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DILMI Fatiha

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Presented by
DILMI Fatiha

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THEME

ISOLATION AND CHARACTERIZATION OF OIL DEGRADING MICROORGANISMS FROM ARZEW OIL REFINERY

Publicly Examined on : 19 / 06 / 2019 in front of the following examiners' Committee

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DEDICATIONS

I dedicate this thesis to:

My Mother

Source of light of my life

My Father

God bless his age

My husband for his help, supports, understanding and encouragement

during my study

My children Joumana, Anes and Raihana, source of happiness and love

My brother and my sisters

To all my family

To all my friends

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ملخص

تعد تسربات البترول ومنتجاته التي تحدث أثناء النقل، التخزين والتكرير من أبرز الملوثات البيئية نظرا للضرر الكبير الذي تحدثه في النظام البيئي. وتعتبر المعالجة الحيوية باستخدام الكائنات الدقيقة طريقة فعالة لمعالجة التربة الملوثة بالنفط.

تهدف هذه الدراسة الى عزل و تشخيص وتحديد البكتيريا المحللة للنفط من تربة ملوثة بهذا الأخير؛ وخلال هذه الدراسة تم جمع خمسة عشر عينة من التربة الملوثة بالنفط من سبعة مواقع في مصفاة أرزيو، الكائنة شمال غرب الجزائر. وبمعاينة الخصائص الفيزيائية والكيميائية لعينات التربة وجد أن الرمل والطين هما الجزءان السائدان فيها، وأنها تحتوي على نسبة ملوحة منخفضة، وهي ملوثة بنسب عالية بمجموع هيدروكربونات نفطية تتراوح من 2 إلى 86 غ / كلغ . في حين تراوح التعداد البكتيري من 1.6×10^5 إلى 1.4×10^8 وحدة قادرة عى النمو لكل غرام واحد من التربة مما يشير أنها تحتوي على تعداد بكتيري عالٍ مقارنة مع التربة غير الملوثة.

تم عزل 78 مستعمرة بكتيرية ذات أحجام وألوان مختلفة باستخدام وسط معدني يحتوي على 1٪ من النفط الخام. وقد تم انتقاء وتحديد اثنين وعشرين سلالة بكتيرية بالنظرالى قدرتها العالية على تحليل النفط الخام، ومن ثم تحديدها من خلال الخصائص المرفولوجية و البيو كيميائية. وكانت 15 سلالة بكتيرية فقط موضوع التوصيف الجزيئي، وذلك وفقا لطريقة التسلسل الجيني لمورثة الحمض النووي الريبي ARNr 16S و قد وصفت السلالات البكتيرية المعزولة على أنها الأجناس و / أو الأنواع التالية :

Pseudomonas aeruginosa, Achromobacter xylosoxidans, Staphylococcus haemolyticus, Enterococcus faecalis, Bacillus cereus, B. anthracis, B. subtilis, Exiguobacterium aurantiacum, Lysinibacillus macroides, P. fluorescens, Burkholderia cepacia, Staphylococcus hominis و *Lysinibacillus sphaericus.*

في حين أبدت 6 سلالات بكتيرية من نوع *P. aeruginosa* نتيجة إيجابية مع البادئة الأولى المطابقة للمورثة alkane 1 monoxygenase (434bp)، وقد تم اختبار قدرة 12 سلالة معزولة على تحليل النفط في وسط معدني من خلال قياس الكثافة الضوئية والتحليل الكتلي. حيث أشارت النتائج إلى أن جميع السلالات المعزولة لها القدرة على استخدام النفط الخام كمصدر وحيد للكربون. كما لوحظ أن أعلى معدل نمو كان لدى سلالات *Pseudomonas aeruginosa* (P2.1 وP2.3)، *Lysinibacillus macroides* B4.2 و Achromobacter xylosoxidans P2.2 وهي السلالات نفسها التي حققت أعلى مستويات التحليل البيولوجي، بحيث جاءت نسبها على التوالي 76.37%، 60.92%، 47.46% و45.20% .

ومن جانب آخر تم اختبار قدرة السلالات المعزولة (منفردة ومجتمعة) على تحليل الديزل من خلال قياس الكثافة الضوئية،التحليل الكتلي وتحليل GC-MS. وقد دلت النتائـج على أن أعلى مستويات النمو في وسط معدني مزود بالديزل كان لدى المجمع البكتيري وسلالة P2.1 *Pseudomonas aeruginosa*. وأن أعلى معدلات التحليل البيولوجي كانت للمجمع البكتيري بنسبة 79.62٪ وتليها سلالة *Pseudomonas aeruginosa* P2.1 بنسبة 64.81٪، وخلصت النتائج أن لدى المجمع البكتيري قدرة عالية على التحليل البيولوجي للديزل مقارنة بالسلالات الفردية، بينما كشف تحليل GC-MS أن المجمع البكتيري والسلالات الفردية يحللان بشكل أكبر الجزء الأليفاتي للديزل مقارنة بالجزء العطري وأن هناك انخفاض في جل مركبات

الديزل و بشكل خاص عند استخدام **التجمع البكتيري** وجد أن هناك انخفاض كلي لمركبات n-alkane (من C9 إلى C26) وهذا بعد 15 يوما من التحضين. وأخيرا تم تحديد الظروف المثلى (درجة الحرارة و درجة الحموضة و الملوحة و التهوية) التي تمنح السلالات البكتيرية قدرة عالية على التحليل البيولوجي للنفط.

خلصت الدراسة إلى أن عينات التربة المدروسة تحتوي على مجموعة متنوعة من البكتيريا المحطمة للنفط ويمكن استخدام هذه السلالات في المعالجة الحيوية للتربة الملوثة بالنفط والنفايات النفطية الأخرى.

الكلمات المفتاحية: العزل، التوصيف، التحليل البيولوجي، الكائنات الحية الدقيقة، النفط الخام، الديزل، تسلسل الجين 16S ARNr ، التربة الملوثة بالنفط ، مصفاة أرزيو النفطية.

ABSTRACT

The spills of petroleum and petroleum product, which occur during transport, storage and refining, are a major contaminant in the environment, as they produce harm to the surrounding ecosystem. Bioremediation is an efficient method used to treat petroleum hydrocarbon contaminated soil using indigenous microorganisms. The purpose of our study was to isolate, screen and identify the hydrocarbon degrading bacteria from oil polluted soil. Fifteen Oil-contaminated soil samples were aseptically collected from seven sites in Arzew oil refinery, North-West of Algeria. Physico-chemical parameters of soil samples showed that sand and loam were the predominant fractions. The soils were neutral to slightly alkaline pH, have a low salinity and highly polluted with total hydrocarbon content ranging from 2 to 86 g/kg soil. Bacterial enumeration ranged from 1.6×10^5 to 1.4×10^8 CFU/g soil indicating high bacterial count in oil-contaminated soils compared with non-contaminated one.

Seventy-eight bacterial colonies with different size and color were isolated using mineral salt media supplemented with 1% of crude oil. Twenty-two bacterial isolates were screened for their best degradative abilities of crude oil, and then they were identified based on morphological and biochemical characterizations. Fifteen isolates were identified using 16S rRNA gene sequence analysis; the isolates were identified as the following genera and/or species: *Pseudomonas aeruginosa, Achromobacter xylosoxidans, Staphylococcus haemolyticus, Enterococcus faecalis, Bacillus cereus, B. anthracis, B. subtilis, Exiguobacterium aurantiacum, Lysinibacillus macroides, P. fluorescens, Burkholderia cepacia, Staphylococcus hominis* and *Lysinibacillus sphaericus*. Six bacterial isolates (identified as *P. aeruginosa*) showed a positive result with primer pair specific to alkane 1 monoxygenase gene (434bp).

The ability of 12 isolated strains to degrade crude oil was carried out in a liquid medium by measuring optical density and gravimetric analysis. Results indicated that all the isolated strains had effectively utilized crude oil as carbon source. *Pseudomonas aeruginosa* (P2.1 and P2.3), *Lysinibacillus macroides* B4.2 and *Achromobacter xylosoxidans* P2.2 had the highest growth in the medium with crude oil and exhibited the highest biodegradation percentage with 76.37%, 60.92%, 47.46% and 45.20% respectively.

The ability of individual pure isolates and bacterial consortia to degrade diesel were determined using the turbidometry method, gravimetric analysis and GC-MS analysis. The results indicate that the bacterial consortium and *Pseudomonas aeruginosa* P2.1 showed the best growth in MSM with diesel and exhibited the highest biodegradation percentage of diesel with 79.62% and 64.81% respectively. The consortium showed the greatest effect of diesel degradation compared to individual strains. The GC-MS analysis revealed that the consortium and individual isolates degrade the aliphatic fraction greater than the aromatic fraction .All compounds in diesel were highly reduced and particularly in bacterial consortium where n-alkanes (from C9 to C26) were almost completely degraded after 15 days of incubation.

The optimal culture conditions (temperature, pH, salinity, agitation speed) for the maximum biodegradation of crude oil by bacterial isolates were determined.

This study indicates that the contaminated soil samples contain a diverse population of hydrocarbon degrading bacteria and these strains could be used for the bioremediation of oil-contaminated soil and other oil waste.

Keywords: Isolation, characterization, biodegradation, microorganism, crude oil, diesel, 16S rRNA, contaminated soil, ARZEW oil refinery.

RESUME

Les déversements du pétrole et des produits pétroliers, qui se produisent pendant le transport, le stockage et le raffinage constituent un contaminant majeur dans l'environnement puisqu'ils nuisent à l'écosystème. La bioremédiation est une méthode efficace pour traiter le sol contaminé par des hydrocarbures pétrolier en utilisant des micro-organismes indigènes. Le but de notre étude était d'isoler, de cribler et d'identifier les bactéries dégradant les hydrocarbures présents dans les sols pollués. Quinze échantillons du sol contaminé par les hydrocarbures pétrolier ont été prélevés de manière aseptique sur sept sites de la raffinerie d'Arzew, dans le nord-ouest de l'Algérie. Les paramètres physico-chimiques des échantillons du sol montrent que le sable et le limon sont des fractions prédominantes. Les sols ont un pH neutre à légèrement alcalin. Nos échantillons de sol ont une faible salinité, ils sont limités en matières organiques et fortement pollués avec une teneur totale en hydrocarbures allant de 2 à 86 g / kg. Le dénombrement bactérien variait de 1.6 x 10^5 à 1.4 x 10^8 UFC/g de sol, indiquant un nombre élevé de bactéries dans les sols contaminés par les hydrocarbures par rapport aux sols non contaminés.

Soixante-dix-huit colonies bactériennes avec différentes tailles et couleurs ont été isolées à l'aide d'un milieu minéral additionnés de 1% de pétrole brut. Vingt-deux isolats bactériens ont été sélectionnés en vertu de leurs meilleures capacités de dégradation du pétrole brut. Ensuite, ils ont été identifiés sur la base de la caractérisation morphologique et biochimique. 15 isolats ont été identifiés par la méthode de séquençage du gène ARNr 16S. Les isolats ont été par la suite identifiés comme appartenant aux genres et /ou espèces suivants: *Pseudomonas aeruginosa*, *Achromobacter xylosoxidans*, *Staphylococcus haemolyticus*, *Enterococcus faecalis*, *Bacillus cereus*, *B. anthracis*, *B. subtilis*, *Exiguobacterium aurantiacum*, *Lysinibacillus macroides*, *P. fluoresoresens*, *Staphylococcus hominis* et *Lysinibacillus sphaericus*. Six isolats bactériens (identifiés comme *P. aeruginosa*) ont montré un résultat positif avec une paire d'amorces spécifique du gène de l'alcane 1 monoxygénase (434 pb).

L'étude de la capacité de 12 souches isolées à dégrader le pétrole brut a été réalisé en milieu liquide par la mesure de la densité optique et l'analyse gravimétrique. Les résultats ont montré que toutes les souches isolées avaient utilisé efficacement le pétrole brut comme source de carbone. *Pseudomonas aeruginosa* (P2.1 et P2.3), *Lysinibacillus macroides* B4.2 et *Achromobacter xylosoxidans* P2.2 présentent la plus forte croissance dans un milieu contenant du pétrole brut et le pourcentage le plus élevé de biodégradation avec 76.37%, 60.92%, 47.46% et 45.20. %, respectivement.

La capacité des isolats purs et de consortium bactérien à dégrader le diesel a été déterminée à l'aide de la méthode de turbidimétrie, d'une analyse gravimétrique et d'une analyse GC-MS. Les résultats ont montré que le consortium bactérien et *Pseudomonas aeruginosa* P2.1 présente une meilleure croissance dans MSM avec du diesel. Ils ont présenté le pourcentage le plus élevé de biodégradation du diesel avec 79,62% et 64,81% respectivement. Le consortium a montré le plus grand effet de dégradation des hydrocarbures par rapport aux souches individuelles. L'analyse GC-MS a révélé que le consortium et les isolats individuels dégradent plus la fraction aliphatique par rapport à la fraction aromatique. Tous les composants du diesel ont été fortement réduits, et en

particulier avec consortium bactérien où n-alcanes (de C9 à C26) ont été complètement dégradé après 15 jours d'incubation.

Dans le but d'améliorer la dégradation des hydrocarbures, les conditions de culture (température, pH, salinité, vitesse d'agitation) ont été optimisées pour les isolats bactériens.

Notre étude confirme que les échantillons du sol contaminé contiennent une population diverse de bactéries capable de dégrader les hydrocarbures. Ces souches peuvent être utilisées pour la bioremédiation des sols contaminés par les hydrocarbures et autres déchets d'huile.

Mots clés: Isolement, caractérisation, biodégradation, microorganisme, pétrole brut, diesel, ARNr 16S, sol contaminé, raffinerie d'ARZEW.

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Analytical Term

GC: gas chromatography

GC-MS: gas chromatography - mass spectrometry

Chemical Terms

PHCs: Petroleum hydrocarbons

AgCl: Silver chloride

$AgNO_3$: Silver nitrate

$BaSO_4$: Barium Sulfate

$BaCl_2$: Barium chloride

$CaCl_2.2H_2O$: Calcium chloride dehydrate

CO_2: Carbon dioxide

DCM: Dichloromethane

EDTA: Ethylene diamine tetra acetic acid

$FeCl_3$: Iron trichloride

HCs: Hydrocarbons

H_2SO_4: Sulfuric acid

K_2HPO_4: Dipotassium hydrogen phosphate

KH_2PO_4: Potassium dihydrogen phosphate

KNO_3: Potassium nitrate

$MgSO_4.7H_2O$: Magnesium sulfate heptahydrate

NaOH: Sodium hydroxide

pH: potential hydrogen

PAH: Polycyclic aromatic hydrocarbon

TPH: Total petroleum hydrocarbons

LPG: Liquid petroleum gas

API: American petroleum institute

SARA: Saturates, aromatics, resins and asphaltenes.

Measurement Terms

bpd: barrels per day

$^{\circ}C$: Degrees Celsius

CFU: Colony Forming Unit

hour: h, min: minute, S: second

kg: kilogram, g: gram, mg: milligram

L: liter, ml: milliliter.

m: meter, cm: centimeter, µm: micrometer, nm: nanometer

M: Molar, mM: millimolar, µM: Micromolar

OD: Optical Density

Rpm: Revolutions per minute

S/m: Siemens per meter, dS: deciSiemens, µS: microSiemens

v/v: volume per volume

w/v: weight per volume

uv: ultraviolet

1 tonne = 308 U.S. gallons = 7.33 barrels = 858 liters

Media

LB: Luria Bertani

MSM: Mineral Salt Medium

NA: Nutrient Agar

TSA: Tryptic Soy Agar

TSB: Tryptic Soy Broth

Molecular Terms

bp: base pair, Kbp: Kilo base pair

BLAST: Basic Local Alignment Search Tool

EMBL: European Molecular Biology Laboratory

DNA: Deoxyribonucleic acid

DNase: Deoxyribonuclease

dsDNA: double-stranded DNA

dNTPs: Desoxyribonucleotides triphosphates

DMSO: Dimethyl sulfoxide

PCR: Polymerase chain reaction

NCBI: National Centre for Biotechnology and Information

MEGA 0.6: Molecular evolutionary Genetics Analysis

RDP: Ribosomal Database Project

rDNA: Ribosomal DNA

RNase: Ribonuclease

SDS: Sodium dodecyl sulfate

TBE: Tris / Borate / EDTA buffer.

TE: Tris/ EDTA buffer

alkB: alkane monooxygenase encoding genes

alkB1: alkane 1 monooxygenase encoding genes

C23O : catechol 2, 3 dioxygenase genes

nahC: 1,2-dihydroxynaphthalene dioxygenase encoding genes

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INTRODUCTION

Introduction

Petroleum hydrocarbons are the main source of energy for industry and daily life. The leakage and accidental oil release occur regularly during the exploration, extraction production, refining, storage and transportation (N. Das & Chandran, 2011; Palanisamy *et al.*, 2014). The spills due the leakage may be small but continuous and prolonged could lead soil and water contamination ending up with health problems (Rahman *et al.*, 2002a; Al-Deeb, 2005).

Environmental pollution by petroleum and petroleum product is a problem for terrestrial and marine ecosystems as well as human health (Zavareh *et al.*, 2016). Uncontrolled liberation of petroleum compounds that are mutagenic, carcinogenic and are potent immunotoxicants into soil and water poses a serious threat to human, animal and plant health (C. Singh & Lin, 2008).

The soil remediation has been tried using different technology including mechanical, evaporation, dispersion, and washing. However, these technologies are expensive and can lead to incomplete decomposition of contaminants (N. Das & Chandran, 2011).

The process of using microorganisms to de grade or remove hazardous component of wastes from the environment is called Bioremediation (Dua *et al.*, 2002; Al-Adwan *et al.*, 2010). It efficiency depends on the presence of appropriate microorganisms and is also affected by the environmental conditions and microbial community composition (Durga *et al.*, 2014).

Biodegradation by natural microorganisms represents one of the primary mechanisms by which oil pollutants can be removed from the environment (Malkawi *et al.*, 2009b; N. Das & Chandran, 2011). Hydrocarbon-contaminated soils biodegradation, which utilize the ability of microorganisms to degrade and/or detoxify organic pollutants, has been established as an efficient, economic and environmentally sound treatment (C. Singh & Lin, 2008). It is considered to be cheaper than other remediation technologies (Leahy & Colwell, 1990).

Salleh *et al.* (2003) reported that the biodegradation is a complete microbial mineralization of complex materials into simple inorganic compound as carbon dioxide, water.

Petroleum hydrocarbon can be degraded by various microorganisms such as bacteria, fungi and yeast (Hamzah *et al.*, 2010). Bacteria are the most active agents in petroleum degradation (Rahman *et al.*, 2003; N. Das & Chandran, 2011). The dominant genera of microorganisms that utilize petroleum hydrocarbons are: *Pseudomonas, Acinetobacter, Nocardia, Flavobacterium, Micrococcus, Arthrobacter, Corynebacterium, Achromobacter, Rhodococcus, Alcaligenes, Mycobacterium, and Bacillus* (Stamenov *et al*., 2015).

Bacterial organisms are the most involved in oil degradation (Rahman *et al.*, 2002a; Malkawi *et al.*, 2009b; N. Das & Chandran, 2011), because they contain different enzymes that are implicated in the oil hydrocarbon metabolism (Al-Deeb & Malkawi, 2009; Hesham *et al.*, 2014). These degradative enzymes are encoded by special genes which are involved in hydrocarbon degradation such as; *alkB*; alkane monooxygenase from *Pseudomonas putida* (C5 to C12 alkane degradation); *alkm*; alkane monooxygenase from *Acinetobacter sp*. strain ADP-l (C10 to C20 alkane degradation); *alkBl* and *alkB2* alkane monooxygenase from *Rhodococcus spp*. (Cl2 to Cl6 alkane degradation); *C23O* catechol-2, 3-dioxygenase from *P. putida* (aromatic hydrocarbons degradation); *ndoB*; naphthalene dioxygenase from *P. putida* (polycyclic aromatic hydrocarbons degradation); *nahC*; 1,2-dihydroxynaphthalene dioxygenase (polycyclic aromatic hydrocarbons degradation) (Margesin *et al.*, 2003; Malkawi *et al.*, 2009a). alkane monooxygenase (*alkB*) and catechol-2, 3-dioxygenase (*C23O*) are the key enzymes involved in n-alkane and aromatic hydrocarbon degradation respectively (Al-Deeb & Malkawi, 2009).

Strategies to enhance the hydrocarbon biodegradation include stimulation of the indigenous microorganisms by optimizing the nutrients, the temperature, the pH and oxygen supply conditions (biostimulation), and through added of an enriched microbial consortium into the existing microbial population (bioaugmentation) (Mariano *et al.*, 2007; N. Das & Chandran, 2011).

The Arzew refinery is one of Algeria's five oil refineries, which could be a source of contamination in air, soil and water. Very few studies have been done in Algeria on the hydrocarbon degradation by microorganisms isolated from oil-contaminated soil. These included, Guermouche (2014) who isolated 15 bacterial strains from oil-contaminated soil at Arzew oil refinery, belonging to the following genera *Pseudomonas, Enterobacter, Serratia* and *Shewanella*. Furthermore, Benchouk (2017)

isolated 5 microbial isolates from oil contaminated soil, these include *Candida sp, Bacillus sp, Pseudomonas aeruginosa, Pseudomonas putida*. The genus *Pseudomonas* and *Rhodotorula* (yeast) were isolated from soil contaminated by ejection of the efflux of drilling fluid from Hassi Messaoud (Akmoussi-Toumi, 2009). Guergouri (2010) isolated 16 bacterial strains from oil-contaminated soil at Skikda oil refinery, these include the genus *Bacillus, Pseudomonas* and *Enterobacter*.

The aims of our study was to isolate and identify petroleum hydrocarbon-degrading microorganisms from different oil-contaminated soils at Arzew refinery using biochemical and molecular methods and to investigate their degradative ability on crude oil and diesel.

This thesis is divided into three parts. The first represents a literature review and which is further subdivided into three parts; the first is the knowledge of hydrocarbons, the bioremediation of soils polluted by hydrocarbons, biodegradation principle, degradative microorganisms and their type of metabolism, and finally factors influence the biodegradation.

The second part is devoted to the presentation of the methodology adopted for the carrying out my work.

The third part of this thesis is devoted to the presentation and discussion of results obtained. The manuscript is completed by a conclusion.

Aims

In the present work, the following objectives have been met:

1. To isolate the oil degrading microorganisms from oil contaminated soil
2. To screening the most potent bacterial isolates for hydrocarbon utilization
3. To identify the bacterial isolates using biochemical and molecular techniques
4. To detect the presence of alkane monooxygenase encoding genes (*alkB1*), catechol 2, 3 dioxygenase genes (*C23O*) and 1,2-dihydroxynaphthalene dioxygenase encoding genes (*nahC*) among certain species of bacteria.
5. To characterize the crude oil degradation potential of bacterial isolate by measuring optical density and gravimetric analysis.
6. Determination of diesel degradation potentials of the pure culture of selected bacterial isolates as well as consortia by turbidometry, gravimetric analysis and Gas Chromatography-Mass Spectrometry analysis.
7. To investigate the effect of nutritional and environmental factors on the biodegradation of crude oil.

I. LITERATURE REVIEW

CHAPTER ONE
Petroleum hydrocarbons

I.1. PETROLEUM HYDROCARBONS

In the world, the petroleum industry is a major participant of pollutants. This participation is due to the increased demand for petroleum and petroleum product as a main source of energy (Mejeha, 2016).

The pollution due to petroleum hydrocarbons (PHCs) has been an environmental problem for a long time. The various uses of crude oil and its derivatives result in increased pollution of lands and marine ecosystems (Su-Chen, 1994).

I.1.1. Crude oil

Crude oil (also called petroleum) is a dark sticky liquid in its unrefined state (A. Kumar *et al.*, 2011). The term petroleum is derived from the Latin derivative *Petra* for rock and *oleum* for oil. Petroleum is largely formed biogenetically at temperatures below 200°C from matter deposited in shallow seas (Hou, 2000). Each petroleum reserve is a unique combination of biomass breakdown products. The petroleum principal chemical elements (90% of the weight of crude oil) are carbon and hydrogen, which are combined in a series of compounds called a hydrocarbon (Salleh *et al.*, 2003). Petroleum is a complex mixture of hydrocarbons and other organic compounds (Van Hamme *et al.*, 2003), is generally in the liquid state, that may also include compounds of sulfur, nitrogen, oxygen, and metals and other elements (El-Naggar *et al.*, 2014).

I.1.1.1. The origin of petroleum

Oils (light and heavy) and gas are originated from organic matter; that is plants and small animals that were once alive that have created the source rock. Source rocks are the rocks that produce hydrocarbons; they are rich in organic matter. Chemical changes after burial convert animal and plant tissue to the complex molecules that eventually produce oil or natural gas by the effects of pressure and heat on sediments trapped beneath the Earth's surface over millions of years. The ancient societies in Egypt, India, and China made limited use of petroleum mainly as fuel for lamps, canoes, boats and medicine. The age of modern petroleum began a century and a half ago. Advances in technology have ameliorated the ability to find and extract oil and gas and to convert them to efficient fuels, lubricants, and other useful products (Aminzadeh & Dasgupta, 2013).

I.1.1.2. Components and classification of petroleum

Petroleum is a complex mixture of more than 17,000 distinct chemical compounds (Head *et al.*, 2006; Mejeha, 2016). These chemical compounds are dominated by hydrocarbons classified as either saturates, aromatics, resins and asphaltenes collectively described as the SARAs. In addition to these dominant constituents, there are other minor components such as nitrogen, sulphur, metals and oxygen (Mejeha, 2016). Table 1 shows the component's percentage of petroleum. Based on hydrocarbon composition, petroleum is classified as naphthenic or paraffinic crude oils. Naphthenic crude oils consist of the high percentage of cyclic aliphatic and aromatics hydrocarbons while paraffinic crude oils consist of a high percentage of saturates hydrocarbons (Simanzhenkov & Idem, 2003). Outside this basic classification, petroleum is also classified based on the non-hydrocarbon components. Thus, the sulphur, nitrogen content and the ratios of the heavy metals nickel and vanadium (the most abundant trace metals in crude oil) have been used to classify petroleum. Petroleum is also classified as sweet or sour oil based on the sulphur content. Sweet oils have sulphur contents less than 1% of the whole petroleum by weight and sour oils have sulphur contents above 1% (Peters *et al.*, 2007; Mejeha, 2016).

Table 1: The component's percentage of petroleum (Gordon, 2013).

Elements	Percentage range (%)
Carbon	83-87
Hydrogen	10-14
Nitrogen	0.1-2
Oxygen	0.1-1.5
Sulphur	0.5-1.6

Oils are classified by several criteria, but among the most important is the specific gravity. The oil industry uses a unit known as API (American Petroleum Institute) gravity, which is defined as [141.5/ (specific gravity)] 131.5 and expressed in degrees. API gravity is related to oil density and specific gravity (Prince & Lessard, 2004). The oils API less than 20° , a petroleum considered heavy oil, bitumen (extra-heavy oil) with a specific gravity usually has an API gravity less than 10° and light oils with the specific gravity range of 2- 8° API (Table 2) (Speight & Ozum, 2001). Light oils have higher proportions of small molecules; heavy oils are rich in larger molecules (Prince & Lessard, 2004).

Table 2: Classification of crude oil (Fahim *et al.*, 2009).

Crude oil categories	Gravity
Light crudes	API > 38
Medium crudes	38 > API > 29
Heavy crudes	29 > API > 8.5
Very heavy crudes	API < 8.5

I.1.2. Definition of Total Petroleum Hydrocarbons

Total petroleum hydrocarbon (TPH) is a term used to describe a large family of several hundred chemical compounds that originally come from crude oil. TPH is a mixture of chemicals, which are made mainly from hydrogen and carbon (A.T.S.D.R, 1999). Crude oil and petroleum products can contaminate the environment; there are many different chemicals in crude oil and other petroleum products, so it is not practical to measure each compound separately. However, it is useful to measure the total amount of TPH at a contaminated site (A.T.S.D.R, 1999; Van Epps, 2006). Speight (2016) also reported that the TPHs term is used because; given the wide variety of chemicals in petroleum and in petroleum products, it is not practical to consider each constituent separately, and after an incident, it is more useful to measure the amount of TPHs at the polluted sites (Speight, 2016).

I.1.3. Definition of Petroleum hydrocarbons

The term hydrocarbons means organic compounds, which contain only carbon and hydrogen (Shah & Patel, 2013). Petroleum hydrocarbons are complex substances formed from hydrogen and carbon molecules and sometimes containing other elements such as oxygen, sulfur, and nitrogen (Aminzadeh & Dasgupta, 2013).

I.1.4. Chemistry of petroleum hydrocarbons

Petroleum hydrocarbons and their refined products originate from a highly complex mixture, although they are comprised mainly of only two elements, hydrogen and carbon (Kostecki *et al.*, 2005; Shah & Patel, 2013). The carbon atoms can be attached or bonded to up to four different atoms, carbon or hydrogen, while the hydrogen atoms are bonded to only one carbon atom. The major method employed to group a hydrocarbon is by the number of carbon atoms that are present in the compound. For example, all of the hydrocarbons that have five carbon atoms can be

designated as C5. Compounds from C1 to C4 are gases at ordinary temperature. They are used as fuels and in the manufacturing of specialty chemicals as plastics. Compounds from C5 to C12 are liquids that will easily convert into gases when heated. These compounds are used as fuels and as cleaning solvents. Compounds from C13 to C17 are generally liquids and are used as fuels and lubricants. Those compounds greater than C17 are usually solids and are used as lubricants, heavy fuels, and as coatings (Kostecki *et al.*, 2005).

I.1.5. Petroleum hydrocarbons classification

Petroleum is a liquid mixture of hydrocarbons (oil) obtained from natural underground reservoirs (A. Kumar *et al.*, 2011). Petroleum hydrocarbons can be categorized for simplicity into four fractions: saturates, aromatics, resins and asphaltenes (Gordon, 2013). In other hand Shah and Patel (2013) reported that hydrocarbon are differentiated on the base of their chain length, saturation or structure.

Based on the general chemical structure of their chemicals constituent, hydrocarbons are divided up into two primary categories, aliphatics and aromatics (Figure 1). Aliphatics contain chains of carbon atoms strung together, while aromatics contain one or more benzene rings bonded together (Van Epps, 2006). Table 3 summarizes the various compounds and gives examples of each.

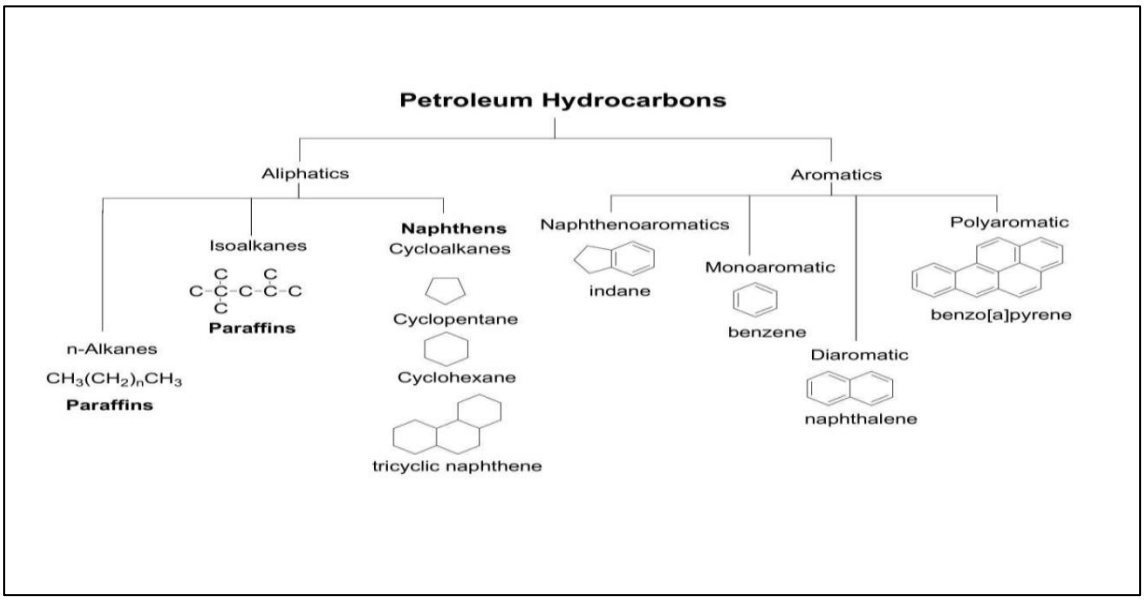
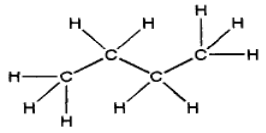
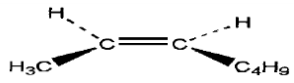
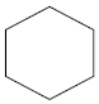
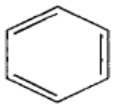
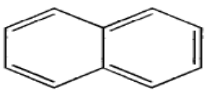


Figure 1: Schematic of hydrocarbons categories (Shah & Patel, 2013).

Table 3: Chemical structure of various categories of hydrocarbons (Van Epps, 2006).

Category	Description	Example Chemical structure
Aliphatics		
Alkanes	Carbon chain with single bond between carbon atoms	n-Butane 
Alkenes	Carbon chains with at least one carbon-carbon double bond	cis-2-Heptene 
Alkynes	Carbon chains with at least one carbon-carbon triple bond (not commonly found in petroleum hydrocarbons)	1-Butyne $HC \equiv CCH_2CH_3$
Cycloalkanes	Single-bonded carbon ring Structure	Cyclohexane 
Aromatics		
Monoaromatics	Primary structure is the benzene ring made up of six carbon atoms with alternating single and double bonds	Benzene 
PAHs	A compound having two or more benzene rings fused together	Naphthalene 

I.1.5.1. Aliphatic

Aliphatic contain chains of carbon atoms strung together, are further divided into families: alkanes, alkenes, alkynes and their cyclic analog.

a. Alkanes *(paraffin)*

Alkanes are also called paraffins or saturated hydrocarbons, they are the major constituents of crude oil and the major constituents of most petroleum products. Alkanes are chemical compounds that consist only of hydrogen and carbon atoms and are bonded exclusively by single bonds (saturated compounds) (Shah & Patel, 2013). These compounds have an empirical formula of C_nH_{2n+2}. The carbon atoms that make up the carbon backbone are linked together to form a chain linear or branched alkanes. Linear alkanes, whose carbon atoms are arranged in a straight chain or row, are called normal paraffins, normal alkanes, or n-alkanes. The branched alkanes are often called isoparaffins or isoalkanes (Kostecki *et al.*, 2005).

b. Alkenes (Olefins)

The second major group of petroleum hydrocarbon is the alkenes. Alkene is an unsaturated chemical compound containing at least one carbon-to- carbon double bond. The simplest acyclic alkenes, with only one double bond and no other functional groups, also known as olefin (Shah & Patel, 2013). These compounds are usually not found in crude oil but are the by-product of a refining process. The alkenes are distinguished from the alkanes by the fact that they contain two less hydrogen atoms than the alkane with the same carbon backbone. Because the alkenes have two fewer hydrogen atoms, they contain a carbon–carbon double bond (Kostecki *et al.*, 2005). Alkenes have the general formula C_nH_{2n}. The simplest alkene is ethylene (C_2H_4) (Shah & Patel, 2013).

c. Alkynes

Alkynes are hydrocarbons that have a triple bond between two carbon atoms, acetylenes is known name of Alkynes, although the name acetylene also refers specifically to with the formula C_nH_{2n-2} (Shah & Patel, 2013).

d. Cycloalkanes

The cycloalkanes are also known as naphthenes, they are similar to the linear and branched alkanes, except that the carbon backbone has the carbon atoms arranged in a circle. These compounds are sometimes called cycloparaffins and have an empirical formula of C_nH_{2n}. They have saturated rings. The carbon atoms that make up the

backbone are arranged in circles containing usually five, six, or seven carbon atoms (Kostecki *et al.*, 2005).

I.1.5.2. Aromatic

Aromatic hydrocarbons typically contain a six-membered carbon ring and they have an empirical formula of C_nH_{2n-6}. The six-membered ring has three carbon–carbon double bonds (Kostecki *et al.*, 2005). The smallest aromatic compounds in petroleum have six carbons in such a ring structure (e.g. benzene and toluene), but other compounds contain multiple rings. Aromatic hydrocarbons can be monocyclic (MAH) or polycyclic aromatic hydrocarbons, often abbreviated (PAH) or polynuclear aromatic HCs (PNA) (Shah & Patel, 2013). The natural crude oil contains significant amounts of polycyclic aromatic hydrocarbons (A. Kumar *et al.*, 2011). Crude oils contain 0.2 to 7% PAHs; PAH content increases with the specific gravity of the oil. PAHs have high boiling points and low solubility in water (Eze, 2010) . The smallest aromatic molecules (one- and two-rings) are both volatile and readily biodegraded, but four-ring and larger aromatic compounds are more resistant to biodegradation. Some larger PAHs are suspected to be potentially carcinogens (Kothari *et al.*, 2013).The United States Environmental Protection Agency (U.S. EPA) has designated 16 PAHs compounds as priority pollutants (Table 4) (A. Kumar *et al.*, 2011; Kothari *et al.*, 2013).

Table 4: The 16 PAH compounds designated by the U.S. EPA as highly toxic (A. Kumar *et al.*, 2011).

Naphthalene	Acenaphthylene	Acenaphthene	Phenanthrene
Anthracene	Benz[a,h] anthracene	Benz[a] anthracene	Chrysene
Pyrene	Benzo[a]pyrene	Indeno[1,2,3-cd] pyrene	Benzo[g, h, i] perylene
Fluorene	Fluoranthene	Benzo[k] fluoranthene	Benzo[b] fluoranthene

I.1.5.3. Non-hydrocarbon polar compounds

Not all compounds in the crude are hydrocarbons consisting of hydrogen and carbon only. The general categories of non- hydrocarbon constituents are presented in Figure 2.

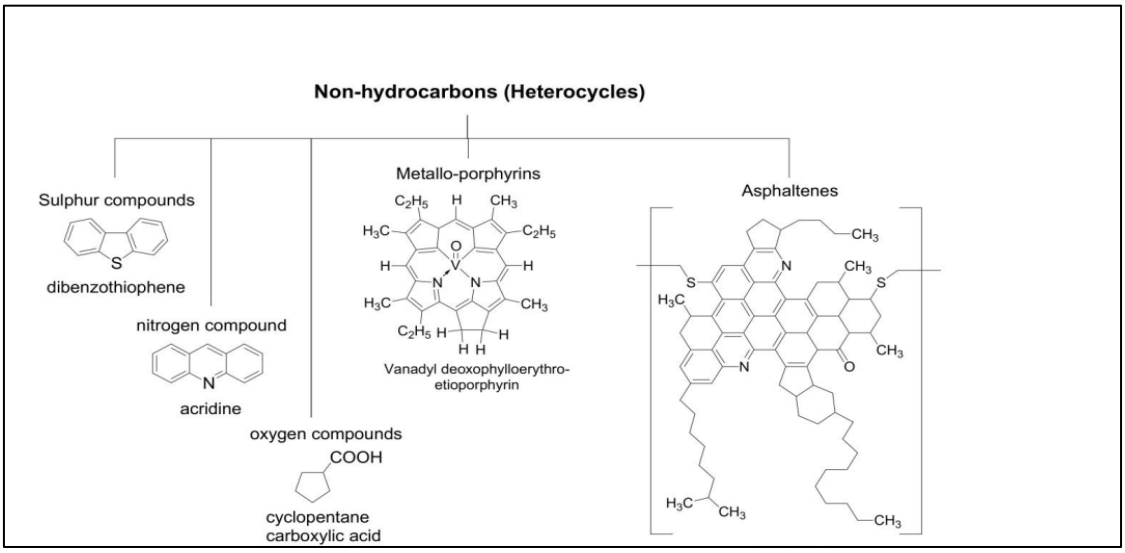


Figure 2: Classification of non- hydrocarbon (heterocyclic) compounds (Shah & Patel, 2013).

a. Resins and asphaltenes

Resins and asphaltenes have very complex and mostly unknown carbon structure with addition of many nitrogen, sulphur and oxygen atoms (Harayama *et al.*, 2004; Shah & Patel, 2013). Resins fraction comprises polar molecules often containing heteroatoms such as nitrogen, oxygen, or sulfur. Resin is a heavier fraction than aromatics and saturates and are composed of fused aromatic rings with branched paraffin and polar compounds. The resin fraction is soluble in light alkanes such as pentane and heptane, but insoluble in liquid propane (Demirbas & Taylan, 2016). Asphaltene constituents are the highest molecular weight heaviest and most polar constituents in crude oil (Speight, 2004). The asphaltenes are polynuclear aromatic structures consisting of 20 or more aromatic rings along with paraffinic and naphthenic chains. The general definition is that asphaltene constituents are insoluble in n-pentane (or in n-heptane) but resins are soluble n-pentane (or in n-heptane) (Speight, 2004).The relative proportions of these fractions are dependent on many factors such as the source, geological history, age, migration and alteration of crude oil (Gordon, 2013). Asphaltenes and resins are high molecular weight compounds. These compounds are non-biodegradable because they are highly insoluble and consist of functional groups protected from microbes attack (Kothari *et al.*, 2013).

b. Sulphur compounds

The sulphur content of crude oil varies from less than 0.05 to more than 10%. Crude oil with less than 1% sulphur is referred to as sweet but sour oils with sulphur contents more than 1%. Crude oils contain sulphur in the form of elemental sulphur S, dissolved H_2S, carbonyl sulphide (Fahim *et al.*, 2009).

c. Oxygen compounds

The oxygen content of crude oil is less than 2%. Crude oil contain oxygen in different forms such as alcohols, esters, ethers, ketones and carboxylic acids (Fahim *et al.*, 2009).

d. Nitrogen compounds

The nitrogen compounds are minor components of crude oil petroleum (Mejeha, 2016).Crude oil contain very low concentration of nitrogen compounds. The nitrogen compounds in crude oil are classified as basic (pyridines) or non-basic (pyrrole) compound (Fahim *et al.*, 2009).

c. Heavy metals

Heavy metals are generally defined as metals with relatively high densities, atomic weights, or atomic numbers. All crude oils contain trace metals, the heavy metals in petroleum has been originated from geochemical processes involved in the generation, migration (to the reservoir) and maturation of petroleum (Mejeha, 2016). Nickel and Vanadium are the most abundant trace metals in crude oil (Okoh, 2006; Mejeha, 2016).

I.1.6. Sources and use of petroleum hydrocarbons

Petroleum products are produced from crude oil by distillation, a process that separates various petroleum fractions by their boiling points. Petroleum hydrocarbons found in the environment are usually originated from crude oil and other petroleum products like diesel, gasoline, lubricating oil (Van Epps, 2006).

Diesel is a complex mixture of hydrocarbons, generally consists of middle distillates of crude oil with boiling points between 200 and 300 °C. Hydrocarbons in diesel tend to be in the C8 to C26 range, Diesel is mainly composed of alkanes (40 to 70 mass%), cycloalkanes (10 to 25 mass%), alkenes (up to 5%) and aromatics (10 to 30%) (Trapp *et al.*, 2001; Van Epps, 2006).

On the other hand gasoline typically comprises the C 4 to C12 crude oil fraction that has a relatively low boiling point (less than 200 °C) (Van Epps, 2006). Gasoline is composed of hydrocarbons, mainly alkanes and cycloalkanes (35 to 65 mass %) and mono-aromatics (25 to 42 mass %) (Trapp *et al.*, 2001).

While polycyclic aromatic (PAHs) may be constituents of crude oil and refined petroleum products, they are a produced during high temperature industrial operations such as petroleum refining (Kothari *et al.*, 2013). PAHs are also widespread in the environment because they are produced by incomplete combustion of coal, oil, wood, or other organic matter (Kanaly & Harayama, 2000). Some of these compounds are suspected to be carcinogens. The increase in molecular weight and number of ring structures of PAHs decreases their volatility and solubility, while increasing adsorption capacity (Kothari *et al.*, 2013).

I.1.7. Crude oil refining processes

Petroleum in the crude state is of limited value, while refining is required to produce products suitable for the market (Speight, 2010). The petroleum refining

process is the separation of the different hydrocarbons present in the crude oil into useful fractions and the conversion of some of the hydrocarbons into products having higher quality performance. The refinery processes convert crude oils to useful products by distillation techniques and separate petroleum products with different boiling point ranges (Hou, 2000). Petroleum products are classified based on the way crude oil is distilled and separated into fractions (called distillates). Refining end-products or the primary end-products produced in petroleum refining may be grouped into three categories as in the below (Speight, 2006):

1. Light distillates (LPG, gasoline and naphtha)
2. Middle distillates (kerosene, diesel)
3. Heavy distillates and residuum (heavy fuel oil, lubricating oils, wax, asphalt).

I.1.7.1. Basic products

There are over 2000 individual refinery products (Fahim *et al.*, 2009). Figure 3 shows the principal refining products with their boiling range, carbon atoms number and their use.

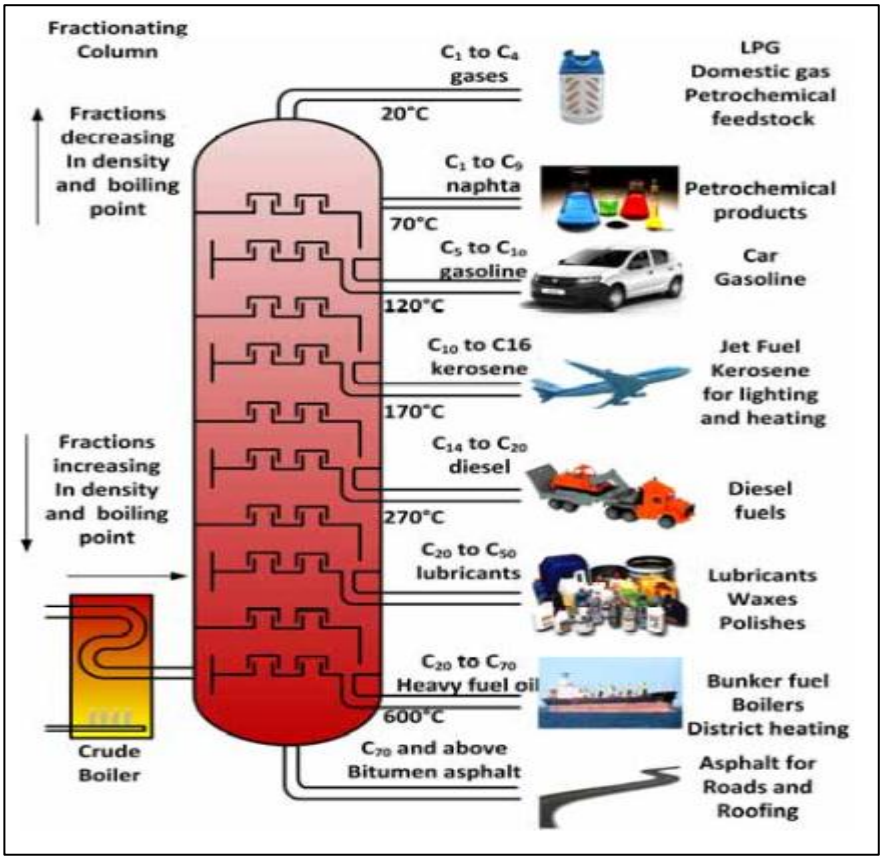


Figure 3: The distillation of petroleum (Devold, 2013).

The basic products from fractional distillation are (Devold, 2013):

- **Liquid petroleum gas (LPG)** has carbon numbers of 1-5 and a boiling point up to 20 °C. Most of the LPGs are propane and butane, with carbon number 3 and 4 and boiling points - 42 °C and -1 °C, respectively. Typical usage is domestic and camping gas, LPG vehicles and petrochemical feedstock.
- **Naphtha** is the fraction with boiling points between 30 °C and 200 °C and molecules generally having carbon numbers 5 to 12. It is used mainly as a feedstock for other processes and for producing additives for high-octane gasoline.
- **Gasoline** has carbon numbers mainly range of 4 to 12 and boiling points up to 120 °C. Its main use is as fuel for internal combustion engines for cars.
- **Kerosene** has main carbon numbers 10 to 16 (range 6 to 16) boiling between 150 °C and 275 °C. Its main use is as aviation fuel and used for lighting (paraffin lamps) and heating.
- **Diesel** is used for diesel engines in cars, trucks, ships, trains and utility machinery. It has a carbon number between 8 and 21 (mainly16-20) and is the fraction that boils between 200 °C and 350 °C.
- **White and black oils**: The above products are often called white oils, and the fractions are generally available from the atmospheric distillation column. The remaining fraction below are the black oils, which must be further separated by vacuum distillation due to the temperature restriction of heating raw crude to no more than 370-380 °C.
- **Lubricating oils** are typically contain 90% raw material with carbon numbers from 20 to 50 and a fraction boiling at 300-600 °C, are used for lubricating waxes and polishes, synthesis of light machine oils, motor oils and greases, as viscosity stabilizers.
- **Fuel oils** is a common term including a wide range of fuels that also comprises forms of diesel, kerosene and the heavy fuel oil as well as bunker that is produced at the low end of the column before bitumen and coke residues. Its main used for ships and power station. The boiling points up to 490 °C.

- **Bitumen** and other residues like coke and tar has carbon numbers above 70 and boiling points above 525 °C. Low sulfur coke can be used for the metals industry (aluminum and steel). Bitumen in the form of asphalt boiling above 525 °C is used for roofing and road paving.

