Republic of Iraq

Ministry of Higher Education

and Scientific Research

University of Misan

College of Science

Identification of Petroleum Biodegradable Bacteria Isolated from Contaminated Soils in Misan Province / Iraq

A Thesis Submitted to

The Council of the College of Science / University of Misan In Partial Fulfillment of the Requirements for the Master Degree in Biology

By

Hiba Naeem Dhaegheem

B.Sc. Biology / University of Misan

(2017)

Supervised by

Prof. Dr. Zahid S. Aziz

Assistant Prof. Dr. Salih H. Jazza

March 2021 A.D

Sha'ban 1442 A.H

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

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صدق الله العظيم

سورة البقرة – أية ﴿32﴾

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This is certify that this thesis entitled (**Identification of Petroleum Biodegradable Bacteria Isolated from Contaminated Soils in Misan Province / Iraq**) was prepared under our supervision at the Department of Biology, College of Science, University of Misan, as partial requirement of the degree of Master in Biology.

Signature

Prof. Dr. Zahid S. Aziz

Assistant Prof. Dr. Salih H. Jazza

Department of Biology

College of Science / University of Misan

Date: 25/ 4 / 2021

Recommendation of Head of Biology Department

In view of the available recommendations, I forward this thesis for debate by the examining committee.

Signature

Assistant Prof. Dr. Maytham Abdul Kadhim Dragh

Head of Department of Biology

College of Science / University of Misan

Date: 25/ 4/ 2021

"Committee certificate"

We the examiner committee, certify that we have read this thesis entitled **(Identification of Petroleum Biodegradable Bacteria Isolated from Contaminated Soils in Misan Province / Iraq)** and examined the student **(Hiba Naeem Dhaegheem)** in its contents and in our opinion it meets the standard of a thesis for the degree of master in Biology with **(Excellent)** estimation.

Signature
Professor
Dr. Bashar Jabbar Jumih
College of Agriculture
University of Misan
Date :25/4/ 2021
(Chairman)

Signature
Assistant Professor
Dr. Waleed Muhssin Ali
College of Science
University of Misan
Date :25 / 4/ 2021
(Member)

Signature
Assistant Professor
Dr. Ahmed Abed Burghal
College of Science / University of Basrah
Date :25 / 4/ 2021
(Member)

Signature
Professor
Dr. Zahid Saadoon Aziz
College of Science
University of Misan
Date :25 / 4/ 2021
(Member and Supervisor)

Signature
Assistant Professor
Dr. Salih Hassan Jazza
College of Science
University of Misan
Date :25/4/ 2021
(Member and Supervisor)

Approved for the College Committee of Graduate Studies

Signature
Assistant Professor
Dr. Sabeeh Jasim Gatea
Date : / / 2021
(Dean)

Acknowledgments

First of all, great thanks for Allah the most merciful the most compassionate, who gave me health, strength, and facilitated the ways for me to accomplish this work, and the prayer and peace of Allah be upon our Master and prophet Muhammad and his divine good family, who guided us to get out of darkness of nescience.

It is a pleasure to express my deep appreciation to my supervisors Professor **Dr. Zahid S. Aziz** and Assistant Professor **Dr. Salih H. Jazza** for highly inspiring guidance, encouragement and support for completing this thesis.

I offer my thanks and appreciation to all members of the Deanery of College of Science /University of Misan and Biology Department for their kind cooperation.

I would like to express my sincere thanks to M.Sc. **Shaima R. Banoon** (College of Science) and Assistant Instructor **Salah M. Salih** (Marine Sciences Center), who really took pains in helping me for completing my work.

My great thanks are going to Mr. **Hassan Al-Shawi** at Nahran Omar Laboratories in Basrah Oil Company, for help me in achieving the sampling analysis in Gas Chromatography.

Also I would like to recognize the positive efforts and invaluable assistance to the staff members of Misan Oil Company in Misan Province for facilitating my work.

Finally, I am sincerely indebted and grateful to my father and my mother, who was a great supporter for me during the most difficult circumstances that I faced, also I would like thank my dear brother Munther and my aunt for support me, and a great tribute with my deep apology to all whom I have not mentioned with my respect.

