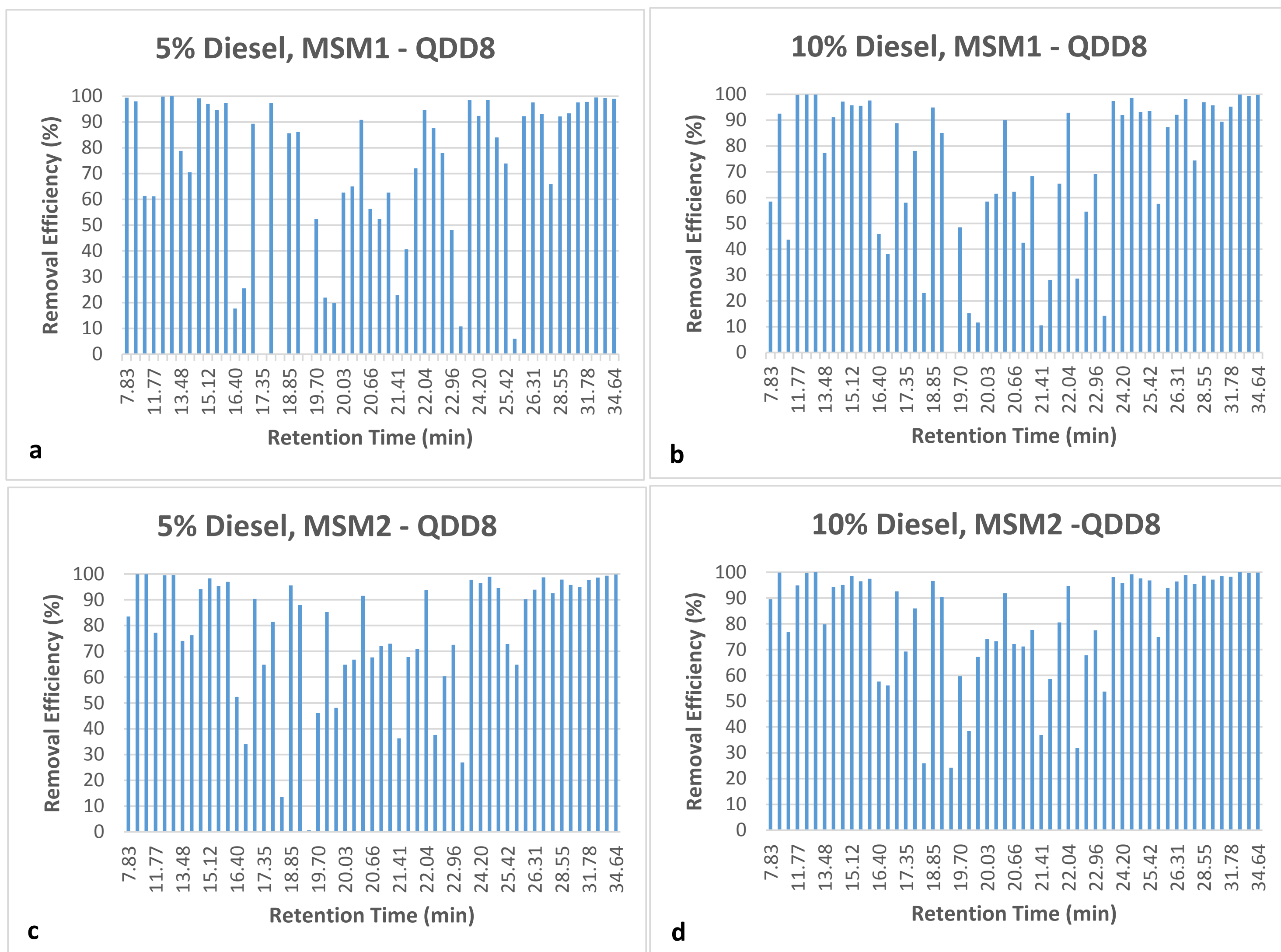

Fig. 9: Percentage of Removal Efficiency (% RE) of selected hydrocarbons by their retention time in the corresponding GC chromatograms over one week period for strain QDD8 under four different growth conditions. (a) MSM1 medium and 5% diesel, (b) MSM1 medium and 10% diesel, (c) MSM2 medium and 5% diesel, (d) MSM2 medium and 10% diesel

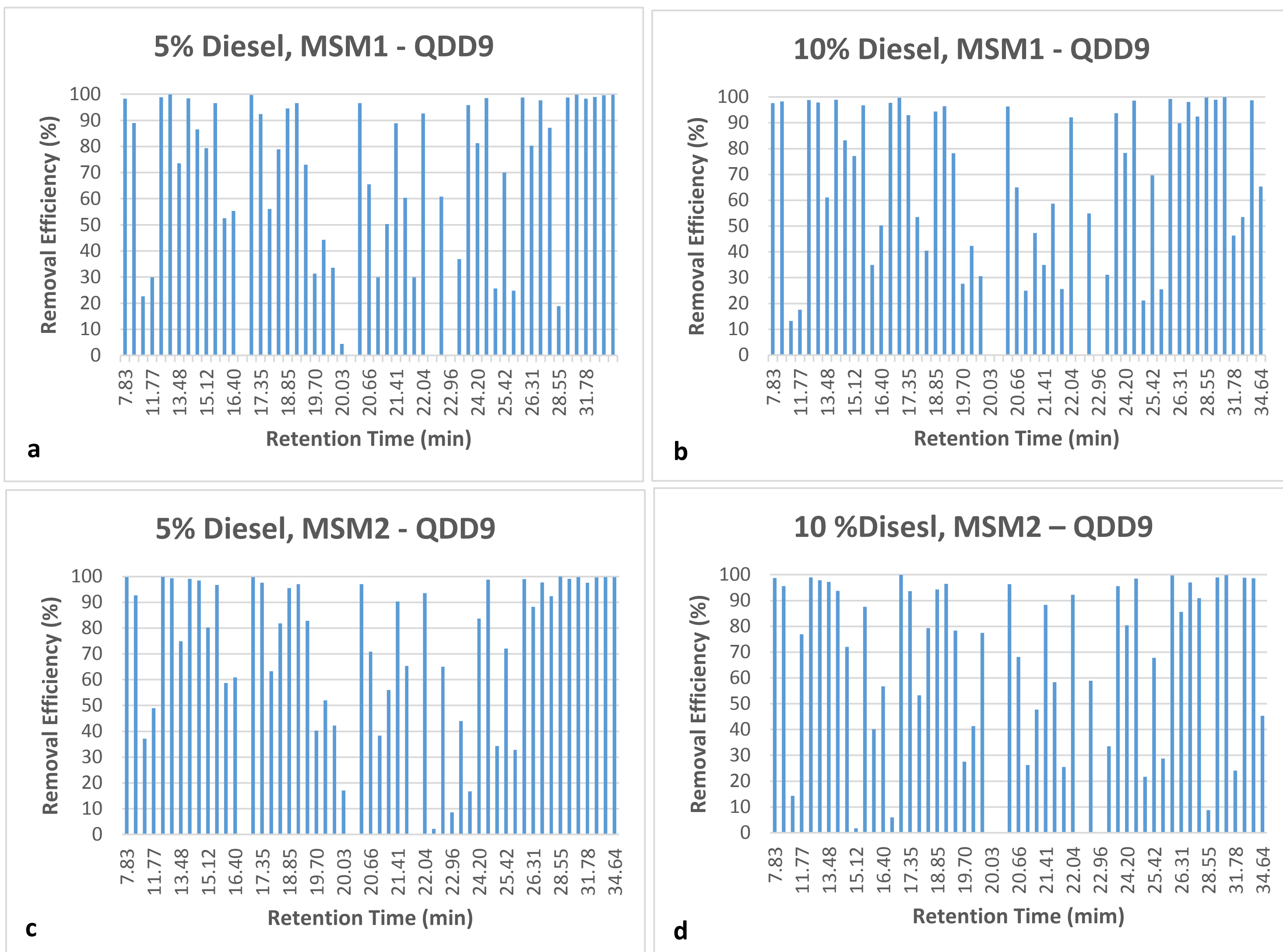

Fig. 10: Percentage of Removal Efficiency (% RE) of selected hydrocarbons by their retention time in the corresponding GC chromatograms over one week period for strain QDD9 under four different growth conditions. (a) MSM1 medium and 5% diesel, (b) MSM1 medium and 10% diesel, (c) MSM2 medium and 5% diesel, (d) MSM2 medium and 10% diesel

The GC analysis results clearly confirm the biological capability of our strains to degrade diesel hydrocarbons under both concentrations 5% and 10%. Still, no specific removal pattern among strains could be derived from these results. The spectrum of hydrocarbons removal is indicative, but cannot be the only information source of the ability of each strain to degrade hydrocarbons. Some hydrocarbons are converted or partially degraded while others are removed. The technique is limited particularly in the case of complex mixtures of hydrocarbons like diesel.

The study of the role of two nitrogen sources at different C/N ratios and two diesel concentrations on growth and biodegradation using our three strains of *P. aeruginosa* spotted the light on how crucial is the nutritional microenvironment in influencing the proliferation as well as the biological activities of bacterial strains. In order to have a deeper and broader understanding of the role of nutritional requirements on hydrocarbon degrading strains, factorial experiment was designed incorporating C, N, and P effects. The following section elaborates the rationale of the experiment besides the main results.

4.2 Factorial Investigation of Strains' Potentiality to Degrade Diesel Hydrocarbons

As far as we know, there is a gab in the published scientific knowledge concerning the role of nutritional elements such as carbon, nitrogen, and phosphorous on orientation of growth and biodegradation potential of bacterial strains. The originality of this work is the integration of the most essential nutritional elements as well as their interactions in one experimental frame. For that target, a three-factor experimental strategy was designed to examine the interaction and effect of carbon, nitrogen, and phosphorous sources on cell growth as well as the degradation of diesel hydrocarbons. Unlike the classical methods of conducting research, factorial experimental design gives researchers the chance to study, analyze, and compare more than one factor at a time.