I.1.7.2. Processes types

Petroleum refining processes can be grouped into three basic operations (Speight, 2010):

1. Separation processes (distillation) are the separation of crude oil into groups of hydrocarbon compounds.

2. Conversion processes change the size and/or structure of hydrocarbon molecules, these processes include.

3. Treatment Processes have been used to remove non-hydrocarbons, impurities, and other constituents that adversely affect the properties of finished products or reduce the efficiency of the conversion processes.

The Refinery is divided up in to process types or units:

- **Desalting**

The crude oil is mixed with a variety of substances: water, soil and gases. Desalting is water washing out salt from the crude oil before it enters the atmospheric distillation unit (Speight, 2010).

- **Distillation (Fractionation)**

The basic refinery uses fractional distillation which is is the separation of crude oil in atmospheric and vacuum distillation towers into groups of hydrocarbon compounds of differing boiling-point ranges called "fractions" (Devold, 2013).

Atmospheric distillation

It is often called the Crude Oil Distillation Unit (CDU), atmospheric distillation is the fractional distillation unit. Operation of a distillation column is based on the difference in boiling temperatures of the liquid mixture components that pass through it.

(Speight, 2010). Raw crude cannot be heated to more than 370-385°C (Fahim *et al.*, 2009).

Vacuum distillation unit (VDU)

To extract more distillates from atmospheric residue, the bottom from CDU is sent to the VDU. It is then heated in several exchangers and pump around of the vacuum unit. Final heating to 380-415 °C (Fahim *et al.*, 2009). Vacuum distillation unit further distills the black oils into fuel oils and residual bitumen and coke to avoid overheating the crude and to extract additional valuable product that could be upgraded (Devold, 2013).

After initial fractional distillation, additional treatment processes may also be included,

- **Naphtha hydrotreater**

Various sulphur compounds are present in the hydrocarbon mixture and, if burnt with the other carbons, will cause sulphuric emissions. The hydrotreater uses hydrogen to desulfurize naphtha fraction (C_5-C_{12}) produced naturally by distillation, must hydrotreat the naphtha before sending to a catalytic reformer unit (Fahim et al., 2009; Devold, 2013).

- **Catalytic reformer unit**

Used to convert the naphtha molecules (C_5-C_{12}) into higher octane reformate. The reformate has higher content of aromatics and cyclic Hydrocarbons. An important by-product of a reformer is Hydrogen released during the catalytic reaction (Devold, 2013).

- **Distillate hydrotreater**

Desulfurizes distillates (such as Diesel) after atmospheric distillation (Devold, 2013).

- **Fluid catalytic cracking (FCC)**

Fluid catalytic cracking play main role for production of gasoline (Fahim et al., 2009). FCC units transform heavier fractions into lighter, more valuable products and

long chain carbon numbers are split into shorter molecules to achieve more of the high value fuel (Devold, 2013).

- **Hydrocracking**

A hydrocracker unit performs the same function as the FCC when more saturated hydrocarbons are desirable in the product. Uses hydrogen to transform aromatics, or more complex molecules with double bonds into alkane carbon chains with single bonds (Devold, 2013).

- **Merox unit** treats LPG, kerosene or jet fuel by oxidizing thiols to organic disulphides.
- **Coking units** transform very heavy residual oils into gasoline and diesel fuel, the coke is a residual product.
- **Alkylation unit** uses sulphuric acid to produce high-octane components for gasoline blending. The main use is to convert isobutane to isooctane or isoheptane by adding an alkyl group.
- **Dimerization unit** is similar to alkylation, but uses a dimer group instead of an alkyl group. For example, butenes can be dimerized into isooctene, which may be hydrogenated to form isooctane.
- **Isomerization unit,** converts linear molecules to higher-octane branched molecules by rearranging the same atoms.
- **Steam reforming unit,** produces hydrogen for the hydrotreater or hydrocracker.
- **Claus unit**, Amine gas treater and tail gas treatment convert hydrogen sulphide from hydro-desulphurization into elemental sulphur.
- **Blending and distribution**

After the refining processes, the various fractions are stored in intermediate tanks, and then blended into marketable products and distribution to gas stations or industries (Devold, 2013).

Figure 4 shows a schematic diagram of a typical modern oil refinery that describes the various unit processes, the intermediate and the end products.

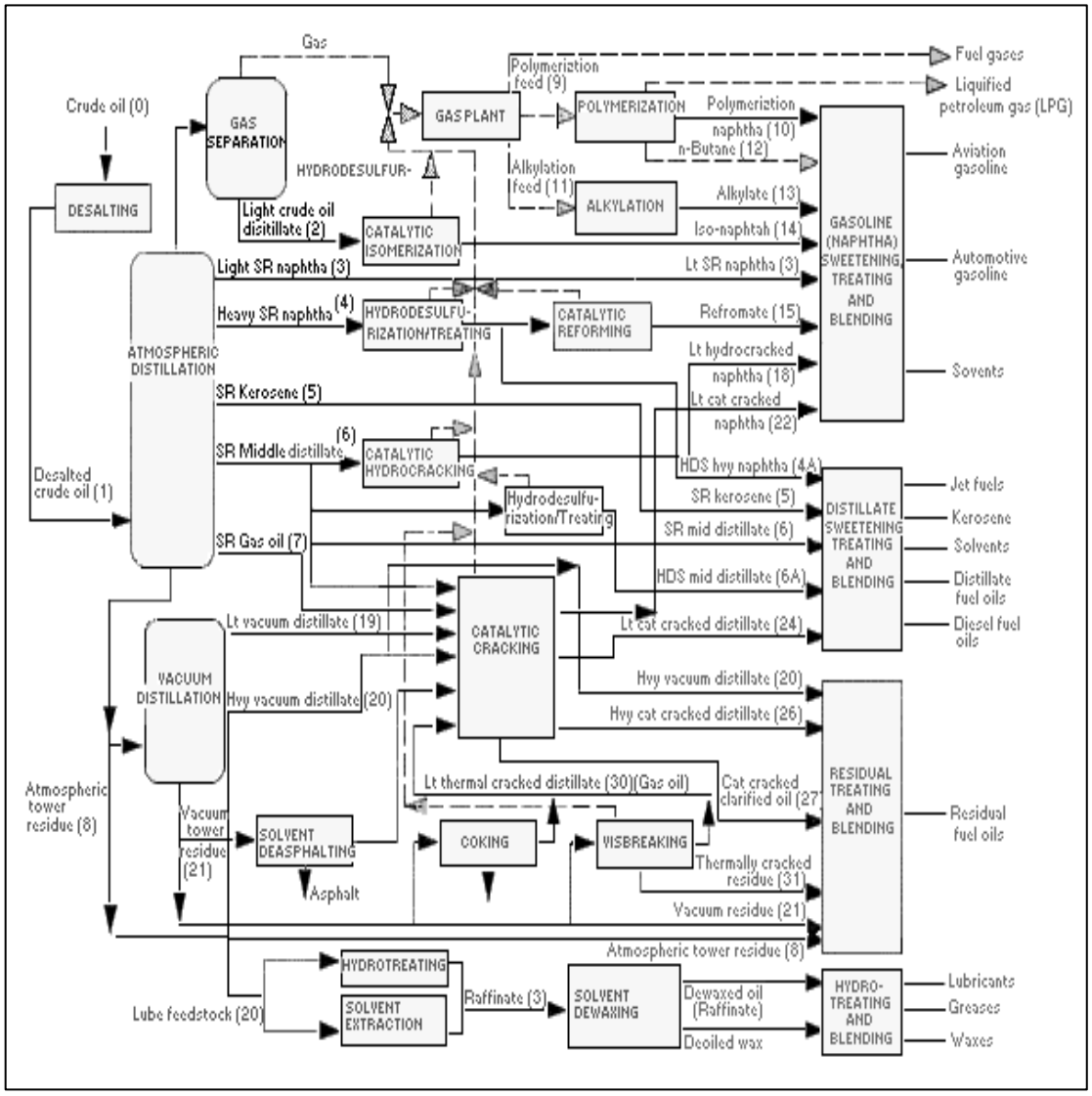


Figure 4: Typical layout for an oil refinery.
(https://www.osha.gov/dts/osta/otm/otm_iv/otm_iv_2.html)

I.1.8. Hydrocarbons in Algeria

Algeria is an important exporter of oil and natural gas and is a member of the Organization of the Petroleum Exporting Countries (OPEC). The hydrocarbons sector is the backbone of the Algerian economy, with reserves of 12.2 billion barrels of oil and 4.5 trillion cubic meters of gas, Algeria holds the world's 14th largest oil reserves and has the ninth-largest reserves of natural gas in the world and is the fourth-largest gas exporter (https://www.agcc.co.uk/uploaded_files/energy_briefing_ukti%20-%20Algeria.pdf).

Algeria is a major refining center with five refineries; Skikda, Algiers, Arzew, Hassi Messaoud and Adrar (Figure 5). With a total output of 350 000 barrels per day of refining oil, Arzew oil refinery have the capacity to process 60 000 barrels of crude oil per day (Chettouh & Hamzi, 2014).

Algeria uses seven coastal terminals for the export crude oil, refined products, liquefied petroleum gas (LPG) and natural gas liquids (NGL). There are facilities located at Arzew (Algeria's largest crude oil export port), Skikda (Algeria's second largest crude oil export port), Algiers, Annaba, Oran, Bejaia, and Skhirra in Tunisia. Arzew handles about 40% of Algeria's total hydrocarbon exports. Algeria's oil pipeline network facilitates the transfer of oil from interior production fields to these export terminals. Sonatrach operates over 2,400 miles of crude oil pipelines in the country.

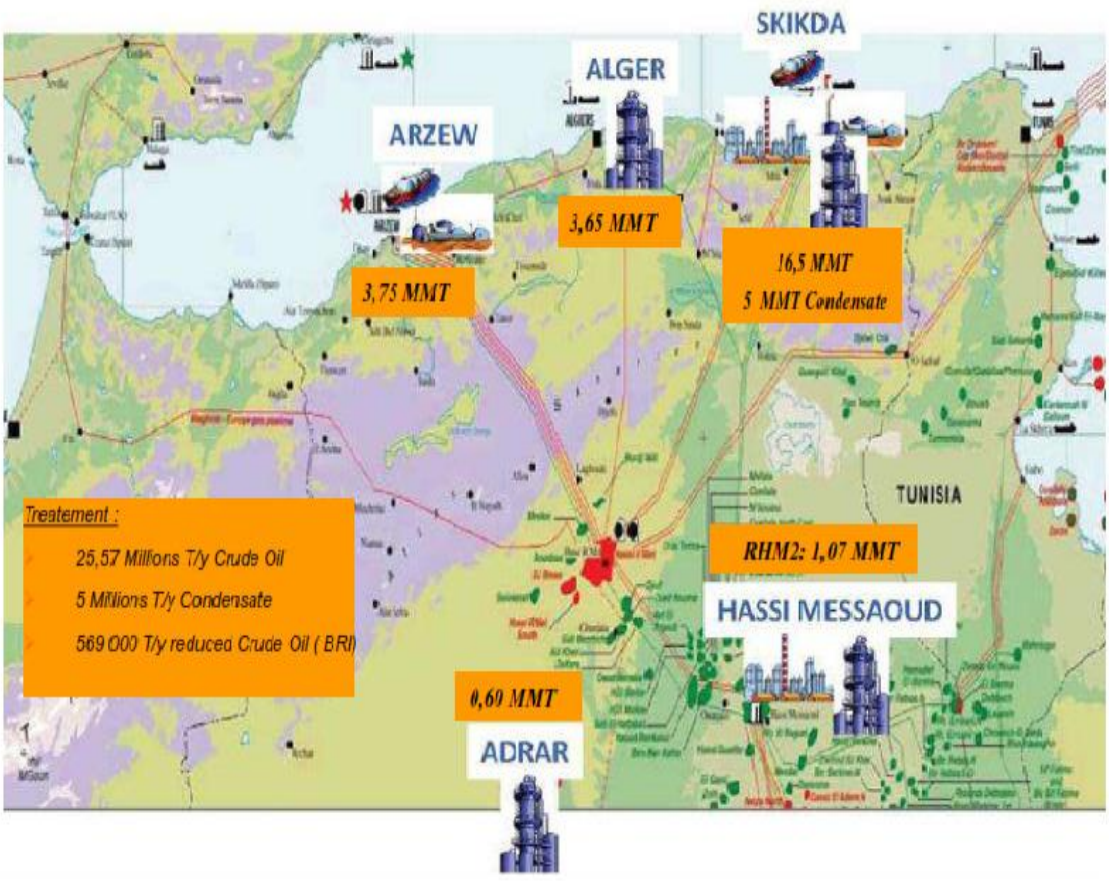


Figure 5: Existing refineries localization.
(http://mem-algeria.org/hydrocarbons/deposits_hydroc.html)

I.1.9. Arzew refinery

Arzew refinery (RA1Z) implanted on the industrial area of Arzew on El-Mahgoun platform, 40 Km from Oran city (Figure 6). It covers a surface of 150 Ha. The construction of the plant has been performed by the Japan Gasoline Corporation Company and started on 19 June 1970.The refinery starts up in March 1973.

Figure 6: Arzew industrial zone (http://www.sonatrach.dz/index.php?option=com).

I.1.9.1. The missions of the refinery

The refinery is designed for the processing of Hassi Messaoud crude oil, meeting national market needs in fuel, lubricants and bitumen, and the exploitation of surplus products mainly kerosene naphtha, fuel oil. Arzew oil refinery have the capacity to process 60 000 barrels of crude oil per day (Chettouh & Hamzi, 2014) and 2,5 million tons /year (http://www.energy.gov.dz/francais/index.php?page=raffinage).

I.1.9.2. Presentation of the refinery units

The Arzew refinery consists of several units:

a. Utilities

Two utilities units (zone 3 and 19) produced and fixed supply and distribution, for process needs: distilled water; electricity; service and instrument air; treated cooling water; fuel gas and steam.

b. Carburants

Atmospheric distillation unit (U.11) it's the main plant unit where Algerian crude oil is processed, after distillation, following products are obtained: Liquefied petroleum gases (LPG); heavy naphtha; light naphtha; kerosene; gas oil and reduced crude which constitutes a charge of vacuum distillation to produce base stock.

Catalytic reforming unit (U.12)**:** Heavy Naphtha of atmospheric distillation unit to obtain a high octane number reformats LPG and gas riches in hydrogen.

Gas plant unit (U.13)**:** Liquefied petroleum gases obtained from the atmospheric distillation unit and catalytic reforming unit are processed in this unit and the following products are obtained: Propane and Butane.

C. Asphalts

Vacuum flashing unit (U.14)**:** Imported reduced oil is fractioned on vacuum gas oil and a viscous product that is obtained in the bottom column; this latter is processed in blowing section to obtain road asphalts.

Oxidized asphalt unit (U.15): Pure asphalt mixed with vacuum gas oil, constitutes the feed of this unit. Oxidized asphalt is obtained from pushed oxidation by air.

d. Lubricants

Arzew refinery is constituted by:

- Two base oil production plants, their annual capacities are 48000 TM and 120000 TM;
- Two lubricating oil blending and filling units;
- Two production and filling grease units;
- A treatment unit and two paraffin-molding units.

The two base oil production plants are set up by the following units: A vacuum distillation unit; a propane desasphalting units; Furfural extraction unit; MEK (methyl - ethyl - ketene) and dewaxing unit and Hydro-finishing of oil.

CHAPTER TWO
Petroleum Hydrocarbon pollution

I.2. Petroleum hydrocarbon pollution

I.2.1. Oil spills

In recent years the demand for petroleum as a source of energy, fuel resources and as a primary raw material for the chemical industry has resulted in an increase in worldwide consumption (Canny, 2002).

The spillages of oil, which occur during routine processes of crude oil extraction, refining, transportation and storage, have increased in incidents of both accidental and deliberate release of petroleum products into environment. Crude oil is the most important organic pollutant in the environment, as 1.7 to 8.8×10^6 tonnes of petroleum hydrocarbons are being released to the marine environments annually (McKew *et al.*, 2007).

Environmental contamination by crude oil is relatively common because of its widespread use and its associated disposal operations and accidental spills. Environmental pollution caused by petroleum is of great concern because petroleum hydrocarbons are toxic to all forms of life. The hydrocarbon contamination in the environment is a very serious problem whether it comes from petroleum, pesticides or other toxic organic matter (Abha & Singh, 2012).

The environmental problems caused by oil spills, is not limited to visible pollution, because there are chronic effects that silently endanger ecosystems, biodiversity and environmental balance, due to bioaccumulation, leaching and extension of contaminants into groundwater with potential effects on all living organisms (Vázquez-Luna, 2014). Oil spills can leave a legacy for decades, their impacts on marine ecosystem (deaths of marine animals), migratory birds and on human health effects are difficult to evaluate (Q. Wang *et al.*, 2011). Table 5 shows major worst oil spills from 1967 to 2010. Natural seeps are found all over the world, and the scale of their releases is quite large.

Table 5: Major oil spills in the world by volume, 1967-2010 (Eze, 2010; Kothari *et al.*, 2013).

Oil spill name	Dates	Site	Quantity Spilled (in millions of gallons)
Arabian Gulf	January 19, 1991	Persian Gulf, Kuwait	520
Mexican Gulf	2010	Mexico	177
Ixtoc 1	June 3, 1979	Bay of Campeche, Mexico	140
Atlantic empress	July 19,1979	Off Tobago	90
Kolva river	Sept. 8, 1994	Kolvar river tributary, Russia	84
Nowruz oil field	Feb. 10, 1983	Persian gulf, Iran	80
Castillo de bellver	Aug. 6, 1983	Off saldanha bay, South Africa	79
Amoco Cadiz	March 16, 1978	Portsall, France	69
ABT summer	May 28, 1991	Off Angola	51-81
Haven	April 11, 1991	Genoa, Italy	45
Odyssey	Nov. 10, 1988	Off Nova Scotia, Canada	41
The sea star	Dec. 19, 1972	Gulf of Oman	35.3
The torrey canyon	March 18, 1967	Scilly Isles, UK	25-36
Prestige	Nov. 13, 2002	Off Spain	20

I.2.2. Petroleum hydrocarbons toxicity

Toxicity is the ability of a chemical substance to alter and damage certain functions of the biological systems (Vázquez-Luna, 2012). There are two type of toxicity; acute toxicity is produced by large, short-termed, accidental polluting agents' discharges (Roth & Baltz, 2009). Furthermore, chronic toxicity is expressed by effects noticed on the long term due to relatively small amounts of a toxic compound found on air, soil or water (Scarlett et al., 2007).

Toxicity of petroleum hydrocarbon is a major concern in environmental hazard management and control. The increasing toxicity order of petroleum hydrocarbons is alkanes< cycloalkanes< alkenes< aromatics. Within each class, toxicity increases as molecular weight decreases (Hou, 2000). Persistence and toxicity of the fuels increased in the order of jet fuel < heating oil < diesel oil, for example diesel oil is more toxic than other petroleum products such as jet fuel to their greater concentration of aromatics (Song *et al.*, 1990; X. Wang & Bartha, 1990). On other hand Vázquez-Luna, 2012 reported that the petroleum hydrocarbons compounds with of the highest molecular weight are the most persistent within the environment.

Short-chain alkanes (< C10) generally are toxic to microorganisms because of their high water solubility and their interaction with membrane lipids (Q. Wang *et al.*, 2011). The aromatics compounds in crude oil produce adverse effects to the local microbial flora. Q. Wang *et al.*, 2011 was found that phenolic and naphthalene derivatives inhibited the growth of the microbial cells. Also, cyclohexane inhibit oxygen uptake in intact cells and isolated mitochondria (Q. Wang *et al.*, 2011). The petroleum aromatic compounds that are carcinogenic and long exposure to these compounds often leads to tumors and cancer, these compounds are an important group of environmental pollutants. The aromatic compounds are introduced into the environment from various sources such as natural or accidental oil spills, refinery waste products, steam and oil storage, etc. Petroleum hydrocarbons can rapidly migrate from the site of contamination and adversely affect aquatic terrestrial and ecosystems and humans (Abha & Singh, 2012). Hou (2000) reported that the mono aromatic (benzene) and di-aromatic (naphthalene) components in crude oil are considered to be the most toxic and most abundant compounds during the initial phases of petroleum spills. However, aromatic compounds tend to be more toxic than aliphatic compounds (Van Epps, 2006).

I.2.3. Environmental impacts of petroleum contamination

Crude oil and its products have aroused much interest in recent years among environmentalists and ecologists in all parts of the world because to the damage on living organisms as well as arable lands by spilled oil. Releases of petroleum into the environment occur naturally from seeps as well as from human sources (Eze, 2010).

The release of petroleum products in large quantities into the environment has impacted negatively on various ecosystems (sea, lands, wetlands and underground water). Their undesirable effects endanger plants and animals lives (Gordon, 2013).

I.2.3.1. Ground ecosystem: soil, microorganisms and plants

Soil is an essential component of the terrestrial ecosystem. It provides the environment for the growth of plants, cycling of nutrients and a living base for microorganisms, insects and animals. Soils are very diverse in composition. (Hou, 2000). Soil contaminations are from industrial accidents, leaky containers trucks, cars, underground storage tanks and poorly disposed of hydrocarbons wastes on land.

There are properties inherent to soils that favor the fixation of the hydrocarbon pollutants. For example soils with a clay texture take a long time to improve from an oil spill, while the thick texture soils improve in short time. The thick texture can favor mobility towards the phreatic surfaces by the infiltration of the pollutant towards the ground water (Figure 7) (Vázquez-Luna, 2012).

Petroleum contamination that lies within unsaturated soil may exist in the form of petroleum vapours in the soil pore space, as residual petroleum adsorbed into soil particles, or as petroleum dispersed in soil moisture. Due to the toxicity of petroleum hydrocarbons, petroleum contamination of soil could be a hazard to plants, animals, and a threat to human health through direct exposure (Hou, 2000).

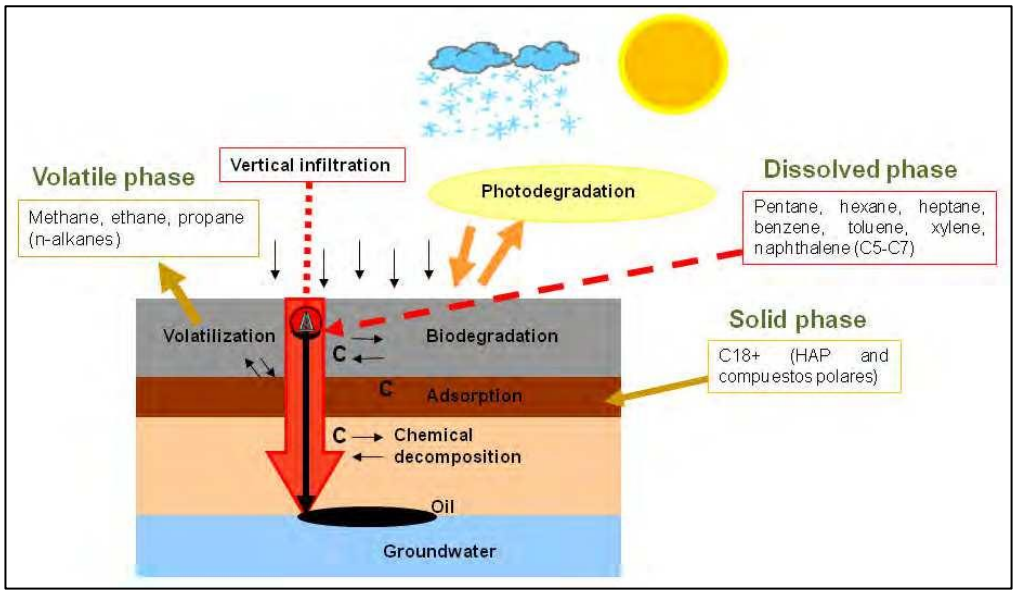


Figure 7: Vertical infiltration process of crude oil (Vázquez-Luna, 2012).

There are several effects due to the crude oil soil pollution, since the oil blocks the gas interchange with the atmosphere (Leitgib *et al.*, 2008) and change the physical and chemical properties of the soils, which leads to diminish the microbial communities

benefic to the soil (Labud *et al.*, 2007). These microbiological variables are indeed a good indicator of the impact of a pollutant on the soil. The toxicity mechanisms caused by the oil on soils is not limited to the microorganisms, since it also includes plants that suffer from hydric stress due to the lack of water and nutrients (Vázquez-Luna, 2012).

a. Microorganisms

Petroleum pollution has been reported to cause microbial population changes such as increases or decreases in microbial numbers (Eze, 2010). Ebuehi *et al.* (2005) reported that the hydrocarbon-utilizing bacterial population increased while the population of heterotrophic bacteria decreased as a result of the crude oil soil pollution. On other hand Ilarionov *et al.* (2003) found that the toxic concentrations of oil on the soil inhibit the development of different species of protozoa, rotifers, algae, fungi, bacteria and actinomycetes.

b. Plants

Oil forms a hydrophobic layer that decreases the water retention. This reduces the plants water retention capability, directly affecting the seeds emergence and germination (Quiñones *et al.*, 2003). Other effect is reflected due to the volatile oil fractions that penetrate and damage the seeds embryo. Other researches find that high hydrocarbons concentrations damage the plants growth, particularly the pollutants diminish the radicle elongation and the vegetative growth (Vázquez-Luna, 2012). Lethal effects of crude oil or oil product on plants are caused by contact with oil or dissolved oil, uptake of oil compounds, blockage of air exchange through surface pores, and by physical or chemical alteration of soil and water (Albers, 1995).

I.2.3.2. Ground water

Groundwater is important as a source of drinking water as well as for irrigation and industrial use. If a significant volume of liquid hydrocarbons is released into the subsurface, the hydrocarbons migrate downward generally under the influence of gravity and capillary forces until they reach the groundwater (Hou, 2000). Some components of petroleum such as toluene, xylenes, benzene and ethyl benzene (BTEX) have high water solubility and poses serious health threat as these contaminants are linked with mutation and cancer (Adebusoye *et al.*, 2006).

I.2.3.3. Marine ecosystems

The rapid increase in the demand for utilisation of petroleum and petrochemicals has resulted in a steadily increasing level of petroleum contamination of marine and estuarine waters (Anderson *et al.*, 1974). Since most oil floats on the surface of the water it can affect many marine animals and sea birds (Shah & Patel, 2013). The oil spill it becomes weathered when introduced into the marine ecosystem (Figure 8), during which several processes take place, such as the evaporation, dispersion, emulsification, dilution, sedimentation and biodegradation (Vázquez-Luna, 2012).

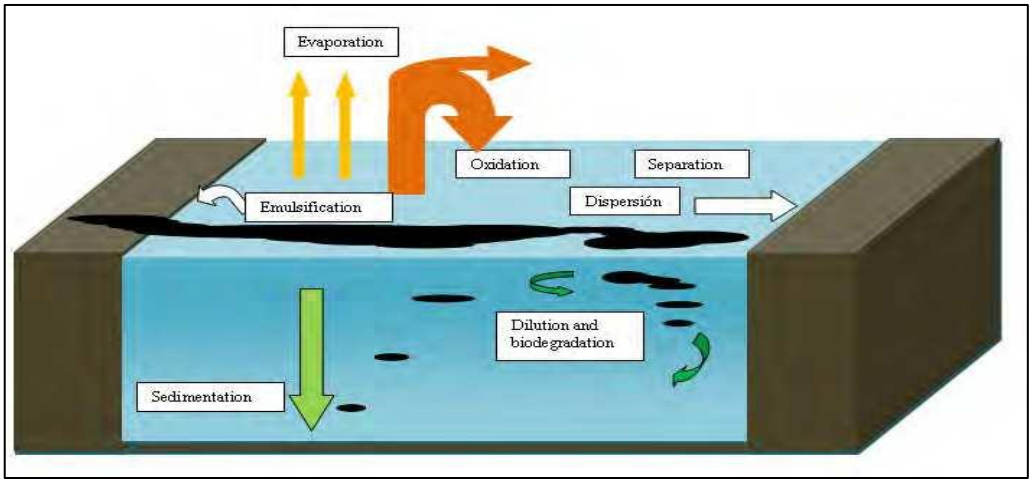


Figure 8: Weathering process of the oil spilled in the sea (Vázquez-Luna, 2012).

I.2.3.4. Human beings

Vázquez-Luna (2012) reported that there is negative effects over the human population health living in areas near oil zones (up to 5 km). In other study, San Sebastián *et al.* (2001) found that women living near oil wells zones presented symptoms such as headache, tiredness, eye irritation, diarrhea and gastritis, nasal and throat irritation. The hydrocarbons pollution might have carcinogen and mutagenic effects on human health (Neff, 2002; Vázquez-Luna, 2012).

I.2.4. Natural fate of spilled oil in the environment

I.2.4.1. Fate and transport of contaminants in the subsurface

Naturally formed soil profiles are containing distinct horizons with specific hydraulic and physical characteristics. The presence of these layers in the soil profile will generally retard movement of contaminant during infiltration (Youdeowei, 2012).

The fate of hydrocarbons in the subsurface depends on the processes of transport, volatilization, dissolution, geochemical reactions, biodegradation, and sorption. Contaminant transport in the subsurface is affected by different processes. They include advection, dispersion, diffusion, adsorption and degradation (Youdeowei, 2012). The general schematic of a hydrocarbon spill is shown in Figure 9.

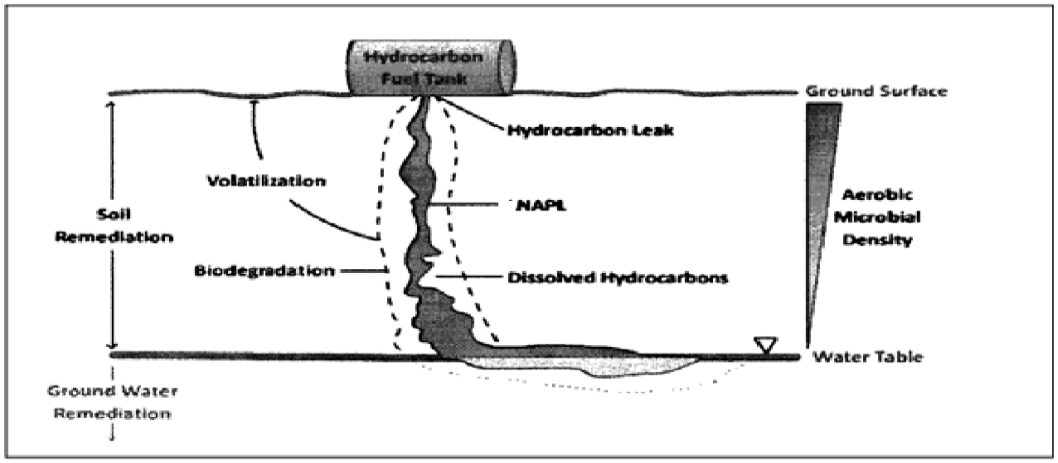


Figure 9: Summary of the major processes to understanding the fate and transport of hydrocarbons in the subsurface (Horel, 2009).

While the bulk hydrocarbon or non-aqueous phase liquid (NAPL) is moving downward toward the water table, part of the hydrocarbon will volatilize and migrate through the soil pores into the air above the ground surface, while part of the compounds will be trapped in the pore space (Figure 10). In hydrocarbon contaminated soils, the hydrocarbon fraction may be present in the pore space as adsorbed fraction to the solid particles (solid phase), dissolved in soil moisture (water phase), vapor of volatile compounds (air phase) or bulk NAPL (free phase) (Horel, 2009).

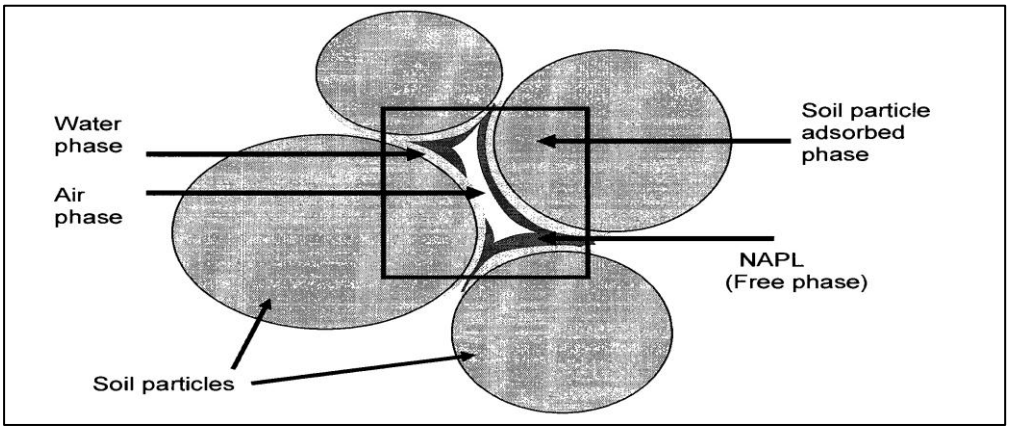


Figure 10: Hydrocarbon distribution in soil (Horel, 2009)

Youdeowei (2012) reported that the adsorption process is the ability of a contaminant to "attach" itself to the soil. It is dependent on the properties of the soil. The fate of hydrocarbons highly depends on the soil water content, where residual saturation can account for a large fraction of the contaminant (Selker *et al.*, 1999).

The degradation is the biological decomposition of dissolved compounds. Biodegradation plays an important role in the fate of crude oil. Many components of petroleum are readily degraded by subsurface micro-organisms (Youdeowei, 2012)

I.2.4.2. Fate of the spilled oil in marine water

After the crude oils are spilled, on the sea surface they spreads to form a thin layer called oil slick. The oil slicks are then acted upon by several natural processes together to degrade the oil slick. These processes are referred as oil weathering processes (OWP). The weathering processes significantly alter the slick properties especially density and viscosity of crude oils (Mishra & Kumar, 2015).

Crude and refined oil spills in the environment change composition on exposure to air, water or sunlight. These conversion into other organic compounds or reduce the severity of an oil are caused by physical, chemical and biological processes (Eze, 2010). These natural processes include: advection, spreading, evaporation, dissolution, dispersion, emulsification, biodegradation and sedimentation (Figure 11) (Spaulding, 1988; Youdeowei, 2012).

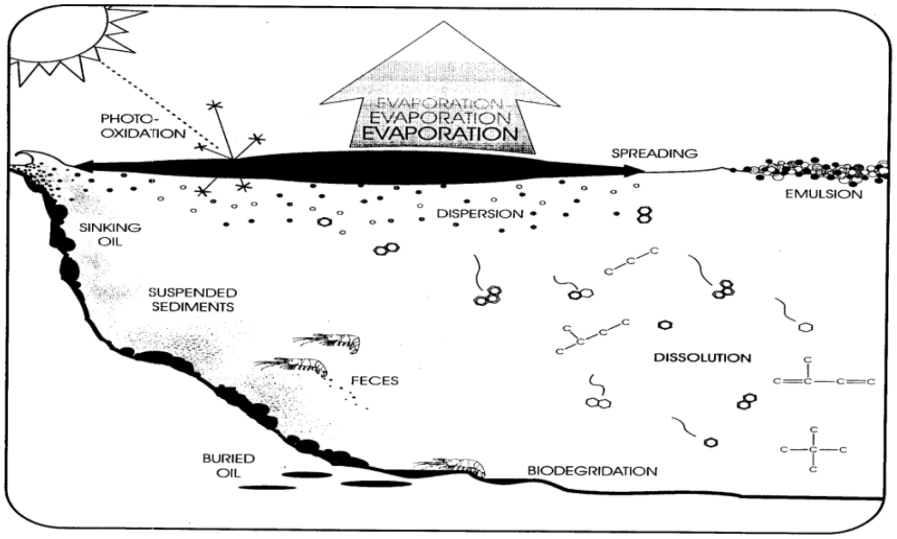


Figure 11: Summary of the major oil weathering processes for oil spilled into the marine environment (Scholz *et al.*, 1999).

a. Advection

Oil moves horizontally in the marine environment under forcing from wind, waves, and currents. The oil is also transported vertically in the water column in the form of droplets of various sizes when its density is only slightly below than water density, both vertical and horizontal transport are therefore important factors in the net move of oil at marine environment (Reed *et al.*, 1999).

b. Spreading

Spreading is the movement of the entire oil slick horizontally on the water surface due to effects of gravity, friction, viscosity, and surface tension (Scholz *et al.*, 1999). If the oil spill is on water, they spread rapidly. This has the effect of providing a larger surface area for evaporation and dissolution (Prince & Lessard, 2004).

c. Evaporation

Evaporation is the preferential transfer of light- and medium weight components of oil from the liquid phase to vapour phase (Scholz *et al.*, 1999). Small hydrocarbons are very volatile, and molecules smaller than those with approximately 15 carbons typically evaporate quite readily if a spill is on the surface (Prince & Lessard, 2004).

d. Dissolution

A few hydrocarbons are sufficiently soluble in water that they transfer from a slick on the surface into the water phase (Scholz *et al.*, 1999). This phenomenon is usually only significant for the BTEX compounds (benzene, toluene, ethylbenzene, and the xylenes) and oxygenated compounds (Prince & Lessard, 2004).

e. Dispersion

Although oils do not dissolve in water, they can disperse. Occurs when wave action breaks up floating slicks into small oil droplets which mix and spread within the water column (Prince & Lessard, 2004). Following evaporation, it is the most important process in the breakup and disappearance of a slick (Scholz *et al.*, 1999).

f. Emulsification

Emulsification is a process that forms emulsions consisting of a mixture of small droplets of oil and water. Emulsions are formed by wave action. Water in oil emulsions

are highly viscous and greatly reduce weathering processes. The largest volume of emulsion is formed within the first week of the spill (Scholz *et al.*, 1999).

g. Photooxidation

Photo-oxidation is when sunlight, in the presence of oxygen, transforms hydrocarbons through photo-oxygenation (increasing the oxygen content of a hydro-carbon component) into new by-products (Scholz *et al.*, 1999). Photooxidation is an important phenomenon not because it removes large volumes of oil from the environment but because it removes the molecules of most toxicological concern such as polycyclic aromatic hydrocarbons (Prince & Lessard, 2004).

h. Sedimentation

Sedimentation is very important process which is incorporation of oil within both suspended and bottom sediment. Sedimentation usually occurs with the heavy-weight components of the oil which do not dissolve in the water. Oil adheres to detrital particles which are mixtures of organic matter, bacteria and small clay (Scholz *et al.*, 1999). At high concentrations of crude oils and heavy refined products interact with sand and gravel so that the oil becomes completely saturated with sediment (Prince & Lessard, 2004).

i. Biodegradation

Biodegradation is the ultimate fate of hydrocarbons released into the environment, although it is sometimes a slow process (Prince & Lessard, 2004). It is a process that occurs when micro-organisms such as bacteria and fungi consume hydrocarbon to use us a food source. Carbon dioxide and water are waste products. Biodegradation is slow process but is significant, it is starts several days following a spill and will continue as long as hydrocarbon persist (Scholz *et al.*, 1999). Biodegradation occur under aerobic and anaerobic conditions. The total biodegradability of the hydrocarbon fraction of different crude oils range from 70 to 97% while the resins, asphaltenes, and other polar compounds in crude oils are not thought to be very susceptible to this process (Prince & Lessard, 2004).

CHAPTER THREE
Bioremediation of oil polluted soil

I.3. Bioremediation of oil polluted soil

Oil spillage and oil pollution in soil and water have been a major threat to the ecosystem and human being through the transfer of toxic organic materials into the food chain. Soil contamination with hydrocarbons causes extensive damage of local system since accumulation of pollutants in animals and plant tissue may cause death or mutations (Clark *et al.*, 1989). The oily wastes are often expensive to destroy, and contaminated areas required expensive remediation processes to reduce the dispersion of contaminants (Otaiku, 2007).

Remediation has been defined as the management of a contaminant at a site so as to prevent, reduce or mitigate damage to human health, or the environment, which can also lead to quick recovery of the affected lands. Physical, chemical and biological processes are employed for remediation (Ebuehi *et al.*, 2005). Physical and chemical methods are neither environment friendly nor cost effective. It is observed that, for the removal of these hazardous products bioremediation is more suitable alternative (Ojo, 2006). Remediation strategies for hydrocarbon polluted soils are:

I.3.1. Physical and chemical methods

Physical and chemical methods for soil remediation are strategies based on the chemical and physical properties of the contaminants and the contaminated media. The viscosity, density, solubility, volatility and physical state of the contaminants and contaminated media are exploited for physical remediation techniques while chemical techniques are often used to convert contaminants into a less harmful form. This techniques could be *in-situ* or *ex-situ* (Mejeha, 2016).

These methods are usually expensive and labor-intensive and most are non-ecofriendly. Some of the physical remediation methods include thermal treatment wich involves the heating of soil to a temperature less than 600 °C to change the pollutant to its gaseous form, air sparging, soil washing, chemical extraction (surfactants or solvent washing), volatilization and steam extraction (Abioye, 2011). Usually, the physical oil clean-up methods limit the migration of the contaminant. This means that the contaminants are not completely eliminated from the environment (Eze, 2010).

The chemical methods that involve the addition of chemicals to convert the contaminant to a less harmful form could generate a more toxic contaminant that may be harmful to the environment (Mejeha, 2016). For example, the use of chemical dispersants (Figure 12) further introduce more contaminants into the environment (Eze, 2010). Hamdan and Fulmer (2011) reported that the combination of Louisiana sweet crude oil with the chemical dispersants (COREXIT® EC9500A) used for the remediation of Deep Water Horizon oil spill in the Gulf of Mexico was more toxic to the aquatic wide life than the crude oil alone and particularly inhibit the hydrocarbon degrading bacteria.

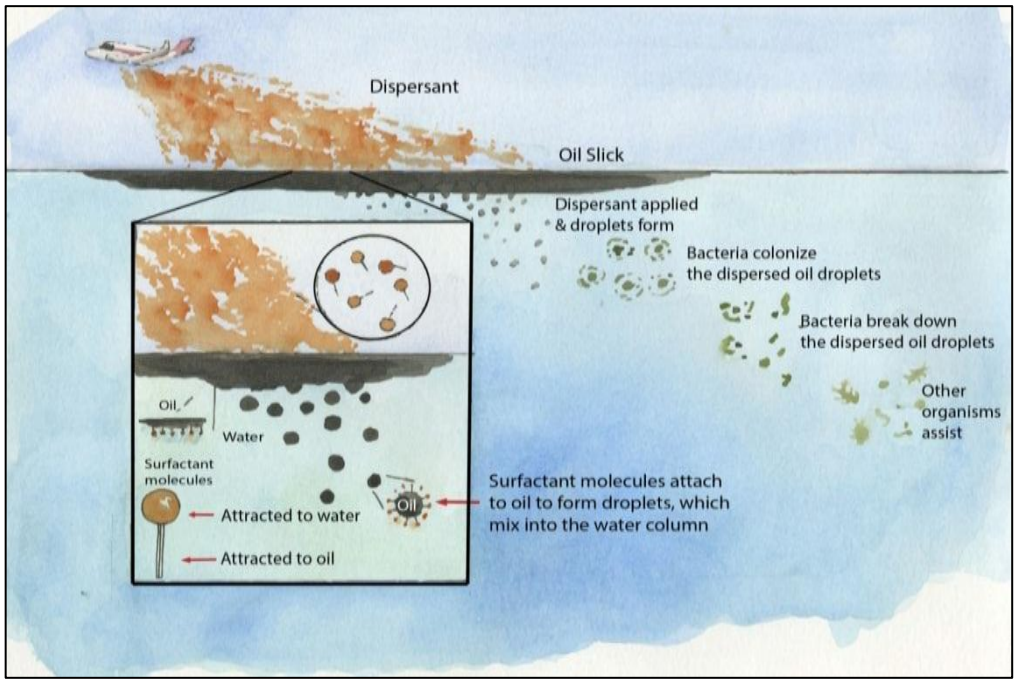


Figure 12: Chemical dispersants and their role in oil spill response.

Dispersants are one strategy used to respond to an oil spill, contain molecules that have one end that is attracted to water and one end that is attracted to oil. When dispersants are applied to an oil slick, these molecules attach to the oil, allowing the oil slick to be broken up into smaller oil droplets. These smaller droplets then mix into the water column and where they are "eaten" and further degraded (broken down) by microbes and other organisms (Graham et al., 2016).

I.3.2. Biological methods (bioremediation)

Bioremediation is one of the most viable processs for remediating soil contaminated by organic and inorganic compounds considered harmfull to environmental health. Biological remediation, a process defined as the use of microorganisms or plants to detoxify or remove organic and inorganic compounds from the environment (Abioye, 2011).

Bioremediation takes place at contaminated sites by using technologies to enhance biodegradation processes. It is a term where natural remedy is applied to a contaminated site cleanup process (Horel, 2009). Usually, microorganisms utilize organic contaminants as carbon sources, energy and thus a complete mineralization of such organic compounds to the simplest compound CO_2 is achieved. This process therefore effectively removes the organic contaminants from the environment (Atlas, 1995). Bioremediation extends the natural processes by which microorganisms consume organic molecules, including hydrocarbons. The microorganisms convert organic molecules to cell biomass and products such as carbon dioxide and water that can be readily accommodated in the environment (Atlas & Cerniglia, 1995).

Bioremediation approach is currently applied to contain contaminants in soil, ground water, surface water and sediments including air. These technologies have become attractive alternatives to conventional cleanup technologies due to relatively low capital costs and their nature is not harmful to the environment (Prasad & Subrahmanyam, 2011). The process of bioremediation are based on **natural attenuation**, which bioremediation occur without human intervention or refers to the enhancement of this natural process, either by introduction of microorganisms to the soil, referred to as **bioaugmentation**, or by adding the appropriate conditions (such as supplying oxygen, moisture and nutrients) for growth of the microorganisms to the soil, referred to as **biostimulation** (Otaiku, 2007; Dolon & Bauder, 2011; Joutey *et al.*, 2013). In the presence of air or oxygen bioremediation process is called aerobic bioremediation and typically proceeds through oxidative processes to render the contaminant either partially oxidized to less toxic by-products or fully oxidized to mineral constituents: carbon dioxide and water. In the absence of oxygen, this processes are called anaerobic bioremediation processes are more complex. In anaerobic respiration, organic contaminants can be mineralized provided sufficient nitrate or sulfate is present. Bioremediation processes involve: complete oxidation of organic contaminants (termed mineralization), biotransformation of organic chemicals into smaller less toxic constituents, or reduction of highly electrophilic halo- and nitro-groups by transferring electrons from an electron donor (typically a sugar or fatty acid) to the contaminant, resulting in a less toxic compound (Rockne & Reddy, 2003).