Hiba

Dedication

To the candles of my life, to the lights by

their inspiration I see my way to the best

my father & my mother

To the ones who stand by me and support

me all my life

my aunt

To those who share each moment of happiness

and sadness as well, to the soul partners

my brothers & my sister

To those who have given me their time,

patience and knowledge

my supervisors & everyone is eager for knowledge

Summary

Oil contaminated soil samples were collected from three differences sites in Misan Province included Bazerkan refinery, South Oil Company and PetroChina Company during November (2019). The chemical and physical properties were determined of oil contaminated soil such as color, temperature and pH, as well as an estimated of aliphatic hydrocarbons (n-alkanes) and aromatic hydrocarbons (polycyclic aromatic hydrocarbons) concentration.

The results were showed varying degrees in color where it graded from brown to black, It is also showed a differences in temperature where ranged from 28 ^{o}C - 30 ^{o}C , while pH values ranged from 7.43- 7.98 for the sites of PetroChina Company, Bazerkan refinery and South Oil Company respectively.

The study also showed there are a differences in the concentration of hydrocarbon components in the oil contaminated soil which analyzed by Gas chromatography, by it is containment on a mixture of n-alkanes and Polycyclic aromatic hydrocarbons. The results were recorded the rate of n-alkanes concentration for these contaminated soils which ranged from 14180.85- 66644.43 µg/gm dry weight, while the rate of Polycyclic aromatic hydrocarbons concentration ranged from 1361.24- 4106.503 µg/gm dry weight.

Oil-biodegradable bacterial strains were isolated from oil contaminated soil samples. Twenty five bacterial isolates were characterized at biochemical levels by ordinary and standard bacteriological tests. The isolates were further identified by the Vitek-2 system and the molecular analysis. Sixteen out of twenty five bacterial isolates were tested their ability to degrade crude oil.

Molecular identified were represented by polymerase chain reaction (PCR) was performed to identify bacterial isolates, where an universal primer for partially amplification of 16s rDNA gene which gave amplification product 1500bp, also universal primer which partially amplify the 16s rDNA gene of *Pseudomonas sp.* had been used to yield amplification product 150bp and their nucleotide sequences were studied in the National Center for Biotechnology Informations (NCBI) by using

the Basic Local Alignment Search Tool (BLAST). The results were revealed the identification of 13 bacterial isolates, 12 by using first universal primer which were (*Bacillus subtilis, Bacillus safensis, Bacillus pumilus, Bacillus sporothermodurans, Sphingomonas paucimobilis , Novosphingobium subterraneum, Brevundimonas sp., Arthrobacter luteolus, Sporosarcina luteola , Acinetobacter junii, Acinetobacter baumannii and Aeromonas salmonicida*) and 1 bacterial isolate was identified as *Pseudomonas putida* by using the second universal primer. Bacterial isolates were recorded in GenBank under different accession numbers (Mk501608.1, KF751673.1, Fj763645.1, EF590133.1, Mk 729043.1, Ks783591, En 430991.1, Mk774b245.1, Kr 999939.1, Kt 984874.1, Kx622562.1, Kj958271.1, Mn589774.1, Kj573537.1, Ky820912.1, Mt730013.1, MK418695.1, Mk829514.1, MK418695.1, Jx6499224.1, Mg 551868.1 and Kx989239.1) which might be for the first time in Iraq. While the others three bacterial isolates (*Pseudomonas aeruginosa, Kocuria kristinae and Pantoea sp*.) were identified depending on morphological characterization and Vitek-2 system.

The results were exhibited in according to the several diagnosis methods used for isolation and identification of bacterial strains from oil contaminated soil that Gram negative bacteria were the most bacterial strains in contaminated soil.

The optimum conditions for growth of oil-degradable bacteria such as pH were ranged from (6-9). The bacterial isolates were showed their ability to grow at temperature ranged from 24-42 ^{o}C , while a few bacterial strains (*Pseudomonas aeruginosa, Brevundimonas sp., Sphingomonas paucimobilis, Novosphingobium subterraneum, Kocuria kristina, Aeromonas salmonicida and Acinetobacter junii*) were also able to grow at 46^{o} C. The optimum growth of these bacterial isolates were at 24^{o} C, 28^{o} C and 37^{o} C, except *Brevundemonas sp.* and *Bacillus sporothermodurans* were also showed optimum growth at 32^{o} C, as well as *Pseudomonas aeruginosa* and *Novosphingobium subterraneum* were also showed optimum growth at 32^{o} C and 42^{o} C.