As elaborated in section (3.2.3), each factor consisted of two levels (high and low concentration); attempting to expose the strains to a variety of conditions ranging from minimal up to excess nutritional requirements. Diesel, as the sole carbon source, was tested at 5% (v/v) and 20% (v/v). Nitrogen, represented in ammonium nitrate (NH_4NO_3), was manipulated between 2 g/l and 8 g/l. While phosphorous source from both sodium phosphate (Na_2HPO_4) and potassium phosphate (KH_2PO_4) varied between 1g/l and 0.265g/l to 4g/l and 1.06g/l respectively. Other components were kept in agreement with the original minimal salt medium (MSM) as described in section (3.2.2). Our *P. aeruginosa* strains were subjected to eight different growth conditions, each of particular C, N, and P

composition. For growth monitoring, the CFU counts were collected after the first and the second week of culturing. The diesel fraction of each experimental condition was sampled at the same time and analyzed using GC in order to correlate the growth results with hydrocarbon removal.

Figure 11 (a, b, c) demonstrates the growth results as CFU counts represented in 10^7 cells/ml. The average of triplicate CFU was plotted versus the time (week1 and week2). The standard error (SE) is also shown in the figure as bars on the head of each column.

Results depict the clear variations of strains' response to the manipulated nutritional parameters in culturing media. The growth of each *P. aeruginosa* strain was affected differently to changing C/N/P ratios. At this point, these dissimilarities cannot be reliable to derive conclusions unless tested for significance. Therefore, the obtained triplicate CFU results were tested for statistical significance using Minitab software (section 3.6). The analysis of variance (ANOVA) examined the individual effect of each nutritional element as well as the first order interactions between each two elements, besides the second order interaction among the three elements together. A summarized ANOVA table was constructed for all strains under the eight different growth conditions and over the two weeks period. Red stars indicated the significant results, while not significant findings were designated by (ns) (Table 4).

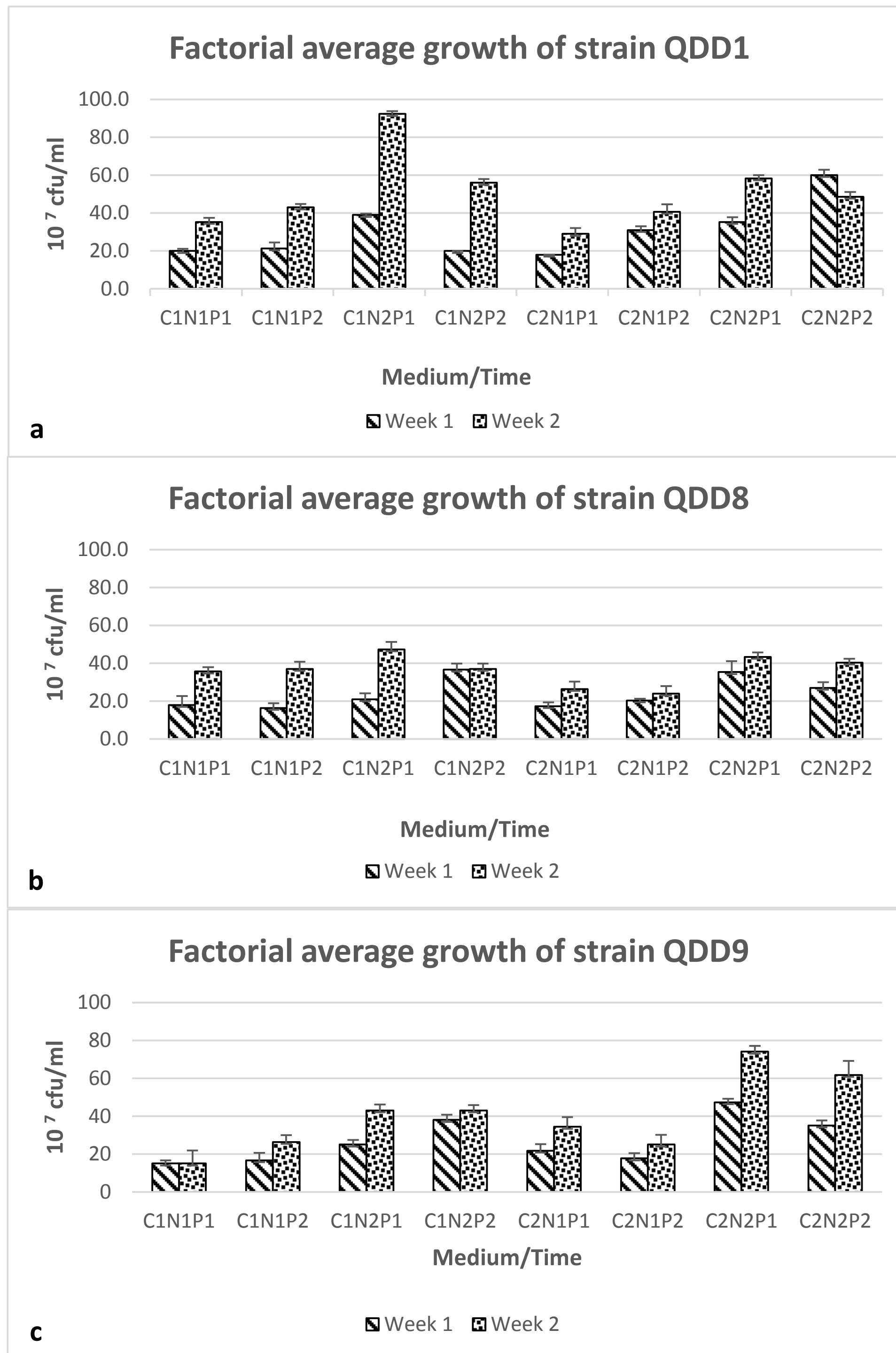

Fig. 11: Growth results of factorial investigation of C, N, and P nutritional elements: average of triplicate cfu (10^7 cells/ml) of three strains (a) QDD1, (b) QDD8 and (c) QDD9 over two weeks period under eight different growth conditions. 1 represents low concentration and 2 represents high concentration as in C1N2P1 for example.

Table 4: Overall ANOVA of three strains QDD1, QDD8, and QDD9 over two-week period. (S.O.V.) is the source of variation. (D.F.) is the degrees of freedom. (MS) is the means of squares. Factors and their interactions are shown as C: carbon, N: nitrogen, and P: phosphorous. One star (*) means significant, two stars (**) mean highly significant, and (ns) means not significant.

		MS					
		Week1			Week2		
S. O. V.	**D.F.**	**QDD1**	**QDD8**	**QDD9**	**QDD1**	**QDD8**	**QDD9**
C	1	726**	24ns	273.38**	937.5**	198.38*	1717**
N	1	1536**	864**	2072.04**	4320.2**	759.38**	5490.4**
P	1	150**	28.17ns	1.04ns	266.7**	77.04ns	40ns
C*N	1	308.17**	0.67ns	51.04ns	400.2**	176.04*	376*
C*P	1	1148.17**	140.17ns	360.38**	352.7**	5.04ns	408.4*
N*P	1	28.17ns	13.5ns	3.37ns	1600.7**	57.04ns	77ns
C*N*P	1	384**	308.17**	145.04*	192.7**	45.38ns	26ns
Error	16	11.46	36.46	24.04	17.7	32.83	72.9

The interpretation of statistical analysis results will be detailed for each strain separately trying to understand how the growth of these hydrocarbon degraders is influenced by the nutritional microenvironment.

4.2.1 Strain QDD9, Week1

The analysis of variance (ANOVA) showed that the effects of both C and N were highly significant while P effect was not significant for Strain QDD9 in week1. When carbon was increased in the media from (Low: 5%) to (High: 20%), the growth was increased significantly from 23.7 to 30.4 10^7 cells/ml. In addition, when nitrogen was increased in the media from (Low: 2 g/l) to (High: 8 g/l), the growth was increased

significantly from 17.8 to 36.3 10^7 cells/ml. However, increasing phosphorus did not affect the growth significantly (Table 5).

The first order interaction between C and P was highly significant while both (C x N) and (N x P) interactions were not significant. The results showed that the combination of high carbon and high phosphorus increased the growth significantly (Table 5). When computing the Least Significant Difference (LSD) between the means, results helped revealing which exact combination was the source of significance. All possible interactions are represented as follows: (C1P1 * C1P2), (C2P1 * C2P2), (C1P1 * C2P1), (C1P2 * C2P2), (C1P1 * C2P2), and (C1P2 * C2P1). The 1 stands for low concentration, while 2 indicates the high concentration. LSD results showed that only when we shift the culture medium from low carbon, high phosphorous to high carbon, high phosphorous, growth was not affected significantly. All other interactional changes between carbon and phosphorous affected growth significantly.

The analysis of variance demonstrated that second order interaction between the three factors (C x N x P) was significant for this case. Hence, adding high carbon, high nitrogen, and high phosphorous to the medium affected growth significantly. The interaction (C1N1P1 * C2N2P1) showed the highest significant difference on growth from 15 to 47.3 10^7 cells/ml (Table 5). Further, the combination of high C, high N, and low P gave the highest cell growth 47.3 10^7 cells/ml.

4.2.2 Strain QDD9, Week2

Here, the analysis of variance is performed for the same strain after the second week of growth. Single factor analysis indicated that the effects of C and N were highly significant, while the effect of P was not. The same result was found in the first week. However, when carbon was increased in the media from (Low: 5%) to (High: 20%), the growth was increased significantly from 31.85 to 48.75 10^7 cells/ml. Further, when nitrogen was increased in the media from (Low: 2 g/l) to (High: 8 g/l), the growth was increased significantly from 25.2 to 55.4 10^7 cells/ml.

On the other hand, the first order interaction (C x N) was significant along with the interaction (C x P) which showed significant effect from the first week. Whilst, the interaction between N and P was not significant. LSD results for (C x N) interaction showed that increasing the carbon from low to high while maintaining nitrogen low had no significant effect on growth; unlike all other combinations. With regard to the (C x P) interaction; the only combinations that were not the source of significant impact on cell growth were (C1P1 * C1P2) and (C1P2 * C2P2) (Table 5).

By the second week of growth, the second order interaction (C x N x P) had no longer significant impact on cells growth of strain QDD9.