I.3.2.1. Types of Bioremediation

There are two main methods to oil spill bioremediation: *In-situ* bioremediation and *Ex-Situ* bioremediation (A. Singh *et al.*, 2009). Figure 13 summary the major bioremediation technologies

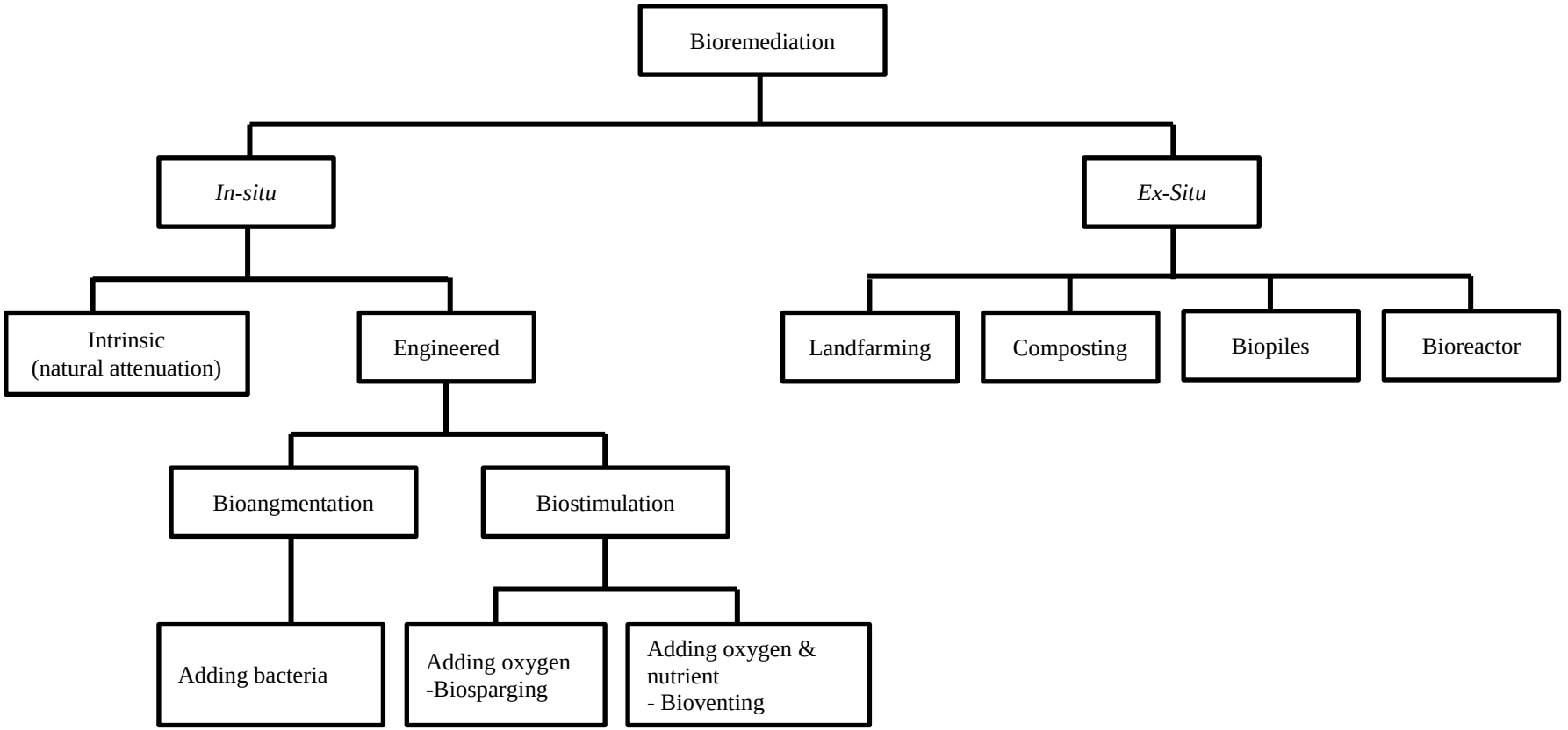


Figure 13: Bioremediation strategies (A. Singh *et al.*, 2009)

i. *In-situ* bioremediation

In-situ bioremediation does not require to excavate or remove soils or water to accomplish remediation and this leads to important cost savings due to the elimination of costs of excavation and transportation of the contaminated soil (Durga *et al.*, 2014). *In-situ* bioremediation also presents some disavantage, such as: it is difficult to control natural conditions (environmenta factors) and it is not suitable for all soils (Coste *et al.*, 2013). Some of the *In-situ* bioremediation biotechnologies of soil contaminated with petroleum hydrocarbons are:

a. Intrinsic bioremediation (natural attenuation)

This is the natural non-engineered process when the contaminants are reduced by the indigenous microbial population without anny human augmentation (Dolon & Bauder, 2011; Joutey *et al.*, 2013).

b. Biostimulation

This process involves the stimulation of indigenous microbial populations microorganisms to degrade the contaminant. The addition of nitrogen, phosphorus and provision of additional oxygen or aeration accelerate the indigenous microorganisms growth and thus bioremediation process (Eze, 2010; Joutey *et al.*, 2013; Durga *et al.*, 2014).

b1. Bioventing

Bioventing is the most common *in-situ* remediation technology that uses microorganisms to decompose organic components in the unsaturated soil (Coste *et al.*, 2013). Bioventing improves the activity of indigenous bacteria by supplying air or oxygen flow and nutrients through wells to contaminated soil (Otaiku, 2007). The bioventing processes are summarized in Figure 14

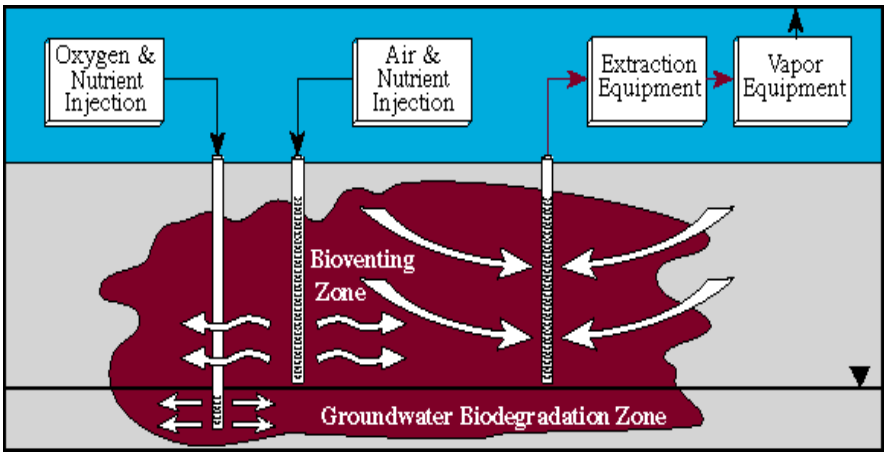


Figure 14: Bioventing (Coste *et al.*, 2013)

b2. Biosparging

Biosparging is an *in-situ* remediation technology that uses indigenous microorganisms in the biodegradation of organic components in the saturated soil (Coste *et al.*, 2013). Biosparging involves the injection of air (or oxygen) under pressure into the saturate zone to increase oxygen concentrations and enhance the rate of biological degradation of contaminants by naturally occurring bacteria (Otaiku, 2007).

c. Bioaugmentation

Bioaugmentation is the introduction of exogenous microorganisms to the contaminated sites for the purpose of facilitating biodegradation process (Dolon & Bauder, 2011; Joutey *et al.*, 2013). Bioaugmentation is also defined as a technique for improvement of the degradative capacity of contaminated areas by introduction of specific competent strains or consortia of microorganisms (Mrozik & Piotrowska-Seget, 2010). These supplemental microorganisms should be more efficient than native flora to degrade the target pollutant. Somteimes genetically engineered microorganisms (GEM) have to be used because have a higher degradative capacity (Joutey *et al.*, 2013; Durga *et al.*, 2014).

ii. *Ex-Situ* bioremediation

This process needs excavation of contaminated soil or pumping of groundwater to facilitate microbial degradation. These techniques involve the excavation or removal of contaminated soil from ground. *Ex-Situ* bioremediation has more disadvantages than advantages (Durga *et al.*, 2014). *Ex-situ* bioremediation include:

a. Landfarming

In this technique the contaminated soil is excavated and spread over a prepared bed and periodically tilled until pollutants are degraded. The purpose is to stimulate indigenous biodegradative microorganisms and facilitate their aerobic degradation of contaminants (Durga *et al.*, 2014).

b. Composting

The Composting technique involves combining contaminated soil with nonhazardous organic amendants such as manure or agricultural wastes. The presence of these organic materials supports the development of a rich microbial population and elevated temperature characteristic of composting (Otaiku, 2007).

c. Biopiles

Biopile technology is an engineered composting system where aeration is provided through a network of pipes and a leachate collection system is used for water-soluble hydrocarbons. Compared to land treatment units, the biopiles require less space. The operation is more controlled, and suited for the treatment of more volatile hydrocarbons such as gasoline and jet fuels (A. Singh *et al.*, 2009).

d. Bioreactors

Slurry reactors or aqueous reactors are used for *ex-situ* treatment of contaminated soil and water. A slurry bioreactor may be defined as a containment vessel and apparatus used to create a three-phase (solid, liquid, and gas) mixing condition to increase the bioremediation rate of soil, sediment, sludge and water-soluble pollutants (Durga *et al.*, 2014). In a bioslurry reactor, water is mixed with the sieved polluted soil to produce slurry treated in a bioreactor. The use of reactors provides rapid degradation of pollutants. The system can be supplemented with nutrients, surfactants and degrading organisms (native or exogenous) (A. Singh *et al.*, 2009).

I.3.2.2. Advantages and disadvantage of Bioremediation

There are several key advantages to using bioremediation as opposed to other, more traditional remediation methods. Firstly, because it can normally be conducted *in-situ*, bioremediation tends to be the most cost effective option, as it does not involve any costly transportation of hazardous materials and it can also reduce the opportunity for contaminants to be spread further during extraction and transportation.

One of the major disadvantages to bioremediation is the limitations on the types of contaminants that it can remove effectively. A second disadvantage to bioremediation, in contrast to other remediation techniques, is its relative sensitivity to environmental factors such as temperature and pH (Kensa, 2011; Durga *et al.*, 2014). Table 6 summarizes the advantages and disadvantages of bioremediation.

Table 6: The advantages and disadvantages of bioremediation (Kensa, 2011).

Technology	Examples	Benefits	Limitations
In-situ bioremediation	Biosparging Bioventing Bioaugmentation	- Most cost efficient, - Non-invasive, - Natural attenuation process treats soil and water.	-The process is sensitive to the environmental, conditions in the ground -Extended treatment time difficulties (The process of bioremediaiton is slow).
	Landfarming Composting Biopiles	- Low cost, - Can be done on site.	- Soil requires excavation -Controlling volatile organic compounds may be difficult.
Ex-situ bioremediation	Bioreactors -Slurry reactors or -Aqueous reactors	-The rate and extent of biodegradation are greater in a bioreactor system, -Optimized environmental parameters environment and is more manageable and hence more controllable.	-Soil requires excavation -Relatively high operating cost.

I.3.3. Biodegradation of petroleum hydrocarbon

Biodegradation is the transformation of a compound through biological activity. It is the breakdown of organic contaminants (hydrocarbons) by microbial organisms into smaller compounds where the microbial organisms transform the contaminants through metabolic or enzymatic processes (Horel, 2009).

Margesin and Schinner (2001) reported that biodegradation is the metabolic ability of microorganisms to transform or mineralize organic contaminants into less harmful, non-hazardous substances, which are then integrated into natural biogeochemical cycles.

Salleh *et al.* (2003) mentioned that the biodegradation denote complete microbial mineralization of complex materials into simple inorganic compound such as carbon dioxide, water and minerals as well as cell biomass (Figure 15).

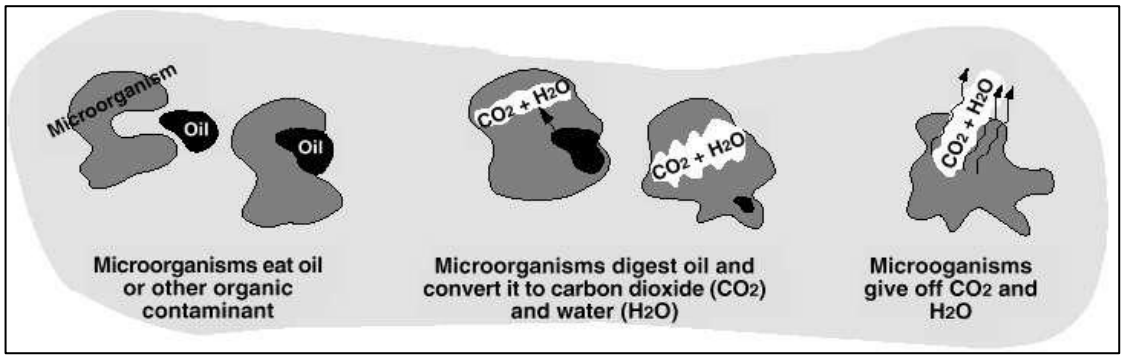


Figure 15: diagram showing mineralization of organic contaminant (Gordon, 2013)

The biodegradation may refer to complete mineralization of the organic contaminants to or to transformation of organic contaminants to other organic compounds. Biodegradation of organic constituents is accomplished by enzymes produced by microorganisms. Many enzymes are not released by microbial cells, substances to be degraded must generally contact or be transported into the cells (Barden, 1994).

In the natural environment, a contaminant may not be completely degraded, but only transformed into intermediate products. As well, many hydrocarbon products that cannot be utilized as a energy or carbon source by microorganisms can be degraded by enzymes generated by microbes to metabolize other compounds. This process is referred to as cometabolism (Barden, 1994).

I.3.3.1. Co–metabolism

Synergistic activities or co–metabolism occurs when an organism is using one compound for growth and gratuitously oxidizes a second compound that are available for use by other microbial populations. The Cometabolism process facilitates the degradation of many recalcitrant pollutants such as PAH compounds (Barden, 1994; Gordon, 2013).

I.3.3.2. Taxonomy of hydrocarbonoclastic microorganisms

Bacteria with the ability to degrade hydrocarbons are named hydrocarbon-degrading bacteria or hydrocarbonoclastic bacteria (Joutey *et al.*, 2013; Dashti *et al.*, 2015; Pasumarthi, 2016). A diverse group of bacteria and fungi is capable of utilizing petroleum hydrocarbons. According to Atlas (1981) more than 100 species representing 30 microbial genera have been shown to be capable of degrading hydrocarbons, there

are at least 22 genera of bacteria and 14 genera of fungi and 1 algal genus which can utilize petroleum hydrocarbon. Based on frequency of isolation, the most important genera of hydrocarbon utilizers in aquatic environments are *Pseudomonas*, *Achromobacter*, *Arthrobacter*, *Micrococcus*, *Nocardia*, *Vibrio*, *Acinetobacter*, *Brevibacterium*, *Corynebracterium*, *Flavobacterium*, *Candida*, *Rhodotorula* and *Sporobolomyces*. In aquatic ecosystems bacteria and yeasts appear to be the prevalent hydrocarbon degraders. Other study listed 11 genera of fungi and 6 genera of bacteria that are hydrocarbonoclastic in soils samples (Atlas, 1981).

Fungi and bacteria all appear to be important hydrocarbon degraders in terrestrial ecosystems (Eze, 2010). Some of the hydrocarbon utilizing microorganisms degrade alkanes, some aromatics, while others utilize both aromatics and paraffinic hydrocarbons, transforming them intocarbon dioxide,water and cell biomass or other less danger end product (Salleh *et al.*, 2003).

I.3.3.3. Distribution of hydrocarbonoclastic microorganisms

Hydrocarbon-degrading bacteria and fungi are widely distributed in soil, freshwater and marine ecosystem (Atlas, 1981). The levels of hydrocarbon-degrading microorganisms and their proportions within the microbial population appear to be a good index of environmental pollution with hydrocarbons. In unpolluted ecosystems, hydrocarbon-degrading microorganisms generally constitute < 0.1% of the microbial populations; in oil polluted ecosystems, they can constitute up to 100% of the viable microorganisms (Eze, 2010).

Atlas (1981) noted that the distribution of hydrocarbon utilizing microorganisms was positively correlated with the presence of hydrocarbons in the environment and also reported that bacteria utilizing a gas-oil as the sole carbon source represented 10% of the heterotrophic bacteria in the area of a refinery effluent compared with 4% in an area not polluted by hydrocarbons.

The degradation rate was the highest in areas of chronic discharge (Tagger *et al.*, 1979). The predominant bacteria in polluted soils belong to a spectrum of genera and species listed inTable 7

Table 7: Predominant bacteria in soil samples polluted with aliphatic and aromatic hydrocarbons, polycyclic aromatic hydrocarbons, and chlorinated compounds (Fritsche & Hofrichter, 2000).

Gram-Negative Bacteria	Gram-Positive Bacteria
Pseudomonas spp.	*Nocardia* spp.
Acinetobacter spp.	*Mycobacterium* spp.
Alcaligenes sp.	*Corynebacterium* spp.
Flavobacterium	*Arthrobacter spp.*
Xanthomonas spp.	*Bacillus* spp.

I.3.3.4. Metabolic pathways of petroleum hydrocarbon degradation

Biodegradation of hydrocarbons can occur under aerobic and anaerobic conditions (Atlas, 1981; Salleh *et al.*, 2003; N. Das & Chandran, 2011).

i. Aerobic biodegradation

The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions. Aerobic degradation means that the degradation of a contaminant occurs in the presence of oxygen. Aerobic microorganisms use oxygen from the air as an electron acceptor to oxidize the contaminant, and give CO_2 as a product (Horel, 2009). Prince and Lessard (2004) reported that more than 60 genera of bacteria and 95 genera of fungi are catalyzed the aerobic biodegradation process. These organisms share the ability to insert one or two of the oxygen atoms of diatomic O_2 into a hydrocarbon molecule, there by activating it and making it accessible to the central metabolism of the organism. Figure 16 shows the main principle of aerobic degradation of hydrocarbons.

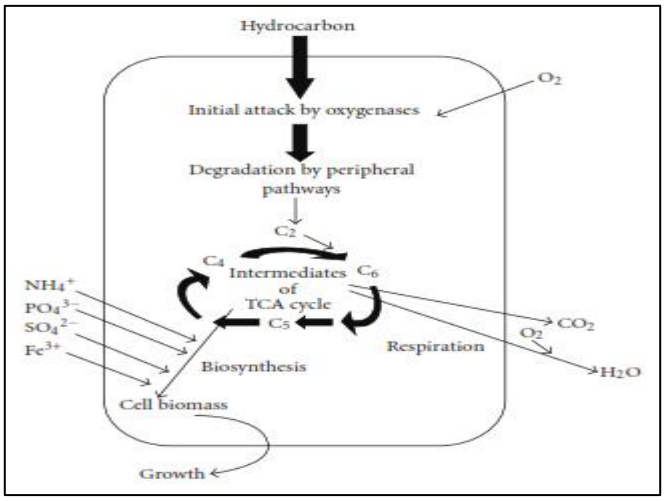


Figure 16: Main principle of aerobic degradation of hydrocarbons by microorganisms (N. Das & Chandran, 2011).

The essential steps of aerobic degradation of hydrocarbons by microorganisms are:

1. Metabolic processes for optimizing the contact between the microbial cells and the organic pollutants. For example, if hydrocarbons are water-insoluble, their degradation requires the production of biosurfactants.

2. The initial intracellular attack of organic pollutants is an oxidative process by the incorporation of oxygen catalyzed by oxygenases and peroxidases.

3. Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, for example, the tricarboxylic acid cycle (Krebs cycle).

4. Biosynthesis of cell biomass occurs from the central precursor metabolites e.g., acetyl-CoA, succinate, pyruvate (N. Das & Chandran, 2011).

The petroleum hydrocarbons degradation can be mediated by specific enzyme system. Figure 17 shows both types of enzymatic reactions involved in these processes.

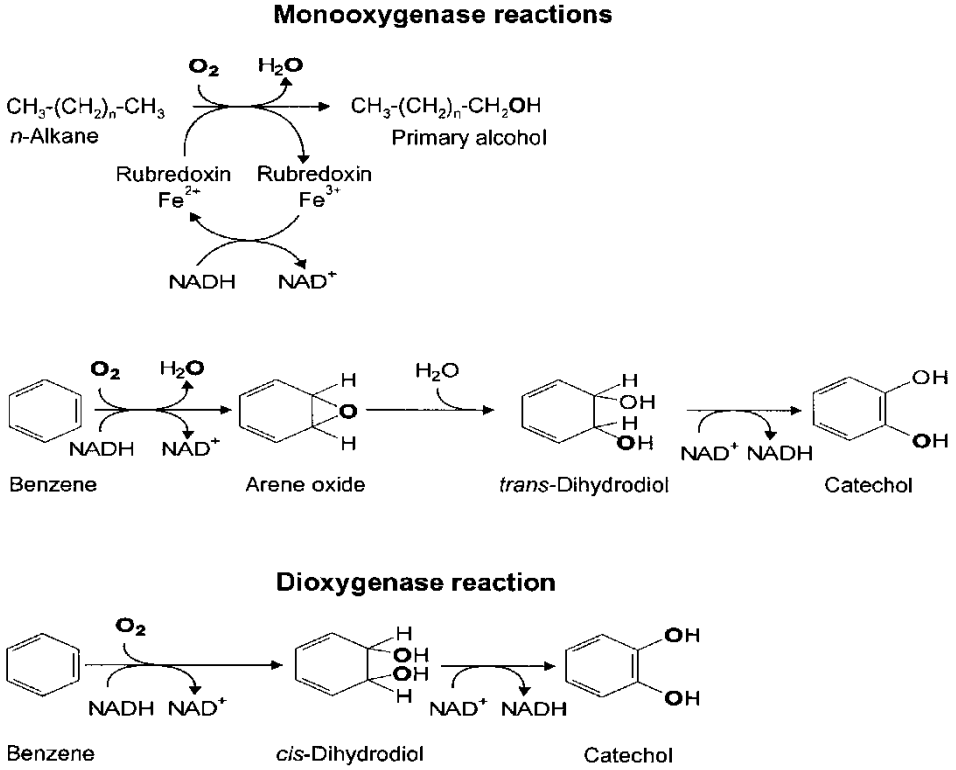


Figure 17: Enzymatic reactions involved in the processes of hydrocarbons degradation.

Monooxygenases incorporate one atom of oxygen of O_2 into the substrate, the second atom is reduced to H_2O. Dioxygenases incorporate both atoms into the substrate (Fritsche & Hofrichter, 2000; N. Das & Chandran, 2011).

a. Aerobic biodegradation of alkanes

The n-alkanes are the most readily metabolized constituents in a petroleum mixture (Atlas, 1981; Salleh *et al.*, 2003). Alkanes with 10 to 24 carbon are the most easily degraded (Salleh *et al.*, 2003). The initial degradation of alkane begins with the oxidation of the terminal methyl group and results in the formation of an alcohol, which is then dehydrogenated via the aldehyde to the corresponding carboxylic acid, Further degradation of the carboxylic acid performed by β-oxidation with the subsequent formation of two-carbon-unit shorter fatty acids and acetyl coenzyme A (Atlas, 1981) which can enter the Krebs cycle to give carbon dioxide and energy while the subterminal oxidation of alkanes sometimes occurs., with formation of a secondary alcohol and subsequent ketone (Atlas, 1981; Al-Deeb, 2005).

Long-chain n-alkanes (C10 - C24) are degraded most rapidly by pathway demonstrated in Figure 18, while short-chain alkanes (less than C_9) are toxic to many microorganisms, but they evaporate rapidly from petroleum-contaminated sites (Fritsche & Hofrichter, 2000).

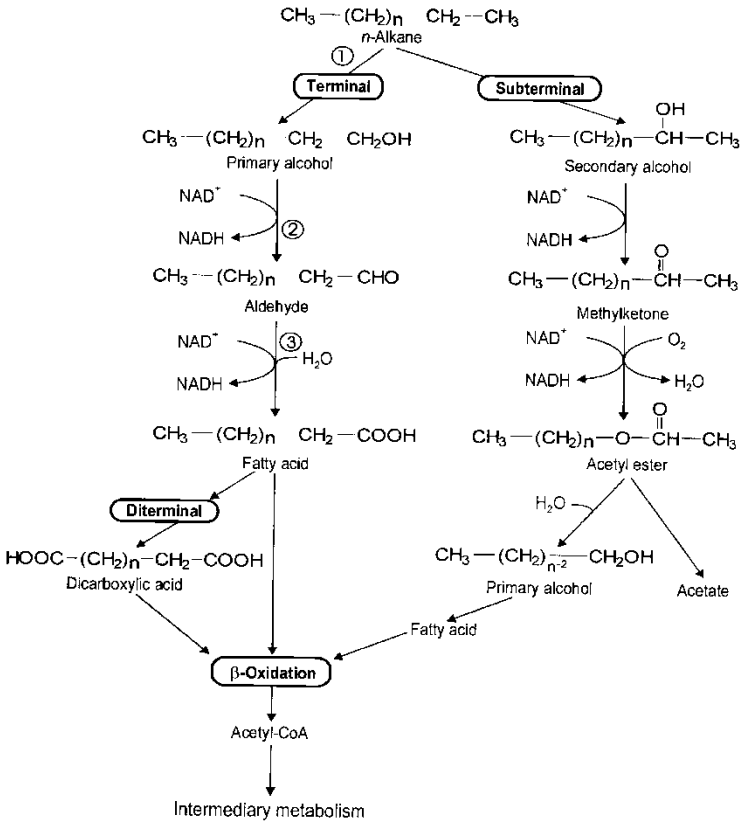


Figure 18: Pathways of alkane degradation.

The main pathway is the terminal oxidation to fatty acids catalyzed by 1. N-alkane monoxygenase, 2. Alcohol dehydrogenase and 3. aldehyde dehydrogenase (Fritsche & Hofrichter, 2000).

b. Aerobic biodegradation of branched alkanes

The branched alkanes are slightly more resistant, but they are still readily degraded. This is because the carbon side group on the carbon chain makes most of the degradation mechanisms of bacteria impossible. This is also the reason why branched alkanes are degraded by other mechanism (Van der Heul, 2011). Highly branched alkanes, such as pristane have been found to undergo omega-oxidation with the formation of dicarboxylic acids as the major degradative pathway. Methyl branching generally increases the resistance of hydrocarbons to microbial attack (Atlas, 1981).

c. Aerobic biodegradation of cyclic alkanes

Cycloalkanes are particularly resistant to microbial attack (Atlas, 1981). The absence of an exposed terminal methyl group complicates the primary attack. A few species are able to use cyclohexane as sole carbon source; more common is its cometabolism by mixed cultures (Fritsche & Hofrichter, 2000). The mechanism of cyclohexane degradation is shown in Figure 19.

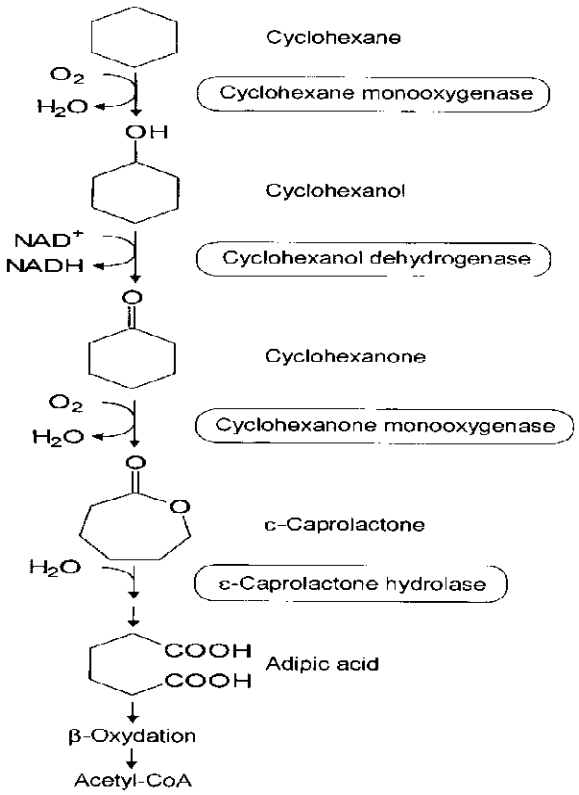


Figure 19: Peripheric metabolic pathway of cycloalkanes (cyclohexane) (Fritsche & Hofrichter, 2000).

d. Aerobic biodegradation of monoaromatic hydrocarbons

The small aromatics (benzene and substituted benzenes) and alkanes are most readily consumed, followed by the larger alkanes and the two- and three-ring aromatics (Prince & Lessard, 2004). Aromatic hydrocarbons, e.g., benzene, toluene, ethylbenzene and xylenes (BTEX compounds), and naphthalene are widely used as fuels and industrial solvents (Fritsche & Hofrichter, 2000). Prokaryotes convert aromatic hydrocarbons by an initial dioxygenase attack, to trans-dihydrodiols that are further oxidized to dihydroxy products, e.g., catechol (Okoh, 2006). These initial reactions, hydroxylation and dehydrogenation, are also common to pathways of degradation of other aromatic hydrocarbons (Fritsche & Hofrichter, 2000). Catechol can be oxidized either via ortho-cleavage pathway which involves cleavage of the bond between carbon atoms of the two hydroxyl groups to yield muconic acid, or via the meta-cleavage pathway which involves cleavage of the bond between a carbon atom with a hydroxyl group and the adjacent carbon atom to yield 2-hydroxymuconic semi aldehyde (Kothari *et al.*, 2013). The general pathways of aerobic degradation of aromatic compounds are demonstrated in Figure 20.

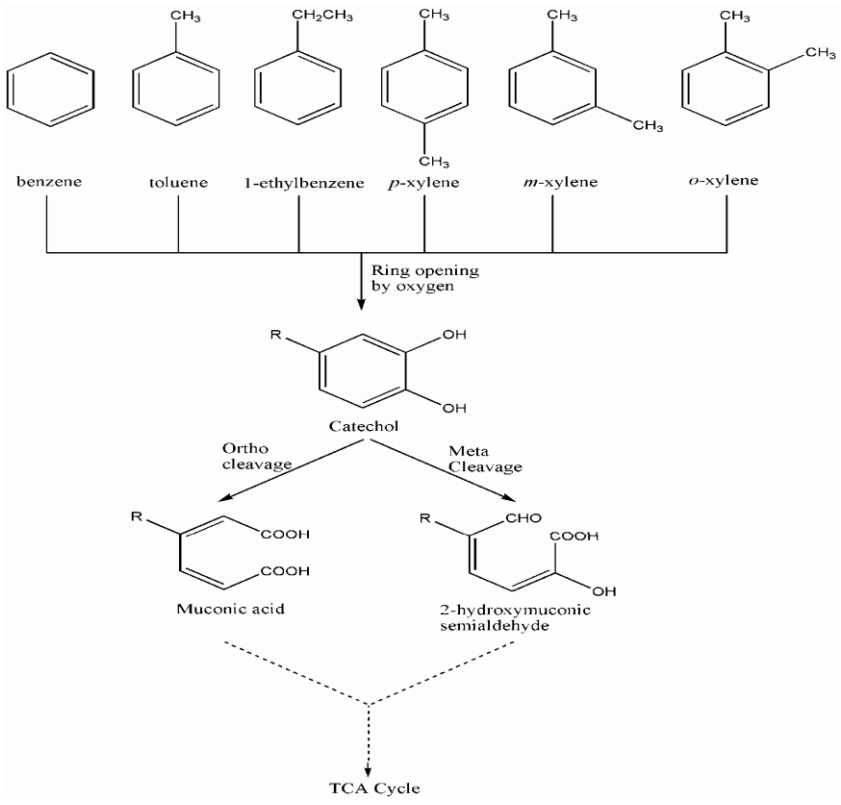


Figure 20: Pathways of aerobic degradation of aromatic compounds (Kothari *et al.*, 2013).

e. Aerobic biodegradation of polyaromatic hydrocarbons

Biodegradability of polyaromatic hydrocarbons (PAHs) is generally inversely related to the number of fused benzene rings (Salleh *et al.*, 2003). Condensed ring aromatic structures are subject to microbial degradation by a similar metabolic pathway as mono aromatic compounds (Atlas, 1981). Catabolism of a PAH molecule starts via the oxidation of the PAH to a dihydrodiol· by a multicomponent enzyme system. The dihydroxylated intermediates may then be processed through either an meta-cleavage or ortho-cleavage type of pathway, leading to intermediates such as catechols, which are then further converted to tricarboxylic acid cycle intermediates (Salleh *et al.*, 2003).

ii. Anaerobic biodegradation

Oxygen is not available in all environments where hydrocarbons occur (e.g., in deep sediments and in oil reservoirs) (Widdel & Rabus, 2001), also most polluted soils are oxygene limited. It has been known that hydrocarbons are also biodegraded under anaerobic conditions (Prince & Lessard, 2004). Since the late 1980s, an increasing number of novel microorganisms have been shown to utilise aromatic and saturated hydrocarbons as carbon source under strictly anoxic conditions (Widdel & Rabus, 2001). These microorganisms use sulfate (reduced to Sulfide), nitrate (reduced to nitrogen), chlorate (reduced to chloride), ferric and manganic ions (reduced to ferrous and manganous ions), and carbon dioxide (reduced to methane) as electron acceptors for anaerobic respiration (Prince & Lessard, 2004). Anaerobic degradation of petroleum hydrocarbons by microorganisms has been shown in some studies to occur only at negligible rates in nature (Atlas, 1981; Leahy & Colwell, 1990).

Anaerobic biodegradation of aromatic and aliphatic hydrocarbons is alternative to aerobic biodegradation treatments in the processes of bioremediation. It is now proved that, benzene, toluene and ethylbenzene can be metabolized under anaerobic conditions. Anaerobic bacteria have also been shown capable of degrading hydrocarbon not only in the pure form, but also in complex hydrocarbon mixtures, such as crude oil (Holliger & Zehnder, 1996). So *et al.* (2003) reported that some bacteria are capable of degrading aliphatic hydrocarbons anaerobically and found that alkanes were oxidized to fatty acids.

Toluene could be biodegraded anaerbically with nitrate, sulfate, Fe, Mn or CO_2 as terminal electron acceptors (Al-Deeb, 2005). In comparison with other aromatic or

saturated hydrocarbons, toluene allows the relatively rapid growth of microorganisms (Widdel & Rabus, 2001). Thus, the anaerobic catabolism of toluene apparently involves the conversion of toluene to benzoate or benzoyl CoA (Figure 21). The first reaction is the addition of toluene to fumarate to form benzylsuccinate (Heider *et al.*, 1998).

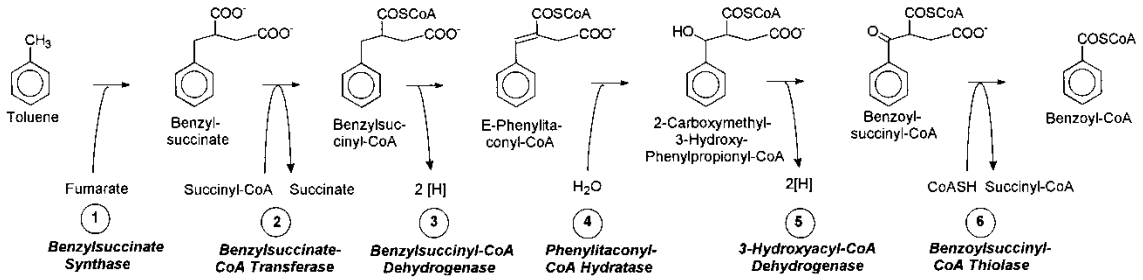


Figure 21: Pathway of anaerobic oxidation of toluene to benzoyl-CoA (Heider *et al.*, 1998).

I.3.3.5. Uptake of hydrocarbons by biosurfactants

Two mechanisms are involved in the uptake of oil substrates: (1) the attachment of microbial cells at oil droplets and (2) the production of biosurfactants. The attachment of the cells is still unknown, whereas the effect of biosurfactants has been studied well (Fritsche & Hofrichter, 2000).

Biosurfactants are surface active chemical compounds produced by a wide variety of microorganisms. They are made up of a hydrophilic moiety and a hydrophobic moiety which enhance solubilization and removal of contaminants. Biodegradation is also enhanced by biosurfactants due to increased bioavailability of oil pollutants (N. Das & Chandran, 2011). Microbial surface-active compounds are a group of structurally diverse molecules produced by different microorganisms and are mainly classified by their chemical structure and their microbial origin. The biosurfactants have a wide range of properties, including the ability to lower surface and interfacial tension of liquids and to form micelles and microemulsions between two different phases (Banat *et al.*, 2010). The micelles (microdroplets) encapsulated in the hydrophobic microbial cell surface are taken inside and degraded.

Pseudomonas are the best known bacteria capable of producing biosurfactants, *P. aeruginosa* is widely studied for the production of glycolipid type biosurfactants (N. Das & Chandran, 2011). Figure 22 demonstrates the emulsifying effect of a rhamnolipid produced by *Pseudomonas spp.* within the oil–water interphase and the formation of

micelles. Table 8 summarizes the type of biosurfactants produced by different microorganisms.

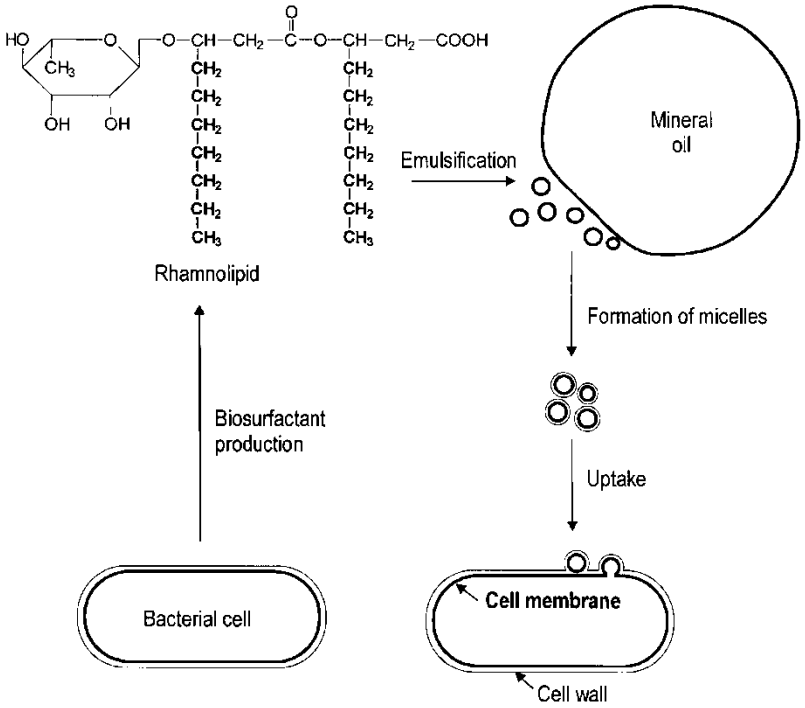


Figure 22: Involvement of biosurfactants in the uptake of hydrocarbons (Fritsche & Hofrichter, 2000).

Table 8: Biosurfactants produced by microorganisms (N. Das & Chandran, 2011).

Biosurfactants	Microorganisms
Sophorolipids	*Candida bombicola*
Rhamnolipids	*Pseudomonas aeruginosa, Pseudomonas fluorescens*
Lipomannan	*Candida tropicalis*
Surfactin	*Bacillus subtilis*
Glycolipid	*Aeromonas sp.* and *Bacillus sp*.

I.3.3.6. Enzymes and genes involved in petroleum hydrocarbon biodegradation

The biodegradation potential had been attributed to the presence of genes that code for oil hydrocarbon degradation enzymes and several genes of oil hydrocarbons degradation have been characterized (Al-Deeb & Malkawi, 2009). Different genera of bacteria are known for their potential oil degradation in which they contain different degradative enzymes involved in the metabolism of hydrocarbons (Malkawi *et al.*, 2009a).

The genes involved in the production of degrading petroleum hydrocarbon enzyme may be located on chromosomal or plasmid DNA (Peixoto *et al.*, 2011). Plasmids probably play important roles in microbial biodegradation process, the ability to degrade petroleum components such, as the aromatic fractions are generally plasmid mediated (Okoh, 2006). The genes responsible for biodegradation pathways are usually arranged in clusters that comprise: (i) catabolic genes encoding the enzymatic steps of the catabolic pathway; (ii) transport genes responsible for active uptake of the compound; and (iii) regulatory genes that adjust expression of the catabolic and transport genes to the presence of the compound to be degraded (Díaz, 2004).

There are several catabolic genes that encode key enzymes involved in a variety of known bacterial hydrocarbon degradative pathways: *alkB*; alkane monooxygenase from *Pseudomonas putida GPo1* (Commonly Known *Pseudomonas oleovorans GPo1*) (C_5 to C_{12} alkane degradation); *alkm*; alkan monooxygenase from *Acinetobacter sp.* strain ADP-1 (C_{10} to C_{20} alkane degradation); *alkB1* and *alkB2* alkane monooxygenase from *Rhodococcus spp.*; *xylE*, catechol-2, 3-dioxygenase (C*23O*) from *P. putida* (xylene and toluene degradation); *nah* naphthalene dioxygenase from *P. putida* (PAH; naphthalene degradation) (Díaz, 2004; Malkawi *et al.*, 2009a). alkB gene are the major genes involved in n-alkanes degradation and are distributed among many different species of bacteria,yeast, fungi, and algae. alkB gene my be located on plasmide or in the genome (Shao & Wang, 2013).

I.3.3.7. Factors influencing the biodegradation of hydrocarbon

The intensity of petroleum hydrocarbons biodegradation in the environment is largely controlled by abiotic and biotic factors which influence the rates of microbial growth and enzymatic activities (Atlas, 1981).

i. Environmental factors

a. Physical state of oil or hydrocarbon

The physical state of hydrocarbons has a significant effect on their biodegradation. In soils, petroleum hydrocarbons are absorbed by soil particles plant and matter, limiting its spreading while in aquatic systems, the oil normally spreads out, forming a thin slick, the degree of spreading is reduced at low temperatures because of the viscosity of the oil (Atlas, 1981). The rate of hydrocarbons spreading in the water

increases the surface area of the oil and thus its availability for microbial attack. The formation of emulsions increases the surface area of the oil and thus making it more available for microbial attack (Leahy & Colwell, 1990). Therefore, the low solubility and dissolution rates of hydrocarbon may limit their availability for biodegradation (Salleh *et al.*, 2003).

b. Chemical composition of oil or hydrocarbons

Because of the variable chemical structures and molecular weights, petroleum hydrocarbons differ in their susceptibility to microbial attack as follows: n-alkanes > branched alkanes> low-molecular-weight aromatics > cyclic alkanes (Leahy & Colwell, 1990; Salleh *et al.*, 2003).

c. Concentration of oil or hydrocarbons

A major factor affecting the rate of biodegradation is the concentration of hydrocarbons in a soil or water system (Eze, 2010). The rates of microbial uptake and biodegradation of organic compounds in aquatic system are usually proportional to the concentration of the compound (Leahy & Colwell, 1990). On the other side, high concentrations of highly soluble organic compounds may be harmful to microbial growth due to their toxicity. Besides to toxicity, high concentrations of petroleum hydrocarbon may also inhibit microbial growth by disturbing the C:N:P ratios and oxygen limitation (Salleh *et al.*, 2003).

Dibble and Bartha (1979) reported that biodegradation rate of oil sludge occurred between oil concentration of 1.25 to 5%, and was optimal at 5%. Oil concentration (> 5%) lead to a decline in microbial activities due to increase in oil toxicity.

d. Temperature

Biodegradation of hydrocarbon is known to occur over a wide temperature range (psychrophilic to mesophilic) from close to zero degrees to up to more than 30°C. The seasonal temperature in the natural environment have been shown to affect the degradation rate (Kothari *et al.*, 2013). The temperature can act on both the metabolic activity of the microbial populations and on the chemical and nature physical of petroleum hydrocarbon. Biodegradation rate decrease under low temperatures and toxic

components in crude oil. At low temperatures, the viscosity of oil increases, reducing the degree of oil spreading in soil and water and retard the volatilization of short chain alkanes ($<C_{10}$),consequently decrease biodegradation rate. With the increase in temperatures lead to increasing in enzymatic activity of mesophilic and thermophilic microorganisms thus increase of biodegradaion rate (Leahy & Colwell, 1990; Salleh *et al.*, 2003). Figure 23 shows that highest degradation rates that generally occur in the range 30–40°C in soil environments, 15–20 °C in marine environments and 20–30 °C in some freshwater environments.

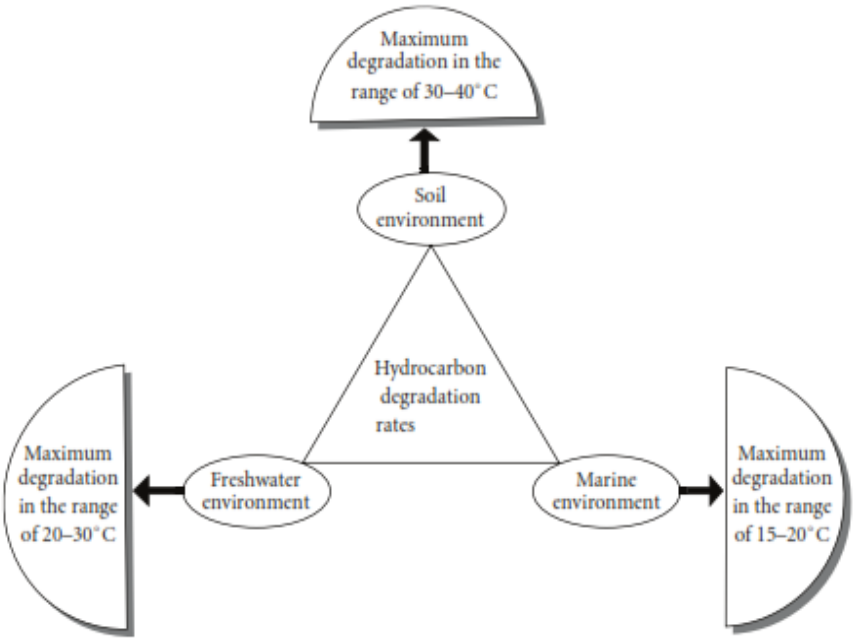


Figure 23: Hydrocarbon degradation rates in soil, fresh water, and marine environments (N. Das & Chandran, 2011).

e. Hydrogen potential (pH)

Soil pH is an indicator of hydrogen ion activity in the soil. A pH in the range of 5 to 9 is generally acceptable for biodegradation; a pH of 6.5 to 8.5 is generally considered to be optimal for biodegradation process (Barden, 1994). Also, Leahy and Colwell (1990) reported that biodegradation rates are highest at a pH near neutrality.

In acidic soil, biodegradation of crude oil is usually dominated by fungal populations, which are generally more tolerant to low pH environments. Alkaliphilic bacteria degrade phenol effectively at pH, ranging from 7.5 to 10.6 (Salleh et al., 2003).

f. Oxygen

The initial steps in the biodegradation of hydrocarbons by bacteria and fungi involve the oxidation of the substrate by oxygenases for which molecular oxygen is required. Aerobic conditions are therefore necessary for this route of microbial oxidation of hydrocarbons in the environment. The availability of oxygen in soils and sediments is dependent and limiting on the type of soil and whether the soil is waterlogged (Leahy & Colwell, 1990).

The major source of oxygen in soil is diffusion from the atmosphere. When soil pores become filled with water, the diffusion of gases through the soil is restricted. Clayey soils tend to retain a higher moisture content, which restricts oxygen diffusion (Barden, 1994). Anaerobic degradation of petroleum hydrocarbons by microorganisms occurs but at very low rate (Leahy & Colwell, 1990; Eze, 2010).

g. Nutrients

The process of biodegradation can be enhanced by addition of essential nutrients such as nitrogen and phosphorous. In any environment, the ratio of C:N:P must be maintained at about 100: 10: 1 to sustain any microbial activity (Kothari *et al.*, 2013). Leahy and Colwell (1990) reported that nitrogen and phosphorus may also be limiting in soils, and the acceleration of the biodegradation of crude oil in soil and groundwater by the addition of urea-phosphate, N-P-K fertilizers, and ammonium and phosphate salts has been demonstrated in several studies. Other nutrients (K, Ca, Mg, S, etc.) are typically found in adequate amounts for metabolic needs in most soils. However, high concentrations of calcium and magnesium may precipitate phosphates, and will reduce the amount available for microbial metabolism. High levels of chlorides may inhibit microbial activity (Barden, 1994).

h. Salinity

Salt concentration has effect the hydrocarbon biodegradation depending on the type of environment and the type of microorganisms involved (Kothari *et al.*, 2013). In a study realized on estuarine sediments noted that a generally positive correlation between salinity and rates of mineralization of phenanthrene and naphthalene. While other study reported that the rates of hydrocarbon degradation decreased with increasing salinity in the range 3.3 to 28.4% (Leahy & Colwell, 1990).

k. moisture content

The moisture content of a soil is expressed in terms of the percentage of its waterholding capacity. Moisture is essential for life processes but excess of it in the soil limit the oxygen availability (Eze, 2010). Soil moisture content also affects the soil aeration status, nature and amount of soluble materials, soil water osmotic pressure, and the pH of the soil. Soil moisture content should be in the range of 25-85% of the water holding capacity; Saturation between of 50-80% is considered optimal for biodegradation (Barden, 1994).

ii. Biological factors

a. Adaptation-effect of prior exposure

Prior exposure to hydrocarbon contamination confers adaptation to indigenous microbial population thus due to enhanced biodegradation rate (Salleh et al., 2003). Adaptation phenomenon can occur by three associated mechanisms as follows (i) induction and/or depression of specific enzymes, (ii) genetic changes which result in new metabolic capabilities, and (iii) selective enrichment of microorganisms able to degrade the hydrocarbon contaminant (Leahy & Colwell, 1990).

b. Petroleum biodegradation bymicrobial consortia

The biodegradations demand more than a single species. Individual microorganisms can metabolize only a limited range of hydrocarbon substrates, so mixture of populations with large enzymatic capacities has a greater petroleum biodegradation rate. Sevral study demonstrated that various microbial genera have been isolated from petroleum-contaminated soil or water. This strongly suggests that ther is cooperation between mixed cultures in a process of biodegradation (Salleh *et al.*, 2003).

MATERIALS AND METHODS

II. Materials and Methods

II.1. Study area

ARZEW oil refinery (RA1Z) is located 40 km from the city of Oran (North-west of Algeria), in an industrial area near the town of Arzew, about 1.7 km west of the Mediterranean Sea (Figure 24). The oil refinery at Arzew, Algeria, is a topping and reforming refinery with a nameplate distillation capacity of 3,000 k tones per annum (60,000 barrels per day).

II.2. Petroleum hydrocarbon

Petroleum hydrocarbon used in this study was crude oil obtained from Arzew oil refinery and diesel was purchased from gas station.

II.3. Soil sampling

Fifteen Oil-contaminated soil samples were aseptically collected from seven sites (ZS1- ZS7) in Arzew oil refinery (Table 9, Figure 25). The Soil samples were obtained at two different depths (5 cm and 10cm) (Fardoux *et al.*, 2000). Around 250 g of soil; were collected per sample in sterile plastic bags and transported immediately in cold storage container to the laboratory.

Other soil sample was taken from a site that has not been subjected to oil spills: from the garden of mascara university.

Soil samples were crushed and sieved through 2mm pore size (Pétard, 1993; Fardoux *et al.*, 2000), then were placed in sterile bags and stored at 4°C for further studies (R Chaussod *et al.*, 1986; Rémi Chaussod *et al.*, 1992; Fardoux *et al.*, 2000).