All the sixteen bacterial isolates were grown on mineral salt media (MSM) supplemented with 0.5% crude oil at three incubation periods (7, 14 and 21) days were showed positive biodegradation of crude oil which composed from n-alkanes and Polycyclic aromatic hydrocarbons.

Therefore, the increasing in the optical density for bacterial growth that measured by spectrophotometer at OD_{620} were indicated to the ability of bacteria to using the hydrocarbons in crude oil as sole carbon and energy sources.

The study showed the ability of bacterial strains to degrade all compounds of crude oil gradually starting from LMW at the first weekly incubation periods (7 days) followed by degradation of HMW at the end periods of incubation (14 and 21 days).

The Gas-chromatography analysis results were exhibited the degradation of n-alkanes by 16 bacterial strains as follows :

Bacillus safensis which degrade 36.25%, 67.47% and 76.51%, *Bacillus subtilis* (71.61%, 79.23% and, 83.39%) , *Bacillus pumilus* (44.92%, 71.76% and 74.54%), *Bacillus sporothermodurans* (57.5%, 71.39% and 77.91%), *Pseudomonas aeruginosa* (69.49%, 76.59% and 84.66%), *Brevundimonas sp.* (62.09%,76.23% and 84.45%), *Arthrobacter luteolus* (53.41%, 73.39% and 77.76%), *Pseudomonas putida* (63%, 77.11% and 84.21%), *Pantoea* sp. (64.63%, 76.36% and 86.3%), *Sphingomonas paucimobilis* (43.1%, 64.01% and 79.63%), *Novosphingobium subterraneum* (70.36%, 76.48% and 78.18%), *Sporosarcina luteola* (69.57%, 74.57% and 87.28%), *Kocuria kristinae* (71.78%, 75.06% and 82.69%), *Aeromonas salmonicida* (68.57%, 69.85% and 77.98%), *Acinetobacter junii* (65.17%, 68.98% and 71.9%) and *Acinetobacter baumannii* (68.35%, 78.97% and 89.18%), this degradation for n-alkanes during weekly incubation periods (7, 14 and 21) days respectively.

While the Gas-Chromatography analysis results were exhibite the degradation of Polycyclic aromatic hydrocarbons by 16 bacterial strains as follows :

Bacillus safensis which degrade 52.96%, 60.13% and 80.85%, *Bacillus subtilis* (63.27%, 85.37% and 85.99%), *Bacillus pumilus* (60.94%, 78.72 %and 87.31%), *Bacillus sporothermodurans* (61.79%, 74.77% and 78.35%), *Pseudomonas aeruginosa* (66.42%, 84.31% and 90.93%), *Brevundimonas* sp. (44.30%, 79.84% and 91.18%), *Arthrobacter luteolus* (40.32%, 73.70% and 86.67%), *Pseudomonas putida* (51.84%, 80.43% and 88.84%), *Pantoea sp*. (74.42%, 78.23% and 88.04%), *Sphingomonas paucimobilis* (56.5%, 83% and 93.39%), *Novosphingobium subterraneum* (43.57%, 83.76% and 92%), *Sporosarcina luteola* (65.1%, 80.74% and 85.82%), *Kocuria kristinae* (69.65%, 86.24% and 87.78%), *Aeromonas salmonicida* (71.95%, 73.94% and 87.55%), *Acinetobacter junii* (70.19%, 74.49% and 85.23%) and *Acinetobacter baumannii* (63.95%, 78.16% and 81.43%), this degradation for Polycyclic aromatic hydrocarbons during weekly incubation periods (7, 14 and 21) days respectively.

Finally, this study showed that the high percentage of the degradation rate of n-alkanes at the end of the periods of incubation (21 days) occurred by *Acinetobacter baumannii* (89.18%), while the high percentage of the degradation rate of PAHs at the end of the periods of incubation were reported by *Sphingomonas paucimobilis* (93.39%).