Statistical Profile of strain QDD9 Week1

I

C	N	P	Mean
C1	N1	P1	15
		P2	16.7
	N2	P1	25
		P2	38
C2	N1	P1	21.7
		P2	17.7
	N2	P1	47.3
		P2	35

II

	N-1	N-2	Mean
C-1	15.8	31.5	23.7
C-2	19.7	41.2	30.4
Mean	17.8	36.3	

III

	P-1	P-2	Mean
C-1	20	27.4	23.7
C-2	34.5	26.4	30.5
Mean	27.3	26.9	

IV

	P-1	P-2	Mean
N-1	18.35	17.2	17.8
N-2	36.15	35.5	35.8
Mean	27.3	26.4	

Statistical Profile of strain QDD9 Week2

I

C	N	P	Mean
C1	N1	P1	15.0
		P2	26.3
	N2	P1	43.0
		P2	43.0
C2	N1	P1	34.3
		P2	25.0
	N2	P1	74.0
		P2	61.7

II

	N-1	N-2	Mean
C-1	20.7	43	31.85
C-2	29.7	67.8	48.75
Mean	25.2	55.4	

III

	P-1	P-2	Mean
C-1	29	34.7	31.85
C-2	54.2	43.3	48.75
Mean	41.6	39	

IV

	P-1	P-2	Mean
N-1	24.7	25.7	25.2
N-2	58.5	52.3	55.4
Mean	41.6	39	

Table 5: Summary of triplicate average cfu/ml results of strain QDD9 over week1 and week2 periods. (I) the mean values of second order interaction (C*N*P), (II) the mean values of first order interaction (C*N), (III) the mean values of the first order interaction (C*P), and (IV) the mean values of the first order interaction (N*P).

4.2.3 Strain QDD8, Week1

For the first week of culture growth, the analysis of variance of strain QDD8 showed that N has highly significant impact on growth while C and P do not. In other words, higher concentration of nitrogen in the medium increased growth from 18 to 30 10^7 cells/ml (Table 6).

According to ANOVA results, none of the first order interactions (C x N), (C x P), nor (N x P) exhibited significant effect on cell growth. However, the second order interaction (C x N x P) was highly significant. This means that supplying the culture medium with high concentrations of carbon, nitrogen, and phosphorous had significant impact on the growth of strain QDD8 cells. The factor combination that showed the highest significant difference was (C1N1P2 * C1N2P2). Under this cultural condition, growth increased significantly from 16.3 to 36.7 10^7 cells/ml (Table 6). The highest cell growth (36.7 10^7 cells/ml) was noticed under the condition C1N2P2.

4.2.4 Strain QDD8, Week2

Strain QDD8 demonstrated different behavior in the second week of growth. The analysis of variance showed that the C effect became significant as well as the N effect. Although, phosphorous effect remained insignificant. Noticeably, growth exhibited a decrease from 39.25 to 33.5 10^7 cells/ml when carbon concentration was increased in the medium from 5% to 20%. However, when nitrogen was increased in the medium from 2 g/l to 8 g/l, growth increased from 30.75 to 42 10^7 cells/ml (Table 6).

The first order interaction of (C x N) showed significant effect by the second week, though; both (C x P) and (N x P) did not. LSD results revealed the sources of significance to be the following combinations: (C2N1 * C2N2), (C1N1 * C2N1), and (C1N2 * C2N1).

As cells grow over a two week period, significant effect of the second order interaction (C x N x P) disappeared. Thus, the combination of high carbon, high nitrogen, and high phosphorous were no longer affecting the growth significantly (Table 4 and 6).

Statistical Profile of strain QDD8 Week1

I

C	N	P	Mean
C1	N1	P1	18.0
		P2	16.3
	N2	P1	21.0
		P2	36.7
C2	N1	P1	17.3
		P2	20.3
	N2	P1	35.3
		P2	27.0

II

	N-1	N-2	Mean
C-1	17.2	28.8	23
C-2	18.8	31.2	25
Mean	18	30	

III

	P-1	P-2	Mean
C-1	19.5	26.5	23
C-2	26.3	23.7	25
Mean	22.9	25.1	

IV

	P-1	P-2	Mean
N-1	17.7	18.3	18
N-2	28.2	31.8	30
Mean	22.95	25.05	

Statistical Profile of strain QDD8 Week2

I

C	N	P	Mean
C1	N1	P1	35.7
		P2	37.0
	N2	P1	47.3
		P2	37.0
C2	N1	P1	26.3
		P2	24.0
	N2	P1	43.3
		P2	40.3

II

	N-1	N-2	Mean
C-1	36.3	42.2	39.25
C-2	25.2	41.8	33.5
Mean	30.75	42	

III

	P-1	P-2	Mean
C-1	41.5	37	39.25
C-2	34.8	32.2	33.5
Mean	38.15	34.6	

IV

	P-1	P-2	Mean
N-1	31	30.5	30.75
N-2	45.3	38.7	42
Mean	38.15	34.6	

Table 6: Summary of triplicate average cfu/ml results of strain QDD8 over week1 and week2 periods. (I) the mean values of second order interaction (C*N*P), (II) the mean values of first order interaction (C*N), (III) the mean values of the first order interaction (C*P), and (IV) the mean values of the first order interaction (N*P).

4.2.5 Strain QDD1, Week1

Strain QDD1 demonstrates quite odd results upon the analysis of variance (ANOVA). Most of its results appeared to be highly significant. Single factor analysis showed that all C, N, and P have highly significant impact on cell growth (Table 4). In the first week of culture, growth has increased from 25.1 to 36.1 10^7 cells/ml when carbon was increased in the medium up to 20% diesel. In addition, high nitrogen concentration caused the cells to grow from 22.6 to 38.6 10^7 cells/ml. Further, when phosphorous represented as [Na_2HPO_4, KH_2PO_4] was increased in the medium from: 1g/l and 0.265g/l to 4g/l and 1.06g/l respectively, cell growth increased significantly from 28.1 to 33.1 10^7 cells/ml (Table 7).

First order interaction of both (C x P) and (C x N) were highly significant, unlike the (N x P) which was not. In both significant interactions, it was interesting to know that the only insignificant combinations were (C1N1 * C2N1) and (C1P1 * C2P1) according to the LSD calculations. In other words, maintaining N and P low makes the increase in carbon concentration ineffective in raising the growth significantly.

Examining the second order interaction (C x N x P), ANOVA results showed that it had significant effect on growth. When computing LSD between the means, the largest significant difference was detected in (C2N1P1 * C2N2P2). Shifting the growth medium conditions from low N and low P to high N and P while keeping C in high concentration (20% diesel) increased the cell growth from 18 to 60 10^7 cells/ml (Table 7). In fact, the

cultural conditions that showed the highest growth of strain QDD1 cells in week 1 was C2N2P2.

4.2.6 Strain QDD1, Week2

By the second week of growth, all the analyzed factors seemed to have highly significant effect on cell growth. This means that single factor effect of C, N, and P, besides the first and the second order interactions were all significant. However, growth decreased significantly from 56.7 to 44.15 10^7 cells/ml when carbon was added to the medium in high concentration. Growth also witnessed a decrease from 53.75 to 47.1 10^7 cells/ml when phosphorous was raised. Dissimilarly, when nitrogen concentration was increased up to 8 g/l, cell growth increased significantly from 37 to 63.85 10^7 cells/ml (Table 7).

In the first order interactions, the source of significance was not from the following combinations (C1N1 * C2N1), (C2P1 * C2P2), or (C1P2 * C2P2). Unlike the case with all other strains; the interaction (N * P) is significant with all its combination in week 2 for strain QDD1. As carbon concentration is kept constant in the medium, (N2 x P1) allowed cells to grow up to 75.3 10^7 cells/ml. LSD results from the second order interaction (C x N x P) brought out that moving cells from medium containing low C, high N, low P to medium containing high C and low N and P had the highest significant difference. However, cells were observed to grow the highest under the interaction (C1 x N2 x P1) (Table 7).

Statistical Profile of strain QDD1 Week1

I

C	N	P	Mean
C1	N1	P1	20.0
		P2	21.3
	N2	P1	39.0
		P2	20.0
C2	N1	P1	18.0
		P2	31.0
	N2	P1	35.3
		P2	60.0

II

	N-1	N-2	Mean
C-1	20.7	29.5	25.1
C-2	24.5	47.7	36.1
Mean	22.6	38.6	

III

	P-1	P-2	Mean
C-1	29.5	20.7	25.1
C-2	26.7	45.5	36.1
Mean	28.1	33.1	

IV

	P-1	P-2	Mean
N-1	19	26.2	22.6
N-2	37.2	40	38.6
Mean	28.1	33.1	

Statistical Profile of strain QDD1 Week2

I

C	N	P	Mean
C1	N1	P1	35.3
		P2	43.0
	N2	P1	92.3
		P2	56.0
C2	N1	P1	29.0
		P2	40.7
	N2	P1	58.3
		P2	48.7

II

	N-1	N-2	Mean
C-1	39.2	74.2	56.7
C-2	34.8	53.5	44.15
Mean	37	63.85	

III

	P-1	P-2	Mean
C-1	63.8	49.5	56.65
C-2	43.7	44.7	44.2
Mean	53.75	47.1	

IV

	P-1	P-2	Mean
N-1	32.2	41.8	37
N-2	75.3	52.3	63.8
Mean	53.75	47.05	

Table 7: Summary of triplicate average cfu/ml results of strain QDD1 over week1 and week2 periods. (I) the mean values of second order interaction (C*N*P), (II) the mean values of first order interaction (C*N), (III) the mean values of the first order interaction (C*P), and (IV) the mean values of the first order interaction (N*P).

4.2.7 General Interpretation of the Statistical Analysis for All Strains

Upon the interpretation of statistical analysis of growth results, several insights can be deduced. Strains' growth was seen to be impacted by altering the carbon concentration from 5% to 20% diesel in the medium. Typically, cells tend to catabolize firstly the readily assimilable hydrocarbons. When we increase the carbon concentration, growth increases as the cell preferred hydrocarbons are present in larger quantities. However, as time passes, cells are obliged to approach the more complex compounds or the recalcitrant hydrocarbons. By the second week of culturing, high concentration of carbon affected the growth of some strains negatively (i.e. growth decreased). This can be explained due to cell consumption of easily degraded hydrocarbon; where the residual recalcitrant compounds became the only C source available. Hence, the rate of energy generation by such substrates is growth limiting as was previously reported by Boethling and Alexander (1979). For other cells, which exhibit what is known as “co-metabolism"; they need certain compounds to co-exist in the medium in order to be able to catabolize others (Horvath, 1972). As growth was inhibited by the increase of diesel concentration (the sole carbon source) in the medium, cells might be claimed to lack the necessary co-metabolism or the necessary co-substrates.