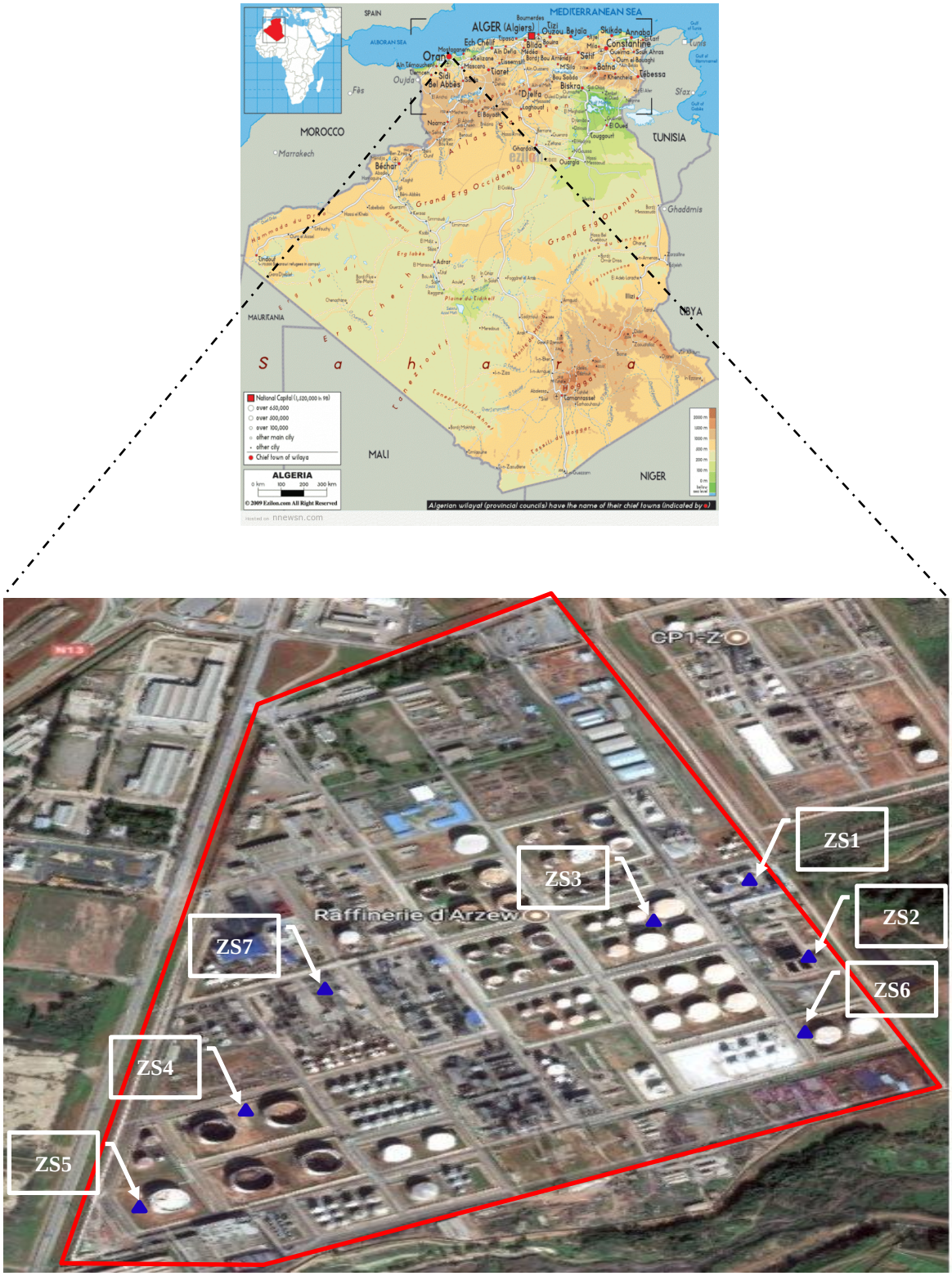


Figure 24: Map of Algeria showing Arzew oil refinery and the different sampling sites of the contaminated soil.
ZS1: Sampling site 1; ZS2: Sampling site 2; ZS3: Sampling site3, ZS4: Sampling site 4, ZS5: Sampling site 5, ZS6: Sampling site 6 and ZS7: Sampling site 7.

Table 9: Presentation of the different sampling sites.

Samples	Depths	Location code	Site	Date of sampling
S1	5cm	ZS1a	Zone 27 (next to a sewer)	18/12/2016
S2	5 cm	ZS1b	Zone 27 (Effluent treatment plant)	18/12/2016
S3	10 cm	ZS1c	Zone 27(Effluent treatment plant)	18/12/2016
S4	10 cm	ZS1d	Zone 27 (next to Sewage Drains)	18/12/2016
S5	5-10 cm	ZS1e	Zone 27 (next to sewer)	18/12/2016
S6	5-10 cm	ZS2a	Zone 28 (next to gasoline storage tank -206-)	19/12/2016
S7	5-10 cm	ZS2b	Zone 28 (next to the storage tank of semi-finished products)	19/12/2016
S8	5-10 cm	ZS3a	Zone 24 (next to valve of diesel storage tank -224-)	19/12/2016
S9	5-10 cm	ZS3b	Zone 24 (under the pipes)	19/12/2016
S10	5-10 cm	ZS4a	Zone 11 (next to crude oil storage tank -101-)	19/12/2016
S11	5-10 cm	ZS4b	Zone 11 (near the vegetation that grows around the crude oil storage tank -101-)	20/12/2016
S12	5-10 cm	ZS5	Zone 13 (near the crude oil storage tank)	20/12/2016
S13	5 cm	ZS6a	Zone 29 (next to Storage tank of reduced imported crude (T302: BRI).	20/12/2016
S14	5-10 cm	ZS6b	Zone 29 (near Storage tank of reduced imported crude Tk2101: BRI)	20/12/2016
S15	5-10 cm	ZS7	Zone 07 (next to tank Storage of base oils)	20/12/2016
S16	5 cm	ZS8	Mascara University (Uncontaminated soil)	22/04/2017

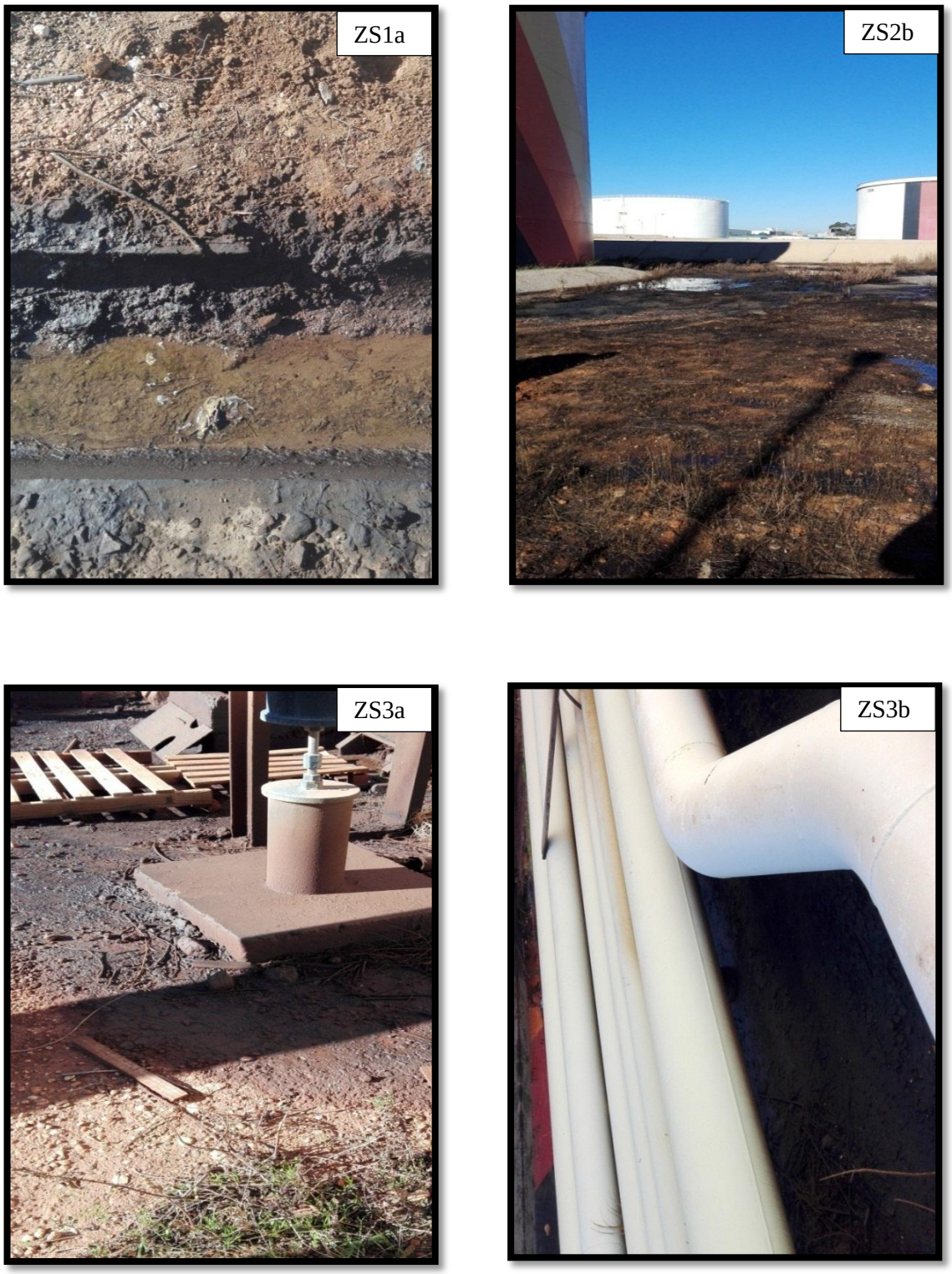


Figure 25: photo of some oil contaminated sites.
ZS1a: site of S1, ZS2b: site of S7, ZS3a: site of S8 and ZS3b: site of S9.

II.4. Soil characterization

Some physicochemical analyses were performed in the laboratory of the "National Institute of Soils, Irrigation and Drainage (INSID of Ksar Chellala, Algeria)", according to the methodology described in (Aubert, 1978): particle size analysis, organic matter, calcium carbonate, soil phosphorus, chloride determination and sulfate determination.

II.4.1. Physical and chemical characterization of soil samples

II.4.1.1. Particle Size Analysis (Granulometry)

The texture analysis is the determination of percentage content of each fraction of clay (< 0.002 mm), silt (0.002–0.05 mm), and sand (0.05–2.0 mm) in total (mineral and organic) soil mass (C Mathieu & Pieltain, 1998). The soil texture was determined using the international Robinson pipette method (Figure 26). A mechanical soil analysis are based on the fact that the smaller particle in water suspension fall more slowly than larger one (Olmstead *et al.*, 1930). Soil particles suspended in solution settle out at a rate that depends on the size of the particles.

To determine the relative masses of sand, silt and clay in the soil sample (the texture) the combined mass of silt plus clay in the first aliquot and the mass of clay in the second must be determined. Also, the initial total volume of suspension and aliquot volume must be known. Transfer aliquot to a weighed evaporating dish and put in oven at 105 °C. Then, fine and coarse sands are measured by sieving (Pétard, 1993; Baize, 2000). The Soils can be further grouped into twelve soil textural classes based on the proportion of sand, silt and clay as defined by using the United States Department of Agriculture (USDA) soil textural triangle (Figure 27) (Brady & Weil, 2002).

Figure 26: Robinson pipette.

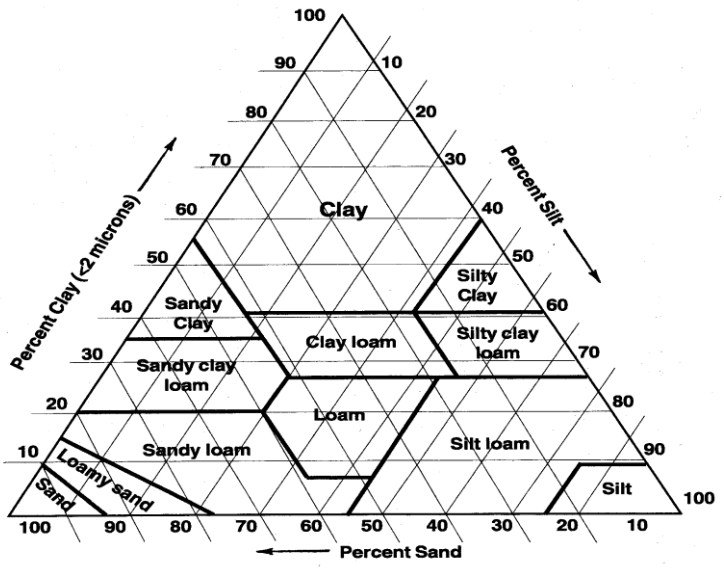


Figure 27: The soil textural triangle Credits: (USDA)

II.4.1.2. Soil water content (moisture content)

Water content or moisture content is the quantity of water contained in a soil. Soil water content is expressed on a gravimetric basis. The method is based on removing soil moisture by oven-drying a soil sample until the weight remains constant. The moisture content (%) is calculated from the sample weight before and after drying (Pétard, 1993; Zaiad, 2010).

a. In the precision balance at tare a capsule was weighed

b. In a capsule air dried soil was weighed

c. The capsule with the sample was taken in an oven (105 °C) for 48 h.

d. The capsule with the dried sample was weighed.

Calculations

$$MC\% = \frac{W2 - W3}{W3 - W1} \times 100$$

Where:

W_1: Weight of capsule (g)

W_2: Weight of air dried soil + capsule (g)

W_3: Weight of dried soil at 105°C + capsule (g)

II.4.1.3. pH

pH values were measured using a digital pH meter (HANNA) as described by Shrivastava and Kanungo (2014). For this 20 g soil sample was mixed with 50 ml distilled water in2:5 ratio of soil/water. The suspension was stirred intermittently with glass rod for 30 minutes and left for one hour. The combine electrode was inserted into supernatant and pH was recorded. pH value as a measure of the hydrogen ion activity of the soil water system and expresses the acidity and alkalinity of the soil.

II.4.1.4. Electrical conductivity

Electrical conductivity (EC) expresses ion contents of solution which determine the current carrying capacity thus giving a clear idea of the soluble salts present in the soil (Wagh *et al.*, 2013). The electrical conductivity of a soil sample was determined with digital electrical conductivity meter for which 20 g soil was added in 100 ml distilled water (in a 1:5 ratio of soil/water). The suspension was stirred intermittently for half an hour and kept it for 30 minutes without any disturbances for complete dissolution of soluble salts. The soil was allowed to settle down and then conductivity cell was inserted in solution to take the reading to record the EC values at 25°C. The values were expressed in µS/cm (Zaiad, 2010).

II.4.1.5. Determination of organic matter

The Walkley-Blak method was used for the determination of soil organic matter (Walkley & Black, 1934; Maral, 2010). In Walkley-Black determinations of organic matter, organic carbon is determined and it is converted to organic matter using the Van Bemmelen correction factor of (100/58=1.724) which assumes soil organic matter is 58 % (w/w) carbon. In this procedure, 1g of finely ground and sieved dry soil sample was taken into 500ml conical flask. To this 10 ml of 1N potassium dichromate and 20 ml of sulfuric acid were added and the contents were shaken for a minute and allowed to set aside for 30 minutes and then 200 ml distilled water, 10 ml of pure phosphoric acid and 1ml diphenylamine indicator were added. The whole contents were titrated with ferrous ammonium sulfate till the color changes from blue violet to green. The blank titration was also carried without soil.

$$\%\text{Organic Matter (W/W)} = \%\ \text{Organic carbon (W/W)} \times F$$

F: Van Bemmelen factor, assuming soil organic matter contains 58 % (w/w) organic carbon.

II.4.1.6. Soil phosphorus

Phosphorus is an essential element for the life of organisms and soil. Phosphorous is never found in pure form in the nature, but only as phosphates, which consists of a phosphorous atom bonded to four oxygen atoms in the phosphate ion and oxides (Maral, 2010). The only form of phosphate that can be detected is orthophosphate. All other forms must undergo pretreatment in order to be transformed into orthophosphate before being analyzed. Phosphorus was estimated by Joret-Hebert method (standard AFNOR NF X 31-161) (Duval, 1963). The phosphoric acid is extracted by 100ml ammonium oxalate at 0.1 N (in 1:25 ratio of soil/water). The dosage is based on the formation and reduction of phosphomolybdic complex. In ammonium oxalate solution containing phosphate, the addition of a sulfomolybdic reagent and ascorbic acid solution causes, by heating, the development of a blue color whose intensity is proportional to the concentration of orthophosohates. The determination of the phosphorus extracted is carried out by spectrocolorimetry at 825 nm after development of the coloration of a phosphomolybdic complex according to the protocol described by Duval (1963).

II.4.1.7. Determination of calcium carbonate in Soils

Gasometric method involves the determination of lime content of the soil based on a volumetric method. In this method the CO_2 liberated by the action of the acid is measured in a closed system volumetrically (Polyzopoulos, 1959). Bernard calcimeter (Figure 28) was used for this study. The percentage of calcium carbonate, $CaCO_3$ (%), is defined as the total carbonates contained in 100 g of dry soil. The determination of $CaCO_3$ (%) is based on the volumetric analysis of carbon dioxide CO_2, which is liberated during the application of hydrochloric acid solution HCl in soil's carbonates and is described with the following reaction (Maral, 2010):

$$\mathbf{CaCO_3 + 2\ HCl \rightarrow CaCl_2 + H_2O + CO_2 \uparrow}$$

Calculus:

$$CaCO3(\%) = \frac{\mathrm{WT} \times \mathrm{V1}}{\mathrm{W} \times \mathrm{V}}$$

Where

W_T: weight of pure $CaCO_3$.

W: weight of soil.

V_1: volume of CO_2 produced by the soil sample.

V: volume of CO_2 produced by pure $CaCO_3$.

Figure 28: Bernard calcimeter

II.4.1.8. Determination of chloride

For this study the chloride ion was analyzed by the argentometric precipitation method (Mohr's Method). The chloride element is present in soil in the form of different salts that are soluble in water. In the presence of silver nitrate the chloride is precipitated in the form of AgCl, the potassium chromate solution is added the soil extract sample, the latter is converted to red silver chromate as soon as all Cl- ions have been precipitated. Silver chloride is precipitated quantitatively before red silver chromate is formed as shown by the following reactions (Maral, 2010).

$$Ag^{+} + Cl^{-} \rightleftharpoons AgCl$$
$$2Ag^{+} + CrO_4^{-2} \rightleftharpoons Ag_2CrO_4$$

10 to 50ml of the soil extract sample was titrated using standard 0.1 N $AgNO_3$. A few drops of 5 % (w/v) potassium chromate was added to the sample and it was titrated with $AgNO_3$ solution till the color turns to reddish brown, in the pH range of 7 to 10 (Clément Mathieu *et al.*, 2003), distilled water was used as a control.

The concentration of Cl^- ion was calculated as follows:

$$Cl^{-}\ (mg/l) = (n - n1) \times N\frac{D}{P} \times 100$$

Where

n: volume in ml of silver nitrate used for the sample.

n1: volume in ml of silver nitrate used for the control.

N: normality of silver nitrate used for titration (0.1).

P: volume of the test sample.

D: dilution factor inverse of the titrated solution.

II.4.1.9. Determination of sulfate

The extract sample is dried at 60 - 65 ° C in a water bath or sand bath. Barium chloride solution was added in excess to precipitate barium sulfate, and the precipitate is digested in a hot solution. The sulfate ion SO_4^{-2} reacts with the barium ion to form a precipitate of barium sulfate. (Anonymous; Clément Mathieu *et al.*, 2003):

$$\mathbf{SO_4^{2-} + BaCl_2 ---> BaSO_4 + 2Cl^-}$$

From the volume of the sample and weight of the precipitate, the concentration of sulfate in the sample is calculated as follows:

$$SO_4\,(mg/l) = \frac{8.56 \times D \times P}{V} \times 1000$$

Where

P: weight of $BaSO_4$ precipitated.

D: inverse of the dilution factor.

V: volume of the sample extract in ml.

II.4.1.10. The total petroleum hydrocarbons (TPH) content

The evaluation of total petroleum hydrocarbons (TPH) in soil samples was determined using the gravimetric analysis as described by Ould Rabah (2012). For solid-liquid extraction, 5g of soil were extracted with twice 50 ml of hexane (Márquez-rocha *et al.*, 2001; C. Singh & Lin, 2008). 50 ml of hexane was Added to 5g of each soil sample, and then covered with aluminum foil, wich was shaken for two hours away from light and was centrifuged for 10 minutes at 5000 rpm. The supernatant was

recovered and the oil-solvent mixture was removed using a separatory funnel. Then the solvent was removed by evaporation using a rotatory evaporation at 37°c (Hamzah *et al.*, 2010). After evaporation, the quantities of hydrocarbon extracted are weighed using a precision balance (Diaz-Ramirez *et al.*, 2008; Ould Rabah, 2012).

II.4.2. Microbiological analysis of soil

II.4.2.1. Bacterial count

The total heterotrophic bacterial count was determined by spread-plating technique using nutrient agar medium.10g of each soil sample was suspended in 90ml sterile physiological saline (0.9% NaCl) and vortexing (Youssef *et al.*, 2010). Serial dilutions were conducted and 0.1ml of the serially diluted samples was plated on nutrient agar plates (Appendix A) in duplicates. The plates were incubated at 30°C for 24 h (Chikere *et al.*, 2009; Boboye *et al.*, 2010; Youssef *et al.*, 2010; Salam *et al.*, 2011; Obuotor *et al.*, 2016). At the end of the incubation period, All plates yielding between 30 and 300 colonies were counted (Chikere *et al.*, 2009). The number of colonies (CFU) was Calculated per gram of soil by dividing the number of colonies by the dilution factor multiplied by the amount of volume added to nutrient agar, according to the following formula (Marchal *et al.*, 1982):

$$N = n / d. v$$

N: number of colonies in CFU / ml.

n: number of colonies counted.

v: volume added (0.1ml).

d: dilution factor.

II.4.2.2. Enrichment and isolation of petroleum hydrocarbon degrading bacteria

Petroleum hydrocarbon degrading bacteria were isolated from oil-contaminated soil as described above. Media used in this study was Bushnell-Hass medium (mineral salts medium), which contained per liter: 1.0 g K_2HPO_4,1.0 g KH_2PO_4, 1.0 g NH_4NO_3,0.05 g $FeCL_3$, 0.2 g $MgSO_4.\ 7H_2O$ and 0.02 g $CaCl_2 \cdot 2H_2O$ and the pH was adjusted to 7 (Youssef *et al.*, 2010). The mineral salts medium (MSM) was

supplemented with 1% (V/V) filter-sterilized crude oil as the sole source of carbon (Verma *et al.*, 2006; Hamzah *et al.*, 2010; Erdogan *et al.*, 2012; Jyothi *et al.*, 2012). For the enrichment procedure: 10g oil-polluted soil samples was added to 100 ml sterile liquid MSM and incubated for 7 days at 30°C on an orbital shaker at 180 rpm in the dark (C. Singh & Lin, 2008; Erdogan *et al.*, 2012). After one week incubation, 1 ml of enriched media was transferred into a fresh MSM and incubated in the same conditions. After four consecutive transfers (each with an incubation interval of one week) (Salam *et al.*, 2011; Guermouche *et al.*, 2013), 100 µl of culture were plated on MSM agar (20 gl^{-1}) supplemented with 1% filter-sterilized crude oil and incubated at 30°C for 3 to 5 days (Verma *et al.*, 2006; Meenakshisundaram & Bharathiraja, 2014).

II.4.2.3. Purification and conservation of isolates

a. Purification

The resulting colonies were repeatedly subcultured onto minimal salt agar supplemented with 1% filter-sterilized crude oil to confirm their oil degrading ability (Al-Adwan *et al.*, 2010). Single colonies distinguished by notable difference in morphological characteristics were streaked onto Luria Bertani agar (LB) plates and tryptic soy agar (Appendix A) plates, then incubated at 30°C overnight. These colonies were cultured onto *Pseudomonas aeruginosa* selective media for rapid detection of this bacterium.

b. Conservation of purified isolates

Bacterial colonies were maintained by streaking on tryptic soy agar slant medium for a period of 4 to 6 weeks. Such medium was prepared in screw-capped vials containing 10-15 ml of the medium. The isolates were streaked on the slant and incubated at 37°C for 24h. Slants were wrapped with Para film and stored at 4°C.

For long-term preservation, pure isolates were maintained in tryptic soy broth with 30% glycerol at -20°C.

II.4.2.4. Screening of the most potent bacterial isolates for hydrocarbon utilization

The method described by El-Khawaga *et al.* (2015) was used. The ability to degrade petroleum hydrocarbon was confirmed by inoculating of 5 ml cultured bacterial isolates (18 to 24 h old) were transferred individually to 100 ml conical flask, containing 50 ml of sterile mineral salts medium and 0.5 ml of petroleum oil was added

as a sole carbon source. Un-inoculated flasks served as control. All flasks were incubated at 30°C on an orbital shaker at 150 rpm for 7 days. The growths of the isolated bacterial species were monitored at regular intervals by measuring the optical density at 600 nm. Based on the growth of bacterial species, the best degrader of petroleum oil was selected and identified (Guermouche *et al.*, 2013; Palanisamy *et al.*, 2014; El-Khawaga *et al.*, 2015; Obuotor *et al.*, 2016).

II.4.2.5. Identification of screened isolates

a. Morphological characteristics

The Morphological characteristics of a bacterial species are based on many factors, including cell and colony morphology.

a_1. Macroscopic observations

Each colony has a characteristic size, form or shape, edge, texture, degree of opacity, and color. These characteristics describe the morphology of a single colony and may be useful in the preliminary identification of a bacterial species (Christopher & Bruno, 2003).

a_2. Microscopic observations

The wet mount (Distilled water motility test)

It is the simplest way and very useful test to determine motility is to place a loop-full of growth from a nutrient agar subculture in a drop of sterile distilled water on a clean slide, cover it with a cover glass and examine microscopically using the 40**x** magnification. In addition of being able to determine the presence or absence of motility, this method is useful in determining cellular shape (rod, coccus, or spiral) and arrangement (irregular clusters, packets, pairs, or long chains) (Aygan & Arikan, 2007).

Gram stain reaction

The Gram stain reaction is dependent on the cell wall structure of the bacteria. The Gram stain is a differential stain because it divides bacteria into two groups: Gram-positive and Gram-negative, where Gram-positive bacteria stain purple and Gram-negative bacteria stain pink. Smears of the isolated colonies of pure culture were

prepared and stained with Gram stain and the characteristics of the organisms were recorded as its being Gram-negative or Gram-positive. For Gram's staining, the following solutions (Appendix A) were used: a basic dye: crystal violet, a mordant: Gram's iodine, a decolorizing agent: ethanol and a counter stain: safranin (Hucker & Conn, 1923; Chelton & Jones, 1959).

No staining method (KOH solubility test)

A simple and rapid (< 60 s) no staining technique for distinction between gram-negative and gram-positive bacteria, is to test for solubility of the bacteria in 3 % potassium hydroxide (Gregersen, 1978; Buck, 1982; Powers, 1995).

A drop of potassium hydroxide (KOH) (3 % w/v) was placed, using a pasteur pipette on a microscope slide, a part of a single colony is removed using a cooled sterile loop, from agar medium and then bacteria are mixed into KOH solution until an even suspension is obtained there after the loop is lifted from the slide. If a mucoid thread can be lifted with the loop it is a gram-negative bacterium, if a watery suspension is produced, it is a gram-positive bacterium (Goszczynska *et al.*, 2000).

Endospore stain

Endospores are highly resistant, dehydrated cells with thick walls and additional layers. The ability of bacterial cells to form endospores is another characteristic used in identification. A sample of bacterial cells that contains endospores will be able to with stand extreme conditions, such as high temperatures (Christopher & Bruno, 2003). Endospore staining (Appendix B) uses two stains to differentiate endospores from the rest of the cell. The Schaeffer-Fulton method (the most commonly used endospore-staining technique) uses heat to push the primary stain (malachite green) into the endospore. Washing with water decolorizes the cell, but the endospore retains the green stain. The addition of a counterstain or secondary stain (safranin) is used to stain the decolorized vegetative cells (Larpent & Larpent-Gourgaud, 1985).

b. Biochemical characterization

After Gram staining we were oriented towards the cultivation of different types of isoltes on selective media: Gram-negative rods in the cetrimide (Appendix A), King A and King B media which are selective for *Pseudomonas*, Cocci Gram positive in the Chapman medium (Appendix A) which is selective for *Staphylococcus* (Marchal *et al.*, 1982).

In bacteria, morphological features alone are of little taxonomic value, because they are too simple to provide enough taxonomic information. Bacteria are mainly distinguished by their physiological and biochemical characteristics (Marchal *et al.*, 1982; Holt *et al.*, 1994). Besides classical biochemical tests, Analytical Profile Index kit (API 20NE/20E and API Staph) (BioMérieux) was also used for further identification with the help of Bergey's Manual, also using ABIS 7 online software and APIweb™software.

API20 NE for Gram-negative rods and API Staph for cocci gram-positive. API 20NE/20E and API Staph strips were inoculated and the tests performed and recorded according to the instructions of the manufacturer (Appendix C).The bacterial isolates were characterized using the following biochemical and physiochemical test:

b_1. Motility test:

Tubes containing motility media were stabbed once of the center with an inoculating ance, then, incubated at 30°C for 24 h. The motile bacteria spread out from the line of inoculation.

b2. Respiration determination

Meat-Liver Agar (Appendix A) favors the growth of most microorganisms, specially designed for the growth and isolation of anaerobic bacteria growing in the depth of the medium. It can also be used to elucidate the type of bacterial respiration (Marchal *et al.*, 1982), growth in the upper zone (obligate aerobes), growth in the deep zone (obligate anaerobes), growth throughout the height of the tube (facultative anaerobes) and growth as a ring in the intermediate zone (microaerophilic bacteria).

b_3. Respiratory enzymes

Catalase test

The test detects the ability of the organisms to produce the enzyme catalase. The catalase is an antioxidant enzyme responsible for eliminating molecules of H_2O_2 (hydrogen peroxide) from the cells that are produced during respiration. The reaction results the release of water and free oxygen. A small amount of pure culture was transferred to a microscope slide using a sterile loop, then 2 -3 drops of 3% hydrogen peroxide were added, immediately bubbling will take place and the test is considered to be positive. The presence of gas bubbles formation indicates catalase activity (Marchal *et al.*, 1982; Collee *et al.*, 1996).

Oxidase test

The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase. A small portion of the colony to be tested was picked up and placed on oxidase disk impregnated with reagent (tetramethyl-p-phenylene diamine hydrochloride). The color change to blue or purple within 30 second indicates a positive test (Marchal *et al.*, 1982; Holt *et al.*, 1994).

Nitrate reduction test

Certain bacteria reduce nitrate (NO_3) to nitrite (NO_2) while others are capable of further reducing nitrite to form nitrogen or ammonia. The nutrient broth was inoculated with a pure isolate . After the incubation period about 5 drops of sulphanilic acid and α-naphthylamine (Appendix) were added to the medium. The appearance of red or pink color indicate the positive test for nitrate reduction. If the color did not change, the results were indecisive. Then small amount of Zinc was added to the broth. If the solution remained colorless, then both nitrate reductase and nitrite reductase were present. If the solution turned red, nitrate reductase was not present (Clarke & Cowan, 1952; Marchal *et al.*, 1982).

b_4. Carbohydrate metabolism

ONPG (O-Nitrophenyl-β-D-Galactopyranoside) test

Fermentation of lactose depends on the presence of two enzymes: permease, which allows the lactose to enter the bacterial cell, and beta-galactosidase, which splits lactose into glucose and galactose, which are subsequently metabolized. O-Nitrophenyl-β-D-Galactopyranoside (ONPG) is structurally similar to lactose except that o-nitrophenol has been substituted for glucose. On hydrolysis through the action of the enzyme β-galactosidase, ONPG (a colorless compound) is cleaved into two residues, galactose and o-nitrophenol which is yellow in color (Figure 29). Development of yellow color provides visual evidence of hydrolysis of ONPG (Marchal *et al.*, 1982).

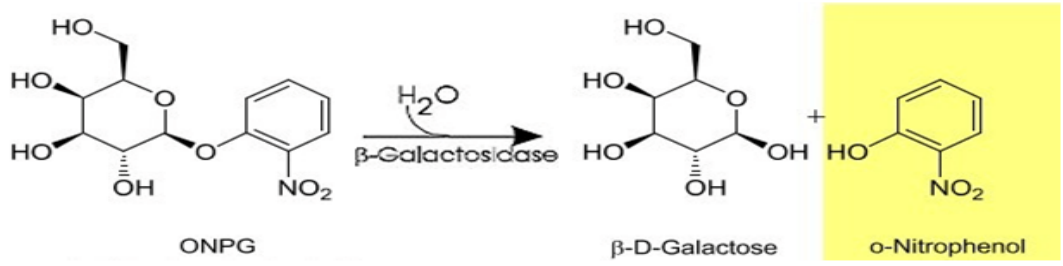


Figure 29: Hydrolysis reaction of ONPG (Marchal et al., 1982).

Citrate Test

The citrate test screens a bacterial isolate for the ability to utilize citrate as its carbon and energy source. A positive diagnostic test rests on the generation of alkaline by-products of citrate metabolism. The citrate test is performed by inoculating microorganism into in an organic synthetic media, "Simons Citrate agar" when sodium citrate is the only source of carbon and energy. Bromothymol blue is used as an indicator when the citric acid is metabolized, carbondioxide is generated which combines with sodium and water to form sodium carbonate which is an alkaline product which is responsible for change in color from green to blue and this constitute positive test. (Marchal *et al.*, 1982; Hemraj *et al.*, 2013).

Methyl Red Test

The red methyl test determines the ability of an organism to produce and maintain stable acid end products from glucose fermentation capacity. This is a qualitative test for acid production.

MR-VP broth was inoculated with a pure culture of each isolate and incubated at 30°C for 24h. Five drops of methyl red reagent were added, mixed, and result was read immediately. A bright red color indicates a positive reaction (Marchal *et al.*, 1982).

Voges-Proskauer Test

The Voges-Proskauer test determines the ability of organisms to produce neutral end product acetyl methyl carbinol (acetoin) from glucose fermentation.

MR-VP broth was inoculated with a young culture of each isolate and incubated at 30°C for 24h. One ml of 40% KOH and 3ml of 5% solution of α –naphthol were added. A positive reaction was indicated by the development of a pink to red color in 2-5 min (Marchal *et al.*, 1982).

Triple Sugar Iron Agar (TSI) test

TSI Agar is used for the determination of carbohydrate fermentation and hydrogen sulfide production in the identification of gram-negative bacilli. The TSI slant is a test tube that contains agar, a pH-sensitive dye (phenol red), 1% lactose, 1% sucrose, 0.1% glucose, sodium thiosulfate and ferrous sulfate. Carbohydrate fermentation is detected by the presence of gas and a visible color change (from red to yellow) of the pH indicator, phenol red. The production of hydrogen sulfide is indicated by the presence of a precipitate that blackens the medium in the butt of the tube (Marchal *et al.*, 1982).

Levan production

Levan is a substance produced through the action of the enzyme levan sucrase. Most fluorescent *Pseudomonas* that utilize sucrose as a sole carbon source, produce this enzyme. Streak-inoculate nutrient agar with 5 % sucrose and incubate for 3–5 days. Levan is produced when colonies are convex, white, domed and mucoid (Goszczynska *et al.*, 2000).

b_5. Protein metabolism

Urease test

The test is carried out in order to know wether a given organism produces the enzyme urease or not. Urease is a hydrolytic enzyme, which attack the amide linkage liberating ammonia (Figure 30). Urease test is performed by growing test organisms on urea broth medium containing a pH indicator phenol red. During incubation

microorganism processing urease will produce ammonia that raises the pH of the medium. The pH shift is detected by the color change of phenol red from light orange to pink. The development of pink color was taken as positive test (Clarke & Cowan, 1952).

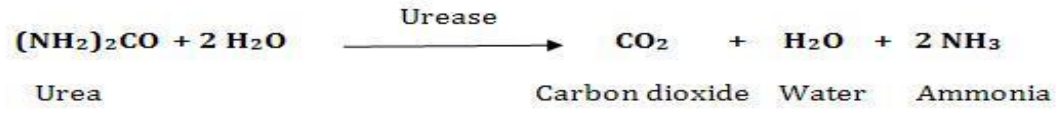


Figure 30: Hydrolysis reaction of urea

Indole test

The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole (Figure 31). urea-indole broth was inoculated with an overnight culture of each suspected isolate and incubated at 30°C for 24h to 48 h. To test for indole production, 5 drops of Kovác's reagent were added directly to the tube. If the culture produces tryptophanase, a positive indole test is indicated by the formation of a pink to red color ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent. If a culture is indole negative, the reagent layer will remain yellow or be slightly cloudy (Clarke & Cowan, 1952).

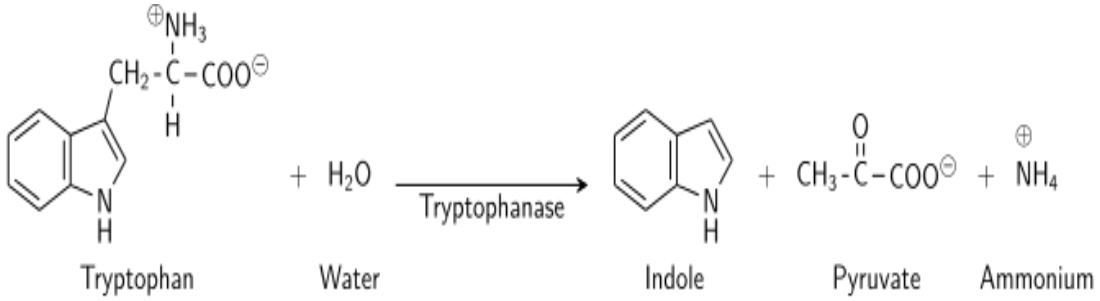


Figure 31: Indole production

Tryptophan deaminase test (TDA)

The presence of tryptophan deamination activity can be detected by addition of TDA reagent indicated by dark brown coloration. Tryptophan deamination is of reductive type where the 'NH_2' group of tryptophan is removed and released as ammonia and energy, which is utilized by bacteria, deamination of the amino acid results in a colored compound with the addition of 10% ferric chloride (Marchal *et al.*, 1982).

Detection of bacterial decarboxylases and dihydrolases:

Bacteria with fermentative metabolism ferment glucose, which induces acidification of the medium and a yellow color of the medium in the presence of bromocresol purple (pH indicator). Decarboxylases and dihydrolases present a maximum activity at acidic pH. When the strains examined possess the appropriate decarboxylase or dihydrolase, their enzymatic activity is demonstrated by the formation of aminated metabolites, which alkalinize the medium, inducing another color change of the medium to pale purple. Moeller Decarboxylase Broth Base with the addition of appropriate L-amino acid is used to differentiate bacteria on the basis of their ability to decarboxylate the amino acids (Larpent & Larpent-Gourgaud, 1985; Goszczynska *et al.*, 2000).

b_6. Decomposition of macromolecules

Starch hydrolysis

This test is performed to check the bacteria utilization of starch by producing the enzyme Amylase.Sterile starch agar medium is prepared by adding 10 g of starch to 1000 ml nutrient agar basal medium is poured on to the sterile petriplates and allowed to solidify (Larpent & Larpent-Gourgaud, 1985). After inoculation and growth, test for starch hydrolysis by flooding the plate, which has been streaked across the center line, with dilute (Lugol's) iodine. Absence of the bluish-purple color characteristic of the starch-iodine complex (a clear zone around growth) indicates starch hydrolysis (amylase activity) (Clarke & Cowan, 1952; Pelczar & Chairman, 1957; Goszczynska *et al.*, 2000).

Gelatin liquefaction

Gelatin hydrolysis represents an enzymatic action upon a protein (Proteolysis). Inoculate a heavy inoculum of test bacteria (18- to 24-hour-old) by stabbing 4-5 times on the tube containing nutrient gelatin medium. Then incubate the inoculated tube along with an uninoculated medium at 35°C for up to 2 weeks. Remove the tubes daily from the incubator and place in ice bath or refrigerator (4°C) for 15-30 minutes (until control is gelled) every day to check for gelatin liquefaction (Gelatin normally liquefies at 28°C and above, so to confirm that liquefaction was due to gelatinase activity, the tubes are immersed in an ice bath or kept in refrigerator at 4°C).Tilt the tubes to observe if gelatin

has been hydrolyzed (Clarke & Cowan, 1952; Pelczar & Chairman, 1957; Larpent & Larpent-Gourgaud, 1985).

Lecithinase production

To determine the ability of microorganisms to produce the enzyme lecithinase this is shown by the appearance of egg yolk opacity. Commonly found in *Bacillus cereus*, *Pseudomonas fluorescens* and some other bacteria. Bacterial lecithinase breaks down lecithin (a normal component of egg yolk) to insoluble diglycerides, resulting in an opaque halo surrounding the colony when grown on egg yolk agar. The yolk agar was prepared by mixing 100 ml Egg Yolk emulsion (used the yolk of one egg in 250 ml saline water) in 900 ml of sterile nutrient agar at 45°C with sterile precautions and immediately pouring into sterile Petri dishes. Inoculate Egg Yolk Agar, which has been streaked across the center line, and following incubation for 24 to 48h. Cultures should not be discarded as negative until after 7 days of incubation (McGaughey & Chu, 1948; Larpent & Larpent-Gourgaud, 1985; Larpent-Gourgaud *et al.*, 1988).

b_7. Other tests

Coagulase test

The coagulase test differentiates strains of *Staphylococcus aureus* from other coagulase- negative species. *S. aureus* strains are capable of coagulating plasma in the tube test and is the most commonly isolated coagulase positive organism, the tube coagulase test is performed by mixing bacterial cells into a larger volume of plasma in a small test tube. As the bacteria multiply in the plasma, they secrete staphylocoagulase. which initiates blood coagulation by activating prothrombin. Staphylocoagulase adheres to fibrinogen, forming a complex that cleaves fibrinogen into fibrin, bypassing the blood clotting cascade and directly causing a clot of fibrin to form. Formation of a clot will be noted within 24 h for a positive response (Williams & Harper, 1946).

DNAse

Deoxyribonuclease (DNase) is an enzyme that breaks down DNA. Certain species of bacteria have the ability to produce the DNase enzyme (*Staphylococcus aureus*). This activity is demonstrated by culturing organisms on an agar medium containing DNA and a dye, which changes color in the presence of the degraded DNA. Toluidine blue DNA agar medium (Appendix A) was prepared according to the

manufacturer's instructions. Approximately 15 to 20 ml of the medium was poured into sterile plastic petri dishes, followed by incubation of plates at 35 to 37 °C for 18 to 24 h. Two millimeter wells were made in the agar and the strain was gently transferred to each well. The development of pink zones around the wells was the positive indication of DNase production. DNase activity results in the production of a bright pink reaction due to the metachromatic property of toluidine blue (Waller *et al.*, 1985; Lior & Patel, 1987; Larpent-Gourgaud *et al.*, 1988).

Test for Growth at 42°C and 4°C

The test strain was inoculated into nutrient agar slant, incubated at 42 °C and at 4 °C for for 24 h. Growth indicate positive test (Larpent-Gourgaud *et al.*, 1988; Goszczynska *et al.*, 2000).

Pigment production

The test tubes containing sterilized King B and King A medium (Appendix A) were inoculated with the isolate of *Pseudomonas* and then incubated for 3 to 5 days and observed. The presence of a green bluish color was interpreted as indicating the production of pyocyanine (King A). The presence of a yellowish green fluorescent pigment observed under UV light (365 nm) pigment only was interpreted as indicating the production of pyoverdin, or fluorescein (King B) (Wahba & Darrell, 1965; Reyes *et al.*, 1981).

Growth at 6.5% of NaCl

This test performed to check the capacity of bacteria to to grow in a nutrient broth medium to which has been added 6.5% sodium chloride (Holt *et al.*, 1994).

b_8. Analytical Profile Index (API)

API test strips consists of microtubes (cupules) containing dehydrated substrates to detect the enzymatic activity or the assimilation / fermentation of sugars by the inoculated organisms. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The reactions are read according to the Reading Table and the identification is obtained by referring to the Analytical Profile Index or using the identification software.

For identification of non-enteric gram-negative rods bacteria, isolated colonies from Cetrimide agar plates were tested using API 20 NE strips, gram-positive cocci bacteria isolated from Chapman plate agar were identified using API staph and API 20E for Gram positive (appendix C).

C. Molecular characterization

The molecular identification was supported by"McGill university and genome québec innovation centre, montreal canada".

C_1. DNA extraction

Extraction of genomic DNA from the selected bacterial isolates was done using genomic DNA purification kit (Thermo Fisher Scientific) according to the manufacturer's instructions (appendix D). The Genomic DNA Purification Kit is a simple and rapid system for high quality genomic DNA purification from various sources, including: bacterial cells, whole blood, serum, cell lines, plant and mammalian tissues. The kit is based on selective detergent-mediated DNA precipitation from crude lysate.

C_2. DNA concentration analysis

The concentration of extracted genomic DNA was measured by UV spectrophotometry at 260nm. This method gives an approximate value of the DNA concentration of samples. It is important to understand that this method may overestimate the DNA concentration, because any free molecule containing an aromatic ring will create some absorbance at 260nm.

The DNA concentration of our sample was measured by Pico-Green method using Quant-iT™ Pico-Green™ dsDNA assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions (appendix D). Quant-iT™ PicoGreen® dsDNA reagent is an ultra-sensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA) in solution. Detecting and quantitating small amounts of DNA is extremely important in a wide variety of biological applications. This quantification method measures only double-stranded DNA in our samples and is not affected by the presence of contaminating molecules (Singer *et al.*, 1997). The Extracted DNA was stored at -20°C (Maloy, 1990).

C_3. DNA quality analysis

The quality of extracted genomic DNA was evaluated by UV spectrophotometry at 260 and 280nm. The 260/280 ratio gives an idea of the quality of the DNA extracted and if it is contaminated with RNA or proteins (Al-Deeb, 2005). The A260/A280 ratio should be 1.7 to 1.9. Smaller ratios usually indicate contamination by protein or organic chemicals. A sample with a ratio close to 1.8 is generally considered as “pure DNA" (Krebs *et al.*, 2009).

C_4. PCR amplification of 16S rRNA gene

Bacterial 16S rRNA gene was amplified using the following two primers: Forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and Reverse primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), PCR amplification was done according to Mullis *et al.* (1986). Each PCR reaction mixture contained (10µl) 1µl DNA template, 2 U of Q5® High-Fidelity DNA Polymerase (New England Bio-labs Ltd) (appendix D), 0.2mM dNTP, 2µl buffer 5X Q5 (New England Bio-labs Ltd), 5% DMSO and 0.2µM of each primer. PCR amplification Conditions were as follows: Initial denaturation for 30 S at 98°C, followed by 30 cycles (98°C for 10 S, 62°C for 15 S and 72°C for 30 S) and a final extension at 72°C for 2 min.

C_5. Gel electrophoresis

PCR products were determined using electrophoresis through 1% agarose gel (Guermouche *et al.*, 2013; Abd El-Aziz, 2015) containing ethedium bromide and visualized with a UV light. Thermo Scientific™ GeneRuler™ 100 bp Plus DNA Ladder, ready-to-use, is designed for sizing and approximate quantification of PCR products on agarose gels. The ladder is composed of fourteen chromatography-purified individual DNA fragments (in base pairs): 3000, 2000, 1500, 1200,**1000**, 900, 800, 700, 600, **500**, 400, 300, 200, 100. It contains two reference bands (1000 and 500 bp) for easy orientation. The ladder is premixed with 6X DNA Loading Dye (Appendix D) for direct loading on the gel.

C_6. Sequencing of 16S rDNA and phylogenetic analysis

The purified PCR product was sequenced by a Sanger dideoxy method using Big-Dye Terminator v3.1 cycle sequencing kit in 96-well plate on 3730 DNA Analyzer (Applied Biosystems) according the following protocol (appendix D). Sequences were

then compared to those in the GenBank database in the National Centre for Biotechnology Information (NCBI) using the website for BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were aligned using Clustal W. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987), The evolutionary distances were computed using Jukes-Cantor method (Jukes & Cantor, 1969) and a phylogenetic tree was constructed by MEGA6 (Tamura *et al.*, 2013). In addition, all 16S rRNA gene sequences were confirmed and classified using the RDP Naïve Bayesian rRNA classifier tool.

II.5. Detection of gene encoding enzymes involved in hydrocarbon degradation

II.5.1. Detection of alkane monooxygenase encoding genes (*alkB* and *alkB1*):

For the detection of genes encoding alkane oxygenase (genes involved in the degradation of n-alkanes), primers used are listed in Table 10. PCR was run with a mixture containing of a 1 µl of the DNA template, 1x Qiagen Buffer (10X), 0.4 µM of each primer, 0.2mM dNTP and 5 U of Qiagen enzyme (Taq DNA polymerase) (Qiagen, Germany). PCR amplification was performed as follows: Initial denaturation for 15 S at 96°C, followed by 30 cycles (96°C for 30 S, 55°C for 30 S and 72°C for 60 S) and a final extension at 72°C for 10 min.

II.5.2. Detection of 1,2-dihydroxynaphthalene dioxygenase encoding genes (*nahC*)

The primers *nahC* has been sepecially designed using the PubMed software of NCBI (Table 10) were used to detect the 1,2-dihydroxynaphthalene dioxygenase genes, PCR was run with a mixture containing of a 1 µl of the DNA template, 1x Qiagen Buffer (10X), 0.4 µM of each primer, 0.2mM dNTP and 5 U of Qiagen enzyme (Taq DNA polymerase-Qiagen, Germany-). PCR amplification was performed as follows: Initial denaturation for 15 S at 96°C, followed by 30 cycles (96°C for 30 S, 55°C for 30 S and 72°C for 60 S) and a final extension at 72°C for 10 min.

II.5.3. Detection of catechol 2, 3 dioxygenase genes (*C23O*)

For PCR amplification of the sequence of catechol 2, 3 dioxygenase genes, pair of primer DEG-F and DEG-R (Table 10). The PCR mixture was containing the following: 1µl DNA template, 2 U of Q5® High-Fidelity DNA Polymerase (New England Bio-labs Ltd), 0.2mM dNTP, 5µl buffer 5X Q5 (New England Bio-labs Ltd),

5% DMSO and 0.4µM of each primer. Cycling was performed with initial denaturation for 0.5 min at 89°C followed by 35 cycles (98°C for 10 S, 58°C for 10 S and 72°C for 30 S) and a final elongation at 72°C for 2 min.

Table 10: List of primers used in PCR reactions for detection of gene encoding enzymes involved in hydrocarbon degradation.