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List of Abbreviations

Abbreviations	Key
PAHs	Polycyclic aromatic hydrocarbons
TPH	Total petroleum hydrocarbons
GC	Gas Chromatography
LMW	Low Molecular Weight
MMW	Medium Molecular Weight
HMW	High Molecular Weight
CFU	Colony Form Unit
MSM	Mineral Salt Medium
OD	Optical Density
NCBI-BLAST	National Center for Biotechnology Information-Basic Local Alignment Search Tool
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
-ve	Gram negative
+ve	Gram positive
bp	base pair
rpm	rotation per minute
UV	Ultra Violet
d.w	dry weight
µg/gm	Microgram/gram
µl	Microliter
ml	Milliliter
N	Normality

CHAPTER ONE

INTRODUCTION

&

LITERATURE REVIEW

1.1 : Introduction :

At the present time, the increasing environmental pollution with toxic and dangerous materials has become a global problem, as the spontaneous and routine spills of petroleum-derived compounds pollute the groundwater, seas, rivers, air and soil (Abha and Singh, 2012 ; Okoh *et al*., 2020). One of the most common environmental pollutants in the soil are petroleum hydrocarbons due to the increased use of petroleum as a source of energy (Yu *et al*., 2011). Hall *et al*. (2013) indicated that petroleum hydrocarbons are veritable and important source in society, especially in the form of crude oil in relation to development and economic growth from energy perspective, it produces and its industrial importance.

The accumulation of hydrocarbons in the soil causes many serious problems that negatively affect the balance and stability of the ecosystem and may cause harm to animals and humans (Chekroun *et al*., 2014). It may lead to delayed plant growth, soil fertility and changes in soil physicochemical and microbiological properties.

There are several methods were developed to clean the sites contaminated with hydrocarbons, including incineration of hydrocarbon pollutants, as well as the development of large-scale boring stations (Al-Majed *et al*., 2012 ; Lim *et al*., 2016). Although the soil treatment period with these methods is a short period but the system requires large amounts of heavy oils in addition to the need to use huge machines (Ivshina *et al*., 2015).

Crude oil occurs naturally and is a complex mixture of non-hydrocarbon compounds and hydrocarbon compounds of appropriate concentration possessing measurable toxicity to living system (Nwakanma *et al*., 2016). Adams *et al*. (2015) explained that the toxicity of petroleum products or crude oil varies widely depending on environmental factors, the nature of formation and concentration of compounds, as well as depending on the biological state of bacteria at the time of contamination, which sparked the attention of researcher to study the field of microorganisms and learn about the role of bacteria in reducing the damage caused by soil pollution (Van Agteren *et al*., 2013). Ghosal *et al*. (2016) showed the advantage of soil contaminated with

hydrocarbons is that these compounds are toxic and mutagenic such as aromatic substances or polycyclic compounds.

To removal petroleum hydrocarbons from the soil, biological treatment techniques were developed using strategies to provide nutrients, ventilation and moisture to improve bacterial activity in pollutant degradation (Chorom *et al*., 2010 ; Fuentes *et al*., 2014). Many types of bacteria were analyzed and isolated that capable of degrading petroleum hydrocarbons biologically, in order to build vital treatment system and use it as a source of energy and carbon (Xu *et al*., 2018).

The most important bacterial species that isolated from oil-contaminated soil which capable of biodegradable of petroleum are *Pseudomonas* sp., *Bacillus* sp., *Acinetobacter* sp., *Flavobacterium*, *Aeromonas* sp., *Micrococcus* sp., *Staphylococcus* sp., *Vibrio* and *Xanthomonas* (Abdul-Ameer, 2019).

The specificity of the biodegradation process of petroleum hydrocarbons is associated with the genetic potential of the biodegradable bacteria for introducing oxygen into the hydrocarbons and generating intermediates which subsequently enter the general metabolic pathway of the energy-generating cell (Millioli *et al*., 2009). With the presence of carbon sources, the bacteria produce the enzymes responsible for attacking the hydrocarbon molecules, many of which are involved in breaking down the hydrocarbons present in the oil, but the lack of appropriate enzymes will act as a barrier or prevent any attack to complete the biodegradation of the hydrocarbons (Peixoto *et al*., 2011 ; Parthipan *et al*., 2017b).

Among the general of bacteria were studied in terms of developing its susceptibility to biodegradation, are *Pseudomonas* sp., *Mycobacterium* sp., *Beijerinkia* sp., *Sphingomonas* sp., *Terrabacter* sp. and *Staphylococcus* sp. (Obayrori and salam, 2010). Kadri *et al*. (2018) suggested that *Alcanivorax borkumensis* is a potential hydrocarbon-degrading bacterium with higher enzymatic capacities for biodegradation of hydrocarbon-polluted environment.