On the other hand, it was clearly observed that higher nitrogen concentration increased cells growth significantly of all strains both in the first and second week of incubation. In other words, all strains were positively sensitive to nitrogen concentration

in the medium. However, growth of strains generally seemed less affected by phosphorous concentration variation as was similarly found by Coulon *et al.,* (2005). In fact, it is known that attempts to enhance hydrocarbon degradation were usually reported by the addition of supplemental nitrogen and to a lesser degree phosphorous (Walworth and Reynolds, 1995; Braddock *et al.,* 1997).

Some strains were found more sensitive (e.g., QDD1) to C/N/P ratio than others (e.g., QDD8). Yet, C/N/P ratios were generally more limiting in the first week of growth. The growth medium might turn into saturation and the three-factor interaction is no longer significant by the second week of growth.

From another perspective, we yet have little knowledge regarding how strains approached diesel hydrocarbons as they grow in all these different conditions. Several questions can be raised: Did strains show behavioral patterns of attacking high MW or low MW hydrocarbons? How could the incubation period change/ remain stable/ enhance the biological activity from one week to two weeks?

In correlation with the growth results analysis, diesel removal was assessed using GC analysis. A sample of the diesel layer from each growth condition was obtained and prepared for GC. Following the GC run, retrieved chromatograms were analyzed and the RE was calculated. Figures 12 and 13 demonstrate the RE of our strains under two selected representative growth conditions C1N1P2 and C2N2P2, respectively. As the coding implies, condition C1N2P1 contains low carbon concentration (5% (v/v) diesel),

low nitrogen concentration (2 g/l NH_4NO_3), and high phosphorous concentration (4g/l and 1.06g/l of Na_2HPO_4 and KH_2PO_4 respectively). While condition C2N2P2 consists of high carbon concentration (20% (v/v) diesel), high nitrogen concentration (8 g/l NH_4NO_3), and high phosphorous concentration (4g/l and 1.06g/l of Na_2HPO_4 and KH_2PO_4 respectively).

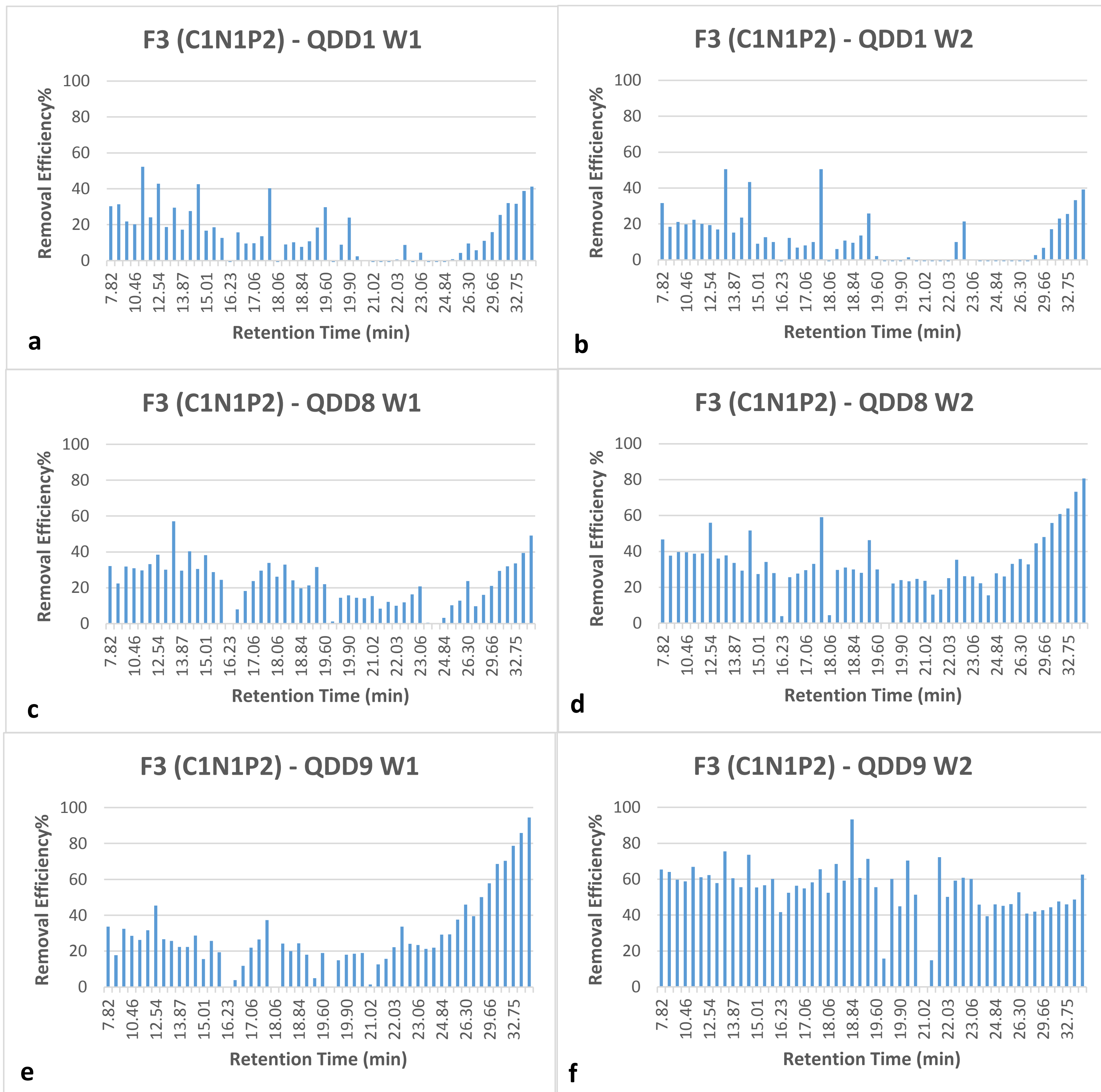

Fig. 12: Percentage of Removal Efficiency (% RE) of selected hydrocarbons by their retention time in the corresponding GC chromatograms over two week period for strains QDD1 (a and b), QDD8 (c and d), and QDD9 (e and f) under selected growth condition of low carbon, low nitrogen, and high phosphorous (C1N1P2).

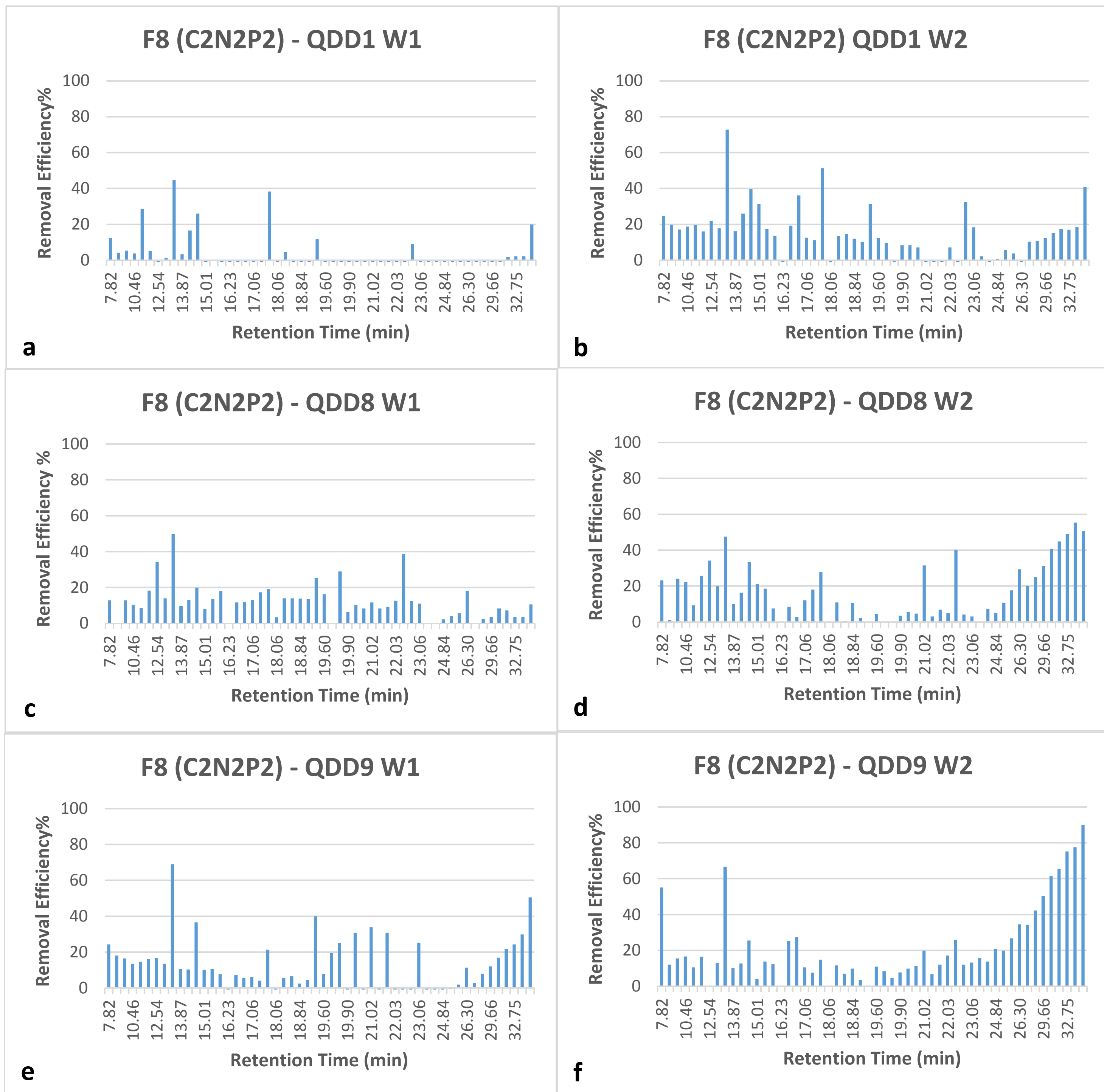

Fig. 13: Percentage of Removal Efficiency (% RE) of selected hydrocarbons by their retention time in the corresponding GC chromatograms over two week period for strains QDD1 (a and b), QDD8 (c and d), and QDD9 (e and f) under selected growth condition of high carbon, high nitrogen, and high phosphorous (C2N2P2).