Primer	Sequence 5'→3'	Fragment size (bp)	Reference
alkB-F	TGGCCGGCTACTCCGATGATCGGAATCT GG	870	(Kok *et al.*, 1989; Whyte *et al.*, 2002; Aboulkacem *et al.*, 2014)
alkB-R	CGCGTGGTGATCCGAGTGCCGCTGAAGGTG		
alkB 1-F	CGGGGTTCAAGGTCGAGCAT	434	(Smits *et al.* (1999); Aboulkacem *et al.*, 2014)
alkB 1-R	CAGGACCAGGTTGGTGAAGA		
nahC –F	CTGCAGGCGTTTATGACAGC	407	PubMed software of NCBI
nahC –R	CTGACGCCCCAAGGAATCAA		
C23O: DEG-F	CGACCTGATCATCGCATGACCGA	238	(Eltis & Bolin, 1996; Jyothi *et al.*, 2012)
C23O :DEG-R	TCTAGGTCAGTACACGGTCA		

II.5.4. Gel electrophoresis

PCR products were determined using electrophoresis through 2% agarose gel containing ethedium bromide and visualized with a UV light. Thermo Scientific™ GeneRuler™ 100 bp Plus DNA Ladder (appendix D).

II.5.5. Sequencing of alkane monooxygenases encoding genes and phylogenetic analysis

The amplified PCR products obtained were sequenced to validate their identities by a Sanger dideoxy method using Big-Dye Terminator v3.1 Cycle Sequencing Kit in 96-well plate on 3730 DNA Analyzer (Applied Biosystems) according the following protocol (appendix D). By using the same primers used for PCR amplification. The nucleotide and deduced protein sequences were then compared to those in the GenBank database in the National Centre for Biotechnology Information (NCBI) using the website for BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).The sequences were aligned using Clustal W. The evolutionary history was inferred using the Neighbor-

Joining method (Saitou & Nei, 1987), The evolutionary distances were computed using Jukes-Cantor method (Jukes & Cantor, 1969) and a phylogenetic tree was constructed by MEGA X (S. Kumar *et al.*, 2018).

II.6. Biodegradation studies

II.6.1. Characterization of bacterial degradation potential on crude oil

II.6.1.1. Bacterial growth determination (Turbidometry)

The ability to de grade crude oil was performed as described by Boboye *et al.* (2010). The turbidometry was used to determine the bacterial growth in the presence of crude oil as sole carbon source in MSM broth. A single colony of the isolate was inoculated into 10ml LB broth at 30 °C overnight. The overnight culture was centrifuged for 15 min at 6000 rpm. The cell pellet was washed three times with MSM and was re-suspended in MSM until OD was equivalent to 0.5 at wavelength 600 nm (El-Khawaga *et al.*, 2015).

One ml of bacterial inoculum (0.5 OD_{600} equivalents) was transferred into 100ml MSM with 1% of crude oil and non-inoculated flasks were also used as controls. The cultures were incubated in the dark at 30°C on a rotary shaker 150 rpm for 15 days. The growth of the bacterium was measured by taking the optical density (OD) readings at 600 nm for 15 days at regular 2-day intervals by a spectrophotometer, against sterile mineral salt medium as a blank (C. Singh & Lin, 2008; Jyothi *et al.*, 2012; Guermouche *et al.*, 2013; Meenakshisundaram & Bharathiraja, 2014).

II.6.1.2. Gravimetric Analysis

The amount of oil degradation was determined using the gravimetric analysis (Márquez-rocha *et al.*, 2001; Verma *et al.*, 2006; Diaz-Ramirez *et al.*, 2008; C. Singh & Lin, 2008). Biodegradation of crude oil was investigated using pre-screened bacterial isolates in 250 ml flasks containing 100ml MSM supplemented with 1% (w/v) filtered oil as sole carbon source and 1% inoculum (v/v) of each isolates. Medium without bacteria were used as abiotic control. Abiotic controls were prepared to evaluate the hydrocarbon evaporation. Cultures were incubated on a rotary shaker at 30°C and 150 rpm for two weeks. After 15 days, cultures were centrifuged at 6000 rpm for 30min to pellet the bacteria. The residual oil in the supernatant of cultures and non-inoculated controls was extracted sequentially with hexane by adding a solvent volume to each sample with 50% of ratio (v/v)(Adebusoye *et al.*, 2006; Abid *et al.*, 2014). The oil-solvent mixture was removed using a separator funnel. Then the solvent was removed by evaporation using a rotary evaporator at 37°C (Hamzah *et al.*, 2010; Erdogan *et al.*,

2012; Guermouche *et al.*, 2013). After the complete evaporation, the hydrocarbon residuel obtained was weighed and compared to the control. The percentage of degraded oil was determined from the following formula (John & Okpokwasili, 2012; W. t. Liu *et al.*, 2012; Barakat, 2017).

$$\textbf{Percentage of degraded oil} = [(W_c - W_r)/ W_c] \times 100\%$$

W_r: weight of residual oil in cultures.

W_c: weight of residual oil in controls.

II.6.2. Characterization of degradation potential of selected individual bacterial isolates and consortia on minimal media containing diesel.

II.6.2.1. Determination of biodegradative activity by turbidometry

Ability to degrade diesel was performed as described by Jyothi *et al.* (2012). Growth of the defined microbial consortium and three individual pure isolates was monitored in 250-mL flasks containing 100 ml MSM with 1mL of sterile diesel as substrate and one ml of bacterial inoculum (0.1 OD_{600} equivalent) was added. Bacterial consortia were formulated by mixing equal proportions of the three pure bacterial cultures. Non-inoculated flasks were also used as controls. The cultures were incubated at 30°C at 150 rpm for 15 days. The growth of the bacterium was measured by taking the optical density (OD) readings at 600 nm for 15 days at regular 2-day intervals by a spectrophotometer, against sterile mineral salt medium as a blank (C. Singh & Lin, 2008; Jyothi *et al.*, 2012; Guermouche *et al.*, 2013; Meenakshisundaram & Bharathiraja, 2014).

II.6.2.2. Gravimetric Analysis

The level of diesel degradation was determined using the gravimetric analysis (Márquez-rocha *et al.*, 2001; Verma *et al.*, 2006; Diaz-Ramirez *et al.*, 2008; C. Singh & Lin, 2008).Growth of the defined microbial consortium and three pure isolates was monitored in 250-mL flasks containing 100 ml MSM with 1mL of sterile diesel as substrate. Medium without bacteria were used as abiotic control. Abiotic controls were prepared to evaluate the hydrocarbon evaporation. Undegraded diesel was extracted from inoculated and un-inoculated culture flasks with hexane by adding a solvent volume to each sample with 50% of ratio (v/v) (Adebusoye *et al.*, 2006; Abid *et al.*, 2014). The organic phase was drawn off (as described above in gravimetric analysis of

crude oil) , and thereafter quantified gravimetrically and chromatographically (Adebusoye *et al.*, 2006). The percentage of degraded diesel was calculated compared to the control using the formula described by (John & Okpokwasili, 2012; W. t. Liu *et al.*, 2012; Barakat, 2017) as mentioned above. The residual diesel of bacterial consortium and control was extracted sequentially at the end of 5 and 15 days. 10 µL of oil sample were directly diluted with 10 mL of hexane (Tzing *et al.*, 2003). The residual diesel were transferred to vials were and kept at 4°C until the gas chromatographic analysis performed (Palanisamy *et al.*, 2014).

II.6.2.3. Analysis of residual diesel in liquid culture by GC-MS

Gas Chromatography-Mass Spectrometry GC-MS analysis was done in order to detect the degradation of diesel oil (Zhenle *et al.*, 2006; Palanisamy *et al.*, 2014). At the end of incubation period, residual diesel after extraction (as described in the gravimetric analysis section) was quantified chromatographically.

1 µl sample of hexane extract were then analyzed by GC-MS (GCMS-TQ8030 from SHIMADZU) with a Rtx®-1 capillary non-polar column (RESTEK-USA-) of 30m length , 0.25mm internal diameter and 0.25 µm film thickness. The gas chromatograph equipped with a split-less injector (split ratios of 50:1) was used for the GC-MS analysis. The oven temperature was initially at 40°C and then programmed to rise up to 270°C at a rate of 8°C/min where it was held for 5 min. The temperatures of injector, transfer line and ionization source were all 250°C. The carrier gas was Helium at flow rate of 1 $ml.min^{-1}$ (Palanisamy *et al.*, 2014). The diesel degradation percentage was calculated by using the following formula (Michaud *et al.*, 2004; Chandran & Das, 2010; Obuotor *et al.*, 2016):

$$\mathbf{BE\ (\%) = 100 - [\ (As \times 100) / Aac]}$$

Where; BE (%) = Biodegradation efficiency, Aac = Total area of peaks in the appropriate abiotic control and As = Total area of peaks in each sample.

II.7. Optimization of bacterial biodegradation of crude oil under different nutritional and environmental conditions:

The most potent oil degrading bacterial isolates were selected for investigating the effect of pH, temperature, different sodium chloride concentrations, substrate

concentrations, speed of agitation, Influence of inoculum size, different intervals time effect of different carbon and nitrogen source on the growth of the bacterial isolates on crude oil.

II.7.1. Effect of different initial pH

The effect of initial pH on the growth of the selected bacterial isolates was investigated. In 100 ml conical flasks 50 ml MSM supplemented with 1% crude oil was prepared at pH 5, 6, 7, 8, 9 and 10 using 1N HCl and 1N NaOH. Each flask was inoculated with 1 % (v/v) standard inoculum ($O.D_{600}$ = 0.5). The flasks without bacteria were kept as control. All flasks were incubated under shaking conditions at 150 rpm at 30ºC for 7 days (El-Khawaga *et al.*, 2015). At the end of the incubation period, the bacterial growth was determined spectrophotometricaly at 600nm and the optimum pH was determined (Bayoumi & Abul-Hamd, 2010; Hamzah *et al.*, 2012; Khan *et al.*, 2015).

II.7.2. Effect of different incubation temperatures

Four different incubation temperatures (25, 30, 35 and 40 °C) were used to investigate the effect of temperature on the growth of the selected crude oil degrading bacterial isolates. MSM was supplemented with 1% crude oil at the optimum pH determined previously and 1% (v/v) standard inoculum. The growth conditions were performed as described above, and the bacterial growth was determined as previously mentioned.

II.7.3. Effect different sodium chloride concentrations

The different sodium chloride concentrations (1, 2, 5 and 10 %) were added to MSM supplemented with crude oil as sole carbon sources, the pH of MSM was adjusted at the optimum pH determined previously. The inoculated flasks were incubated at optimum temperature determined previously for 7 days. The growth was determined at 600 nm as previously mentioned.

II.7.4. Effect of different agitation speed

To check the effect of aeration rate, the flasks were incubated in orbital shaker at different agitation speed values (0,100 and 150 rpm). The growth conditions were performed as described above, for each agitation value, the flasks were incubated at

optimum temperature for 7 days. At the end of the incubation period, the optimum agitation speed value was determined by spectrophotometricaly at 600nm (Bayoumi & Abul-Hamd, 2010).

II.7.5. Effect of initial crude oil concentrations

The isolate was grown in MSM supplemented with different concentrations of petroleum oil (1%, 2% and 5%) (v/v) at the optimum pH (Palanisamy *et al.*, 2014). The flasks were incubated in orbital shaker for 7 days at optimum temperature**.**

II.7.6. Effect of different carbon and nitrogen source

The influence of different carbon and nitrogen source (peptone, yeast extract, glucose and maltose) on the growth of the selected utilizing crude oil bacterial cultures was investigated using MSM supplemented with 1% of crude oil at optimal pH. The nitrogen sources were added at equimolecular amount by nitrogen content located in MSM- Control (Bayoumi & Abul-Hamd, 2010). The following carbon source were supplied independently to MSM broth at 0.2% concentration (Al-Maabreh, 2005). All flasks were incubated at optimum temperature for 7 days at optimum shaking conditions. The bacterial growth was determined as previously mentioned.

II.7.7. Influence of initial inoculum size

0.1 ml, 1 ml and 3 ml, of bacterial inoculum were introduced, respectively, to 100 ml of MSM containing 1% substrate (crude oil) with optimal pH. After these flasks were cultured at optimum temperature for 7 days in a shaker with an optimum agitation speed (Dongfeng *et al.*, 2011). The growth rate was measured at 600nm (Bayoumi & Abul-Hamd, 2010).

II.7.8. Influence of initial inoculum concentration

To study the effect of initial inoculum concentration on degradation, MSM supplemented with 1% of crude oil was inoculated with different inoculum quantity such as 0.1, 0.5, 1 and 1.5 OD at 600 nm (Palanisamy *et al.*, 2014). All conical flasks were incubated at optimum temperature for 7 days in a shaker with a optimum agitation speed.

II.7.9. Effect of different interval times of incubation

50 ml of MSM broth medium supplemented with petroleum oil (1% v/v) and optimum pH were introduced into 100 ml conical flask. All flasks were cultured at optimum temperature in a shaker with an optimum agitation speed for different

intervals time: 0, 5, 7,10, 20 and 30 days (El-Khawaga *et al.*, 2015). The growth rate were determined at the end of each time interval.

The controls, without inoculation of strain were kept under similar conditions with inoculated flasks.

CHAPTER THREE
Results & Discussion

III. Results and discussion

III.1. Physical and chemical characterization of soil samples

III.1.1. Particle size analysis (granulometry)

In this study the soil samples were collected from different sites in Arzew oil refinery. Table 11 present clay, silt and sand percentage data determined by Pipette hydrometer methods. Table 11 and Figure 32 also present the textural classes of the contaminated and control soil samples.

Table 11: Content of particles size and texture of soil samples.

Samples	Granulometry Parameters					Texture
	Clay %	Fin silt %	Coarse silt %	Fine sand %	Coarse sand %	
S1	9,69	9,18	3,04	18,79	59,29	Sandy loam
S2	8,66	1,53	8,05	29,07	52,70	Loamy sand
S3	33,13	16,82	3,46	15,76	30,83	Sandy clay loam
S4	12,65	7,59	8,54	22,50	48,72	Sandy loam
S5	29,56	28,54	3,61	17,06	21,22	Clay loam
S6	27,52	21,92	4,10	22,17	24,28	Sandy clay loam
S7	30,14	5,62	22,90	23,24	18,10	Clay loam
S8	16,32	34,67	8,63	20,99	19,39	Loam
S9	16,25	19,80	2,66	23,80	37,48	Sandy loam
S10	14,77	20,88	7,63	20,47	36,26	Sandy loam
S11	40,33	14,62	7,13	13,56	24,35	Clay
S12	25,32	24,30	12,92	15,66	21,80	Loam
S13	25,30	23,78	6,15	20,36	24,42	Loam
S14	41,48	15,18	26,03	10,26	7,05	Silty clay
S15	21,71	21,21	4,25	20,66	32,16	Sandy clay loam
S16	27,21	8,06	29,32	28,28	7,13	Clay loam

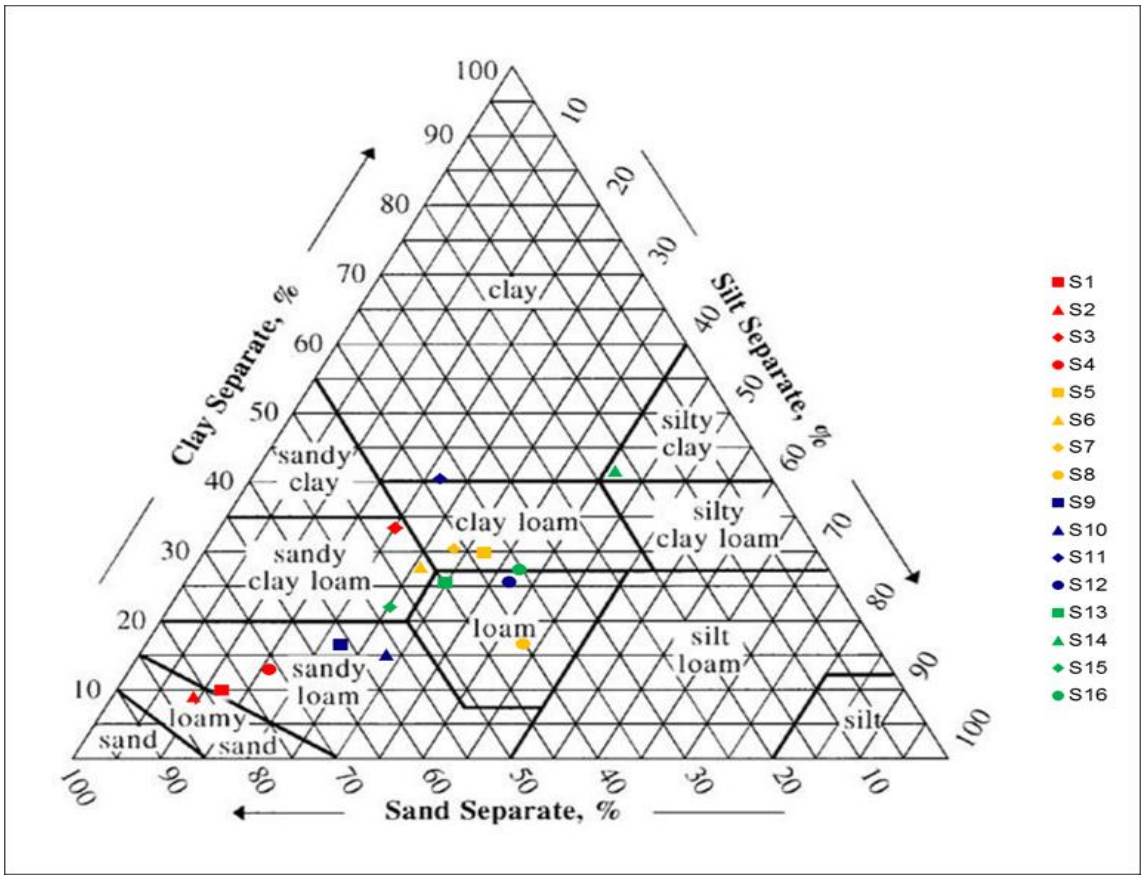


Figure 32: Localization of soils studied on the texture triangle (USDA).

There are variations in the texture between the soil samples. Granulometry analysis shows that sand is the predominant fraction. Thus, samples S1, S2, S3, S6, S9, S10 and S15 have sandy and loamy nature while the rest of the samples have clay and loam nature.

The physical properties of a soil influence its ability to cycle nutrients, hold water and act as an environmental filter (Shober, 2008). The soil texture greatly influences water availability (Zaiad, 2010). According to Bouderhem *et al.* (2016) and Greer *et al.* (1998), the sandy and loamy texture facilitates fluids circulation; that contain nutriments and oxygen which are accessible to microorganisms if the medium is permeable. In another work carried out by Kogbara *et al.* (2015) who studied the effects of different soil textures on the petroleum hydrocarbons degradation, and they found that the sandy loam had the highest, while the clay soil had the least total hydrocarbon content (THC) reduction. However, the increasing in clay content decreased the rate of THC reduction, whereas a better aeration of the coarse soil textures (sand and loamy sand) would increase the microbial survival compared with the fine soil textures (silty

clay and clay) because oxygen is a limiting nutrient in oil-contaminated soils (Kogbara *et al.*, 2015).Q. Liu *et al.* (2017) reported that total petroleum hydrocarbons degradation in sand exhibited greatest microbial mineralization while the petroleum hydrocarbons biodegradation was negatively correlated with clay contents.

III.1.2. Hydrogen ion concentration (pH)

The pH of 16 soils ranged from to 7.55 to 8.16 (Figure 33) indicating that the soils are neutral to slightly alkaline. Soil pH is important because most microbial species can survive only within a certain pH range. Furthermore, soil pH can affect the availability of nutrients, for example the solubility of phosphorus is maximized at a pH 6.5(Barden, 1994). Biodegradation rate is higher at pH near neutrality (Leahy & Colwell, 1990; Salleh *et al.*, 2003). Dolon and Bauder (2011) reported that Biodegradation of petroleum hydrocarbons is optimal at a pH 7 (neutral); the acceptable range is pH 6 to 8. Biodegradation can occur under a wide-range of pH however; a pH of 6.5 to 8.5 is generally optimal for biodegradation in most aquatic and terrestrial systems (Joutey *et al.*, 2013). Our results are in agreement with previous findings, Q. Wang *et al.* (2011) noted that a pH 7 is an optimal for the best degradation of crude oil by a consortium of *Pseudomonas* strains. On the other hand, biodegradation of crude oil is dominated by fungal populations in acidic soil (Leahy & Colwell, 1990; Salleh *et al.*, 2003).

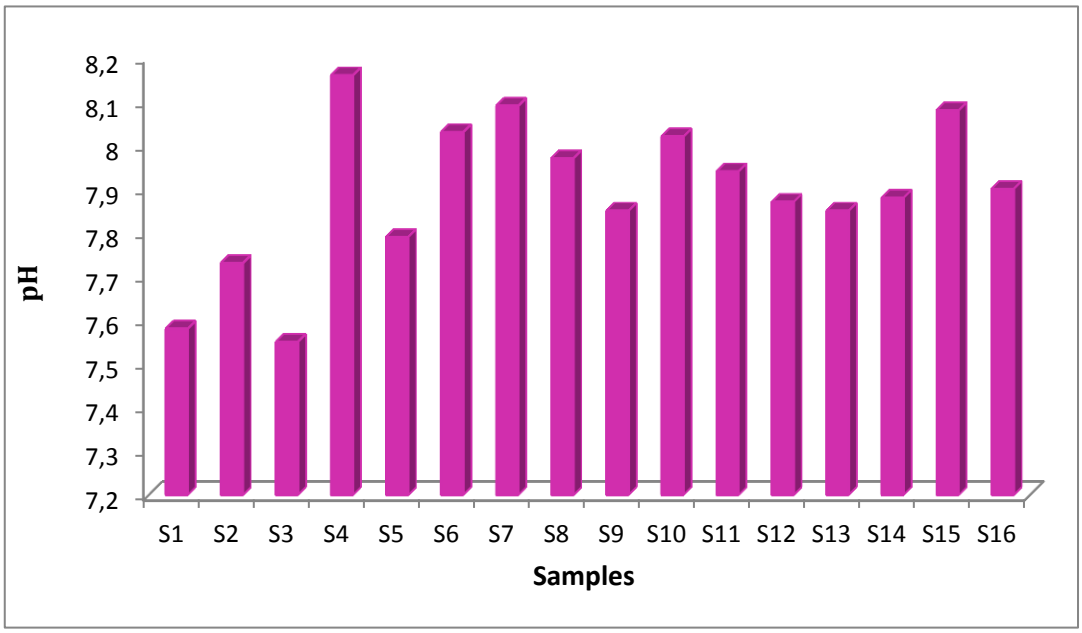


Figure 33: Hydrogen potential of the soils samples

III.1.3. Moisture content

Water content value ranges from 3.29% to 7.47% (Figure 34), however sample S8 and S13 showed the highest water content. Soil moisture is important to biodegradation because the majority of microorganisms live in the water film surrounding the particles of soil. The water content has been greatly affected by soil texture. The sandy soil can be recharged with soil moisture quickly, but is unable to hold water, as the soil with heavy textures (Zaiad, 2010). Dibble and Bartha (1979) reported that oil sludge biodegradation was optimal at 30 to 90% water-holding capacity. Water play major roles in the degradation operation, because is essential to all biological activity. Cabridenc (1985) noted that moderate humidity is desired for optimal rate of microbial degradation in the soil, while the microbial activity slows at a low levels of humidity.

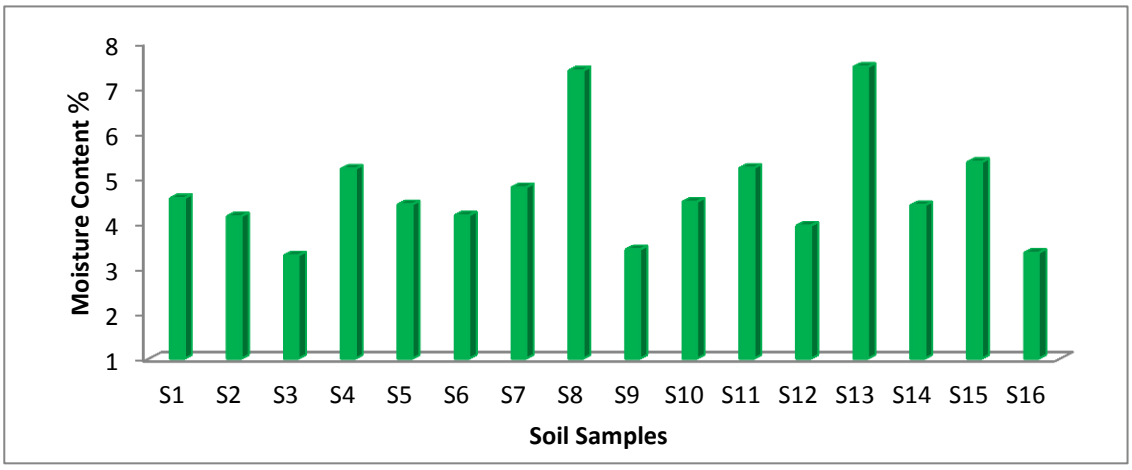


Figure 34: Moisture content of the soil samples.

III.1.4. Electrical conductivity

The electrical conductivity (EC) of our samples ranged from 0.0578 dS/m to 1.794 dS/m (Figure 35). Electrical conductivity is used to estimate the soluble salt concentration in soil, and is commonly used as a measure of salinity. soil with EC below 0.4 dS/m is considered non-saline, while soils above 0.8 ms/cm are considered severe saline (Zaiad, 2010; Wagh *et al.*, 2013). Therefore, our samples have a low salinity; however sample S12 shows excess content of soluble salts. Ward and Brock (1976) found that the hydrocarbon metabolism and microbial metabolic rates are decreased with increasing salinity.

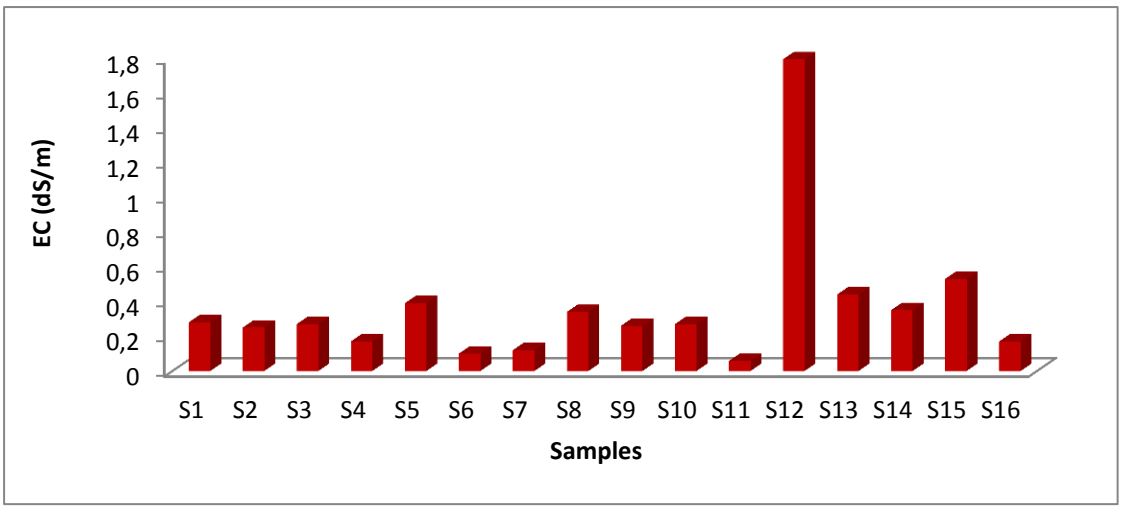


Figure 35: Electrical conductivity of the soil samples

III.1.5. Chemical characteristics of the soil samples

The results of organic carbon, organic matter, calcium carbonate ($CaCO_3$), phosphorus, chloride ion concentrations and sulfate of soil samples are given in Table 12.

Table 12: Chemical characteristics of the soil samples.

Samples	Organic carbon %	Organic matter %	Calcium carbonate $CaCo_3$ %	Phosphorus ppm	Chloride ppm	Sulphate ppm
S1	1,05	1,81	46,48	136,71	83,68	164,35
S2	0,94	1,62	06,34	86,02	129,32	164,35
S3	0,99	1,70	41,41	113,67	114,11	143,81
S4	0,56	0,97	52,39	92,93	230,75	143,81
S5	0,99	1,70	34,65	226,57	266,25	164,35
S6	0,99	1,71	24,51	249,62	153,83	164,35
S7	1,11	1,92	28,31	158,22	248,50	308,16
S8	1,02	1,75	22,31	148,23	40,83	267,07
S9	0,77	1,33	54,93	175,12	44,38	308,16
S10	0,94	1,61	55,77	145,16	365,00	123,26
S11	0,63	1,08	38,63	199,69	281,46	102,72
S12	1,05	1,81	57,53	101,38	244,95	123,26
S13	0,97	1,66	20,96	66,05	266,25	82,18
S14	0,97	1,67	0,82	93,70	97,63	143,81
S15	0,79	1,36	52,60	72,96	44,38	143,81
S16	0,58	1,00	2,88	142,09	63,08	184,90

Organic matter of soil is defined as a group of animal and plant remains at different stages of decomposition. Organic matter enhance the physical properties of soil (resistance to erosion and moisture holding capacity) (McCauley *et al.*, 2003; Wagh *et al.*, 2013). Organic matter keeps the soil open, allowing infiltration of water and air (Wagh *et al.*, 2013) , Soil samples were classified according to their organic matter contents as follows: 0.0-1.0 too low, 1.1-2.0 low, 2.1-3.0 medium, 3.1-4.0 good and >4 high (Maral, 2010).

The organic carbon (%) ranges from 0.56 to 1.11 %, the organic soil matter range from 0.97 to 1.92 %. Most samples have low organic matter content while two samples (S4 and S16) have too low organic matter content. It means the soils are limited in their organic matter.

The $CaCO_3$ % (lime content) values of the soil samples range from 0.82 to 57.53%. It means that all of the soil samples are high calcareous except sample S2 which is moderately calcareous, sample S14 is calcareous and sample S16 is slightly calcareous. The $CaCO_3$% levels and classification of soil were evaluated using the following criteria: slightly calcareous (0 to 1.0 %), calcareous (1.1 to 5.0 %), a moderately (5.1-15 %) and high calcareous (above 15%) (Maral, 2010).

The soil must contain an adequate concentration of all the mineral elements which are necessary to the microbial activity; these include phosphorus, sulfate, nitrogen, potassium, calcium and oxygen in the condition of aerobic degradation. For the optimal microbial degradation rate, the concentration of the biodegradable carbon and of assimilable phosphorus and nitrogen must give the (C:P:N) ration of 100:1:5. (Cabridenc, 1985). Phosphorus is one of the essential macronutrient for metabolism and plant growth (Wagh *et al.*, 2013).

Phosphorous in our soil samples varies from 66.05 to 249.62 ppm. The phosphorus concentration of soil samples S5, S6 and S11 are high but soil samples S1, S3, S2, S4, S12, S13, S14 and S15 have low, soil samples S7, S8, S9, 1S0 and S16 have medium phosphorus content.

Soil samples were classified according to their phosphorus concentration contents as follows: <100 too low, 100 to 140 low, 140 to 180 Moderately, 180 to 220 good and >220 high (Anonymous). Atlas (1981) noted that the addition of phosphorus and nitrogen containing fertilizers can be used to enhance microbial hydrocarbon

degradation. The chloride concentration of the soil samples varies from 40,83 to 365,00 ppm and the sulphate range from 82,18 to 308,16 ppm. Chloride is important to plants in very low quantities, but at high concentrations it can cause toxicity to sensitive crops (Maral, 2010).

III.1.6.The total petroleum hydrocarbons (TPH) content

The total hydrocarbon content of our samples range from 2 to 86 g/kg of soil (**Error! Reference source not found.**) these results are greater than the value cited by utch standard (0.1 g hydrocarbon/kg of soil) (Akmoussi-Toumi, 2009). This result confirms that these soils samples are highly polluted by hydrocarbon compounds. This pollution, which can be attributed to oil spills occur during the refining, transportation and storage of oil. The soil environmental factors were the most dominant factors of bacterial community distribution and abundance (Zhang *et al.*, 2018).

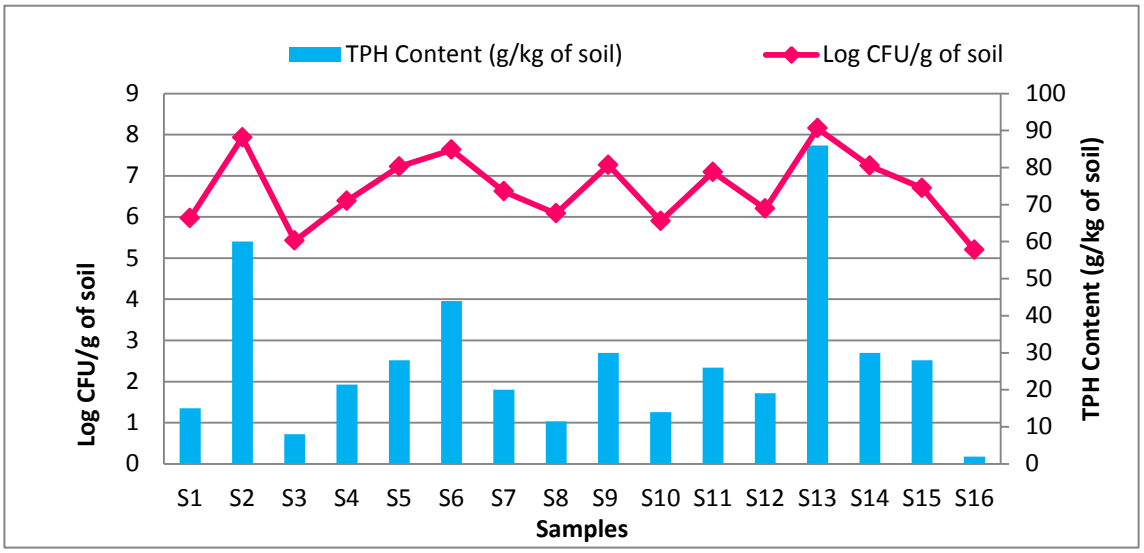


Figure 36: Total bacterial count (Log CFU/g soil) and total petroleum hydrocarbons (TPH) content in the soil samples.

III.2. Microbiological analysis of soil

III.2.1. Bacterial count

Total aerobic bacterial count on nutrient agar for each soil sample is presented in **Error! Reference source not found.**. The total aerobic counts ranged from 1.6 x 10^5 to .4 x 10^8 CFU/g of soil, with different colony type. The highest bacterial counts were in the soil samples (S13 and S2, location code ZS6b, ZS1b respectively) in which these sites had the darker black-color soil. While the lowest bacterial counts were found in the soil samples (S16, S3 and S10 location code ZS8, ZS1c and ZS4a respectively) in

which these sites the lighter soil color. Also results showed high bacterial count that were found in oil contaminated soil compared with the negative control soil (S16) that were taken from non-contaminated soil.

The variations in the number of bacterial colonies between soil samples may be due to possibility that the higher content of TPH may have greater number and diversity of microorganisms, in contrast with low content of TPH might be due to lower number of microorganisms as shown in Figure 36.

These results agree with (Youssef *et al.*, 2010), who reported that petroleum contaminated sites have greater bacterial count abundance and large number of the hydrocarbon degrading bacteria than the un-contaminated sites. This also agree with Hood *et al.* (1975),who noted that, the content of hydrocarbons represents a nutrient enrichment where lower content may be limiting the bacterial growth. However, a positive correlation was noted between ratios of hydrocarbonoclastes and total aerobic bacteria and the concentration of hydrocarbons.

Also, we observed that the number of colonies that were isolated from the subsurface soil (5cm) was slightly greater than that were isolated from the deep soil (>5-10 cm). This results might be originated from the level of organic nutrient available at subsurface soil (5cm) was greater than that are present at the deeper soil (>5 cm-10cm), as reported by Al-Deeb (2005). Furthermore Al-Deeb and Malkawi (2009) and Leahy and Colwell (1990) found that the variation in the bacterial count between oil contaminated samples may be due to the possibility that the lower level of aged contamination may have greater number and diversity of microorganisms in contrast with fresh spilled oil with high contaminant which might kill large number of soil microorganisms. Also, our results coincide with (Salleh *et al.*, 2003), who reported that a high concentrations of highly soluble or volatile organic compounds may be detrimental to microbial forms due to their toxicity.

The abundance of metabolically active fungal and bacterial population in sites that are polluted with hydrocarbons strongly suggests that these microorganisms utilize hydrocarbons as carbon and energy source (Salleh *et al.*, 2003). Hydrocarbon utilizing microorganisms are ubiquitous in nature but are found at relatively higher number in petroleum polluted areas (El-Khawaga *et al.*, 2015)

III.2.2. Isolation of hydrocarbon degrading bacteria

Hydrocarbon degrading bacterial strains were isolate by enrichment technique. A total of 78 bacterial colonies (appendix E) with different morphologies were isolated on MSM agar.

III.2.3. Screening of the most potent hydrocarbon degrading bacteria

Seventy eight bacterial isolates were isolated from oil contaminated soil, and then purified. The isolated bacteria were tested for their growth ability on MSM supplemented with 1% crude oil as sole carbon source. The growths of the isolated bacterial species were monitored at regular intervals (2 days) by measuring the optical density at 600 nm.

Based on the growth of bacterial species, 22 bacterial isolate were screened with the best degradative abilities on crude oil, the isolates show diverse forms, colors, margins, and shapes, suggesting bacterial diversity among isolates.

III.2.4. Identification of screened isolates

III.2.4.1. Morphological characterization

III.2.4.1.1. Macroscopic observations

Bacterial colonies were grown several times in MSM agar supplemented with 1% crude oil and showed that those bacterial isolates were able to utilize crude oil as sole carbon and energy source. The selected bacterial colonies were cultured on nutrient agar (NA), tryptic soy agar (TSA) and/or cetrimide. The morphological description of bacterial isolates is indicated in (Table 13). The morphology of bacterial colonies obtained from different contaminated soil sites have ranged from circular, irregular while the color of the isolates was diverse: some were cream, some exhibited yellow color, while others cream white, orange or even green and light-yellow indication different pigments production by some isolates.

Table 13: Macroscopic characterization of isolated bacterial strains.

N°	Bacterial isolates	Colony size	Colony color	Colony shape	Colony elevation	Colony margin	Colony surface	Colony optical property	Colony consistency
1	P1.2	Small	Yellow	Circular	Raised	Entire	Smooth	Opaque	Mucoid
2	P2.1	Small	Light-yellow	Circular	Convex	Entire	Smooth Shiny	Translucent	Viscous
3	P2.2	Small	Cream	Circular	Raised	Entire	Smooth	Opaque	Buttery
4	P2.3	Small	Green	Circular	Convex	Irregular	Smooth Shiny	Translucent	Viscous
5	P2.8	Small	Yellow	Circular	Convex	Entire	Smooth Shiny	Opaque	Viscid
6	P4.2	Small	Yellow	Irregular	Convex	Entire	Smooth Shiny	Opaque	Viscid
7	P6.3	Medium	Yellow	Circular	Convex	Entire	Smooth	Translucent	Buttery
8	P13.1	Medium	Yellow	Circular	Convex	Irregular	Smooth	Opaque	Viscous
9	P14.1	Large	Yellow	Circular	Raised	Entire	Smooth Shiny	Opaque	Mucoid
10	P14.2	Small	Cream White	Circular	Flat	Irregular	Smooth	Opaque	Viscous
11	E1.1	Small	White	Circular	Raised	Entire	Smooth	Opaque	Buttery
12	E2.5	Small	Cream	Circular	Raised	Entire	Smooth	Opaque	Buttery
13	S15.1	Small	White	Circular	Convex	Entire	Smooth	Opaque	Buttery
14	S15.3	Small	Yellow-Orange	Circular	Convex	Entire	Smooth, dull	Opaque	Buttery
15	B3.1	Large	White	Irregular	Flat	Undulate	Dull	Opaque	Buttery
16	B3.2	Small	White	Circular	Flat	Entire	Rough	Opaque	Buttery
17	B4.1	Small	Cream	Slightly Irregular	Flat	Irregular	Rough	Opaque	Buttery
18	B4.2	Small	Cream White	Irregular	Raised	Undulate	Smooth	Opaque	Buttery
19	B6.2	Medium	White	Irregular	Flat	Irregular	Rough	Opaque	Brittle
20	B7.1	Small	White	Circular	Flat	Entire	Rough	Opaque	Buttery
21	B15.2	Large	White	Circular	Umbonate	Undulate	Rough	Opaque	Dry
22	B15.3	Medium	Orange	Circular	Convex	Entire	Smooth Shiny	Opaque	Buttery

II.2.4.1.2. Microscopic observations

All the twenty-one isolates were studied for their Gram reaction property; cell morphology using standard Gram's staining procedure (Figure 37) and endospore staining (Figure 38). The data are presented in Table 14. The isolates were found to be purple and pink colored, Gram-positive and Gram-negative respectively, of varying size and shape under microscope using oil immersion technique. The majority of the bacterial isolates were gram negative coccobacilli and bacilli (45.45%), some were gram positive bacilli (36.36%), and some were gram positive cocci (18.18%).

Table 14: Microscopic characterization of isolated bacterial strains.

N°	Isolate	Mobility	Gram	Shape	Arrangement	Endospore	Endospore position
1	P1.2	+	-	Rod	Single Bacilli	-	-
2	P2.1	+	-	Rod	Single and Diplobacilli	-	-
3	P2.2	+	-	Rod	Single, Diplobacilli and Bacilli in clusters	-	-
4	P2.3	+	-	Coccobacillus	Single Bacilli	-	-
5	P2.8	+	-	Rod	Single and Diplobacilli	-	-
6	P4.2	+	-	Coccobacillus	Single and Diplobacilli	-	-
7	P6.3	+	-	Coccobacillus	Single Bacilli	-	-
8	P13.1	+	-	Rod	Single Bacilli	-	-
9	P14.1	+	-	Coccobacillus	Single Bacilli	-	-
10	P14.2	+	-	Rod	Single Bacilli	-	-
11	E1.1	-	+	Cocci	Short chains, Diplococci and single Cocci	-	-
12	E2.5	-	+	Cocci	Short chains, Diplococci and single Cocci	-	-
13	S15.1	-	+	Cocci	Singl cocci, Diplococci, tetrad and in clusters	-	-
14	S15.3	-	+	Cocci	Single Cocci, pairs and in grape-like clusters	-	-
15	B3.1	+	+	large-rod	Diplobacilli	+	Central non deformante
16	B3.2	-	+	Rod	Bacilli in short chain	+	Sub-terminal non-deformante
17	B4.1	+	+	Long-rod	Single and Diplobacilli	+	Sub-terminal
18	B4.2	+	+	Long-Rod	Single Bacilli	+	Terminal
19	B6.2	-	+	large-rod	Short chains and Single Bacilli	+	Sub-terminal and Central, non deformante
20	B7.1	+	+	Rod	Single Bacilli	+	Sub-terminal and Terminal non deformante
21	B15.2	+	+	Rod	Single and Diplobacilli	+	Sub-terminal deformante
22	B15.3	+	+	Short Rods to almost coccoid	Singly or in clusters	-	-

(+): positive; (-): negative

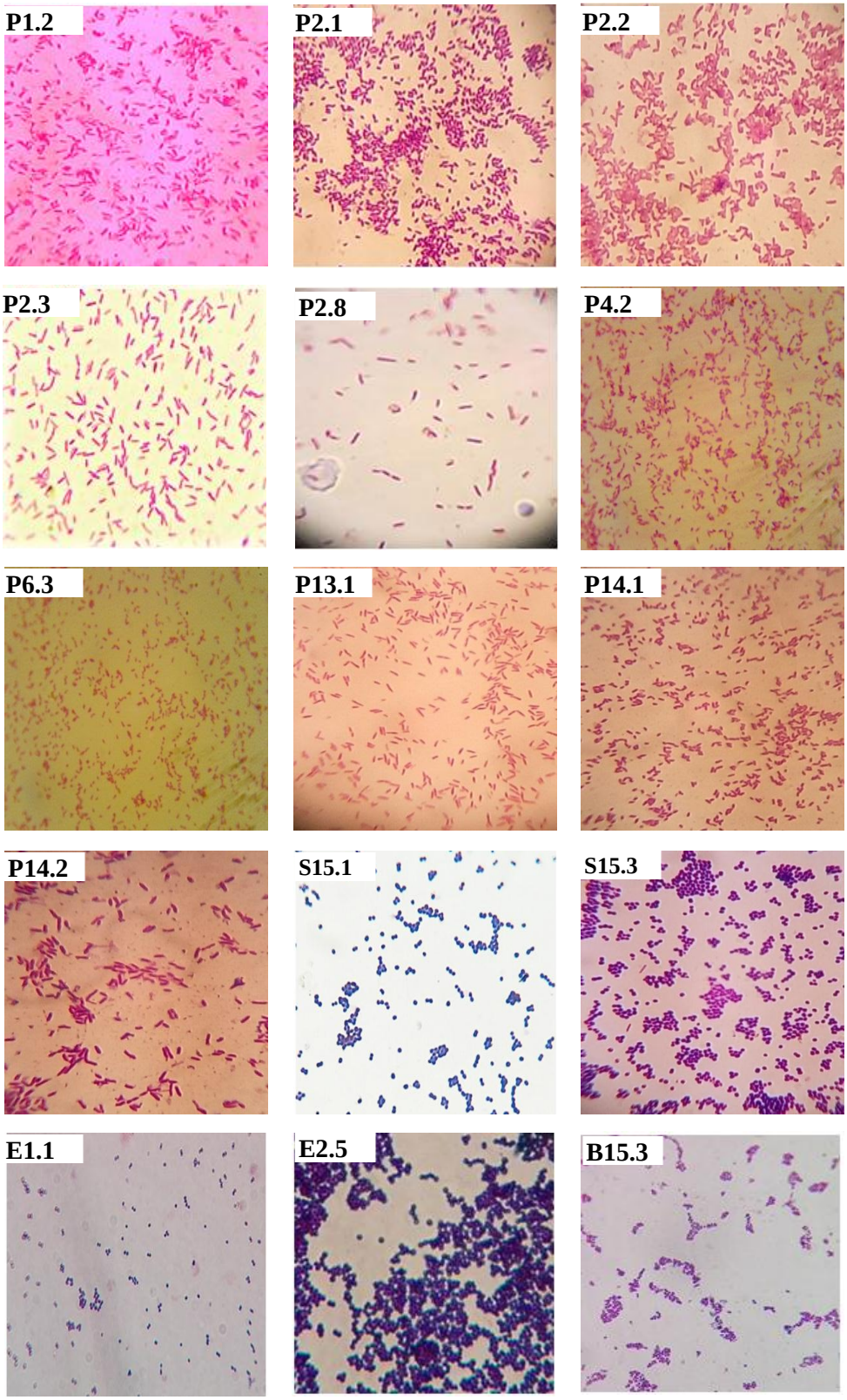


Figure 37: Microscopic aspect of isolated bacterial strains (G x 1250), Gram stain.

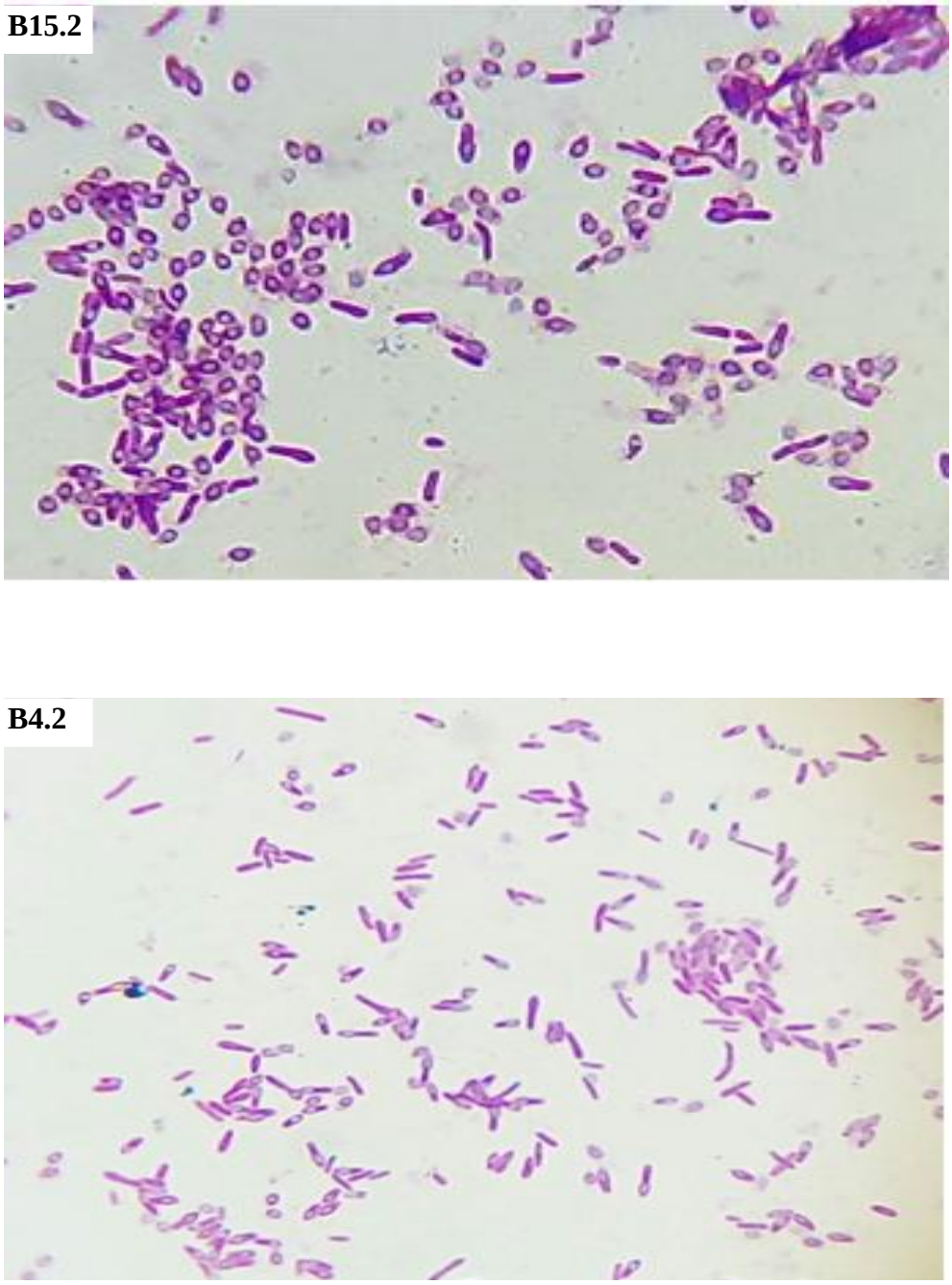


Figure 38: Microscopic aspect of some isolated bacterial strains (G x 1250), endospore staining.

III.2.4.2. Biochemical characterization of the bacterial isolates

More biochemical and physiological tests were carried out to identify the isolates precisely, and were tentatively given only at this point. The results of the biochemical tests and bio-Mérieux API kits (20NE, STAPH and 20E) for all isolates are indicated in Table 15, 16, 17 and 18.

Table 15: Biochemical characterization of the bacterial isolates S15.1 and S15.3.

	Bacterial isolates	
Tests	**S15.1**	**S15.3**
Oxidase	-	-
Catalase	+	+
3% KOH	-	-
Respiration	Aero-anaerobe facultatif	Aero-anaerobe facultatif
GLU	+	+
FRU	+	+
MNE	-	-
MAL	+	+
LAC	+	-
TRE	+	+
MAN	-	-
XLT	-	-
MEL	-	-
NIT	+	+
PAL	-	-
VP	-	-
RAF	-	-
XYL	-	-
SAC	+	+
MDG	-	+
NAG	+	+
ADH	+	-
URE	-	+
DNase	-	+
Coagulase	-	-
Hemolysis	+	-
Bacterial Species	***Staphylococcus haemolyticus***	***Staphylococcus hominis***

(+): positive reaction; (-): negative reaction

Table 16: Biochemical characterization of the gram-negative bacilli isolates.

		Tests																												
		Oxidase	Catalase	3% KOH	Respiration	NO3	TRP	GLU (fermentation)	ADH	URE	ESC	GEL	PNPG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	Pyocyanine	Pyoverdine	Growth at 42 °C	Growth at 4 ° C	TSI	Levane Production	Bacterial Species
Bacterial isolates	P1.2	+	+	+	Obligate Aerobe	+	-	-	+	+	-	+	-	+	-	-	+	-	-	+	+	+	+	+	-	+	+	+	-	S^-/L^- H^-/G^-	+	*Pseudomonas aeruginosa*
	P2.1	+	+	+	Obligate Aerobe	+	-	-	+	-	-	+	-	+	-	-	+	+	-	+	+	+	+	+	-	+	+	+	-	S^-/L^- H^-/G^-	+	*Pseudomonas aeruginosa*
	P2.2	+	+	+	Obligate Aerobe	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	S^-/L^- H^-/G^-	-	*Achromobacter xylosoxidans*
	P2.3	+	+	+	Obligate Aerobe	+	-	-	+	-	-	+	+	+	-	-	+	+	-	+	+	+	+	+	-	+	+	+	+	S^-/L^- H^-/G^-	+	*Pseudomonas aeruginosa*
	P2.8	+	+	+	Obligate Aerobe	-	+	-	+	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+	-	+	S^-/L^- H^-/G^-	+	*Pseudomonas fluorescens*
	P4.2	+	+	+	Obligate Aerobe	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	-	S^-/L^- H^-/G^-	+	*Pseudomonas fluorescens*
	P6.3	+	+	+	Obligate Aerobe	+	-	+	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-	-	-	S^-/L^- H^-/G^-	-	*Burkholderia cepacia*
	P13.1	+	+	+	Obligate Aerobe	+	-	-	+	+	-	+	-	+	-	-	+	-	-	+	+	+	+	+	-	+	+	+	-	S^-/L^- H^-/G^-	+	*Pseudomonas aeruginosa*
	P14.1	+	+	+	Obligate Aerobe	+	-	-	+	+	-	+	-	+	-	+	+	-	+	+	+	+	+	+	-	+	+	+	-	S^-/L^- H^-/G^-	+	*Pseudomonas aeruginosa*
	P14.2	+	+	+	Obligate Aerobe	+	-	-	+	+	+	+	+	+	-	+	+	-	-	-	-	+	+	+	-	+/-	+	+	-	S^-/L^- H^-/G^-	+	*Pseudomonas aeruginosa*

(+): positive reaction; (-): negative reaction; (S): Sucrose; (G): Glucose; (L): Lactose; (H): H_2S.

Table 17: Biochemical characterization of the bacterial isolates E1.1 and E2.5.

	Bacterial isolates	
Tests	**E1.1**	**E2.5**
Oxidase	-	-
Catalase	-	-
3% KOH	-	-
Respiration	Aero-anaerobe facultatif	Aero-anaerobe facultatif
ONPG	-	-
ADH	+	-
LDC	-	-
ODC	-	-
CIT	-	-
H_2S	+	+
URE	-	-
TDA	-	-
IND	-	-
VP	+	+
GEL	+	-
GLU	+	+
MAN	+	+
INO	-	-
SOR	+	+
RHA		
SAC	+	+
MEL	+	+
AMY	-	-
ARA	+	+
NIT	+	+
Growth in 6.5%	+	+
Hemolysis	+	+
Lecithinase	-	-
Starch Hydrolysis	-	-
Coaglase	-	-
Bacterial Species	***Enterococcus faecalis***	***Enterococcus faecalis***

(+): positive reaction; (-): negative reaction

Table 18: Biochemical characterization of the gram-positive bacilli isolates.

Bacterial isolates								
Tests	**B3.1**	**B3.2**	**B4.1**	**B4.2**	**B6.2**	**B7.1**	**B15.2**	**B15.3**
Oxidase	-	-	+	-	-	+	-	-
Catalase	+	+	+	+	+	+	+	+
3% KOH	-	-	-	-	-	-	-	-
Respiration	aero-anaerobe facultatif	aero-anaerobe facultatif	Obligate Aerobe	Aerobe	aero-anaerobe facultatif	Aerobe	aero-anaerobe facultatif	aero-anaerobe facultatif
ONPG	-	-	-	-	-	+	+	-
ADH	+	-	-	-	-	-	+	+
LDC	+	-	-	-	-	-	-	-
ODC	+	-	-	-	-	-	-	-
CIT	+	+	-	+	+	+	+	-
H_2S	-	-	-	-	-	-	-	-
URE	+	-	+	-	-	+	+	-
TDA	+	-	-	-	-	-	-	+
IND	-	-	-	-	-	-	-	+
VP	+	+	-	+	+	-	+	-
RM	-	-	+	-	-	+	-	+
GEL	+	+	-	-	+	+	+	+
GLU	+	+	-	+	+	-	+	+
MAN	-	-	-	+	-	-	+	+
INO	-	-	-	+	-	-	+	-
SOR	+	-	-	-	-	-	+	-
RHA	-	-	-	-	-	-	-	-
SAC	+	+	-	+	+	+	+	+
MEL	-	-	+	-	-	-	+	-
AMY	+	-	-	-	-	-	+	+
ARA	-	-	-	-	-	-	+	-
NIT	+	+	+	-	+	-	+	+
Growth in 6.5%	+	+	+	-	+	+	+	+
Hemolysis	+	-	-	+	-	-	+	-
Lecithinase	+	+	-	-	+	-	-	-
Starch Hydrolysis	+	+	-	+	+	-	+	+
Bacterial Species	***Bacillus cereus***	***Bacillus anthracis***	***Bacillus pasteurii***	***Bacillus sp***	***Bacillus anthracis***	***Lysinibacillus sphaericus***	***Bacillus subtilis***	***Exigobacterium sp***

(+): positive reaction; (-): negative reaction

The isolates were identified by morphological and Biochemical techniques using the taxonomic scheme of Bergey's Manual of determinative bacteriology (Holt *et al.*, 1994; Brenner *et al.*, 2005; Vos *et al.*, 2009). As indicated in Table 15, 16, 17 and 18, the isolates were tentatively identified as the following bacterial genera and/or species: *Pseudomonas aeruginosa* (P1.2), *Pseudomonas aeruginosa* (P2.1), *Achromobacter xylosoxidans* (P2.2), *Pseudomonas aeruginosa* (P2.3), *Pseudomonas fluorescens* (P2.8), *Pseudomonas fluorescens* (P4.2), *Burkholderia cepacia* (P6.3), *Pseudomonas aeruginosa* (P13.1), *Pseudomonas aeruginosa* (P14.1), *Pseudomonas aeruginosa* (P14.2), *Staphylococcus haemolyticus* (S15.1), *Staphylococcus hominis* (S15.3) *Enterococcus faecalis* (E1.1), *Enterococcus faecalis* (E2.5), *Bacillus cereus* (B3.1), *Bacillus anthracis* (B3.2), *Bacillus pasteurii* (B4.1), *Bacillus sp* (B4.2), *Bacillus anthracis* (B6.2), *lysinibacillus sphaericus* (B7.1), *Bacillus subtilis* (B15.2) and *Exigobacterium sp* (B15.3). According to the results in this study the most dominant genus among bacterial isolates was the genus *Pseudomonas* followed by the genus *Bacillus* then the genus *Staphylococccus*, *Burkholdriale* and *Enterococcus*. Previous studies had reported the dominance of the genus *Pseudomonas* among oil degrading bacteria (Al-Deeb, 2005; Ben Said *et al.*, 2008; Al-Adwan *et al.*, 2010; Guermouche *et al.*, 2013; Benchouk & Chibani, 2016). Similarly, Sibi *et al.* (2015) isolated *Pseudomonas aeruginosa* and *Achromobacter* from oil contaminated soil. Furthermore, Al-Adwan *et al.* (2010) isolated sixteen bacterial genera include *Pseudomonas*, *Staphylococcus* and *Bacillus* from AL-Rwuayashid (the northeast part of Jordan) oil contaminated soil. K. Das and Mukherjee (2007) isolated from a petroleum contaminated soil samples of North-East India strains of *Pseudomonas aeruginosa* and *Bacillus subtilis*, these bacterial strains could utilize crude petroleum-oil hydrocarbons as a sole source of carbon and energy. Moreover, Erdogan *et al.* (2012) isolated 33 strains of hydrocarbon-degrading bacteria include *Pseudomonas spp*. from the contaminated soil in Turkey. These results confirm that *Pseudomonas sp*. is the most common bacteria capable of degrading hydrocarbon (Salleh *et al.*, 2003; Boboye *et al.*, 2010). Further study revealed that the biodegradation ability was detected in following species and/or genera; Achromobacter xylosoxidans (De Vasconcellos *et al.*, 2009; Ebrahimi *et al.*, 2012; Meenakshisundaram & Bharathiraja, 2014), *Enterococcus faecalis* (Boontawan & Boontawan, 2011; Obuotor *et al.*, 2016), *Pseudomonas fluorescens* (Adebusoye *et al.*, 2006; Al-Deeb & Malkawi, 2009; El-Khawaga *et al.*, 2015), *Burkholderia cepacia*, *Staphylococcus* (Chadli et al., 2013), *Staphylococcus*

haemolyticus (Xiaolu *et al.*, 2010) *Bacillus cereus* (Kebria *et al.*, 2009; Meenakshisundaram & Bharathiraja, 2014), *Exigobacterium sp* (Arora & Bae, 2015; Yang *et al.*, 2015) and *lysinibacillus* (Liang *et al.*, 2009; Yao *et al.*, 2011; Roselin, 2012; Ren *et al.*, 2015).

III.2.4.3. Molecular characterization of bacterial isolates

Molecular identification was performed using PCR-amplified 16S rDNA sequences which currently used as a sensitive and specific detection method for microorganisms (Malkawi *et al.*, 2009a; Al-Adwan *et al.*, 2010). The Genomic DNA was extracted from 15 bacterial isolates and positive control (*Pseudomonas aeruginosa* ATTC 27853) and universal primers 27F and 1492R were used for the amplification and sequencing of the 16S rRNA gene fragment.

III.2.4.3.1. Bacterial identifiction using 16S rDNA universal primer

Using 16S rDNA universal primer pair (conserved sequence among all bacteria), all bacterial isolates showed positive results. PCR products of 1500 bp of the isolates were analyzed on 1% agarose gel electrophoresis (Figure 39). Positive control (*Pseudomonas aeruginosa* ATTC 27853) showed also a 1500 bp amplification product.

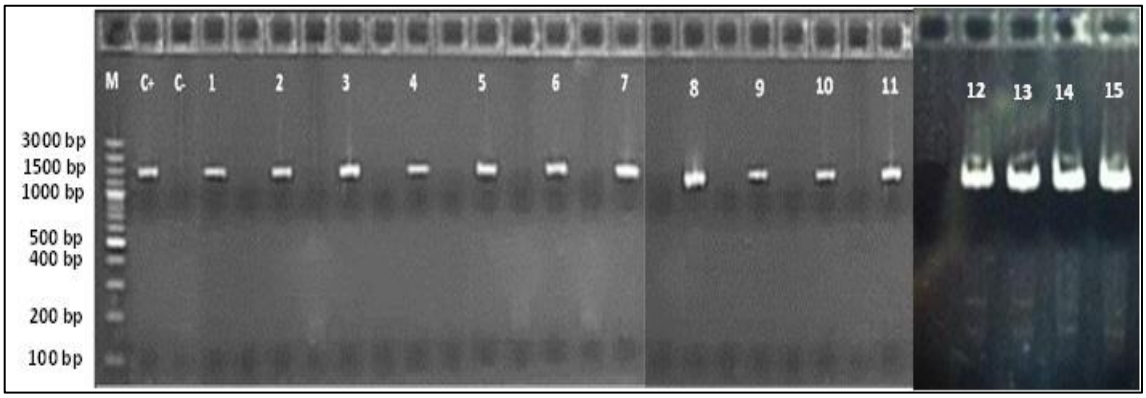


Figure 39: Agarose gel electrophoresis of PCR amplification product of bacterial isolates using universal primer pair of 16S rDNA.

Lane M: Gene Ruler 100 bp Plus DNA Ladder (Thermo Scientific), lane C+: positive control with *Pseudomonas aeruginosa* ATTC 27853, lane C-: negative control without DNA, lane 1: E2.5, lane 2: P2.1, lane 3: B4.2, lane 4: B3.1, lane 5: B6.2, lane 6: B15.3, lane 7: P13.1, lane 8: isolate P14.2, lane 9: isolate P14.1, lane 10: isolate P1.2, lane 11: isolate B15.2, lane12: isolate P3.2, lane 13: isolate P2.2, lane 14: isolate S15.1 and lane 15: isolate E1.1.

III.2.4.3.2. 16S rDNA sequencing and phylogenetic analysis

Based on the partial 16S rDNA sequencing and phylogenetic analysis (Figure 40), the isolates were identified compared to the closest relative species in the GenBank database with 95-100% similarity (Table 19). The 16S rDNA sequences revealed that the isolated strains (P1.2, P2.1, P2.3, P13.1, P14.1 and P14.2) belong to phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Pseudomonadales*, family *Pseudomonadaceae* and genus *Pseudomonas*. The strains (P1.2, P2.1, P2.3, P13.1, P14.1 and P14.2) were highly similar (99-100%) with *Pseudomonas aeruginosa* strain DSM 50071 (NR 117678), *Pseudomonas aeruginosa* strain NBRC12689 (NR 113599) and *Pseudomonas aeruginosa* strain ATCC10145 (NR 114471). Thus this newly isolated strain was identified as a *Pseudomonas aeruginosa* strain. This result strongly supported (95% confidence) by the RDP Naïve Bayesian rRNA classifier tool. The phylogenetic analysis (Figure 40) revealed that the strains (P1.2, P2.1, P2.3, P13.1, P14.1 and P14.2) formed a monophyletic group with *Pseudomonas Otitidis* strain MCC10330 (NR043289).

The P2.2 isolate has 95% identity with *Achromobacter xylosoxidans* and classified as the order *Burkholderiales*, Family *Alcaligenaceae* and genus *Achromobacter* with RDP classifier tool (95% confidence).

On the basis of 16S rRNA gene sequence analysis, B15.3 is a member of the genus *Exiguobacterium*, this isolate was highly similar (99%) with *Exiguobacterium aurantiacum* strain NBRC 14763 (NR113666) and *Exiguobacterium aurantiacum* strain DSM 6208 (NR 043478).The *Exiguobacterium aurantiacum* strain B15.3 formed a robust cluster with *Exiguobacterium mexicanum* strain 8N (NR 042424) and *Exiguobacterium aquaticum* strain IM TB-3094 (NR 109413).

The B4.2 isolate was identified as *Lysinibacillus macroides* strain B4.2 on the basis that it exhibited 99% sequence similarity with *Lysinibacillus macroides* strain LMG 18474 (NR 114920) and it's classified as the genus *Lysinibacillus* (95% confidence, RDP Naive Bayesian rRNA Classifier). The phylogenetic tree (Figure 40) indicated that the strain B4.2 formed a phyletic group with *Lysinibacillus bronitolerans* strain 10a (NR 041276) and were then grouped with *Lysinibacillus macroides* strain LMG 18474 (NR 114920).

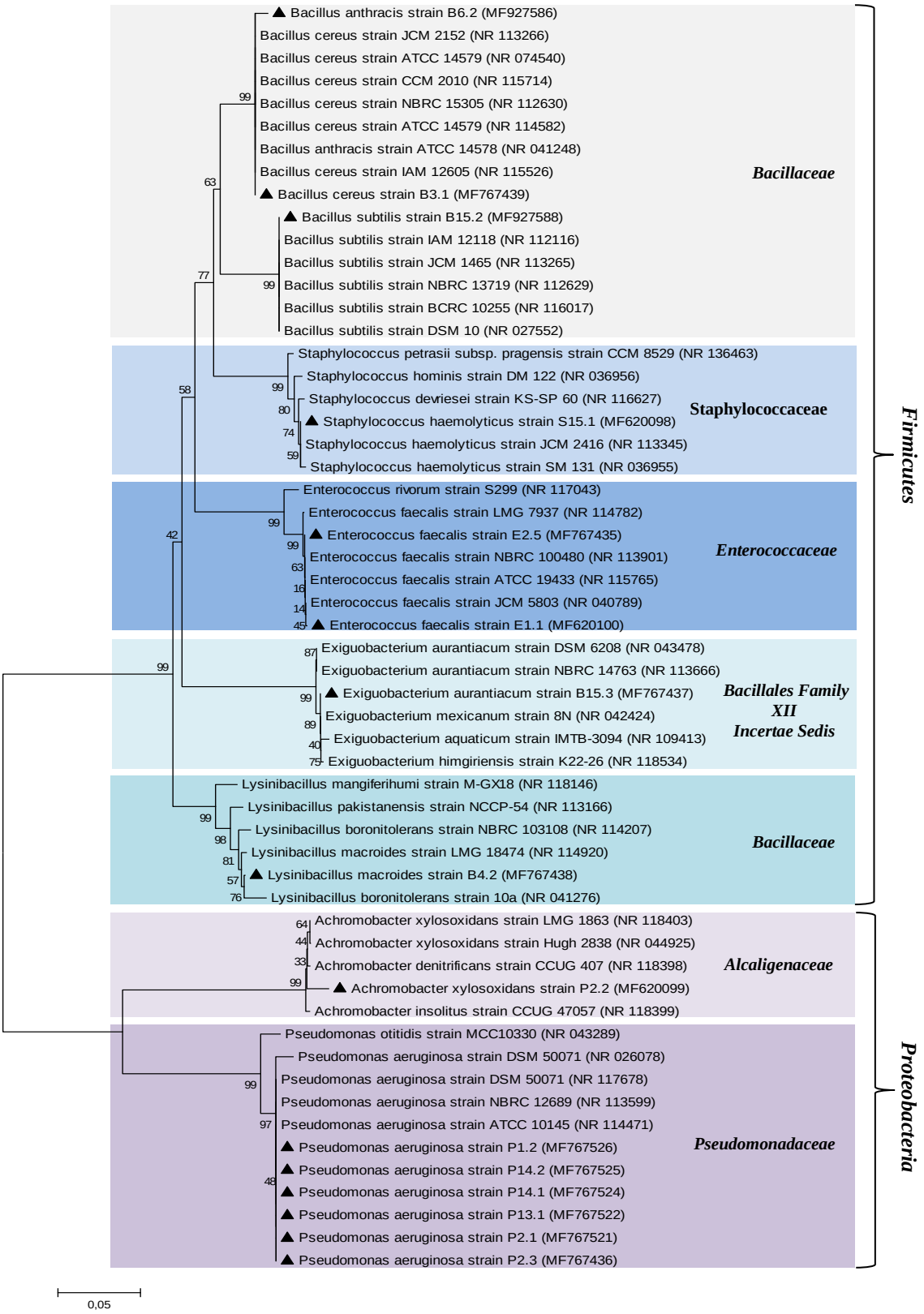


Figure 40: Phylogenetic tree based on the partial 16S rRNA gene sequences of isolates and related species found by a BLASTn database search.

(GenBank accession numbers are listed after species between parentheses). Our sequences are marked by triangles.

Table 19: Closest relative, sequence similarity, GenBank accession number and identification of the bacterial isolates.

Isolate code	Closest relative	Query cover (%)	Identity (%)	Identification	GenBank accession number
P1.2	*Pseudomonas aeruginosa*	100%	99%	*Pseudomonas aeruginosa strain P1.2*	MF767526
P2.1	*Pseudomonas aeruginosa*	100%	100%	*Pseudomonas aeruginosa strain P2.1*	MF767521
P2.3	*Pseudomonas aeruginosa*	100%	100%	*Pseudomonas aeruginosa strain P2.3*	MF767436
P13.1	*Pseudomonas aeruginosa*	100%	99%	*Pseudomonas aeruginosa strain P13.1*	MF767522
P14.1	*Pseudomonas aeruginosa*	100%	100%	*Pseudomonas aeruginosa strain P14.1*	MF767524
P14.2	*Pseudomonas aeruginosa*	100%	99%	*Pseudomonas aeruginosa strain P14.2*	MF767525
P2.2	*Achromobacter xylosoxidans*	100%	95%	*Achromobacter xylosoxidans strain P2.2*	MF620099
B3.1	*Bacillus cereus*	100%	99%	*Bacillus cereus strain B3.1*	MF767439
B4.2	*Lysinibacillus macroides*	99%	99%	*Lysinibacillus macroides strain B4.2*	MF767438
B15.2	*Bacillus subtilis*	100%	100%	*Bacillus subtilis strain B15.2*	MF927588
B6.2	*Bacillus anthracis*	100%	99%	*Bacillus anthracis* B6.2	MF927586
B15.3	*Exiguobacterium aurantiacum*	100%	99%	*Exiguobacterium aurantiacum strain B15.3*	MF767437
S15.1	*Staphylococcus haemolyticus*	100%	99%	*Staphylococcus haemolyticus strain S15.1*	MF620098
E1.1	*Enterococcus faecalis*	100%	100%	*Enterococcus faecalis strain E1.1*	MF620100
E2.5	*Enterococcus faecalis*	100%	99%	*Enterococcus faecalis strain E2.5*	MF767435

There are limited reports describing the involvement of *Lysinibacillus macroides* in biodegradation of hydrocarbon. In previous study, the biodegradation of petroleum was studied by growing *Lysinibacillus fusiformis* in MSM supplemented with petroleum as the sole carbon source. Bacteria were grown by assimilating the carbon present in the crude oil for their metabolism and the biodegradation process of petroleum was occurred (Roselin, 2012). Results show that the 16S rRNAgene sequence from the isolate E1.1 and E2.5 was closely related to *Enterococcus faecalis* with a homology of 100%. As show in Figure 40, the strains **(**E1.1 and E2.5) shared one cluster with *Enterococcus faecalis* ATCC19433 (NR115765), *Enterococcus faecalis* NBRC 100480 (NR113901) and *Enterococcus faecalis* JCM 5803 (NR040789). S15.1 isolate was identified as *Staphylococcus haemolyticus* strain S15.1 and was found to be related (100% identity) to *Staphylococcus haemolyticus* strain SM131 (NR036955) and

Staphylococcus haemolyticus strain JCM2416 (NR113345) and was assigned to the family *Staphylococcaceae*.

The 16S rRNA gene sequence was also compared using BLASTN, and B15.2 showed high similarity to *Bacillus subtilis* (100%), Hence, B15.2 could be a *Bacillus subtilis* (MF927588). B3.1 strain was highly homologous to *bacillus cereus* with 99% sequence similarity. As show in phylogenetic tree the B3.1 strain and *Bacillus cereus* shared one cluster. Therefore, the strain B3.1 was identified as *Bacillus cereus* strain B3.1; B6.2 strain was highly homologous to *Bacillus anthracis* with 99% sequence similarity.

The *Bacillus anthracis* strain B6.2 formed a robust cluster with *Bacillus cereus* strain JCM 2152 (NR113266), *Bacillus cereus* strain ATCC 14579 (NR074540), *Bacillus cereus* strain CCM 2010 (NR115714) and *Bacillus anthracis* strain ATCC 14578 (NR041248)

These results highlight the different groups of bacterial genera involved in hydrocarbon degradation. Many scientists studied the petroleum degradation by various *Bacillus* species. Kulshreshtha *et al.* (2013) isolated *Exiguobacterium alkaliphilum sp. nov* from alkaline wastewater drained sludge of a beverage industry facility located near New Delhi, India. based on phylogenetic analysis Kulshreshtha *et al.* (2013) reported that the strain *Exiguobacterium alkaliphilum sp. nov* was most closely related to *Exiguobacterium aurantiacum* DSM6208^{T} (99.46 *%), Exiguobacterium aquaticum* IMTB-3094^{T} (99.18 %), *Exiguobacterium mexicanum* 8N^{T} (99.06 %). Arora and Bae (2015) was studied the degradation pathway of 4-chloroindole for *Exiguobacterium sp.* PMA. Da Cunha *et al.* (2006) noted that seven *Bacillus* strains isolated from the rock of an oil reservoir located in a deep-water production basin in Brazil showed positive results for oil biodegradation. Ijah and Ukpe (1992) isolated from oil spilled soil *Bacillus strains* 28A and 61B and were identified as efficient crude oil degraders.

The results of microbial isolation and identification confirm literature review about the presence of aerobic bacteria in petroleum contaminated soils, supporting the hypothesis that these bacteria may play a major role in oil biodegradation processes (De Vasconcellos *et al.*, 2009).

III.3. Detection of gene encoding enzymes involved in hydrocarbon degradation

The catabolic genes can be used to detect the specific activities of bacterial strain. We chose three key enzyme coding genes including alkane monooxygenase (*alkB1*) and (alkB) , 1,2-dihydroxynaphthalene dioxygenase (*nahC*) and catechol 2, 3 dioxygenase (*C23O*) responsible for the mineralization of aliphatic and PAHs compounds. Among the 22 isolated strains, we have studied a set of 15 strains for the presence of *alkB1*, alkB, *nahC* and *C23O* genes.

III.3.1. PCR detection of alkane monooxygenase encoding genes (*alkB* and *alkB1*) and phylogenetic analysis:

Genomic DNA extracted from the selected strains was used as templates for the amplification of *alkB1* and *alkB* genes sequences. No PCR product in the presence of alkB primers for all isolated strains (Figure 41). No strain contain *alkB* gene, therefore, all isolates do not degrading of short chains alkanes. Smits *et al.* (1999) and Whyte *et al.* (1996) reported that the probability of detecting the *alkB* gene is too low or even neglected.

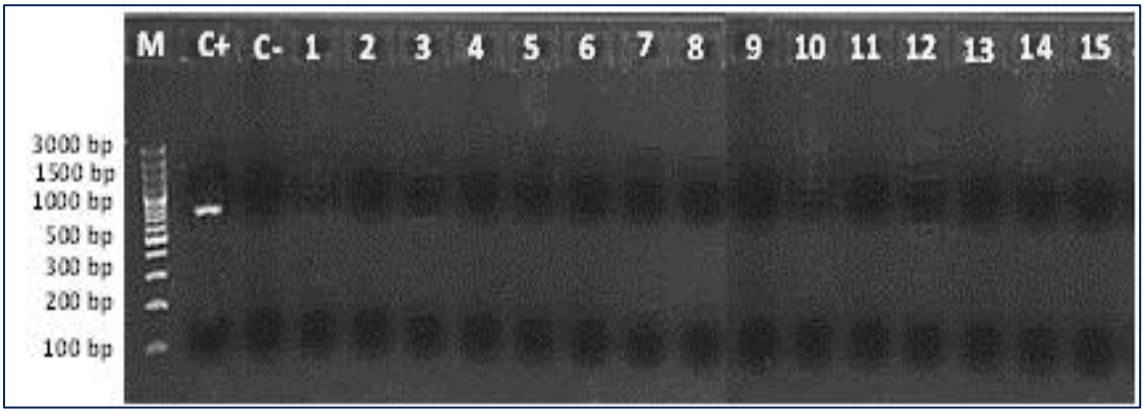


Figure 41: Agarose gel electrophoresis of PCR amplification product of alkane monooxygenase (*AlkB*) gene.

Contrary to *alkB*, The PCR in the presence of primers *alkB1* gave an amplification product of 434 bp (Figure 42) for strains of *P. aeruginosa* (P2.3, P2.1, P14.1, P13.1, P1.2 and P14.1). However, no PCR product was observed with the other isolates (Figure 42). Although these other isolates were negative for *alkB1* genes, they may be containing other alkane monoxygenase or other biodegradation genes.

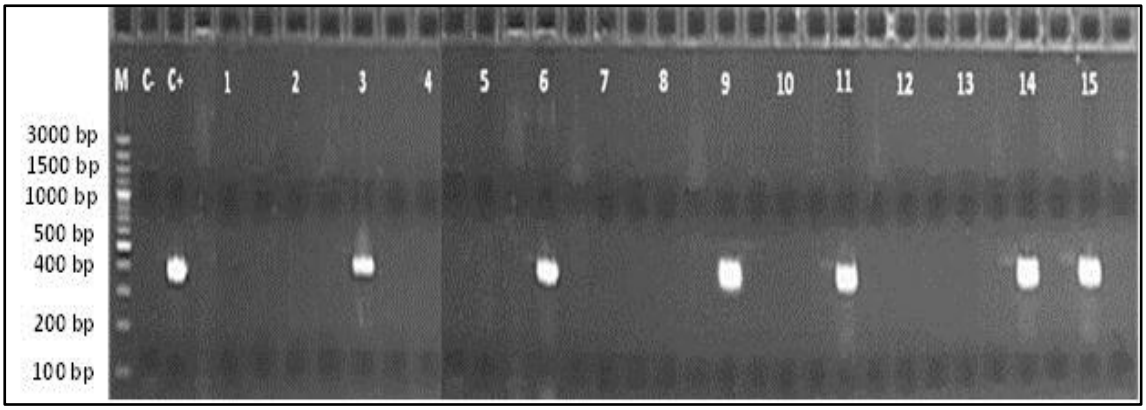


Figure 42: Agarose gel electrophoresis of PCR amplification product of alkane 1 monooxygenase (*AlkB1*) gene.

lane M: Gene Ruler 100 bp Plus DNA Ladder (Thermo Scientific), lane C+: positive control with 20 Strain Even Mix Genomic Material (ATCC® MSA-1002™) see in appendix D, lane C-: negative control without DNA, lane 1, 2, 4, 7, 8, 9, 10, 12 and 13 show that there was no amplification of DNA in bacterial strains (B4.2, B3.1, B15.3, B6.2, B15.2, S15.1, E1.1, E2.5 and P2.2 respectively), lane 3 : isolate P2.3, lane 6: isolate P2.1,lane 9: isolate P14.1, lane 11: isolate P13.1, lane 14: P1.2 and lane 15: P14.2.

The *alkB1* gene sequences and corresponding amino acid sequences have been deposited in the Gene bank database under the accession numbers as show in Table 20.

The phylogenetic analysis of alkane monooxygenase encoding genes (*alkB1*) (Figure 43) indicated that the (P2.3, P2.1, P14.1, P13.1 and P14.1) sequences were most closely related to the *P. aeruginosa partial alkB1 gene for putative alkane-1-monooxygenase, isolate PAO1 (AJ344087)* with 99% similarity. The P1.2 sequence was highly similar (99%) with *P. aeruginosa partial alkB1 gene for putative alkane-1-monooxygenase, isolate UMI-97 (AJ344084).*

Table 20: Closest relative gene, sequence similarity, GenBank accession numbers of bacteria, gene and -corresponding amino acid sequences.

strain	Bacteria accession number	Closest relative gene	Gene sequence identity (%)	Gene Identification	Gene accession number	Amino acid accession number
P1.2	MF767526	*Pseudomonas aeruginosa partial alkB1 gene for putative alkane-1-monooxygenase, isolate UMI-97 (AJ344084)*	99	*Pseudomonas aeruginosa strain P1.2 alkane 1-monooxygenase (alkB1) gene, partial cds*	MH686298	AYA93245
P2.1	MF767521	*Pseudomonas aeruginosa partial alkB1 gene for putative alkane-1-monooxygenase, isolate PAO1 (AJ344087)*	99	*Pseudomonas aeruginosa strain P2.1 alkane 1-monooxygenase (alkB1) gene, partial cds*	MH686295	AYA93242
P2.3	MF767436	*Pseudomonas aeruginosa partial alkB1 gene for putative alkane-1-monooxygenase, isolate PAO1 (AJ344087)*	99	*Pseudomonas aeruginosa strain P2.3 alkane 1-monooxygenase (alkB1) gene, partial cds*	MH442997	AXA19858
P13.1	MF767522	*Pseudomonas aeruginosa partial alkB1 gene for putative alkane-1-monooxygenase, isolate PAO1 (AJ344087)*	99	*Pseudomonas aeruginosa strain P13.1 alkane 1-monooxygenase (alkB1) gene, partial cds*	MH686296	AYA93243
P14.1	MF767524	*Pseudomonas aeruginosa partial alkB1 gene for putative alkane-1-monooxygenase, isolate PAO1 (AJ344087)*	99	*Pseudomonas aeruginosa strain P14.1 alkane 1-monooxygenase (alkB1) gene, partial cds*	MH686297	AYA93244
P14.2	MF767525	*Pseudomonas aeruginosa partial alkB1 gene for putative alkane-1-monooxygenase, isolate PAO1 (AJ344087)*	99	*Pseudomonas aeruginosa strain P14.2 alkane 1-monooxygenase (alkB1) gene, partial cds*	MH686299	AYA93246

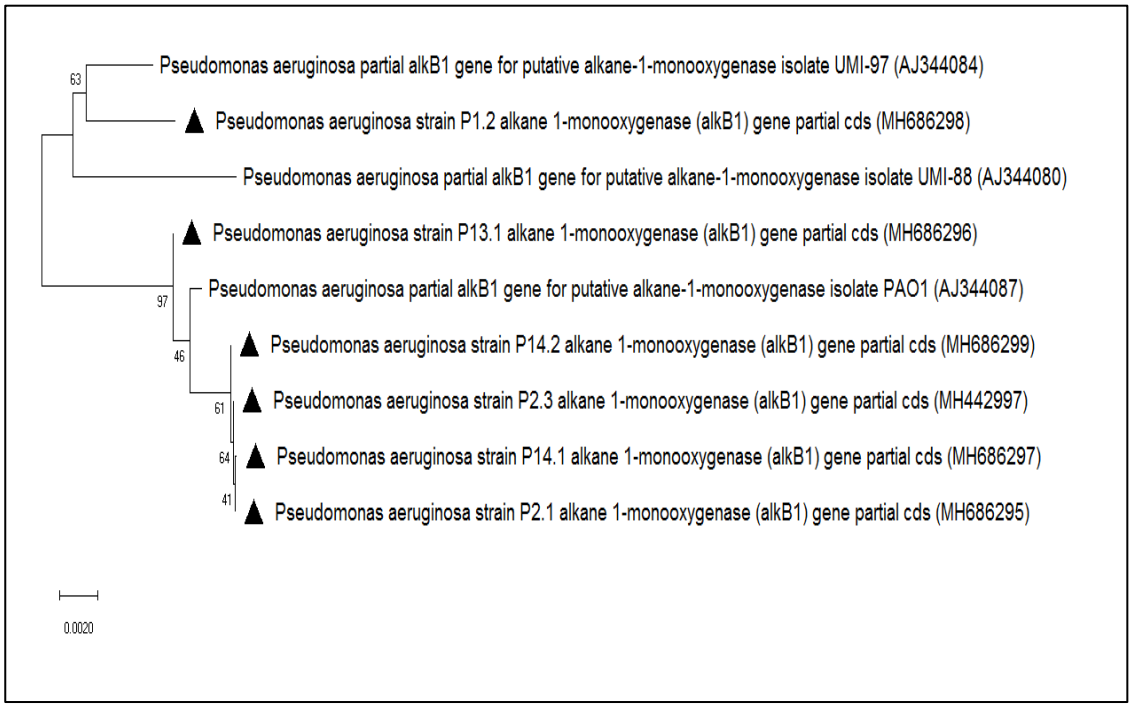


Figure 43: The phylogenetic tree based on alkane 1 monooxygenase *(alkB1)* sequences from the strains of *Pseudomonas aeruginosa* (P2.3, P2.1, P14.1, P13.1, P1.2 and P14.1).

(GenBank accession numbers are listed between parentheses after each sequences). Our sequences are marked by triangles.

The sequence obtained of the six strains were compared to that *P.aeruginosa* PAO-1 (reference strain), the result obtained are shown in Figure 44. The amino acid sequences deduced from nucleotide sequences of *alkB1* gene of 6 strains were presented in Table 21. Figure 44 demonstrated that *alkB1*genes fragments of strains of *P. aeruginosa* (P2.3, P2.1, P14.1, P13.1, P1.2 and P14.1) present a high identity with that of *P. aeruginosa* PAO-1 and also high DNA sequence similarity exist between this six strains. *AlkB1* genes of PAO-1 strain is carried not by a plasmid but by the chromosome (Aboulkacem *et al.*, 2014).

Nucleotide sequencing

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                1…/…/…/…/…/...80
alkB1, P1.2     CCGCATGCCT ACAAGTACAA CTTCCTCAAC GCCTGGCGCC TCGAAGCGGT GCGGCTGCGC AAGAAGTGCC TGCCGGTGTT
alkB1, P2.1     CCGCATACCT ACAAGTACAA CTTCCTCAAC GCCTGGCGCC TTGAAGCGGT GCGGCTGCGC AAGAAGGGCC TGCCGGTGTT
alkB1, P2.3     CCGCATACCT ACAAGTACAA CTTCCTCAAC GCCTGGCGCC TTGAAGCGGT GCGGCTGCGC AAGAAGGGCC TGCCGGTGTT
alkB1, P13.1    CCGCATGCCT ACAAGTACAA CTTCCTCAAC GCCTGGCGCC TTGAAGCGGT GCGGCTGCGC AAGAAGGGCC TGCCGGTGTT
alkB1, P14.1    CCGCATACCT ACAAGTACAA CTTCCTCAAC GCCTGGCGCC TTGAAGCGGT GCGGCTGCGC AAGAAGGGCC TGCCGGTGTT
alkB1, P14.2    CCGCATACCT ACAAGTACAA CTTCCTCAAC GCCTGGCGCC TTGAAGCGGT GCGGCTGCGC AAGAAGGGCC TGCCGGTGTT
alkB1, PAO1     CCGCATGCCT ACAAGTACAA CTTCCTCAAC GCCTGGCGCC TTGAAGCGGT GCGGCTGCGC AAGAAGGGCC TGCCGGTGTT
                81…/…/…/…/…/…/...160
alkB1, P1.2     CGGCTGGCAG AACGAACTGA TCTGGTGGTA CCTGCTGAGC CTGGCGTTGC TGGTCGGTTT CGGTTGGGCG TTCGGCTGGC
alkB1, P2.1     CGGCTGGCAG AACGAACTGA TCTGGTGGTA CCTGCTGAGC CTGGCGTTGC TGGTCGGTTT CGGTTGGGCG TTCGGCTGGC
alkB1, P2.3     CGGCTGGCAG AACGAACTGA TCTGGTGGTA CCTGCTGAGC CTGGCGTTGC TGGTCGGTTT CGGTTGGGCG TTCGGCTGGC
alkB1, P13.1    CGGCTGGCAG AACGAACTGA TCTGGTGGTA CCTGCTGAGC CTGGCGTTGC TGGTCGGTTT CGGTTGGGCG TTCGGCTGGC
alkB1, P14.1    CGGCTGGCAG AACGAACTGA TCTGGTGGTA CCTGCTGAGC CTGGCGTTGC TGGTCGGTTT CGGTTGGGCG TTCGGCTGGC
alkB1, P14.2    CGGCTGGCAG AACGAACTGA TCTGGTGGTA CCTGCTGAGC CTGGCGTTGC TGGTCGGTTT CGGTTGGGCG TTCGGCTGGC
alkB1, PAO1     CGGCTGGCAG AACGAACTGA TCTGGTGGTA CCTGCTGAGC CTGGCGTTGC TGGTCGGTTT CGGTTGGGCG TTCGGCTGGC
                161…/…/…/…/…/....240
alkB1, P1.2     TGGGGATGGT TTTCTTCCTT GGCCAGGCGT TCGTCGCGGT GACCCTGCTG GAAATCATCA ACTACGTCGA GCACTACGGC
alkB1, P2.1     TGGGGATGGT TTTCTTCCTT GGCCAAGCGT TCGTCGCGGT GACCCTGCTG GAGATCATCA ACTACGTCGA GCACTACGGC
alkB1, P2.3     TGGGGATGGT TTTCTTCCTT GGCCAAGCGT TCGTCGCGGT GACCCTGCTG GAGATCATCA ACTACGTCGA GCACTACGGC
alkB1, P13.1    TGGGGATGGT TTTCTTCCTT GGCCAAGCGT TCGTCGCGGT GACCCTGCTG GAGATCATCA ACTACGTCGA GCACTACGGC
alkB1, P14.1    TGGGGATGGT TTTCTTCCTT GGCCAAGCGT TCGTCGCGGT GACCCTGCTG GAGATCATCA ACTACGTCGA GCACTACGGC
alkB1, P14.2    TGGGGATGGT TTTCTTCCTT GGCCAAGCGT TCGTCGCGGT GACCCTGCTG GAGATCATCA ACTACGTCGA GCACTACGGC
alkB1, PAO1     TGGGGATGGT TTTCTTCCTT GGCCAAGCGT TCGTCGCGGT GACCCTGCTG GAGATCATCA ACTACGTCGA GCACTACGGC
                241…/…/…/…/…/...320
alkB1, P1.2     CTGCATCGGC GGAAGGGCGA GGACGGGCGC TACGAGCGGA CCAACCATAC CCACTCCTGG AACAGCAACT TCGTCTTCA
alkB1, P2.1     CTGCATCGGC GAAAGGGCGA GGACGGGCGC TACGAGCGGA CCAACCATAC CCACTCCTGG AACAGCAACT TCGTCTTCA
alkB1, P2.3     CTGCATCGGC GAAAGGGCGA GGACGGGCGC TACGAGCGGA CCAACCATAC CCACTCCTGG AACAGCAACT TCGTCTTCA
alkB1, P13.1    CTGCATCGGC GAAAGGGCGA GGACGGGCGC TACGAGCGGA CCAACCATAC CCACTCCTGG AACAGCAACT TCGTCTTCA
alkB1, P14.1    CTGCATCGGC GAAAGGGCGA GGACGGGCGC TACGAGCGGA CCAACCATAC CCACTCCTGG AACAGCAACT TCGTCTTCA
alkB1, P14.2    CTGCATCGGC GAAAGGGCGA GGACGGGCGC TACGAGCGGA CCAACCATAC CCACTCCTGG AACAGCAACT TCGTCTTCA
alkB1, PAO1     CTGCATCGGC GAAAGGGCGA GGACGGGCGC TACGAGCGGA CCAACCATAC CCACTCCTGG AACAGCAACT TCGTCTTCA
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Figure 44: Comparison of nucleotides sequences of a*lkB1* gene of *Pseudomonas aeruginosa* (P2.3, P2.1, P14.1, P13.1, P1.2 and P14.1) with that of *P. aeruginosa PAO-1***.**

Table 21: The amino acid sequences deduced from nucleotide sequences of *alkB1* gene.

Amino acid sequences accession number	Deduced *alkb1* amino acid sequences
AYA93242	**alkane 1-monooxygenase, partial [*Pseudomonas aeruginosa* P2.1]** ARFGQSVYQFLPHTYKYNFLNAWRLEAVRLRKKGLPVFGWQNELIWWYLLSL ALLVGFGWAFGWLGMVFFLGQAFVAVTLLEIINYVEHYGLHRRKGEDGRYER TNHTHSWNSNFVF
AYA93243	**alkane 1-monooxygenase, partial [*Pseudomonas aeruginosa* P13.1]** SARFGQSVYQFLPHAYKYNFLNAWRLEAVRLRKKGLPVFGWQNELIWWYLLS LALLVGFGWAFGWLGMVFFLGQAFVAVTLLEIINYVEHYGLHRRKGEDGRYE RTNHTHSWNSNFVF
AYA93244	**alkane 1-monooxygenase, partial [*Pseudomonas aeruginosa* P14.1]** PHTYKYNFLNAWRLEAVRLRKKGLPVFGWQNELIWWYLLSLALLVGFGWAF GWLGMVFFLGQAFVAVTLLEIINYVEHYGLHRRKGEDGRYERTNHTHSWNS NFVF
AYA93245	**alkane 1-monooxygenase, partial [*Pseudomonas aeruginosa* P1.2]** SSARFGQSVYQFLPHAYKYNFLNAWRLEAVRLRKKCLPVFGWQNELIWWYLL SLALLVGFGWAFGWLGMVFFLGQAFVAVTLLEIINYVEHYGLHRRKGEDGRY ERTNHTHSWNSNFVFT
AYA93246	**alkane 1-monooxygenase, partial [*Pseudomonas aeruginosa* P14.2]** SSARFGQSVYQFLPHTYKYNFLNAWRLEAVRLRKKGLPVFGWQNELIWWYLL SLALLVGFGWAFGWLGMVFFLGQAFVAVTLLEIINYVEHYGLHRRKGEDGRY ERTNHTHSWNSNFVFT
AXA19858	**alkane 1-monooxygenase, partial [*Pseudomonas aeruginosa* P2.3]** ARFGQSVYQFLPHTYKYNFLNAWRLEAVRLRKKGLPVFGWQNELIWWYLLSL ALLVGFGWAFGWLGMVFFLGQAFVAVTLLEIINYVEHYGLHRRKGEDGRYER TNHTHSWNSNFVFT

Alkane are saturated hydrocarbons that are widespread in environments, they are a major constituents of crude oil. Alkanes are considered as pollutants, with short-chained alkanes acting as solvents toward cellular membranes while longer-chained alkanes may contribute to the formation of oil films that may limit oxygen and nutrient exchange. However, the alkanes also serve as important carbon and energy sources for

some microorganisms (Wasmund *et al.*, 2009). Various microorganisms, including bacteria, yeasts and filamentous fungi, can degrade alkanes (Shao & Wang, 2013). Many bacterial species are able to degrade n alkanes. Concerning the genetics of the degradation of short-chain n-alkanes (C6-C12), only two bacteria have been investigated more in detail until now (*P. oleovorans, P. Stenotrophomonas*), both carrying the OCT-plasmid (Kok *et al.*, 1989; Lee *et al.*, 1996; Vomberg & Klinner, 2000), the first peptide encoded by the *alk*-operon which is localized on the OCT-plasmid of *P. oleovorans* is a membrane-bound alkane monooxygenase (*AlkB*) (Vomberg & Klinner, 2000).

Our results concord with Aboulkacem *et al.* (2014) that found in the search of *alkB* and *alkB1* genes, which are respectively responsible for the degradation of short chain n-alkanes and long chain n-alkanes, the absence of *alkB* gene; however, the *alkB1* gene, strongly present within the population of *P. aeruginosa* isolated, the nucleotide sequencing of a *alkB1* gene fragment for 4 *P. aeruginosa* strains as well as the *P. aeruginosa PAO-1* (reference strain) has shown a highly conserved nucleotide sequences in spite of their heterogeneity origin. Similarly, Smits *et al.* (1999) have found that the alkane hydroxylase genes seem to be quite divergent in different genera. Recently, Throne-Holst *et al.* (2007) found,two genes encoding *AlkB*-type alkane hydroxylase homologues, have been shown to be involved in the degradation of n-alkanes with chain lengths of from 10 to 20 C atoms in *Acinetobacter sp*. strain DSM 17874.

III.3.2. PCR detection of 1,2-dihydroxynaphthalene dioxygenase encoding genes (*nahC*)

Dioxygenases catalyze the incorporation of both atoms of dioxygen into substrates. Cleavage of the aromatic ring is one of the most important functions of dioxygenases. Dioxygenases are classified into two groups according to their mode of ring fission, intradiol enzymes cleave the ring between two hydroxyl carbons and extradiol enzymes in contrast cleave the ring between one hydroxylated carbon and its adjacent nonhydroxylated carbon. 1,2-dihydroxynaphthalene dioxygenase is an extradiol ring-cleavage enzyme that cleaves the first ring of 1,2-dihydroxynaphthalene (Harayama & Rekik, 1989). Polymerase chain reaction amplification was used to detect *nahC* gene sequences in the bacterial isolates strains. Amplification of DNA was not observed in bacterial isolates (Figure 45) revealed no *nahC* genes amongst the isolates.