The illustrated GC results further support the diversity among our *P. aeruginosa* strains. Biodegradation of diesel hydrocarbons represented in hydrocarbons RE varied considerably under different growth conditions. For instance, under growth condition C1N1P2, hydrocarbon removal was much higher for strains QDD8 and QDD9 compared to QDD1. On the other hand, when nutrients were in maximum concentrations, strains QDD1 and QDD8 exhibited lower hydrocarbons RE than QDD9. The shown GC figures are only a representative sample of the entire results under all growth conditions for the three strains.

Generally speaking, there were few noticeable observations among all strains. Under minimal nutrients concentration of C, N, and P as in condition (C1N1P1) strains exhibited low removal activity. However, when nitrogen was supplied in maximum concentration while C and P were minimal as in condition (C1N2P1), removal was improved (data not shown). This further emphasizes the role of nitrogen in enhancing both growth and biological activity of hydrocarbon degrading strains as formerly reported by Braddock *et al.,* (1997).

Mostly, all strains expressed enhanced removal activity by the second week of incubation. This can be explained by CFU results that show improved growth in week 2 (Figure 11). As the biomass increases, larger ratio of diesel could be approached. Furthermore, strains generally were able to attack high MW hydrocarbons only by the second week. As time passes, cells are able to proliferate in higher numbers and produce

larger quantities of bio-surfactants that facilitate the cell/diesel interaction and thus, its degradation (Xia *et al.,* 2014).

Obviously, nutrients concentration plays a critical role in hydrocarbons biodegradation activity of bacterial strains. Nevertheless, neither excess nor minimal concentrations are favorable for strains to express good biological removal of hydrocarbons, which supports the previous observations of Braddock *et al.,* (1997). Hence, maximum growth of a strain is not always translated into maximum RE of many hydrocarbons. In fact, the biological activity involved in degradation of diesel hydrocarbons is very complex. Sure, the objective of the microorganism is to get energy and carbon from the hydrocarbons, but many bioconversion reactions may occur, which can affect the GC chromatogram and cannot give a real idea on biodegradation.

Further, the cultural media may lead to production of different molecules and different concentrations of surfactants, which are key tools involved in hydrocarbons degradation. This may explain the fluctuations between compositions (especially Nitrogen) and between strains. Moreover, GC analysis as shown in the RE may fluctuate from one week to another, one condition to another, and one strain to another within 5 to 8% due to analytical issues.

The screening of strains under wider range of nutritional concentrations is recommended. As experimental ranges encompass the in-between values other than the maximum and minimum ranges, we have investigated. This might lead to a clearer view

of the optimal growth condition under which each strains produce considerable biomass and favor hydrocarbon removal.

4.3 Isolation of Genomic and Plasmid DNA from *Pseudomonas* strains

4.3.1 Genomic DNA Isolation

Having the objective of screening our *Pseudomonas aeruginosa* strains for their genetic potentiality in degrading a variety of hydrocarbons, the genomic DNA was extracted. Knowing that good quality DNA is a fundamental requirement for successful molecular investigations; two genomic DNA isolation methods have been adapted from previous research studies. Several steps have been added or modified in order to obtain high quality DNA accessible to further experiments. However, all other steps were followed precisely as described in references. The intensity of genomic DNA yielded from the first protocol was higher than that of the second (Figure 14). Method-I uses 100% ethanol for DNA precipitation versus isopropanol in the second method. Isopropanol is known to precipitate lower concentrations of nucleic acids particularly at low temperatures (Kennedy, 2009).

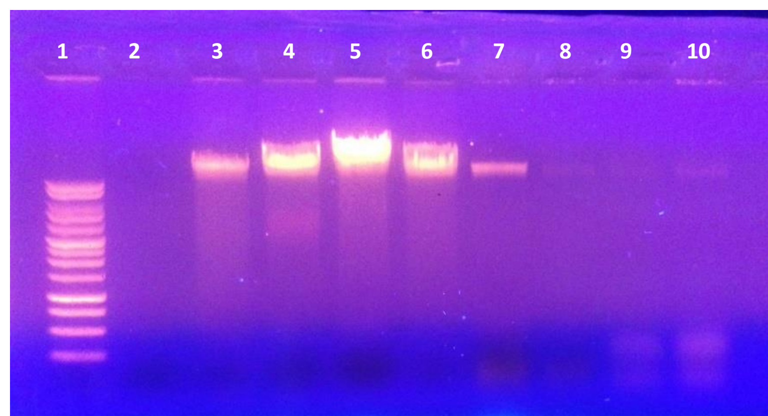

Fig. 14: Agarose gel electrophoresis (0.7%) of genomic DNA: (1) 1 Kb Ladder (250 bp, 500 bp, 750 bp, 1 kb, 1.5 kb, 2 kb, 2.5 kb, 3 kb, 4 kb, 5 kb, 6 kb, 8 kb, and 10 kb), (2) negative control, (3, 4, 5, 6) *E. coli* as standard gram-negative bacteria, QDD1, QDD8, and QDD9 using Method-I, (7, 8, 9, 10) the same strains using the Method-II

4.3.2 Plasmid DNA Isolation

Emerging from the fact that many hydrocarbon-degrading genes are localized on plasmid DNA, it was in our interest to extract the plasmid DNA from our *P. aeruginosa* strains. The goal was to assure the existence of plasmids among our strains, nonetheless; the available adapted extraction protocol has some limitations. Figure 15 demonstrates the isolated plasmid DNA on 0.7% agarose gel with *E. coli* strain serving as positive control of bacteria carrying known plasmid (pUC18).

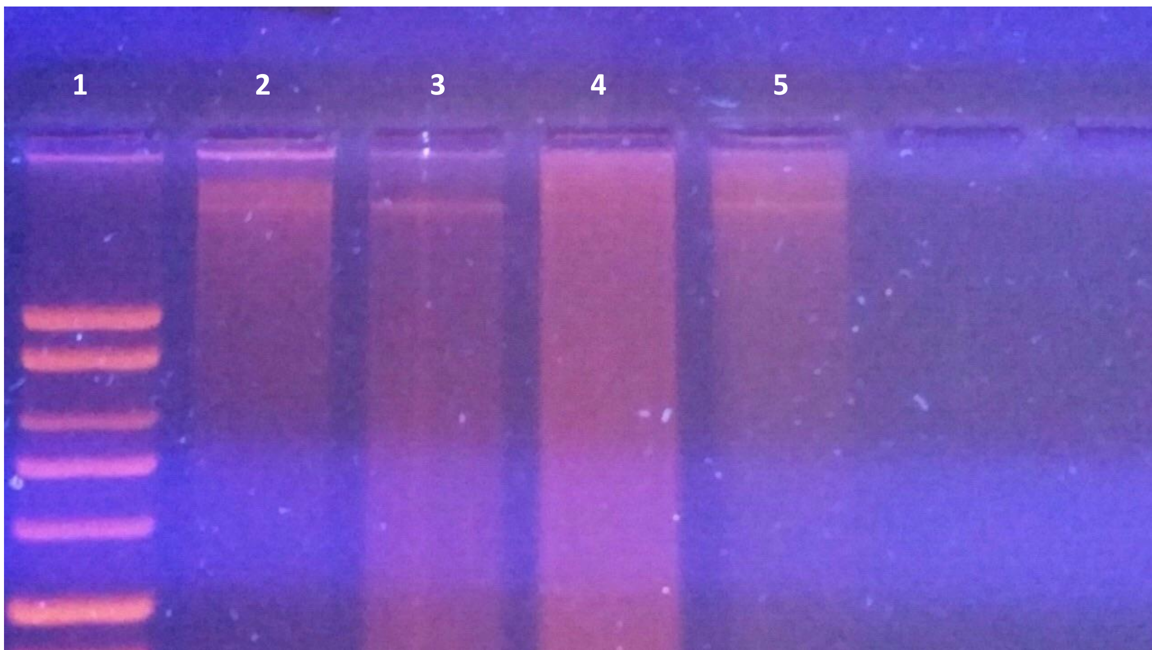

Fig. 15: Agarose gel electrophoresis (0.7%) of plasmid DNA: (1) 1 Kb Ladder (250 bp, 500 bp, 750 bp, 1 kb, 1.5 kb, 2 kb, 2.5 kb, 3 kb, 4 kb, 5 kb, 6 kb, 8 kb, and 10 kb), (2) *E. coli* TOP10 (plasmid pUC18), (3) QDD8, (4) QDD9, (5) QDD1

4.4 Detection of hydrocarbon degrading genes by PCR (optimization study)

Using Polymerase Chain Reaction (PCR), we attempted to screen our *P. aeruginosa* strains for the presence of certain genes encoding for a variety of hydrocarbon catabolic enzymes. As described earlier in the primer selection section (3.9), the genes were chosen over a wide range of hydrocarbons; from aliphatic n-alkanes, to single ring aromatics (BTEX), and to polycyclic aromatic hydrocarbons (PAHs). The template DNA used in these PCR reactions was the extracted genomic DNA, since chromosomal DNA extraction protocols are known to co-precipitate some plasmid DNA as well particularly those of large molecular weight. Therefore, we would be able to detect the presence of a certain gene whether it is chromosomal or plasmid borne.

As a preliminary screening step, our strains have been tested against all primers set under annealing temperature 50 ^{o}C. The reaction conditions and the PCR program were detailed in section (3.10). The ribotyping universal primer pair RipS73 (5' AGAGTTTGATCCTGGCTCAG 3') and RipS74 (5' AAGGAGGTGATCCAGCCGCA 3') which has been used to identify our strains (Xiao and Zhang, 2011) was included in our first screening as sort of positive control of known expected amplicon 1.5 kb. The DNA ladders used were 1 Kb (250 bp, 500 bp, 750 bp, 1 kb, 1.5 kb, 2 kb, 2.5 kb, 3 kb, 4 kb, 5 kb, 6 kb, 8 kb, and 10 kb) and 100 bp (100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1 kb, and 1.5 kb). Figure 16 shows the PCR products of strain QDD1 over the 13 primer

pairs besides the RipS73/ RipS74. In this initial screening step, negative control was run for the Rip primer pair only. For simplicity, primers are referred to as numbers from 1 to 13 as displayed in Table 3.