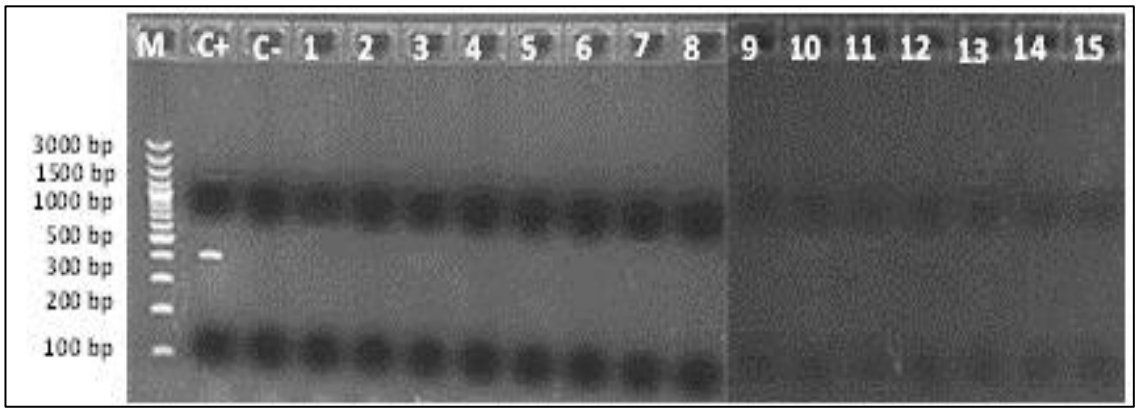

Figure 45: Amplification patterns on agarose gel 2% using *nah C* primers.

Lane M: Gene Ruler 100 bp Plus DNA Ladder (Thermo Scientific), lane C+: positive control with 20 Strain Even Mix Genomic Material (ATCC® MSA-1002™) see in appendix D, lane C-: negative control without DNA. Figure show that there was no amplification of DNA in any of the bacterial isolates.

III.3.3. PCR detection of catechol 2, 3 dioxygenase genes (*C23O*)

Catechol 2,3-dioxygenase, cleaves the aromatic ring of catechol (Harayama & Rekik, 1989). *C23O* dioxygenases play a key role in the metabolism of aromatic rings by bacteria because they are responsible for cleavage aromatic C–C bond at ortho or meta position (Hesham *et al.*, 2014). However, in this study by using the DEG-F and DEG-R primers, no PCR products were seen from DNA extracted from the selected isolates, so no signal for *C23O* gene was detected (Figure 46) indicating the absence of Catechol 2,3-dioxygenase enzyme activities for the hydrocarbon degradation.

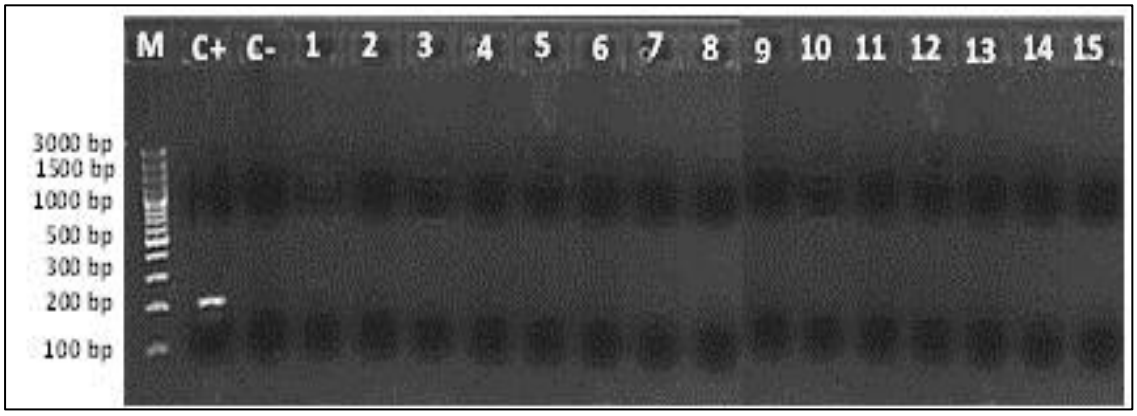

Figure 46: Amplification patterns on agarose gel 2% using *C23O* primers.

Lane M: Gene Ruler 100 bp Plus DNA Ladder (Thermo Scientific), lane C+: positive control with 20 Strain Even Mix Genomic Material (ATCC® MSA-1002™) see in appendix D, lane C-: negative control without DNA. Figure show that there was no amplification of DNA in any of the bacterial isolates.

III.4. Biodegradation studies

III.4.1. Characterization of bacterial degradation potential on minimal media containing crude oil

Among 22 bacterial strains were isolate from oil contaminated soil, purified and identified, 12 were selected for this study.

III.4.1.1. Bacterial growth determination (Turbidometry)

Following a 15 days incubation of the 12 isolates on 1% crude oil as sole source of carbon and energy, the bacterial growth was evaluated by measuring optical density (600nm) at regular intervals (2 days), as indicator for utilization of hydrocarbon. The graphs based on the O.D readings at different time intervals of incubation on the degrading activity of the oil-degrading bacteria are illustrated in the Figure 47, 48, 49 and 50. Our results show that all the isolates had effectively utilized crude oil; the level of degradation differs between isolates due to differences in their growth. Linear increase in OD was observed between days 4 and 8 of all strains but *Staphylococcus* (S15.1, S15.2) and *Enterococcus faecalis* (E1.1, E2.5) attained the decline phase at day 8. *Pseudomonas aeruginosa* (P2.1, P2.3), *Lysinibacillus macroides* B4.2 and *Achromobacter xylosoxidans* P2.2 had the highest growth in the medium with crude oil while the bacteria with the least degrading activities on crude oil was *Staphylococcus haemolyticus* S15.1, *Enterococcus faecalis* E1.1, *Enterococcus faecalis* E2.5 and *Staphylococcus hominis* S15.3 respectively.

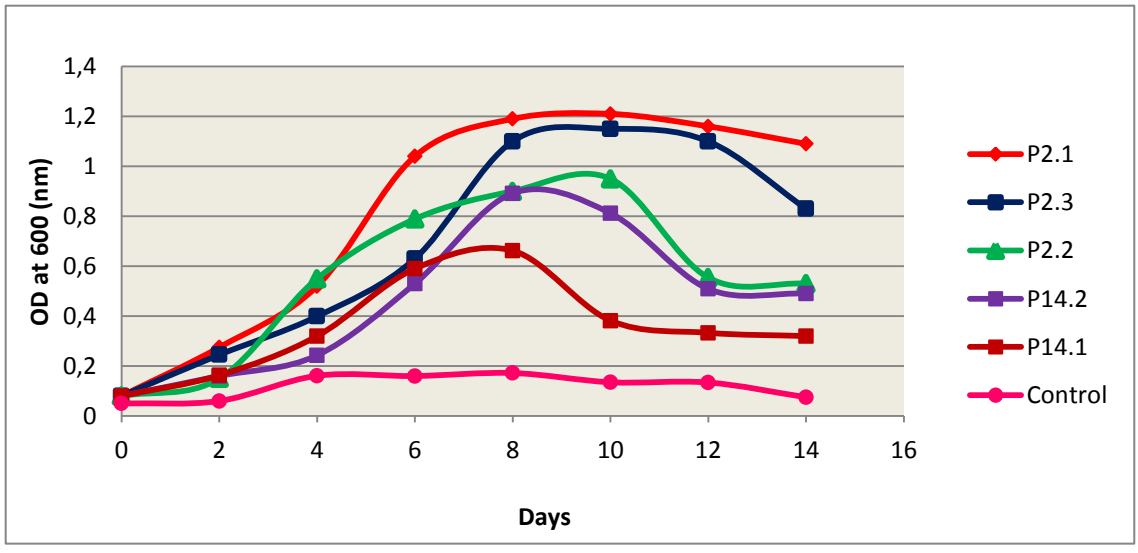

Figure 47: Growth curve (OD values) of P2.1, P2.3, P2.2, P14.2 and P14.1 isolates in MSM broth supplemented with 1% of crude oil for a14 days of incubation.

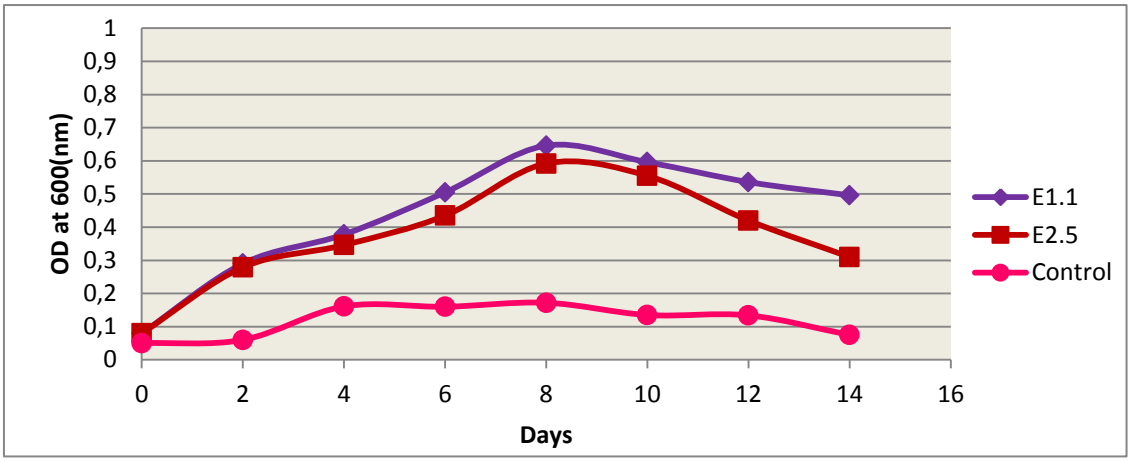

Figure 48: Growth curve (OD values) of E1.1and E2.5 isolates in MSM broth supplemented with 1% of crude oil for a14 days of incubation.

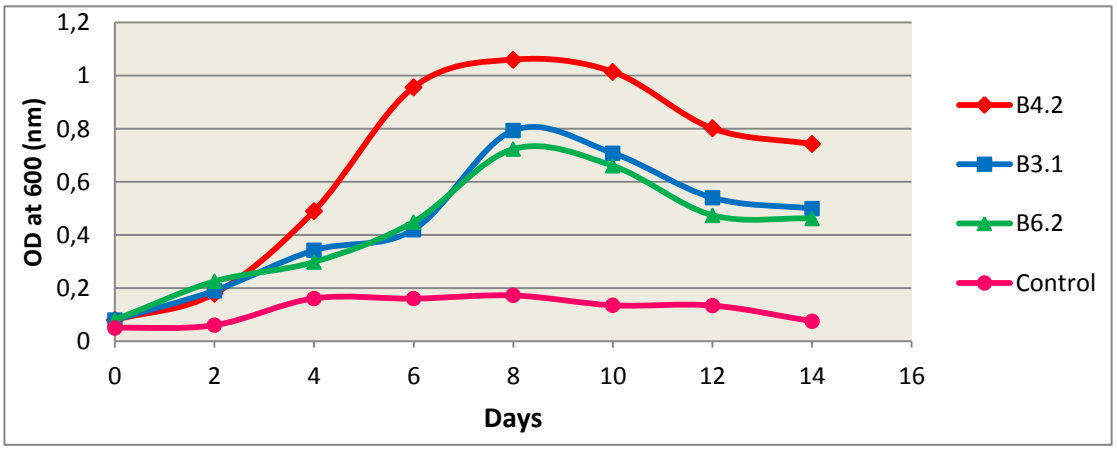

Figure 49: Growth curve (OD values) of B4.2, B3.1 and B6.2 isolates in MSM broth supplemented with 1% of crude oil for a14 days of incubation.

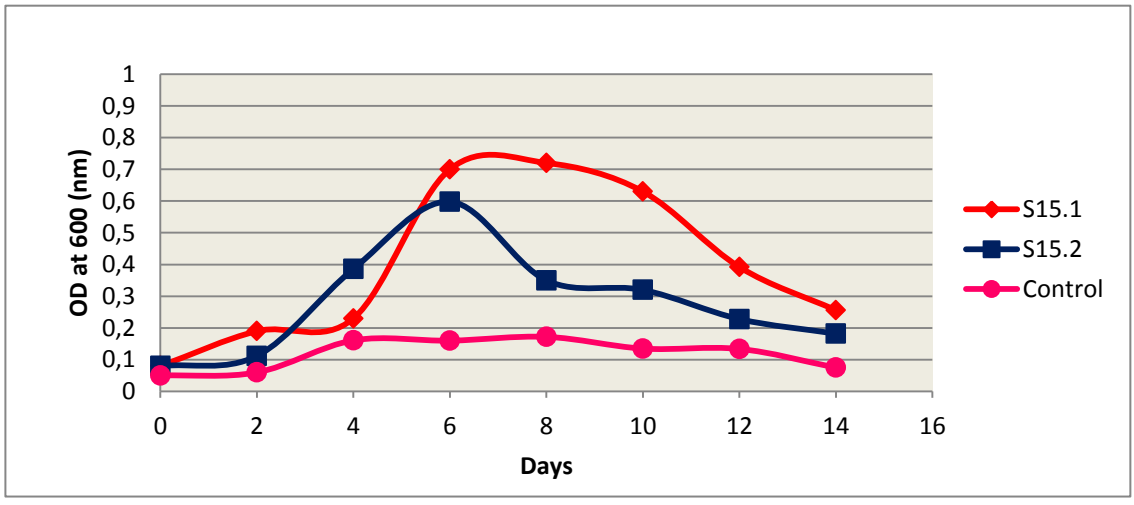

Figure 50: Growth curve (OD values) of S15.1 and S15.2 isolates in MSM broth supplemented with 1% of crude oil for a14 days of incubation.

The bacterial cells were able to multiply within the days of study, indicating that they were able to degrade and utilize the oil for their development and growth, hence the increase in the concentration of the broth (turbidity) indicates growth of bacteria therefore hydrocarbons degradation, more specifically between days 4 and 8 and decline in the optical density of the broth suggests decrease in the bacterial population and that the hydrocarbon has been degraded, particularly between days 10 and 14. Sepahi *et al.* (2008) reported that the utilization of hydrocarbons as a sole carbon source and energy by microbial strains is evident by the increase in the microbial density. Similarly, Sibi *et al.* (2015) reported that *Pseudomonas aeruginosa* and *Achromobacter sp* showed the highest growth in petrol and diesel containing media. In another study realized by Obuotor *et al.* (2016) *Pseudomonas aeruginosa* was able to utilize spent engine oil better than all other isolates. According K. Das and Mukherjee (2007), *Pseudomonas aeruginosa* strains were more efficient than *Bacillus subtilis* strains isolated from a petroleum contaminated soil from north-east India. On the other hand, Boontawan and Boontawan (2011) found that *Enterococcus faecalis* showed a high oil degradation performance. Liang *et al.* (2009) isolated from soil samples an aerobic fomesafen degrading bacterium and identified as a member of the genus *Lysinibacillus*, in that study, *Lysinibacillus sp.* ZB-1 stain was found to be the best degrader of fomesafen (one of the diphenyl ethers). *Lysinibacillus* are potential candidates for cleanup of contaminated systems (Liang *et al.*, 2009; Rajeswari *et al.*, 2014). Originally, this genus was *Bacillus sp*. and was transferred into this genus as *Lysinibacillus sp*. in 2007 (Ahmed *et al.*, 2007).However, the bacterial growth was limited with increasing incubation time which could be possible due to the release of by products, resulting in a decrease of crude oil biodegradation rate (Sibi *et al.*, 2015).

III.4.1.2. Gravimetric Analysis

The present study examined the crude oil biodegradation percentage of the selected bacterial strains that was isolate from contaminated soil using gravimetric analysis. The degradation potential of the selected strains is presented in (Figure 51). *Pseudomonas aeruginosa* P2.1, *Pseudomonas aeruginosa* P2.3 and *Lysinibacillus macroides* B4.2 exhibited the highest biodegradation percentage of crude oil with 76.37%, 60.92% and 47.46% respectively. The crude oil degradation potential of *Achromobacter xylosoxidans* P2.2 was also remarkable 45.20%. The crude oil degradation capability of *Enterococcus faecalis* E1.1, *Enterococcus faecalis* E2.5 and

Staphylococcus hominis S15.3 was the lowest with 16.71%, 16.11% and 16% respectively. Our results agree to report of Obayori *et al.* (2009) that *Pseudomonas aeruginosa* MVL1 utilized the oil substrate with more than 70%. Similar finding have been reported by Obuotor *et al.* (2016) that *Pseudomonas aeruginosa* utilized spent engine oil more effectively than other screened bacteria. Verma *et al.* (2006) reported that Gravimetric analysis showed that *Bacillus sp.* degraded 59% of the oily sludge in 5 days whereas *Pseudomonas sp*. degraded 35%.

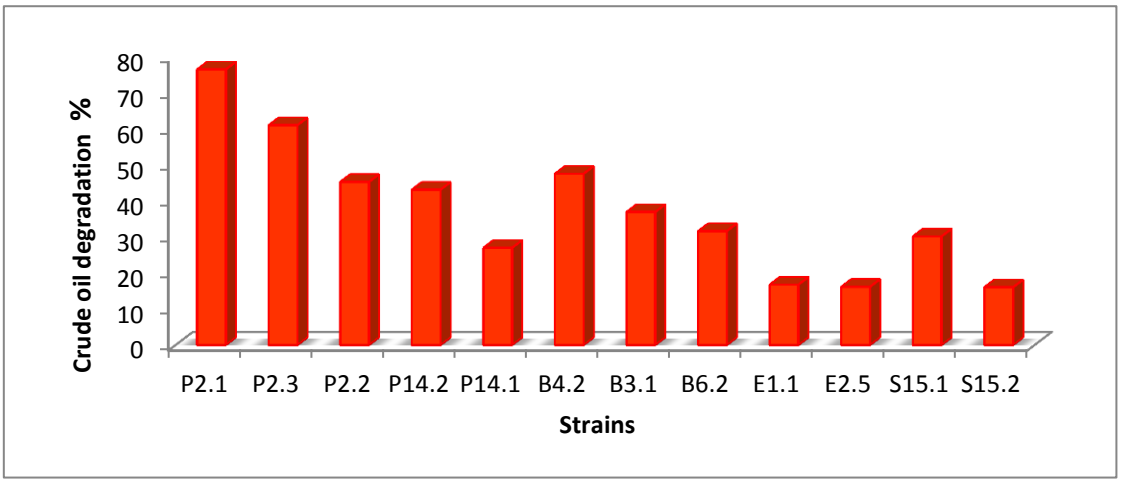

Figure 51: Percentage of crude oil degradation by bacterial isolates during 14 days of incubation in MSM broth supplemented with 1% (v/v) crude oil as sole carbon source at 30°c.

III.4.2. Characterization of degradation potential of selected individual bacterial isolates and consortia on minimal media containing diesel.

For this study, three best potential degrading bacteria were selected. These following three isolates P2.1, B4.2 and S15.1 were cultured separately and mixed in equal proportion for use in culture interactions.

III.4.2.1. Determination of biodegradative activity by turbidometry

Figure 52 shows the OD change with times for each isolates and the mixed cultures grown on diesel. The selected individual bacterial isolates and consortia had effectively utilized diesel. More specifically, linear increase in OD was observed between days 4 and 10 of consortia and *Pseudomonas aeruginosa* P2.1 but *Lysinibacillus macroides* B4.2 and *Staphylococcus haemolyticus* S15.1 growth became limited at days 8. The results indicate that the bacterial consortium and *Pseudomonas aeruginosa* P2.1 showed the best growth in MSM broth with diesel. The results

demonstrate that consortia and *Pseudomonas aeruginosa* P2.1 have the greatest ability to utilize diesel, this results agree with the observation of Guermouche *et al.* (2013) that a mixed culture showed the highest cell density. This can be attributed to the fact that individual microorganisms may metabolize only a limited range of substrates, while the consortium of different bacterial strains with large enzymatic capability has a greater ability to degrade complex mixtures (Guermouche *et al.*, 2013) . Malkawi *et al.* (2009b) was tested the ability of the bacterial consortia to grow on mixed hydrocarbons (Naphthalene, Toluene and Heptane) as the sole carbon source at varying concentrations, this latter found that the bacterial consortia showed better hydrocarbon degradation behavior than the single isolates. A study by Zhen-Yu *et al.* (2008) evaluated the bioremediation potential of bacteria indigenous to soils of the yellow river delta in china as a treatment option for soil remediation. They have reported that the mixed microbial consortia demonstrated wider catabolic versatility and faster overall rate of hydrocarbon degradation than individual isolates.

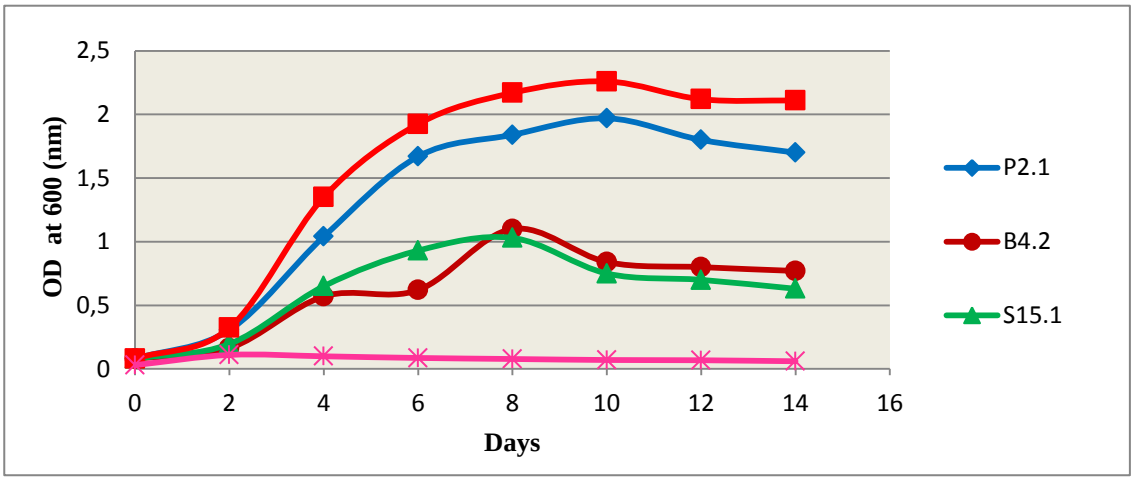

Figure 52: Growth curve (OD values) of P 2.1, B4.2, S15.1 isolates and mixed bacterial consortium in MSM broth supplemented with 1% of diesel for a14 days of incubation.

III.4.2.2. Gravimetric Analysis

This study examined the diesel biodegradative ability of selected individual bacterial strain and consortia that isolate from contaminated soil using gravimetric analysis. The degradation potential of each isolates and the mixed cultures is presented in (Figure 53). The bacterial consortium and *Pseudomonas aeruginosa* P2.1 exhibited the highest biodegradation percentage of diesel with 79.62% and 64.81% respectively. The diesel degradation capability of *Lysinibacillus macroides* B4.2 and *Staphylococcus*

haemolyticus S15.1 was the lowest with 37.03% and 20.37% respectively. Our results agree to report of Wongsa *et al.* (2004) that *pseudomonas aeruginosa* degraded diesel oil most rapidly, 80% after one week. Sibi *et al.* (2015), found that *Pseudomonas aeruginosa* is the best diesel degrader with maximum degradation of 34.4% at the end of 10 days incubation period. Similar finding have been reported by Márquez-rocha *et al.* (2001) that the bacterial consortium was able to remove more than 85% of the diesel (118.3 to 17.5 g diesel and the control lost only 13% of the initial diesel concentration. Al-Wasify and Hamed (2014) that the hydrocarbon biodegradation by the consortia (collection between the three strains) were the highest. The bacterial consortium gave the highest degradation percentage because there is no individual bacterial strain with the metabolic capacity to degrade all the components of crude oil or diesel (Zhu *et al.*, 2001; Al-Wasify & Hamed, 2014). A large variety of physiological and metabolic factors are indispensable for the degradation of various diesel compounds, all of these characteristic are not found in single organism (Bento *et al.*, 2005). A microbial consortium is required to complete oil biodegradation because the mixtures of hydrocarbon markedly differ in solubility, volatility and susceptibility to degradation and the enzymes necessary needed cannot be found in a one organism (Adebusoye *et al.*, 2006; Al-Wasify & Hamed, 2014).

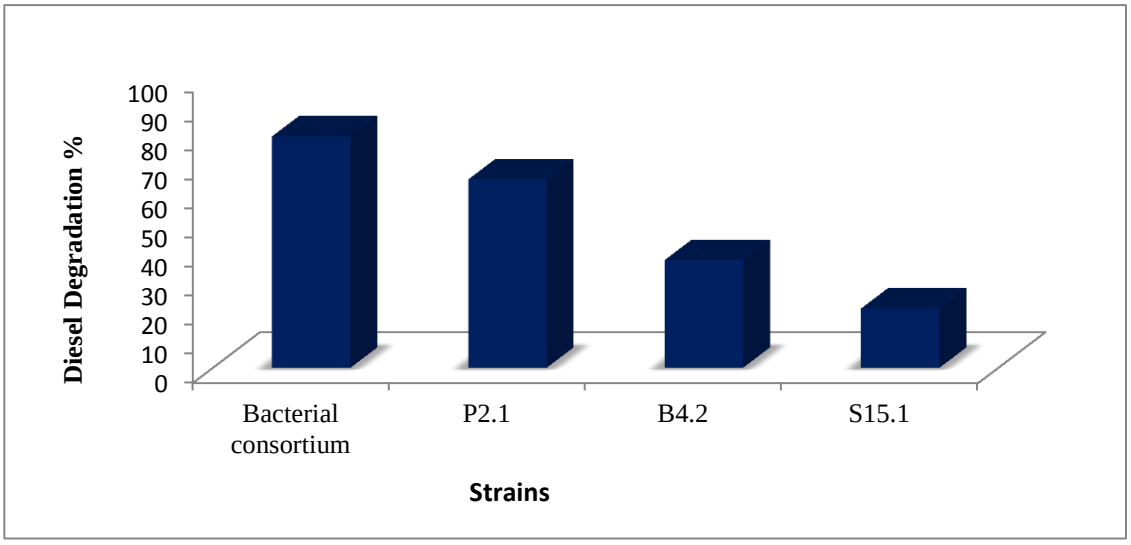

Figure 53: Percentage of diesel degradation by bacterial isolates (P2.1, B4.2 and S15.1) and bacterial consortium in MSM broth supplemented with 1% of diesel for a 14 days of incubation.

III.4.2.3. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Biodegradation of diesel by selected individual bacterial strain and consortium was confirmed by GC-MS analysis. The strain P2.1, B4.2 and S15.1 were grown in MSM in the presence of diesel as only carbon source at 30 °C for 15 days. The consortium of this three isolates and uninoculated sterile controls were kept together for 5 and 15 days under similar condition. The residual diesel at the end of each incubation time was analyzed quantitatively and qualitatively using GC-MS. It must be noted that any reduction in peak area of inoculated sample compared with the control is caused by biodegradation because any loss by non-biological process would affect the uninoculated control to the same as the inoculated sample (Al-Wasify & Hamed, 2014). The GC-MS analysis revealed the presences of aliphatic fraction (*n*-alkanes) range from (C9-C26), branched alkane and aromatic fraction (Figure 54 (A) - (B)). Similarly (Chandran & Das, 2010) found that yeast *Trichosporon asahii* species degraded almost all the hydrocarbon present in diesel (C9 ─ C26), with 95.01% degradation percentage. The percentage of individual compounds was represented in Table 22. The GC analyses showed that the level of alkanes was significantly reduced in inoculated flasks; indicate that the isolates and the consortium degrade the aliphatic fraction greater than the aromatic fraction. This was found early by Verma *et al.* (2006) and Bhattacharya *et al.* (2015). The hydrocarbon most readily degraded by microorganisms are *n*-alkanes (Verma *et al.*, 2006; Chandran & Das, 2010). Diaz-Ramirez *et al.* (2008) reported that biodegradation of aromatic compounds by the defined mixed cultures was significant lower than the obtained for aliphatic compounds. This could be a consequence of low solubility and natural resistance of these fractions of oil (Gutiérrez-Rojas, 2000).Our results indicated that bacterial consortium more effectively degraded diesel in 15 days (Figure 54 (C)) than 5 days (Figure 54 (D)). All compound in diesel were highly reduced by mixed culture with 61.94% after 5 days and 78.26% after 15 days of incubation and particularly the bacterial consortium degraded *n*-alkanes from C9 to C26 almost completely (Figure 54 (D)). Adebusoye *et al.* (2006) found that the growth of bacterial consortium with diesel and crude oil resulted in total disappearance of all the hydrocarbon peaks in 14 days of incubation. The present study showed that the the medium and long chain alkanes was more degraded compared to the short chain alkanes. Similarly, Hamzah *et al.* (2010) reported that *Acinetobacter lwoffii* and *Pseudomonas aeruginosa* attacked the long chain n-alkanes (C12 to C20), possibly due

to their less toxic nature. Short chain alkanes are highly toxic for many microorganisms; they can also evaporate fast into the atmosphere (Horel, 2009); however in spills of terrestrial the movement of contaminant occurs vertically, which reduce the losses in the evaporation process (Leahy & Colwell, 1990). The intermediate chain length (C10-C24) can easily be used as a carbon source for the microorganisms and degrade as quickly as possible (Atlas & Bartha, 1992). This coincide with, Ghazali *et al.* (2004) who reported that the short and medium chain alkanes are generally more easily degraded due to their lower hydrophobicity.

In our study, the most efficient strains for diesel degradation was, *Pseudomonas aeruginosa* P2.1, which can degrade most alkanes, indicating that the microorganism had a very efficient degradative enzyme and also not affected by inhibitory component in diesel. Overall percentage degradation of the compound in diesel by *Pseudomonas aeruginosa* P2.1 was found to be 64.97% (Figure 54 (E)), whereas the isolate *Lysinibacillus macroides* B4.2 and *Staphylococcus haemolyticus* S15.1 showed the least degradation with 31.78%, and 15.20% respectively (Figure 54 (F) and (G)). Similar finding has been indicated by S. Mnif *et al.* (2011) that the *Pseudomonas aeruginosa* strain C450R was the most efficient strains for crude oil degradation, which can degrade 96.2 % of the aliphatic fraction of hydrocarbons, whereas isolate *Bacillus* T4SS10 showed the least degradation (60.9%). Previous study shows that *Acinetobacter baumannii* was able to degrade >99% of the diesel oil within 5 days of incubation (Palanisamy *et al.*, 2014). Ijah and Antai (2003) found that *Bacillus spp.* were predominant bacteria in oil contaminated soil and were able to degrade 30-40% crude oil. *Bacillus spp.* are greater tolerant of high extent of hydrocarbons in soil on account of their resistant endospores (Ghazali *et al.*, 2004; Verma *et al.*, 2006).

The present work has added that the Species *Pseudomonas aeruginosa* P2.1, and the consortium of the three isolates (*Pseudomonas aeruginosa* P2.1, *Lysinibacillus macroides* B4.2 and *Staphylococcus haemolyticus* S15.1), can be considered as a suitable bioremediation agent for the treatment of the area polluted by diesel as well as other oil-waste. Chandran and Das (2010) reported that the diesel is a model hydrocarbon to study the hydrocarbon degradation because it composes of a variety of molecules.

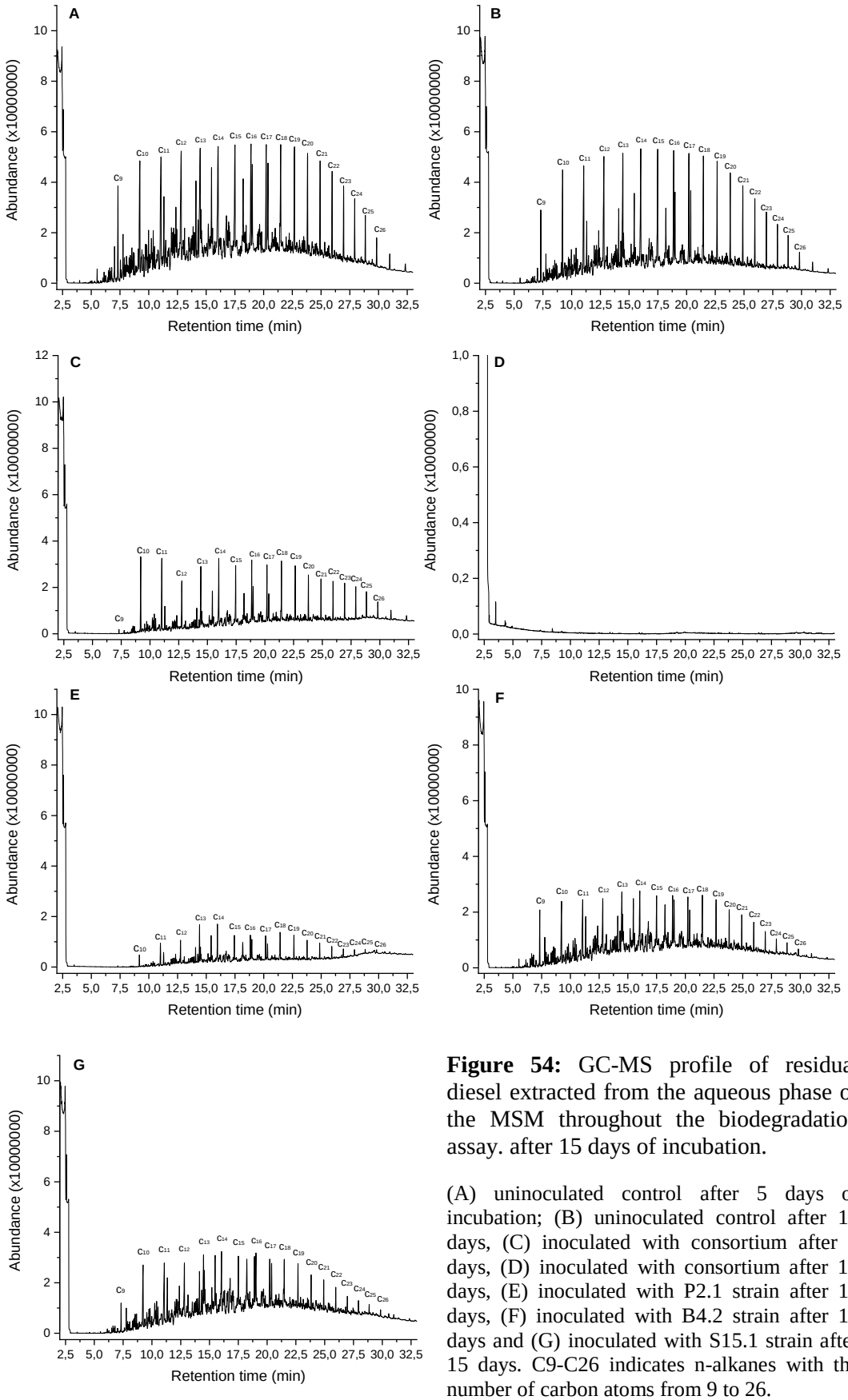

Figure 54: GC-MS profile of residual diesel extracted from the aqueous phase of the MSM throughout the biodegradation assay. after 15 days of incubation.

(A) uninoculated control after 5 days of incubation; (B) uninoculated control after 15 days, (C) inoculated with consortium after 5 days, (D) inoculated with consortium after 15 days, (E) inoculated with P2.1 strain after 15 days, (F) inoculated with B4.2 strain after 15 days and (G) inoculated with S15.1 strain after 15 days. C9-C26 indicates n-alkanes with the number of carbon atoms from 9 to 26.

Table 22: Percentage of biodegradation efficiency of individual hydrocarbon compound in diesel by individual selected isolates and bacterial consortium.

Major Components of diesel	Name	BE (%)				
		After 15 days of Incubation				After 5 days of incubation
		P2.1	B4.2	S15.1	BC	BC
C9H20	Nonane	98.26	28.27	58.69	99.94	96.28
C10H22	Decane	89.25	46.79	39.75	99.91	58.74
C11H24	Undecane	80.06	47.08	40.17	99.95	68.26
C12H26	Dodecane	79.75	50.09	44.31	99.99	79.27
C13H28	Tridecane	68.57	47.18	39.80	99.98	72.62
C14H30	Tetradecane	69.60	48.68	39.32	99.95	67.44
C15H32	Pentadecane	78.56	51.22	42.37	99.97	69.84
C16H34	Hexadecane	78.57	50.76	41.60	99.96	68.57
C17H36	Heptadecane	79.02	50.58	42.80	99.82	66.90
C18H38	Octadecane	76.18	48.41	41.66	99.95	64.48
C19H40	Nonadecane	77.88	49.73	42.94	99.95	67.10
C20H42	Eicosane	80.66	52.29	46.78	99.88	70.81
C21H44	Heneicosane	81.70	51.03	45.36	99.90	69.40
C22H46	Docosane	83.45	51.05	45.34	99.90	60.03
C23H48	Tricosane	99.84	53.54	48.22	99.46	59.84
C24H50	Tetracosane	83.21	55.36	44.20	94.79	59.14
C25H52	Pentacosane	79.12	52.91	39.15	87.70	43.94
C26H54	Hexacosane	67.03	45.52	24.39	82.76	34.94
Overall*		64.97	31.78	15.20	78.26	61.94

(BE): Biodegradation Efficiency.
(BC): Bacterial Consortium
(*): Represents overall degradation of all the diesel compound considering the total area of control and samples.

III.5. Optimization of biodegradation condition of crude oil

The present study was conducted to optimize the different factors for the biodegradation rate of crude oil by most potent bacterial strains (P2.1, B4.2 and S15.1). These including pH, temperature, sodium chloride concentrations, substrate concentrations, speed of agitation, inoculum size, intervals time, effect of different carbon and nitrogen source. All previously mentioned factors were determined by bacterial growth (OD at 600nm) (Bayoumi & Abul-Hamd, 2010; Hamzah *et al.*, 2012; Khan *et al.*, 2015). MSM medium with crude oil (without inoculation) was used as control.

III.5.1. Effect of different initial pH

The pH value is an important environmental factor affecting the microbial growth (Dongfeng *et al.*, 2011). The optimization of initial medium pH is important for the bacterial growth and for increased biodegradation rate. The data presented graphically in Figure 55 showed that optimum initial pH value for strains (P2.1, B4.2 and S15.1) was 7 at which these bacterial strains gave the highest growth rate (OD600 were 1.17, 1.04 and 0.76 respectively). The growth of the bacterial strains was decreased while increasing or decreasing this optimal pH. This study confirmed that these species grew better in neutral condition , this agree with El-Khawaga *et al.* (2015) who found that *P.aeruginosa* and *P. fluorescens* isolated from petroleum contaminated soil have a maximum biomass at pH 7. Palanisamy *et al.* (2014) reported that optimal diesel oil biodegradation by *Acinetobacter baumanii* occurred at pH 7, while increasing or decreasing the pH reduce the degradation rate. Also Bayoumi and Abul-Hamd (2010) noted that the optimum pH value for biodegradion rate of toluene and phenol by both *B. subtilis*-EPRIS12 and *B.laterosporous*-EPRIS41 was also 7 at which these bacterial species gave high growth rate (0.98 and 0.65 respectively). Most heterotrophic bacteria and fungi favor a pH near neutrality (Leahy & Colwell, 1990).

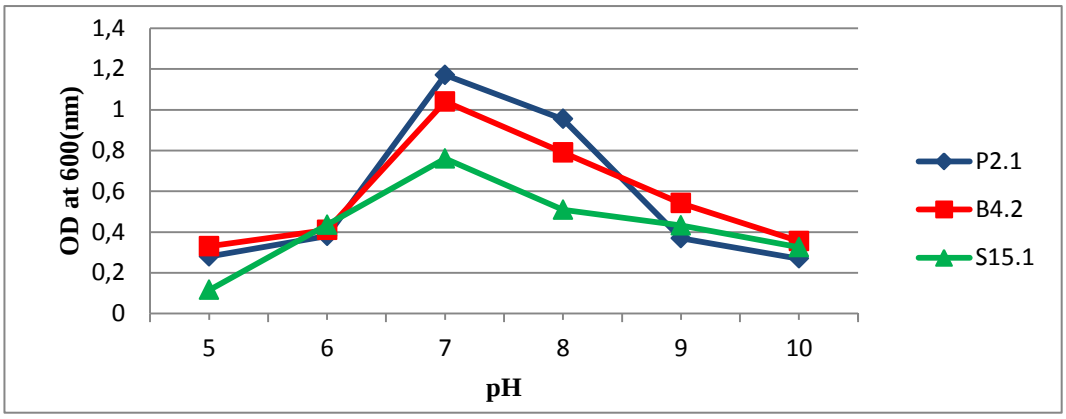

Figure 55: Effect of different pH value on the crude oil degradation by three isolate P2.1, B4.2 and S15.1.

III.5.2.Effect of incubation temperatures

The effect of temperature on the bacterial growth in MSM supplemented with crude oil was evaluated. In this study, the strains were able to grow up to 40°C. Figure 56 showed that the optimum temperature for the crude oil degradation by the three strains (P2.1, B4.2 and S15.1) was 30°C. The minimum growth was observed at 20 °C. The biodegradation rate decreased gradually above and below the optimal temperature. Hamzah *et al.* (2012) found that *Trichoderma virens* UKMP-1M, fungus isolated from oil-polluted waste water produced maximum biomass (0.23g/l) when incubated at 30°C. I. Mnif *et al.* (2014) reported that 30°C was the optimal temperature for the diesel degradation by *B. subtilis* SPB1. The temperature influences petroleum biodegradation by its effect on the physical state and chemical nature of oil, the hydrocarbon metabolism rate and composition of microbial community (Atlas, 1981). At low temperature, the oil viscosity increases, the toxic short-chain alkanes volatilization is reduced and rates of enzymatic activity. Generally, the degradation rate is decreased with decreasing temperature (Leahy & Colwell, 1990).

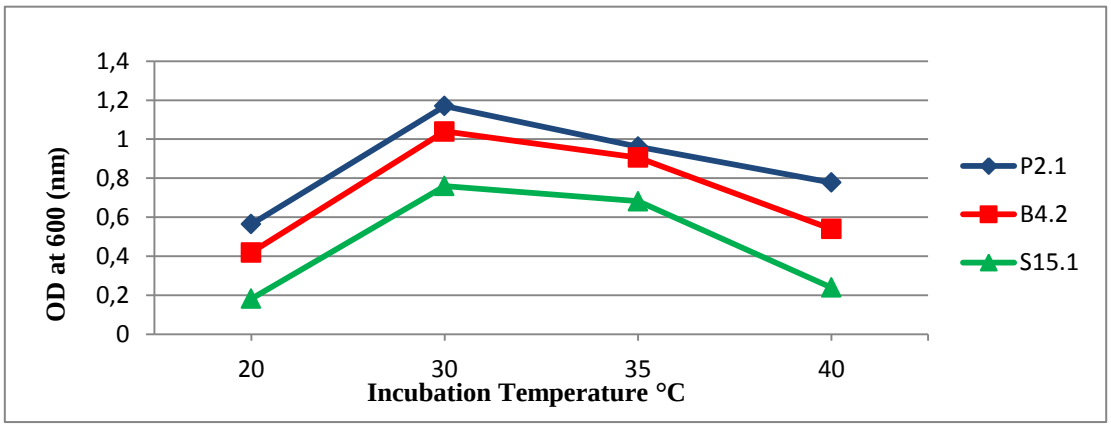

Figure 56: Effect of different incubation temperatures on the crude oil degradation by three isolate P2.1,B4.2 and S15.1.

III.5.3. Effect of sodium chloride concentrations

This experiment was performed to determine the optimum sodium chloride concentrations. Results presented in Figure 57 showed that the growth rate of both strains (P2.1 and B4.2) was decreased when sodium chloride concentrations increased. The growth of S15.1 was increased with increasing of sodium chloride concentrations up to 5%, but the growth of this strain decreased at 10% NaCl concentration. Bayoumi and Abul-Hamd (2010) noted that the bacterial growth decreased when sodium chloride concentrations increased. The hydrocarbon metabolism decreased with increasing salinity (Leahy & Colwell, 1990). This finding coincide with Palanisamy *et al.* (2014), who reported that biodegradation increased when NaCl concentration increased.

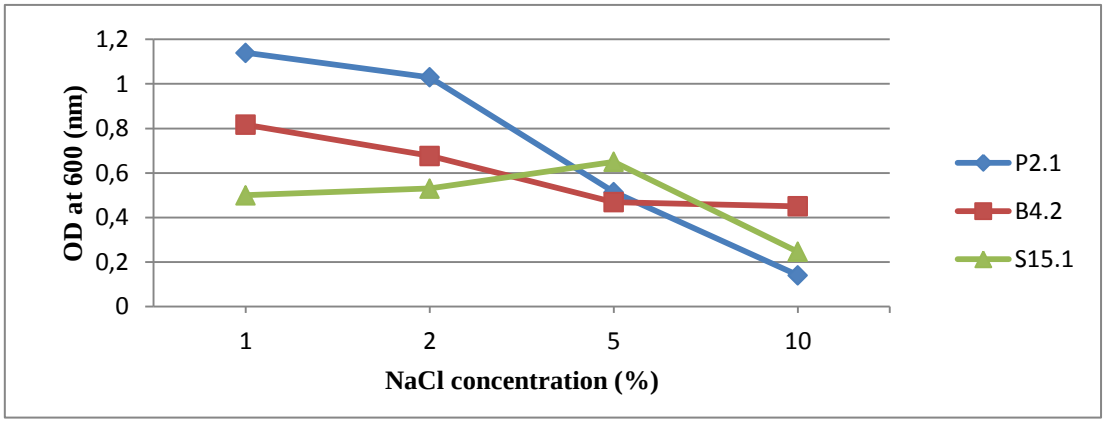

Figure 57: Effect of different sodium chloride concentrations on the crude oil degradation by three isolate P2.1, B4.2 and S15.1.

III.5.4. Effect of agitation speed

The data presented in Figure 58 showed that the bacterial growth of all isolates increased by increasing agitation speed and reduced when the strains incubated without shaking; therefore, the optimum agitation speed was 150 rpm. This result agree with El-Khawaga *et al.* (2015) who noted that *P.aeruginosa* and *P. fluorescens* isolated from oil contaminated soil have a highest value of microbial population at 150 rpm. Hamzah *et al.* (2012) showed that an increase of *Trichoderma virens* UKMP-1M biomass as the rate of agitation increased up to 200 rpm, then reduced when the speed of agitation increased up to 250 rpm. The aerobic pathway is the most effective strategy for bioremediation (Trindade *et al.*, 2002; Chokshi, 2003). Aerobic conditions are therefore necessary for the microbial oxidation of hydrocarbons in the environment (Leahy & Colwell, 1990). In addition, the continuous agitation is necessary to increase the surface

area of the oil droplets exposed to bacteria and increased exposure to oxygen (El-Khawaga *et al.*, 2015).

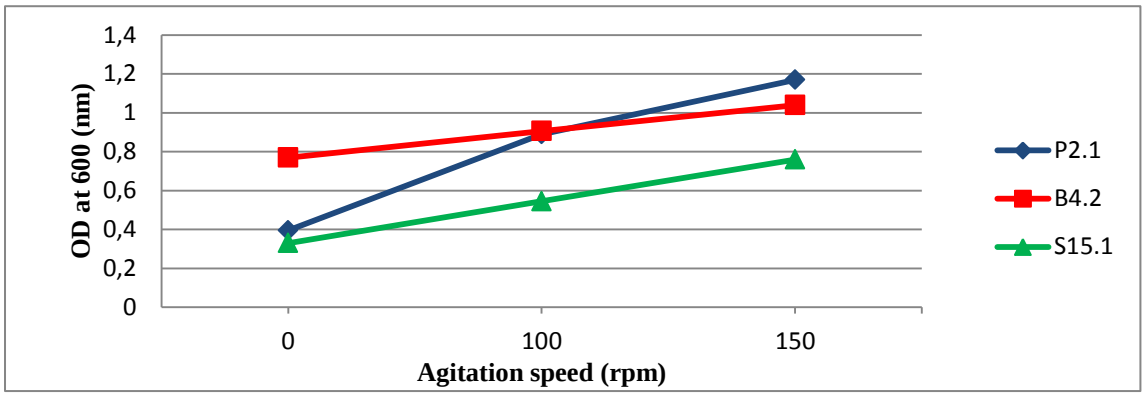

Figure 58: Effect of different agitation speed on the crude oil degradation by three isolate P2.1, B4.2 and S15.1.