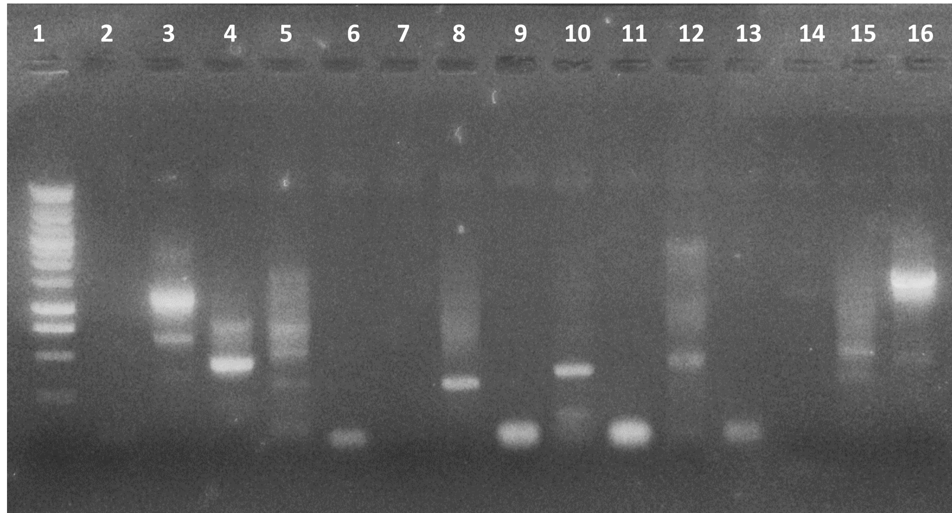

Fig. 16: PCR amplification of the indicated catabolic genotypes using selected primers set in this study for strain QDD1, T_H = 50 °C. 1% agarose gel under 100 Volts/ cm. (1) 1 kb DNA ladder (250 bp, 500 bp, 750 bp, 1 kb, 1.5 kb, 2 kb, 2.5 kb, 3 kb, 4 kb, 5 kb, 6 kb, 8 kb, and 10 kb), (2) Negative control of Rip, (3) P1, (4) P2, (5) P3, (6) P4, (7) P5, (8) P6, (9) P7, (10) P8, (11) P9, (12) P10, (13) P11, (14) P12, (15) P13, (16) Rip (1.5 kb)

Figure 17 (a, b) illustrates the PCR amplification results for strain QDD8 under the same previous conditions for primer pair 1 (P1) to primer pair 13 (P13) plus the Rip primer.

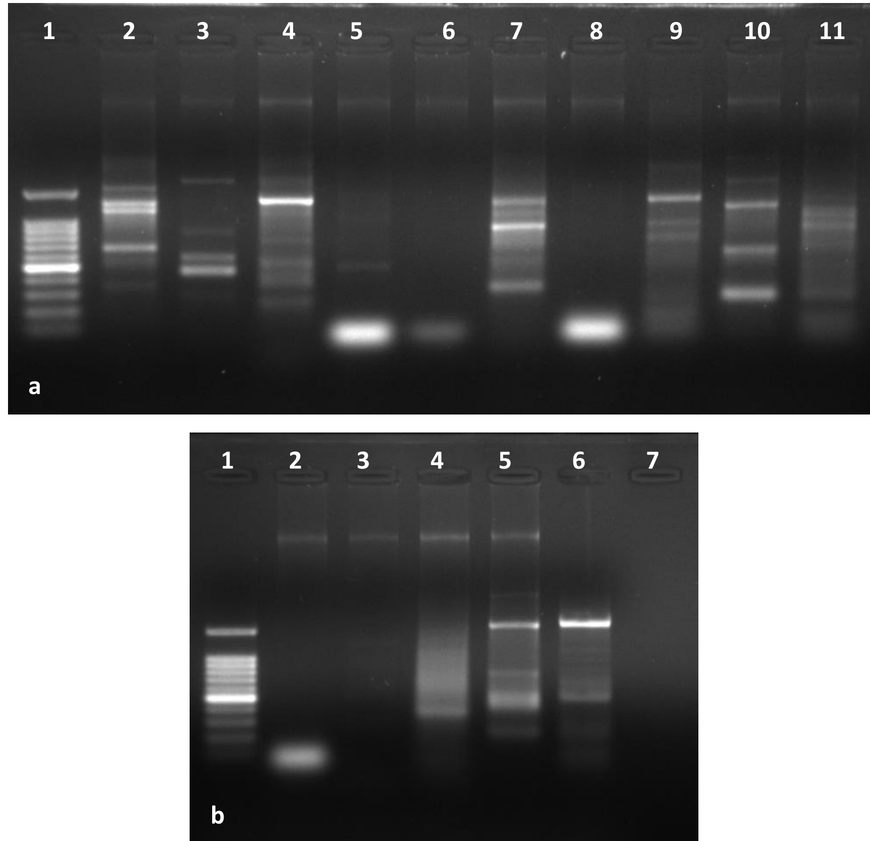

Fig. 17: PCR amplification of the indicated catabolic genotypes using selected primers set in this study for strain QDD8, T_H = 50 ^oC. 1.2% agarose gel under 50 Volts/ cm.

a: (1) 100 bp DNA ladder (100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1 kb, and 1.5 kb), (2) P1, (3) P2, (4) P3, (5) P4, (6) P5, (7) P6, (8) P7, (9) P8, (10) P9, (11) P10

b: (1) 100 bp DNA ladder, (2) P11, (3) P12, (4) P13, (5) Rip 1.5 kb, (6) Rip 1.5 kb [repeated], (7) negative control of Rip

Figure 18 (a, b) demonstrates the PCR amplification results for strain QDD9 under the same reaction conditions as the other strains QDD1 and QDD8.

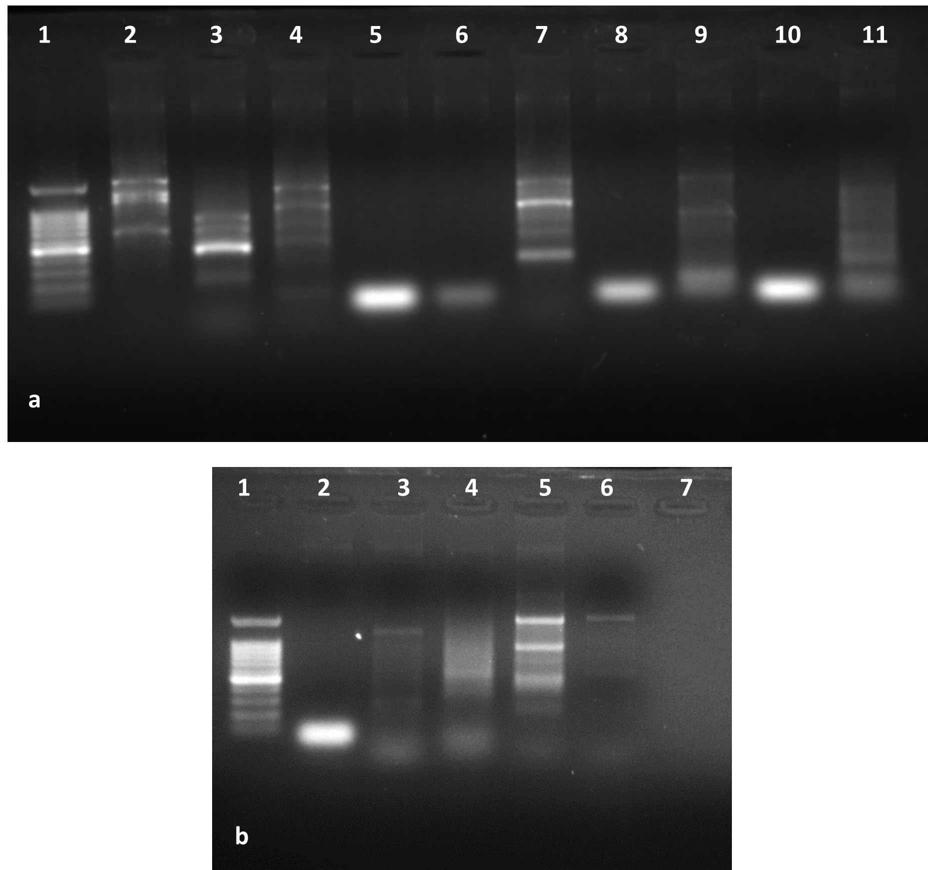

Fig. 18: PCR amplification of the indicated catabolic genotypes using selected primers set in this study for strain QDD9, T_H = 50 ^{o}C. 1.2% agarose gel under 50 Volts/ cm.

a: (1) 100 bp DNA ladder (100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1 kb, and 1.5 kb), (2) P1, (3) P2, (4) P3, (5) P4, (6) P5, (7) P6, (8) P7, (9) P8, (10) P9, (11) P10

b: (1) 100 bp DNA ladder, (2) P11, (3) P12, (4) P13, (5) Rip 1.5 kb, (6) Rip 1.5 kb [repeated], (7) negative control of Rip

The analysis of gel figures of our *P. aeruginosa* strains under 50 ^{o}C annealing temperature shows several non-specific amplified bands for some primers while clear negative results for others. Apparently, those primer pairs, which did not show any amplified bands at this low temperature will not improve at higher degrees. For example, primer pairs 4, 5, 7, 11, and 12 had clearly no amplified bands. However, we were suspicious regarding other primers that might need more optimization in order to make sure of any specific amplification of the expected size. On the other hand, primer pair 6 corresponding to gene *alkB1* encoding for alkane hydroxylase enzyme showed an intense band of expected size (330 bp) with strain QDD1, but bands of lower intensity and specificity with QDD8 and QDD9. As described in the reference (Kohno *et al*., 2002), this primer pair along with P4 and P5 corresponding to *alkB* and *alkM* genes, repectively work well at annealing temperature as low as 40 ^{o}C. Here, we can consider P4 as clear internal negative control, whilst P6 is a clear internal positive control for our *P. aeruginosa* strains. Since Kohno *et al*., (2002) have used *Pseudomonas aeruginosa* RWTH529 and *Pseudomonas aeruginosa* PAO as reference strains for *alkB* (P4) and *alkB1* (P6) genes respectively.

The second step was to confirm and compare all three strains under the same conditions in one PCR run. The first primer pairs batch (P1 to P7) was used in this experiment as illustrated in figure 19 (a, b, c, d).

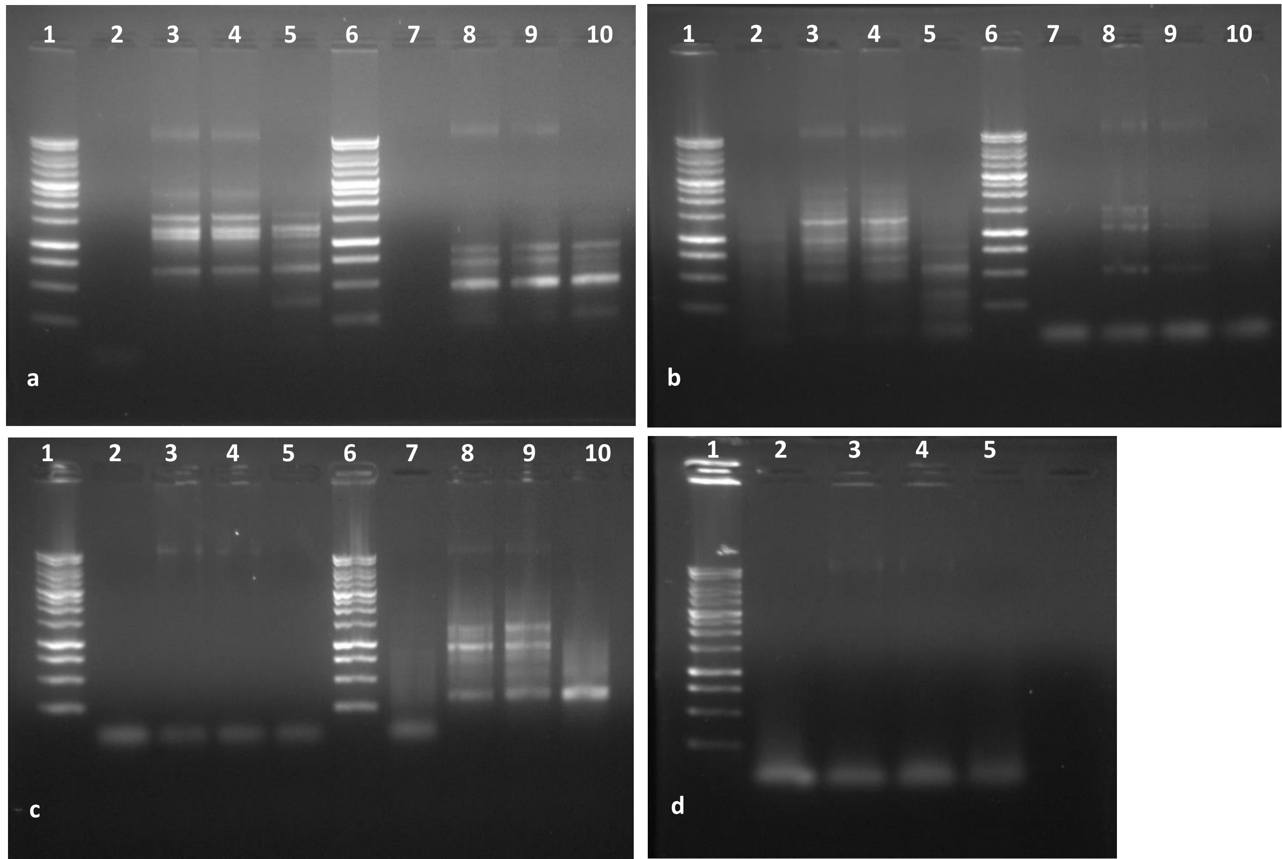

Fig. 19: Confirmation PCR amplification of the indicated catabolic genotypes using first batch of primers from P1 to P7 for strains QDD1,QDD8, and QDD9, T_H = 50 ^{o}C. 1.0% agarose gel under 100 Volts/ cm.

a: Primer pair 1 and 2 showed in order for all strains as follows (1) 1 kb DNA ladder (250 bp, 500 bp, 750 bp, 1 kb, 1.5 kb, 2 kb, 2.5 kb, 3 kb, 4 kb, 5 kb, 6 kb, 8 kb, and 10 kb), (2) negative control (no template DNA), (3) QDD8, (4) QDD9, (5) QDD1 [P1]. (6) 1 kb DNA ladder, (7, 8, 9, 10) the same order for [P2]

b: Primer pair 3 and 4 showed in order for all strains as follows (1) 1 kb DNA ladder, (2) negative control (no template DNA), (3) QDD8, (4) QDD9, (5) QDD1 [P3]. (6) 1 kb DNA ladder, (7, 8, 9, 10) the same order for [P4]

c: Primer pair 5 and 6 showed in order for all strains as follows (1) 1 kb DNA ladder, (2) negative control (no template DNA), (3) QDD8, (4) QDD9, (5) QDD1 [P5]. (6) 1 kb DNA ladder, (7, 8, 9, 10) the same order for [P6]

d: Primer pair 7 showed in order for all strains as follows (1) 1 kb DNA ladder, (2) negative control (no template DNA), (3) QDD8, (4) QDD9, (5) QDD1

The results of this PCR amplification confirmed the results obtained in the first screening step for all strains. Emerging from these results, we thought of optimizing the PCR reaction via increasing the annealing temperature by 2 degrees (52 ^{o}C). For this experiment, the second batch of primer pairs (P8 to P13) was selected (Figure 20 (a, b, c). Under 50 ^{o}C annealing temperature, primer pairs 8, 10, and 13 showed suspected bands for strain QDD1, while primer pair 9 showed a light band of expected size (291 bp) for strain QDD8 (Hendrickx *et al*., 2006). Increasing the temperature might exclude the non-specific amplified bands.

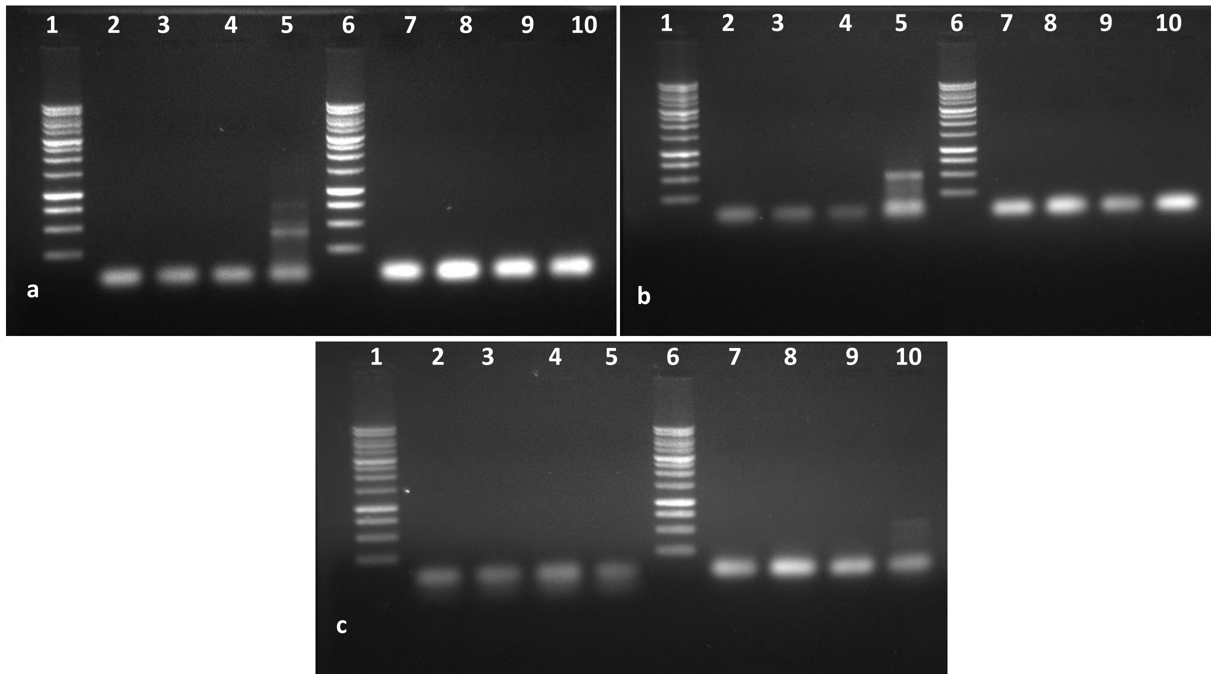

Fig. 20: Optimization PCR amplification of the indicated catabolic genotypes using second batch of primers from P8 to P13 for strains QDD1,QDD8, and QDD9, T_H = 52 ^{o}C. 1.0% agarose gel under 100 Volts/ cm.

a: Primer pair 8 and 9 showed in order for all strains as follows (1) 1 kb DNA ladder, (2) negative control (no template DNA), (3) QDD8, (4) QDD9, (5) QDD1 [P8]. (6) 1 kb DNA ladder, (7, 8, 9, 10) the same order for [P9]

b: Primer pair 10 and 11 showed in order for all strains as follows (1) 1 kb DNA ladder, (2) negative control (no template DNA), (3) QDD8, (4) QDD9, (5) QDD1 [P10]. (6) 1 kb DNA ladder, (7, 8, 9, 10) the same order for [P11]

c: Primer pair 12 and 13 showed in order for all strains as follows (1) 1 kb DNA ladder, (2) negative control (no template DNA), (3) QDD8, (4) QDD9, (5) QDD1 [P12]. (6) 1 kb DNA ladder, (7, 8, 9, 10) the same order for [P13]

Obviously, many non-specific amplifications disappeared as temperature increased. Nevertheless, primer pairs 8 and 10 for strain QDD1 are still showing some bands. The band of primer 8 corresponding to gene *tmoA* encoding for toluene monoxygenase might be of the expected size (505 bp) (Hendrickx *et al*., 2006). While the light band of primer pair 9 (291 bp) that was evident in the previous experiment with strain QDD8 has disappeared in this optimization step. It is deducible that this band is not the expected one since primer 9 (*xylA* gene) proved to work well under annealing temperatures up to 64 ^{o}C (Hendrickx *et al*., 2006). The strain served as positive control in the reference article for this primer pair was *Pseudomonas putida* mt-2.