III.5.5. Effect of initial crude oil concentrations

The results presented graphically in Figure 59 indicate that the three isolates are able to degrade the crude oil, and tolerate petroleum oil up to 10%. It can be seen from Figure 59 that when substrate concentration was equal to 1%, both strains (P2.1 and B4.2) could achieve a high growth rate. Also, data indicated that the isolate S15.1 was slightly higher in 2% of initial crude oil concentration. The microbial populations decreased with increasing the crude oil concentration. These results are in agreement with Hamzah *et al.* (2012) who reported that *Trichoderma virens* UKMP-1M grow in a wide range of crude oil concentrations. The production of biomass increased to 2.25 g/L in the presence of 1% (v/v) heavy crude oil. The production of biomass decreased when the concentration of crude oil increased beyond 1% (v/v). El-Khawaga *et al.* (2015) found that bacterial growth of the two isolates *P.aeruginosa* and *P. fluorescens* decreased when petroleum oil concentration increased from 1 to 9%. Bayoumi and Abul-Hamd (2010) noted that the biodegradation by *B.subtilis*-EPRIS12 and *B.laterosporous*-EPRIS41 decreased when the toluene and phenol concentrations increased. Rahman *et al.* (2002b) found that the percentage of degradation by the bacterial consortium decreased from 78% to 52% as the concentration of crude oil was increased from 1 to 10%. Our result coincide with Okoh (2006) who noted that the amount of heavy crude oil metabolized by some bacterial strains increased with increasing concentration of initial oil up to 0.6% (w/v). Furthermore, higher concentration of crude oil could have a toxic effect on microorganisms and lead to

decrease the normal growth of strains and so reduce the hydrocarbon degradation rate (Dongfeng *et al.*, 2011). The toxicity of crude oil or petroleum products varies, depending on their concentration, composition, environmental factors and on the biological state of the organisms at the time of the contamination (Obire & Anyanwu,

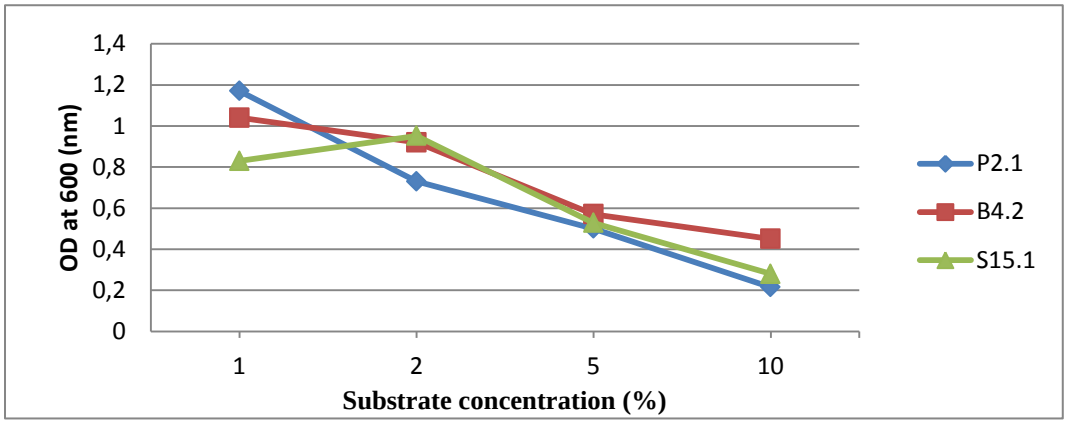

Figure 59: Effect of initial crude oil concentrations on the crude oil degradation by three isolate P2.1, B4.2 and S15.1.

2009).

III.5.6. Effect of different carbon and nitrogen source

Replacement of ammonium nitrate (NH_4NO_3) at 1 g/l in MSM with other nitrogen sources showed that bacterial growth rate was more enhanced in the presence of NH_4NO_3 than with yeast extract and peptone (Figure 60). On other hand, the lowest bacterial growth for these isolates was observed when inoculated in MSM with peptone as nitrogen source. This result disagree with Hamzah *et al.* (2012) who reported that the presence of peptone in MSM produced better biomass growth than with urea, yeast extract, KNO_3 and $(NH_4)_2SO_4$. Palanisamy *et al.* (2014) found that the addition of yeast extract to MSM medium containing 4% diesel enhanced the diesel oil degradation and reported that the addition of external nutrient enhanced the degradation of crude oil. In addition, Figure 60 show that the presence of maltose in MSM produce more biomass than with glucose. This finding agree also with Palanisamy *et al.* (2014) who reported that *Acinetobacter baumannii* degrade diesel more effectively in MSM medium supplemented with maltose. Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants, especially nitrogen, phosphorus (Okoh, 2006). In the environment, C: N: P ratio must be maintained at about 120:10:1 to maintain any microbial activity (Salleh *et al.*, 2003).

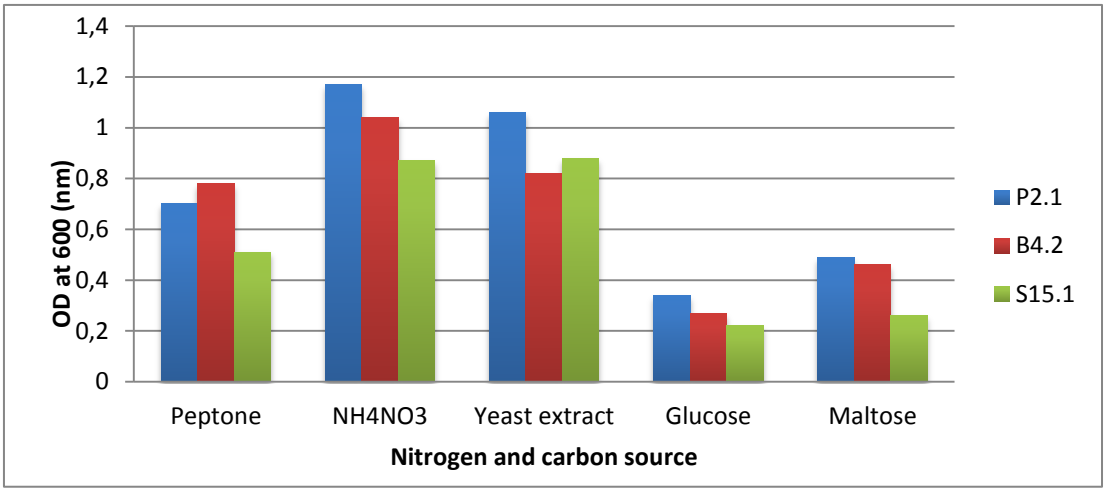

Figure 60: Effect of carbon and nitrogen sources on the crude oil degradation by three isolate P2.1, B4.2 and S15.1.

III.5.7. Influence of initial inoculum size

The influence inoculum size on crude oil degradation by the three strains (P2.1, B4.2 and S15.1) is presented in Figure 61. The results illustrated in Figure 61 showed that bacterial growth rate increased with an increasing inoculum amount (the inoculum size ranged between 0.1%- 3%). Therefore, the hydrocarbon degradation rate increased with increasing the inoculum size. Our results concord with Dongfeng *et al.* (2011) who found that the rate of hydrocarbon degradation increased with an increasing inoculum size, where it reached 54.22% at 3% inoculum size. Nevertheless, in the range of 3 ml to 7 ml of inoculum added, the hydrocarbon degradation rate decreased, thus inoculum size could not realize a high hydrocarbon degradation rate (Dongfeng *et al.*, 2011) .

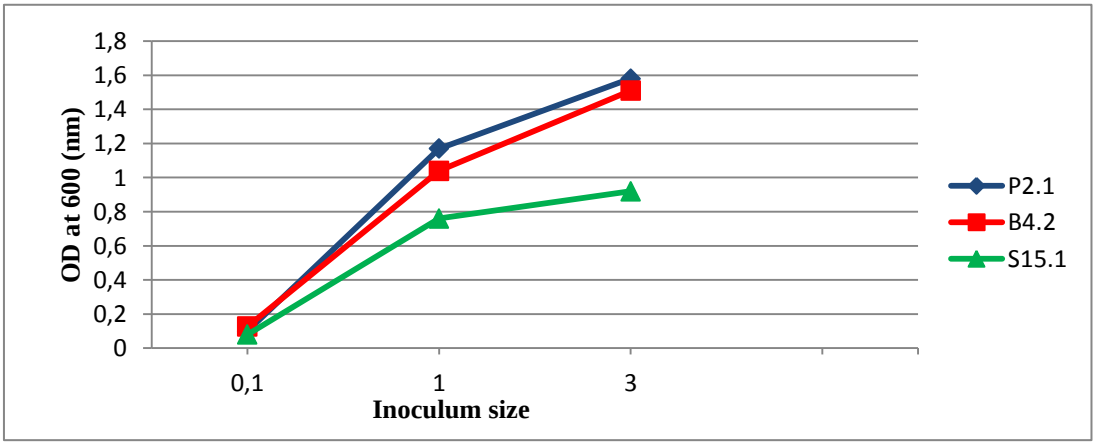

Figure 61: Influence of inoculum size on the crude oil degradation by three isolate P2.1, B4.2 and S15.1.

III.5.8. Influence of initial inoculum concentration

MSM medium was inoculated with initial inoculum concentration of 0.1, 0.5, 1 and 1.5 optical density at 600 nm. It can be seen from Figure 62 that the bacterial growth increased with an increasing the initial inoculum concentration. When inoculum of the three strains (P2.1, B4.2 and S15.1) increased up to 1.5 optical density, the bacterial growth reached a maximum value (1. 825, 1.51 and 1. 44 respectively) which indicate a higher crude oil degradation. These results agree with (Palanisamy *et al.* (2014)) that noted, when inoculum of *Acinetobacter baumannii* increased up to 1.5 optical density, resulted in higher growth and indicates higher diesel oil degradation.

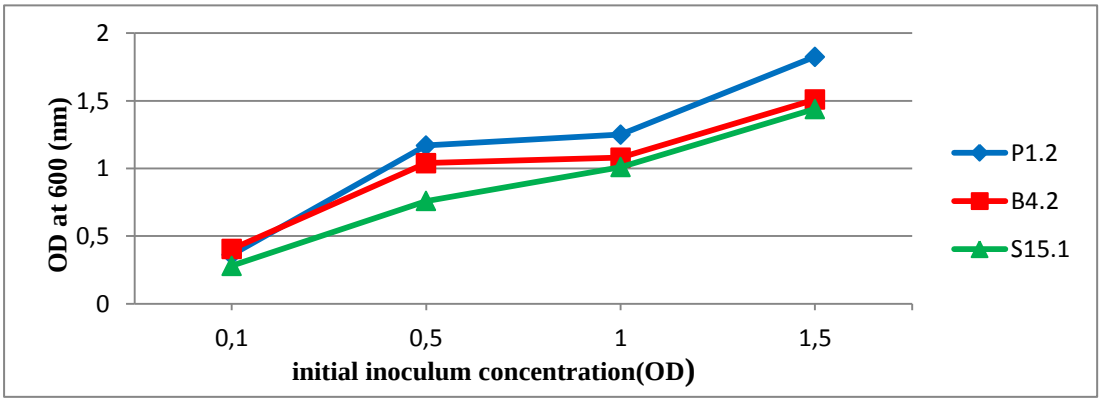

Figure 62: Influence of initial inoculum concentration on the crude oil degradation by three isolate P2.1, B4.2 and S15.1.

III.5.9. Effect of interval times of incubation

The results presented Figure 63 may indicate that the strain P2.1 obtain a maximum growth rate (OD = 1.21) at day 10, while both B4.2 and S15.1 strains reached higher growth at day 7. Also the bacterial count decrease after day 10 for P2.1 isolate and after day 7 for the two strains (B4.2 and S15.1). These result concord with those noted by El-Khawaga *et al.* (2015) who reported that the maximum growth of *P. aeruginosa* and *P. fluorescens* were 9.12 and 8.97 Log CFU/ml culture, respectively obtained at 10 day. Also, the bacterial count decreased after 10 day for the two strains.

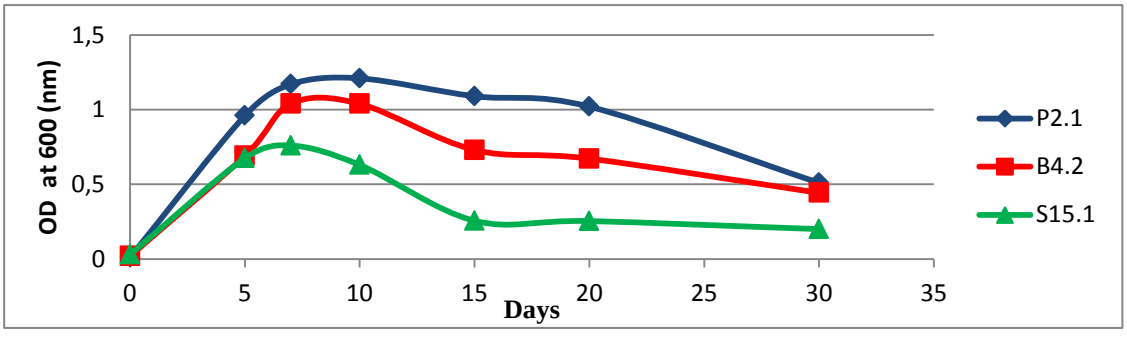

Figure 63: Effect of different time intervals on the crude oil degradation by three isolate P2.1, B4.2 and S15.1.

CONCLUSION AND RECOMMENDATION

Conclusion and recommendation

The pollution due to petroleum hydrocarbons has been an environmental problem for a long time. Indigenous microorganisms play important role in the bioremediation of petroleum and petroleum based product.

The present study focused on the degradation of crude oil and diesel by a bacterial species isolated from oil-contaminated soil collected from Arzew oil refinery, Algeria. These soils have been subjected for several decades to chronic and high levels of hydrocarbon contamination. They thus constitute a model of choice to understand the microbial mechanisms occur in the presence of hydrocarbons.

The current study aimed to isolate, characterize the oil-degrading microorganisms from the Arzew refinery using biochemical and molecular methods, and to characterize the petroleum oil and diesel degradation potential of the bacterial isolates.

In this study, 15 oil-contaminated soil samples were collected from Arzew oil refinery, these samples were highly polluted by hydrocarbon compounds and showed high bacterial count.

Seventy-eight bacterial isolates were isolated from oil-contaminated soil at Arzew oil refinery. These strains have significant ability to utilize a crude oil as sole source of carbon and energy. Twenty-two bacterial isolates were screened with the best degradative abilities on crude oil; the isolates show diverse forms, colors, margins, and shapes, suggesting bacterial diversity among isolates. They have been identified using morphological and biochemical characterization. Among 22 bacterial isolates, only 15 isolates were identified using 16S rRNA gene sequence analysis.

The isolates were identified as the following genera and/or species: *P. aeruginosa, Achromobacter xylosoxidans, Staphylococcus haemolyticus, Enterococcus faecalis, B.cereus, B.anthracis, B. subtilis, Exiguobacterium aurantiacum, Lysinibacillus macroides, P. fluorescens, Burkholderia cepacia, Staphylococcus hominis* and *Lysinibacillus sphaericus.*

Six bacterial isolates (identified as *P. aeruginosa*) showed a positive result with primer pair specific to alkane 1 monoxygenase gene (434bp).

The ability of 12 isolated strains to degrade crude oil was carried out in a liquid medium by measuring optical density and gravimetric analysis. The turbidometry results show that all the isolates had effectively utilized crude oil; the level of degradation differs between isolates, *Pseudomonas aeruginosa* (P2.1 and P2.3), *Lysinibacillus macroides* B4.2 and *Achromobacter xylosoxidans* P2.2 had the highest growth in the medium with crude oil while *Staphylococcus haemolyticus* S15.1, *Enterococcus faecalis* E1.1, *Enterococcus faecalis* E2.5 *and Staphylococcus hominis* S15.3 respectively had the lowest ability to utilize crude oil. The gravimetric analysis demonstrate that *Pseudomonas aeruginosa P2.1, Pseudomonas aeruginosa P2.3, Lysinibacillus macroides B4.2 and Achromobacter xylosoxidans P2.2* exhibited the highest biodegradation percentage of crude oil respectively.

The ability of three individual pure isolates P2.1, B4.2, S15.1 and bacterial consortium to degrade diesel was determined using the turbidometry method, gravimetric and GC-MS analysis. The selected individual bacterial isolates and consortia had effectively utilized diesel as sole carbon source and energy, the results indicate that the bacterial consortium and *Pseudomonas aeruginosa* P2.1 respectively showed the best growth in MSM broth supplemented with diesel.

The gravimetric analysis also indicates that the bacterial consortium and *Pseudomonas aeruginosa* P2.1 exhibited the highest biodegradation percentage of diesel. This bacterial consortium could potentially use for bioremediation of oil contaminated soil. Therefore, bioaugmantation enhances bioremediation process.

The GC-MS analysis revealed that the individual isolates and the consortium degrade the aliphatic fraction greater than the aromatic fraction, and indicated that bacterial consortium degraded diesel more effectively in 15 days than 5 days of incubation. All compound in diesel were highly reduced by mixed culture compared to the individual isolates.

The optimum parameters for crude oil degradation by three bacterial strains were: pH, 7; temperature, 30°C ; petroleum oil concentration, 1%; agitation speed, 150 rpm; sodium chloride concentrations, 1% for two strains (P2.1 and B4.2) but the growth of S15.1 strain was increased with increasing of sodium chloride concentrations up to 5% ; ammonium nitrate were found to be the best nitrogen sources for the three bacterial strains and addition of external nutrient (Maltose) enhanced the degradation of

crude oil. The hydrocarbon degradation rate increased with increasing the inoculum size and concentration of bacterial strains.

The present work showed that the isolated strains have a significant ability to utilize a crude oil and diesel as a sole carbon source and energy. Therefore, the degradative capabilities of these strains suggest that they could be used for the bioremediation of other oil wastes in the environment.

- The isolated oil degrading bacteria could be used to remediate oil-contaminated soil at Arzew region and other polluted soil.
- Additional works will be suggested in our future research in order to use the oil degrading strains as future solution for not only oil contamination cleaning up but also the removal of different pollutants from our environment.
- Further studies have to be done on the degradation of polycyclic aromatic hydrocarbons, which are not easily degraded by microorganisms.
- Further, work to investigate the effect of UV irradiation on the biodegradation efficiency of the bacterial isolates.
- Further analysis and studies at the genetic levels had to be done such as sequencing of PCR product of bacterial isolates before and after UV irradiation.
- The genes coding for the enzymes from the strains that exhibited the highest enzyme activity can be cloned in suitable vectors and transformed to fast growing bacteria such as *E. coli*.

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APPENDICES

Appendices

Appendix A: Media Formulations.

The Gram stain solutions

The Gram stain solutions can be made using the recipes below or a kit can be purchased.

Crystal Violet stain

(a) 8 g crystal violet dissolved in 80 ml 98% ethanol

(b) 3.2 g ammonium oxalate dissolved in 320 ml ddH_2O

Let stand 24 h, mix (a) and (b), then filter.

Gram's Iodine

(a) 1.2 g iodine dissolved in 20 ml 98% ethanol

(b) 2.4 g potassium iodide dissolved in 340 ml ddH_2O

Mix (a) and (b)

Safranin

1.0 g Safranin dissolved in 40 ml 98% ethanol

Dilute with 400 ml ddH_2O, and then filter

Milieu Bushnell and Haas (MSM)

KH_2PO_4……..	1.0g/l
K_2HPO_4……	1.0g/l
$FeCl_3$……..	0.05g/l
NH_4NO_3……	1.0g/l
$CaCL_2$. $2H_2O$…….	0.02g/l
MgSO4. $7H_20$……	0.2g/l

Nutrient Agar

Beef Extract.....	1.5g/l
Yeast Extract.....	1.5g/l
Pepton.....	5g/l
Sodium chloride…….....	5g/l
agar ……	0.2g/l

King B

Mixed peptone.....	20g/l
Dipotassium hydrogen phosphate…….....	1.5g/l
Magnesium sulfate.....	1.5g/l
Agar ……	15g/l

King A

Peptone.....	20g/l
Magnesium chloride ……....	1.4g/l
Potassium sulfate.....	10g/l
Agar ……	15g/l

LB agar

Tryptone.....	10g/l
Yeast extract.....	5.0g/l
Sodium chloride.....	5.0g/l
Agar.....	15g/l

LB Broth

Tryptone.....	l0g/l
Yeast extract.....	5g/l
Sodium chloride.....	5g/l
Tryptone.....	l0g/l

Meat-Liveragar

Meat-liver Peptone.....	30.0 g/l
Glucose.....	2.0 g/l
Bacteriological agar.....	6.0 g/l
Meat-liver Peptone.....	30.0 g/l

DNase Agar Base

DNase Agar toluidine blue is recommended for the detection of deoxyribonuclease activity of bacteria and fungi, and especially for identification of pathogenic Staphylococci.

Composition

Tryptose……..	20.0 g/l
Deoxyribonucleic acid (DNA)……	2.0 g/l
Sodium chloride……..	5.0 g/l
Agar…….....	15.0 g/l

Preparation

Suspend 42 grams in 1000 ml distilled water. Heat with frequent agitation to dissolve the medium completely. Sterilize by autoclaving at 12 to 15 lbs pressure (118°C to 121°C) for 15 minutes. Cool to 45°C and pour into sterile petri plates. Add 0.1 g Toluidine Blue before sterilizing the medium or flood the plates with 0.1% Toluidine Blue solution after incubation as desired.

Blood Agar Base (BBL)

Heart Muscle, Infusion from (solids).....	2.0 g/l
Pancreatic Digest of Casein.....	13.0 g/l
Yeast Extract.....	5.0 g/l
Sodium Chloride.....	5.0 g/l
Agar ……	15.0 g/l

Preparation

Suspend 40 g of the powder in 1 L of purified water, autoclave at 121°C for 15 minutes.For preparation of blood agar, cool the base to 45-50°C and aseptically add 5% sterile, defibrinated blood. Mix well.

Chapman Medium

Sodium Chloride……..	75.00 g/l
Beef Extract …….....	1.00 g/l
D-Mannitol ……..	10.00 g/l
Phenol Red ……...	0.025 g/l
Pancreatic Digest of Casein …….	5.00 g/l
Bacteriological Agar ……	15.00 g/l
Peptic Digest of Animal Tissue ……...	5.00g/l

Tryptic Soy Agar

Tryptone (Pancreatic Digest of Casein)……	17.0 g/l
Soytone (Peptic Digest of Soybean)…….……	3.0 g/l
Glucose (= Dextrose) ……..…….....	2.5 g/l
Sodium Chloride……	5.0 g/l
Dipotassium Phosphate……....	2.5 g/l
Agar……	15 g/l

Cetrimide Agar

Pancreatic digest of gelatin …….....	20 g/l
Magnesium chloride……	1.4 g/l
Potassium sulfate……..	10 g/l
Cetrimide……..	0.3 g/l
Agar…….	15 g/l

Appendix B:The Gram stain and the endospore stain procedure

Gram staining procedure

1. A drop of distilled water was placed on the glass slide.

2. A pure colony was picked-up from Petri dish and mixed with distilled water, dry it and fixed by Bunsen burners flame passed for three times.

3. Few drops of crystal violet were applied for 30 seconds. The slide was then washed in running tape water.

4.The smeared slide was then covered with lugol’s iodine solution for 30 seconds a washed in running tape water.

5.The slide was decolorized with 95% alcohol. As soon as the stain ceased to pour-out (2-3 seconds), washed immediately in running tape water for further 30 seconds.

6.A fuchsine was applied for 30 seconds.

7. A fuchsine was washed in running tap water, then blot dried on filter paper and left at room temperature for the residual moisture to evaporate.

8.A drop of oil immersion was placed on stained part of the slide, and then the slide was examined under the oil immersion objective.

The endospores stain procedure:

1. Prepare a heat fixed smear on a clean microscope slide of the bacteria. Place a small drop of sterile water on the slide and add a very small aliquot of the bacteria to the water drop with an inoculating needle. Use a sterile inoculating loop to make a very thin smear of the bacteria on the surface of the slide. Allow the smear to dry completely.

2. Place a small piece of blotting paper over the smear and place the slide (smear side up) on a staining rack that has been placed over a boiling water bath in the corner of the room.

3. Completely cover the blotting paper that covers the smear with malachite green and allow the slide to heat thoroughly over the water bath for 10 minutes.

5. After 10 minutes carefully remove the slide from the rack using a clothespin and allow the slide to cool to room temperature for 2 minutes.

7. Use water to wash the malachite green from the microscope slide.

8. Stain the smear with safranin for 2 minutes.

9. Rinse the slide to remove the secondary stain.

10. Observe the bacteria under 1000X (oil immersion) total magnification

Appendix C: API galleries

API 20 E

API 20 E is a standardized identification system for enterobacteriaceae and other non-fastidious, Gram-negative rods, which uses 21 miniaturized biochemical tests and a database.

The 20 tests were performed according to the manufacturer's instructions as follows:

1. Preparation of the galleries

Five ml of tap water dispensed in the inoculation tray to provide a humid atmosphere during incubation.

2. Preparation of bacterial suspension

Open an ampule of API Suspension Medium, or use any tube containing of sterile

saline or sterile distilled water, without additives. Using a pipette, remove a single well isolated colony from an isolation plate. It is recommended to use young cultures (18-24 h old). Carefully emulsify to achieve a homogeneous bacterial suspension. The suspension turbidity should be equal to a 0.5 McFarland standard. This suspension must be used immediately after preparation.

3. Inoculation of the galleries

The twenty micro tubes were inoculated. According to the manufactures instructions.

Using the same pipette, fill both tube and cupule of the tests CIT, VP and GEL with the bacterial suspension.

Fill only the tube (and not the cupule) of the other tests.

Create anaerobiosis in the tests ADH, LDC, ODC, H2S and URE by overlaying with mineral oil.

Close the incubation box. Incubate at 36°C ± 2°C for 18-24 h.

Reading table

Tests	Substrates	Reactions/enzymes	Results	
			Negative	**Positive**
ONPG	2-nitrophenyl-ßD-galactopyranoside	ß-galactosidase (Ortho NitroPhenyl-ßd-Galactopyranosidase)	colorless	yellow
ADH	L-arginine	Arginine DiHydrolase	yellow	red/ orange
LDC	L-lysine	Lysine DeCarboxylase	yellow	red/ orange
ODC	L-ornithine	Ornithine DeCarboxylase	yellow	red/ orange
CIT	Tri sodium citrate	CITrate utilization	pale green/yellow	blue-green/ blue
H2S	sodiumthiosulfate	H2S production	colorless / grayish	black deposit/ thin line
URE	Urea	UREase	yellow	red / orange
TDA	L-tryptophane	Tryptophane DeAminase	**TDA/ immediate** Yellow	Reddish brown
IND	L-tryptophane	INDole production	**JAMES/ immediate** colorless pale green/yellow	pink
VP	Sodium pyruvate	Acetoin production (Voges Proskauer)	**VP1+VP2/ 10min** colorless	pink / red
GEL	Gelatin (bovineorigin)	GELatinase	no diffusion	diffusion of black pigment
GLU	D-glucose	fermentation/ oxidation (GLUcose)	blue/ blue-green	yellow/ greyish yellow
MAN	D-mannitol	fermentation/ oxidation (MANnitol)	blue/ blue-green	yellow
INO	Inositol	fermentation/ oxidation (INOsitol)	blue/ blue-green	yellow
SOR	D-sorbitol	fermentation/ oxidation (SORbitol)	blue/ blue-green	yellow
RHA	L-rhamnose	fermentation/ oxidation (RHAmnose)	blue/ blue-green	yellow
SAC	D-sucrose	fermentation/ oxidation (SACcharose)	blue/ blue-green	yellow
MEL	D-melibiose	fermentation/ oxidation (MELibiose)	blue/ blue-green	yellow
AMY	Amygdalin	fermentation/ oxidation (AMYgdalin)	blue/ blue-green	yellow
ARA	L-arabinose	fermentation/ oxidation (ARAbinose)	blue/ blue-green	yellow
OX	cytochrome-OXidase	(see oxidase test package insert)		

API 20 NE

1. Preparation of inoculum

- Open an ampule of API Suspension Medium (5 ml), or use any tube containing of sterile

- Saline or sterile distilled water, without additives. Using a pipette, remove a single well isolated colony from an isolation plate. It is recommended to use young cultures (18-24 h old). Carefully emulsify to achieve a homogeneous bacterial suspension. The suspension turbidity should be equal to a 0.5 McFarland standard. This suspension must be used immediately after preparation.

2. Preparation of the Strip

- Prepare an incubation box, tray and lid, and distribute about 5 ml of distilled water or demineralized water tray to create a humid atmosphere.

3. Inoculation of the galleries

- Inoculate tests NO3 to PNPG by distributing the suspension into the tubes (and not the cupules) using the same pipette.

- Fill the tubes and cupules of tests GLU to PAC with the suspension.

- Add mineral oil to the cupules of the 3 underlined tests (GLU, ADH and URE) until a convex meniscus is formed.

- Close the incubation box and incubate at 29°C ± 2°C for 24 h (± 2 h).

Reading Table

Tests	Substrates	Reactions/enzymes	Negative results	Positive results
$N0_3$	Potassium nitrate	reduction of nitrate to nitrites	**NIT 1 + NIT 2 / 5 min** colourless	pink-red
		Reduction of nitrates to nitrogen	**Zn / 5 min** pink	colourless
TRP	Tryptophane	indole production	**JAMES / immediate** colourless / pale green / yellow	pink
GLU	Glucose	Acidification	blue to green	yellow
ADH	Arginine	arginine dihydrolase	yellow	orange/pink/red
URE	Urea	Urease	yellow	orange/pink/red
ESC	Esculin	hydrolysis (β-glucosidase)	yellow	grey/brown/black
GEL	Gelatin (bovine origin)	hydrolysis (protease)	no pigment diffusion	diffusion of black pigment
PNPG	p-nitrophenyl-β-D-galactopyranoside	β-galactosidase	colourless	yellow
[GLU]	Glucose	Assimilation	transparent	opaque
[ARA]	Arabinose	Assimilation	transparent	opaque
[MNE]	Mannose	Assimilation	transparent	opaque
[MAN]	Mannitol	Assimilation	transparent	opaque
[NAG]	N-acetyl-glucosamine	Assimilation	transparent	opaque
[MAL]	Maltose	Assimilation	transparent	opaque
[GNT]	Gluconate	Assimilation	transparent	opaque
[CAP]	Caprate	Assimilation	transparent	opaque
[ADI]	Adipate	Assimilation	transparent	opaque
[MLT]	Malate	Assimilation	transparent	opaque
[CIT]	Citrate	Assimilation	transparent	opaque
[PAC]	phenyl-acetate	Assimilation	transparent	opaque
OX	see oxidase test	cytochrome oxidase	colorless/ light purple	dark purple

API Staph

- API Staph is a standardized system for the identification of the genera Staphylococcus, Micrococcus, which uses miniaturized biochemical tests and a specially adapted database.

Preparation of the inoculum

- Open an ampule of API Staph Medium, prepare a homogeneous bacterial suspension with a turbidity equivalent to 0.5 McFarland. It is recommended to use

young cultures (18-24 h old). This suspension must be used immediately after preparation.

Inoculation of the strip

- Using a pipette, fill the microtubes with the inoculated API Staph Medium. Only fill the tube portion of the microtubes, not the cupules (slightly underfill the microtubes).

- Ensure anaerobiosis in the ADH and URE tests by filling the cupules with mineral oil to form a convex meniscus.

- Close the incubation box. Incubate at 36°C ± 2°C for 18-24 h.

Reading table

Tests	Active ingredients	Reactions/ enzymes	Result	
			Negative	Positive
0	No substrate	Negative control	red	—
GLU	D-glucose	(Positive control)(D-GLUcose)	red	yellow
FRU	D-fructose	acidification(D-FRUctose)		
MNE	D-mannose	Acidification (D-ManNosE)		
MAL	D-maltose	Acidification (MALtose)		
LAC	D-lactose	acidification (LACtose)		
TRE	D-trehalose	acidification (D-TREhalose)		
MAN	D-mannitol	acidification (D-MANnitol)		
XLT	xylitol	acidification (XyLiTol)		
MEL	D-melibiose	acidification (D-MELibiose)		
			NIT1+NIT2/10min	
NIT	Potassium nitrate	reduction of NITrates to nitrites	colorless-light pink	red
			ZYMA+ZYMB/10 min	
PAL	ß-naphthyl phosphate	Alkaline Phosphatase	yellow	violet
			VP1 + VP2/10min	
VP	Sodium pyruvate	Acetyl-methyl-carbinol production (Voges Proskauer)	colorless-light pink	violet-pink
RAF	D-raffinose	acidification (RAFfinose)	Red	yellow
XYL	D-xylose	acidification (XYLose)		
SAC	D-saccharose(sucrose)	acidification (SACcharose)		
MDG	methyl-αD-glucopyranoside	acidification (Methyl-αD-Glucopyranoside)		
NAG	N-acetyl-glucosamine	acidification(N-Acetyl-Glucosamine)		
ADH	L-arginine	ArginineDiHydrolase	Yellow	orange - red
URE	Urea	UREase	Yellow	red-violet

Appendix D: Kit and Reagents

- **Genomic DNA purification kit (Quant-iT™ Pico Green ® ds DNA Kits)**

Quant-iT™ Pico Green® ds DNA reagent is an ultra-sensitive fluorescent nucleic acid stains for quantitating double-stranded DNA (ds DNA) in solution.

Table1. Contents and storage information

Material	Amout	Concentration	Storage	Stability
Quant-iT™ Pico Green® ds DNA reagent (Component A)	1mL	Solution in DMSO	• 2–6ºC • Desiccate • Protect from light	When stored as directed product stable for at least 6 months
20XTE (Component B)	25mL	200mMTris-HCl,20 mM EDTA,pH7.5	• Room temperature*	
Lambda DNA standard (Component C)	1mL	100µg/mL in TE	• 2–6ºC*	

- **Q5® High-Fidelity DNA Polymerase**

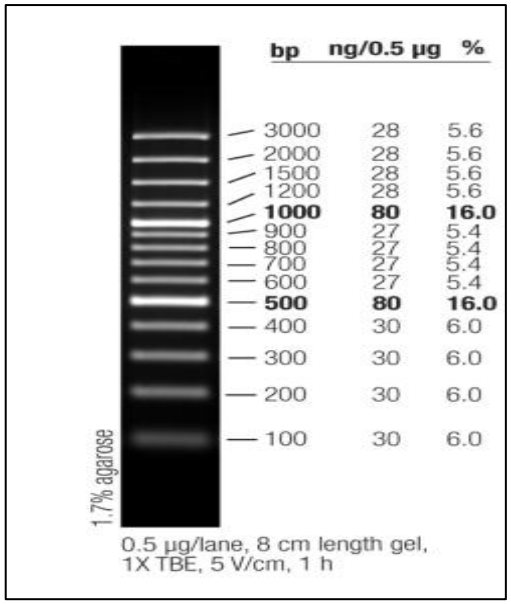
Q5® High-Fidelity DNA Polymerase sets a new standard for both fidelity and robust performance. With the highest fidelity amplification available (~280 times higher than *Taq*), Q5 DNA Polymerase results in ultra-low error rates. Q5 DNA Polymerase is composed of a novel polymerase that is fused to improving speed, fidelity and reliability of performance.

Q5 High-Fidelity DNA Polymerase is ideal for cloning and can be used for long or difficult amplicons. Q5 High-Fidelity DNA Polymerase is supplied with an optimized buffer system that allows robust amplification. The 5X Q5 Reaction Buffer contains 2 mM Mg at final (1X) reaction concentrations and is recommended for most routine applications.

Product Source

An *E. coli* strain that carries the Q5 High-Fidelity DNA Polymerase gene.

GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific)



6X DNA Loading Dye

10 mM Tris-HCl (pH 7.6),

0.03% bromophenol blue,

0.03% xylene cyanol FF,

60% glycerol and

60 mM EDTA

I. Sequencing with the BDT v3.1 kit in 96-well plate

I.1. Procedures

I.1.1. Material

I.1.1.1. Consumables

One semi-skirted 96-well PCR plate (Fisher Scientific).

0.5-10 µl universal Pipet tips (VWR).

200 µl presterilized filter LTS tips (Rainin).

Adhesive Sealing Sheets (Life Technologies).

I.1.1.2 Solutions

- Sequencing master mix, stored in the in the 4ºC. They are kept in a dark brown 50ml tube identified by **MASTER MIX BDT0.8** (See Sequencing Master Mix 0.8 BDT preparation below).

I.1.1.3 Equipment

- Electronic pipette EDP3-Plus 20-200 µl from Rainin.
- Multichannel pipette m10 (0.5-10 µl) from Biohit.
- Thermo-sealer (ABgene).
- Centrifuge: model 5430, Eppendorf.
- Mini centrifuge (model C-1200 from National Labnet Co.) for single or strip tube.

Thermocycler:

- 96-Well GeneAmp® PCR system 9700 from Applied Biosystems or
- Veriti 96 Well Thermal Cycler from Applied Biosystems or
- Mastercycler pro S vapo. protect from Eppendorf

I.1.2. Preparation

I.1.2.1. PCR product sequencing

- PCR products must be a minimum 7.5 µl of purified PCR product or a minimum of 20 µl of unpurified PCR product. Before sequencing, all PCR products must be purified to remove primers and unincorporated dNTPs.

- In each well of the 96-well plate, add in the following order with a single or a multichannel pipette m10 from Biohit or an electronic pipette:

- 2.5 µl primer (5 µM)
- 2.0 µl PCR product *

* If the PCR product is longer than 500 base pairs or the band on the gel is not bright use 4 to 6 µl of the PCR product instead of 2.0 µl.

I.1.2.2. Sequencing reaction

1. Once all the wells are filled with primer and DNA template (PCR products), add the appropriate sequencing master mix.

2. With the electronic pipette EDP3-Plus 20-200µl from Rainin, add 5.4 µl of "**sequencing master mix**" to each well of the plate for a final volume of 9.90 µl. Note: 5.4 µl is used instead of 5.5 µl due to a restriction of the model of the pipette used.

3. Seal the plate with an adhesive sealing sheet and centrifuge it for 30 Sec at 100 g.

- Put the plate in a thermocycler and close the lid.

- Choose program BDT25 or BDT40 for resequenced samples, enter 10 or 15 µl for the reaction volume and start the program.

4. The programs BDT25 and BDT40 correspond to:

- An initial denaturation at 96°C for 1 minute.

- 25 or 40 cycles: 96°C for 10 seconds (denaturation), 50°C for 5 seconds (hybridization) and 60°C for 4 minutes (annealing).

5. When the 25 or 40 cycles are finished, the thermocycler cools down at 4°C.

II. Sequencing Master Mix 0.8 BDT preparation

II.1. Procedures

II.1.1. Materials

II.1.1.1. Consumables

- Brown 50 ml Centrifuge Tube Flat Top Cap (Illumina)

- 100-1000 µl Universal Fit Pipette Tips (Fisher Scientific)

- 5000 µl BIOHIT Tip Bulk (VWR)

- BigDye® Terminator v3. 1 Cycle Sequencing RR-2500 from Applied Biosystems (Life Technologies), Quantity: 0.8 ml and storedin freezer.

- 5X Sequencing Buffer,Quantity: 1.6 ml,4ºC walk-in fridge.

- DMSO (Sigma),Quantity: 0.5 ml.

II.1.1.2. Solutions

- Milli-Q water

II.1.1.3 Equipment

- Pipet-Lite Pipette SL1000 from Rainin

- mLINE pipettor 500-5000 µl from BIOHIT

II.1.2. Preparation

Preparation of the Sequencing Master Mix:

- Label a 50 ml tube with "Sequencing Master Mix BDT 0.8".

- Thaw out a 1 ml tube of BDT v3.1. (The rest will be kept at -20 ºC for future use.)

- Add every component as shown in the following table using the 5000 µl and 1000 µl pipettes with their corresponding tips:

Sequencing Master Mix for 1000 reactions

	Per unit (µl)	Total volume (ml)
dH2O	2.6	2.6
5X buffer	1.6	1.6
DMSO	0.5	0.5
BDT v3.1	0.8	0.8
Subtotal	5.5	5.5

Close the tube and mix gently.

-Positive Control: 20 Strain Even Mix Genomic Material (ATCC® MSA-1002™)(Purchase from One Codex platform).

ATCC® Microbiome Standards are mock microbial communities that mimic mixed metagenomic samples. This product comprises genomic DNA prepared from fully sequenced, characterized, and authenticated ATCC Genuine Cultures® that were selected based on relevant phenotypic and genotypic attributes, such as Gram stain, GC content, genome size, and spore formation.

Components

5% *Acinetobacter baumannii* (ATCC 17978)

5% *Actinomyces odontolyticus* (ATCC 17982)

5% *Bacillus cereus* (ATCC 10987)

5% *Bacteroides vulgatus* (ATCC 8482)

5% *Bifidobacterium adolescentis* (ATCC 15703)

5% *Clostridium beijerinckii* (ATCC 35702)

5% *Deinococcus radiodurans* (ATCC BAA-816)

5% *Enterococcus faecalis* (ATCC 47077)

5% *Escherichia coli* (ATCC 700926)

5% *Helicobacter pylori* (ATCC 700392)

5% *Lactobacillus gasseri* (ATCC 33323)

5% *Neisseria meningitidis* (ATCC BAA-335)

5% *Porphyromonas gingivalis* (ATCC 33277)

5% *Propionibacterium acnes* (ATCC 11828)

5% *Pseudomonas aeruginosa* (ATCC 9027)

5% *Rhodobacter sphaeroides* (ATCC 17029)

5% *Staphylococcus aureus* (ATCC BAA-1556)

5% *Staphylococcus epidermidis* (ATCC 12228)

5% *Streptococcus agalactiae* (ATCC BAA-611)

5% *Streptococcus mutans* (ATCC 700610)

Appendix E: Morphological characteristics of all bacterial isolates.

Morphological characteristics of bacterial isolates cultured on tryptic soy agar medium.

N°	Bacterial isolates	Colony Size	Colony color	Colony shape	Colony elevation	Colony margin	Colony surface	Colony optical property	Colony consistency	Gram stain
1	P1.1	medium	yellow	circular	Flat	entire	smooth shiny	opaque	buttery	-
2	P1.2	small	yellow	circular	Raised	entire	smooth	opaque	mucoid	-
3	P1.3	medium	yellow	circular	Convex	entire	smooth shiny	opaque	viscid	-
4	P2.1	small	light-yellow	circular	Convex	entire	smooth shiny	translucent	viscous	-
5	P2.2	small	cream	circular	Raised	entire	smooth	opaque	buttery	-
6	P2.3	small	yellow	circular	Convex	irregular	smooth shiny	translucent	viscous	-
7	P2.4	small	yellow	circular	Convex	entire	smooth shiny	translucent	viscid	-
8	P2.5	small	yellow	circular	Flat	entire	smooth shiny	opaque	viscid	-
9	P2.6	small	yellow	circular	Flat	entire	smooth shiny	opaque	mucoid	-
10	P2.7	large	brown	circular	Flat	entire	smooth shiny	opaque	mucoid	-
11	P2.8	small	yellow	circular	Convex	entire	smooth shiny	opaque	viscid	-
12	P2.9	small	yellow	circular	Flat	entire	smooth shiny	opaque	buttery	-
13	P2.10	small	yellow	circular	Convex	entire	smooth shiny	opaque	viscid	-
14	P2.11	large	brown	circular	Flat	entire	smooth shiny	opaque	buttery	-
15	P3.1	small	yellow	circular	Flat	entire	smooth	transparent	buttery	-
16	P4.1	medium	yellow	circular	Flat	entire	smooth shiny	opaque	viscid	-
17	P4.2	medium	yellow	irregular	Convex	entire	smooth shiny	opaque	viscid	-
18	P6.1	small	yellow	irregular	Flat	irregular	rough	translucent	viscid	-
19	P6.2	small	yellow	circular	Raised	entire	smooth shiny	opaque	buttery	-
20	P6.3	medium	yellow	circular	Convex	entire	smooth	translucent	buttery	-
21	P7.1	medium	light-yellow	irregular	Raised	irregular	rough	opaque	buttery	-
22	P9.1	medium	yellow	filamentous	Flat	filamentous	rough	opaque	buttery	-
23	P9.2	medium	green	circular	Convex	entire	smooth	opaque	viscid	-
24	P9.3	small	yellow	circular	Flat	entire	rough	translucent	buttery	-

25	P9.4	small	yellow	circular	Flat	entire	smooth	opaque	viscid	-
26	P9.5	medium	brown	circular	Flat	entire	smooth shiny	opaque	buttery	-
27	P11.1	medium	yellow	filamentous	Flat	curled	rough	translucent	viscid	-
28	P11.2	small	yellow	circular	Flat	entire	rough	translucent	viscid	-
29	P11.3	small	white	circular	Flat	entire	rough	translucent	viscid	-
30	P11.4	small	white	circular	Flat	entire	smooth	transparent	dry	-
31	P13.1	medium	yellow	circular	Convex	irregular	smooth	opaque	viscous	-
32	P13.2	small	brown	irregular	Flat	lobate	smooth	opaque	buttery	-
33	P14.1	large	yellow	circular	Raised	entire	smooth shiny	translucent	mucoid	-
34	P14.2	medium	cream white	circular	Flat	irregular	smooth	opaque	viscous	-
35	P14.3	small	white	irregular	Convex	undulate	smooth	translucent	buttery	-
36	P14.4	small	yellow	circular	Convex	entire	smooth	opaque	mucoid	-
37	P14.5	medium	white	filamentous	Flat	irregular	smooth	opaque	dry	-
38	P15.1	medium	white	circular	Flat	entire	smooth	opaque	buttery	-
39	P15.2	small	white	circular	Flat	entire	rough	translucent	dry	-
40	P15.3	medium	blue	filamentous	Flat	irregular	rough	opaque	buttery	-
41	P16.1	small	yellow	circular	Convex	undulate	smooth shiny	translucent	viscid	-
42	P16.2	medium	yellow	circular	Flat	undulate	smooth	opaque	buttery	-
43	P16.3	medium	green	filamentous	Flat	curled	rough	opaque	buttery	-
44	S15.1	small	white	circular	Convex	entire	smooth	opaque	buttery	+
45	S15.3	small	yellow	circular	Convex	entire	smooth, dull	opaque	buttery	+
46	E1.1	small	white	circular	Raised	entire	smooth	opaque	buttery	+
48	E2.5	small	cream	circular	Raised	entire	smooth	opaque	buttery	+
49	B1.1	medium	white	irregular	Flat	undulate	rough	opaque	buttery	+
50	B1.2	medium	white	irregular	Flat	undulate	rough	opaque	buttery	**+**
51	B1.3	small	yellow	circular	Flat	entire	rough	opaque	buttery	+
52	B2.1	small	yellow	circular	Flat	entire	smooth	opaque	buttery	+
53	B2.2	medium	yellow	irregular	Flat	undulate	rough	opaque	buttery	+
54	B2.3	small	yellow	circular	Flat	entire	rough	opaque	buttery	+

55	B3.1	large	white	irregular	Flat	undulate	dull	opaque	buttery	+
56	B3.2	medium	yellow	irregular	Flat	undulate	smooth	opaque	dry	**+**
58	B4.1	small	cream	slightly irregular	Flat	irregular	rough	opaque	buttery	+
59	B4.2	small	cream white	irregular	Raised	undulate	smooth	opaque	buttery	+
60	B5.1	medium	white	irregular	Flat	undulate	rough	translucent	buttery	**+**
61	B5.2	medium	white	irregular	Flat	irregular	rough	opaque	buttery	+
62	B6.2	medium	white	irregular	Flat	irregular	rough	opaque	brittle	+
63	B6.3	large	white	irregular	Flat	undulate	rough	opaque	buttery	**+**
64	B7.1	small	white	circular	Flat	entire	rough	opaque	buttery	+
65	B7.2	medium	white	irregular	Flat	undulate	rough	opaque	buttery	**+**
66	B8	small	orange	circular	Convex	entire	smooth	translucent	buttery	**+**
67	B10	medium	white	circular	Convex	undulate	rough	translucent	buttery	**+**
68	B11	small	orange	irregular	Convex	undulate	rough	translucent	buttery	**+**
69	B12	small	white	circular	Flat	entire	rough	opaque	buttery	**+**
70	B13	medium	white	irregular	Flat	undulate	rough	translucent	buttery	**+**
71	B14	small	yellow with dark center	circular	Convex	entire	smooth	transparent	viscid	**+**
72	B15.1	medium	white	irregular	Flat	undulate	rough	opaque	dry	**+**
73	B15.2	large	white	circular	Flat	lobate	rough	opaque	dry	+
74	B15.3	medium	orange	circular	Convex	entire	smooth shiny	opaque	buttery	+
75	B16.1	medium	cream	filamentous	Flat	filamentous	rough	opaque	dry	**+**
76	B16.2	medium	cream	irregular	Flat	filamentous	rough	opaque	buttery	**+**
77	B16.3	small	white	circular	Convex	entire	rough	transparent	dry	**+**
78	B16.4	small	yellow	circular	Convex	entire	smooth shiny	translucent	buttery	**+**

(+): positive; (-): negative

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Isolation and molecular identification of hydrocarbon degrading bacteria from oil-contaminated soil

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Abstract

The spills of hydrocarbon due to the petrochemical industry are major contaminants in the environment. Bioremediation is an effective, economical and environmentally sound treatment. The purpose of our study was to isolate, screen and identified the hydrocarbon degrading bacteria from oil polluted soil. Three oil contaminated soil were collected from Arzew oil refinery, North-west of Algeria. Sixteen bacterial strains were isolated using mineral salt media supplemented with 1% of crude oil; these isolates were screened for their best degradation abilities. Four selected bacterial strains designated as (P2.3, P2.2, S15.1 and E1.1) were identified on the basis of morphological, biochemical and molecular characterization using 16S rRNA gene sequence analysis. The sequences were compared to the closest relative species in the GenBank database of National Centre for Biotechnology Information. The growths rates of the selected isolates were determined using spectrophotometer at 600nm.Based on the partial 16S rRNA gene sequencing and phylogenetic analysis; the isolates were identified as *Pseudomonas aeruginosa* P2.3, *Achromobacter xylosoxidans* P2.2, *Staphylococcus haemolyticus* S15.1 and *Enterococcus faecalis* E1.1. Results indicated that the isolates strains had effectively utilize crude oil as sole carbon source. Linear increase in Optical Density (OD) was observed between days 4 and 10. *Pseudomonas aeruginosa* P2.3 and *Achromobacter xylosoxidans* P2.2 showed the highest growth in media with crude oil. This study indicates that the contaminated soil samples contain a diverse population of hydrocarbon degrading bacteria and these strains could be used for the bioremediation of oil contaminated soil.

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