Generally noting, strain QDD1 has exhibited different genotypic content from its close relatives QDD8 and QDD9 according to its behavior under the same PCR conditions. The following optimizing experiment was designed after referring to the calculated melting temperature of each primer pair using the formula [T_m = 4(GC) + 2(AT)]. Certain interesting primers were selected to be tested on new different temperatures, including our internal positive control (P6) and internal negative control (P4). The chosen primer pairs were P1 (annealing temperatures: 46 ^{o}C and 52 ^{o}C), P2 (annealing temperatures: 50 ^{o}C and 52 ^{o}C), P3 (annealing temperature: 52 ^{o}C), P4 (annealing temperature: 56 ^{o}C), P6 (annealing temperature: 56 ^{o}C), P8 (annealing temperatures: 50 ^{o}C and 56 ^{o}C), P10 (annealing temperature: 56 ^{o}C), and P13 (annealing temperature: 56 ^{o}C). This PCR test was run for all our *Pseudomonas* strains. The only clear, intense, and highly specific band

appeared was with what we called internal positive control for strain QDD1 with primer pair 6 under annealing temperature 56 ^{o}C (Figure 21).

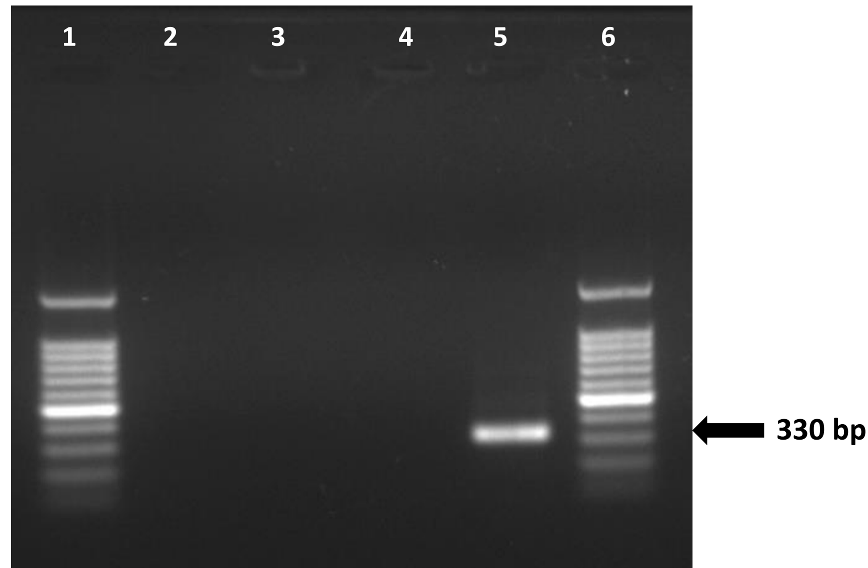

Fig. 21: Optimization PCR amplification for primer pair 6 under annealing temperature 56 ^{o}C. (1) 100 bp DNA ladder (100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1 kb, and 1.5 kb), (2) negative control (no template DNA), (3) QDD8, (4) QDD9, (5) QDD1, (6) 100 bp DNA ladder

As the uniqueness of strain QDD1 genotypic profile became evident, the last PCR optimization experiment was performed on QDD1 alone over primer pairs 1, 2, 6, 8, 10, and 13 under a bit lower annealing temperature 54 ^{o}C. Since many bands disappeared at 56 ^{o}C annealing, we wanted to make sure of any specific amplifications that might occur under intermediate temperature and of the expected sizes as in Table 3. Besides our intense band with P6, this time a specific band appeared with P2, yet with smaller size than expected (around 400 bp) (Figure 22). This band was observable at 50 ^{o}C, but with many other non-specific amplifications (Figure 16 and Figure 19, a, lane 10).

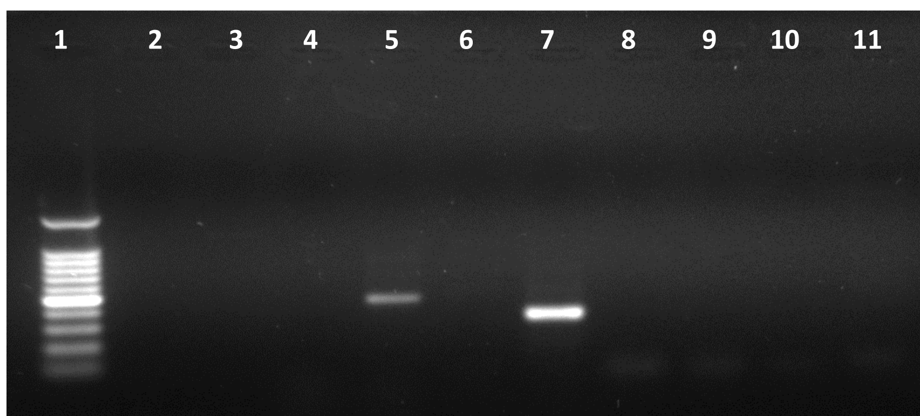

Fig. 22: Optimization PCR amplification for strain QDD1 under annealing temperature 54 ^{o}C. (1) 100 bp DNA ladder (100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1 kb, and 1.5 kb), (2) negative control of P1 (no template DNA), (3) P1, (4) negative control of P2, (5) P2, (6) negative control of P6, (7) P6, (8) negative control of P10, (9) P10, (10) negative control of P13, (11) P13

In summary, upon the screening of three *Pseudomonas aeruginosa* strains, isolated from different contaminated sites in Qatar, for the presence of several hydrocarbon degrading genes, *alkB1* gene was clearly evident and highly specific for strain QDD1 even under high annealing temperatures. Hence, strain QDD1 was proved to possess the gene encoding for alkane hydroxylase enzyme catalyzing the first step in the pathway of n-alkanes breakdown (Kohno *et al.,* 2002; Kloos *et al.,* 2006; Nie *et al.*, 2014). Several *P. aeruginosa* isolates have been recognized to carry *alkB1* and *alkB2* genes for metabolizing medium and long n-alkanes (Striebich *et al*., 2014). However, strains QDD8 and QDD9 lack this *alkB1* gene, which can be explained that they might be carrying different alkane hydroxylases that were not tested or could be some novel genes yet to be discovered. Further optimization experiments are recommended under a wider range of annealing temperatures for the selected primers set. Sequencing the amplified gene of

QDD1 can provide more specific information regarding the homology with reference strains.

For maximizing the capabilities of bacterial strains in biodegradation, particularly in cases similar to ours, when strains possess some genes and lack others, scientists have long been constructing recombinant strains (Mrozik and Piotrowska-Seget, 2010). It was practically possible to insert an entire pathway or catabolic system into a host isolate. *Pseudomonas* strains were the most exploited in this field (Haro and de Lorenzo, 2001). Nonetheless, in genetically modified organisms (GMOs) some complications are associated during application regarding their genetic stability and their ability to compete the wild type native strains.

5. Conclusion

Environmental deterioration resulting from oil liberation incidents has been alerting since the beginning of petroleum industry. Hydrocarbons comprising the petroleum matrix are perceived as toxic and environmentally persistent organic pollutants. Impacted terrestrial and marine ecosystems may take decades to recover and environmental losses may be irreversible. Efforts to remediate polluted soil and water have been looking for more feasible and cost-effective alternatives. Bioremediation is an environmentally friendly solution that utilizes natural means to biodegrade pollutants. Microorganisms, particularly bacteria, are capable of catabolizing hydrocarbons into non-hazardous or less hazardous products.

The research team in our lab has established a database of locally isolated and identified collection of hydrocarbon degrading bacterial strains. A selection of three *Pseudomonas aeruginosa* strains was done based on their ability to grow on diesel hydrocarbons as the sole carbon source. Our initial study emphasized the importance of the role played by medium components on cell growth and biological activity among the selected strains via screening on variant nitrogen sources, C/N ratios as well as 5% and 10% (v/v) diesel concentrations. Growth results have been correlated to biological activities represented by the removal of diesel hydrocarbons using GC analysis. Strains' tolerance to higher diesel concentrations was clearly influenced by medium composition.

Growth dynamics and metabolic variability exhibited by the screened strains led us to investigate more the nutritional profiles.

Three-factor experimental design was implemented in order to examine the effect of carbon, nitrogen, and phosphorous concentrations on the growth and biodegradation potential of the selected strains. According to the statistical analysis of growth results, the single factor effect of carbon seemed to inhibit the growth of some strains by the second week period. Elevated diesel concentrations limited cells growth due metabolic challenges in catabolizing the residual recalcitrant hydrocarbons. Unlike carbon, the effect of nitrogen seemed to be significantly enhancing cell growth under various experimental conditions. However, growth of strains generally seemed less affected by phosphorous concentration variation. In addition, some strains were found more sensitive to changing C/N/P ratios than others. Yet, we can generalize that the C/N/P factorial interaction was more growth influencing in the first week of incubation rather than the second. The capability of our strains to degrade diesel hydrocarbons was confirmed via GC under all different growth conditions. Although our strains belong to the same bacterial group, diversity in their responses to the explored nutritional manipulations were undoubtedly manifested.

In parallel, *P. aeruginosa* strains have been subjected to molecular screening for genes encoding hydrocarbon catabolic enzymes or enzymes' subunits. An optimization study using a selected set of primers corresponding to wide spectrum of degradative

genes in the catabolic pathway of aliphatic n-alkanes, to single ring aromatics (BTEX), and to polycyclic aromatic hydrocarbons (PAHs) was performed. Results demonstrated that one *Pseudomonas aeruginosa* strain (QDD1) possesses the *alkB1* gene encoding for alkane hydroxylase enzyme participating in the catabolic pathway of n-alkanes. In literature, several *P. aeruginosa* isolates have been recognized to carry *alkB1* gene for metabolizing medium and long n-alkanes. The genetic makeup of strain QDD1 was distinguished to be different from other strains as well as its biodegradation potential and its sensitivity to nutritional manipulations.

Further investigations of these three promising strains are greatly recommended. The following research experiments are suggested:

- The screening of strains under wider range of nutritional concentrations for greater optimization of both growth and hydrocarbon degradation.
- The investigation of oxidative metabolism of strains under larger fermenter scale with more controlled conditions.
- The study of other physical parameters such as pH and temperature and their role in biodegradation.
- The analysis of surfactants production by our strains and its impact on hydrocarbon removal.
- Sequencing the amplified *alkB1* gene fragment of the strain QDD1.

- Cloning the whole corresponding gene and hybridize it with the total DNA of the other strains, to look for partially similar genes if existing in the other strains
- Cloning the *alkB1* gene downstream a strong promoter, transfer it to other strains and study the effect on the hydrocarbon degradability.

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