Crude oil degradation processes are ampacted by several factors such as diesel bioavailability, bacteria species, temperature, nutrients availability, oxygen availability, salinity and pH, it will be very useful to

achieve a high removal efficiency and the optimum condition for bacteria are highly correlated with these factors for live in the contaminated environment (Jiang *et al*., 2016 ; Xu *et al*., 2018).

However, the proper and correct performance of bacteria in degradation of hydrocarbons biologically is mainly dependent on the availability of appropriate environmental factors (Akpe *et al*., 2013). In recent year, attention to biodegradation of petroleum pollutants has increased using appropriate and sustainable methods as human kind strives to clean polluted environments (Koukkou, 2011). Ye *et al*. (2017) indicated that the most environmentally safe means of disposal of petroleum pollutants soil is the application of biological techniques compared to other physical, chemical and mechanical methods.

The aim of this study :

In view of the absence or lack of studies in Misan province on the above-mentioned information to find a solution to treatment the soil pollution problem with petroleum compounds, this study was aimed to :

1- Biochemical and Molecular characterization of oil biodegradable bacteria.

2- Determine the ability of bacteria to remove petroleum compounds from soil.

3- Estimation the degree of bacterial activity in biodegradation of crude oil .

4- Determine some factors which impact on the bacterial biodegradation.

1.2 : Literature Review

1.2.1 : Oil Pollution :

Oil pollution is one of the main issues nowaday, which generated the interest of many researchers (especially after the major disasters to which the soil was exposed and caused by oil spills or Leak) for study of type of environmental pollution represented by the presence of hydrocarbons produced from oil or its derivatives (Adekunle and Adebambo, 2007 ; Diyauddeen and Wandaud, 2011 ; Gargouri *et al*., 2011). Sivagamasundari and Jeyakumar (2018) mentioned that oil pollution is one of the most important factors of environmental pollution known today, because it causes a great danger to the environment, which is difficult to control especially in the soil, because of its wide impact on all aspects of life. It was considered the main cause of petroleum hydrocarbon contamination is the presence of heavy molecular weight branching in twenty carbon or more atoms and the presence of polycyclic aromatic hydrocarbons (PAHs) (Bidoia *et al*., 2010). Husain (2008) indicated that these compounds remain for a long time in the soil, because they are highly resistant to degradation which are due to their low solubility in water. The period of petroleum hydrocarbons remaining in the soil depends on their concentration, nature, composition and susceptibility to biodegradation which are among the most prominent determinants of the toxicity of these compounds (Vanishree *et al*., 2014 ; Wuana *et al*., 2014) . The long stay in the soil leads to imbalance of the ecosystem (Tetteh, 2015). Barnier *et al*. (2014) and Wu *et al*. (2014) mentioned that oil pollution affects biological diversity with the decrease in hydrocarbon sources in the soil over time, for this reason soil pollution by oil was considered one of the most worrying forms of pollution (Pinheiro *et al*., 2013).

1.2.2 : Source of oil pollution:

The sources of petroleum hydrocarbons that enter the environment are among the most important of which are the geological factors represented by the presence of source rocks at suitable depths under the surface of the rich hydrocarbons so the ground temperature is sufficient to form oil from them (Caineng *et al*., 2013). The presence of dense forests that occupied some places on the surface of the earth and as a result of the factors of erosion, they were covered by ground layers which led to their decomposition and turning them into oil (Lutgens *et al*., 2014).

The large increase in oil consumption in the world, especially after the development of technology in the twentieth century, led to soil pollution in oil as a result of the spills that occur during the routine processes of producing, distributing and refining crude oil (Panda *et al*., 2013). Where it is extracted in large quantities from the land and then transferred to different regions through land or sea transport, or by long pipes to different regions (Hammadi, 2014). The fact that hydrocarbons are the primary energy sources in the various industries of many chemical products and compounds have increased the demand for petroleum products, which has led to a great exposure of the earth at the environmental level to hydrocarbons (Odell, 2013). Hu *et al.* (2013) pointed out that the increase in pollution is caused by several sources, including manufacturing, extraction, drilling, refining, and transportation, all of which are potential sources of environmental pollution.

The worrying of environmental scientists, governments and societies worried about the environmental pollution resulting from the oil spill, it has become a very common thing that occurs constantly through the failure and leakage of oil from the transport pipelines (lkuesan, 2017). Mukred *et al*. (2008) explained the difference of the oil components in volatility and susceptibility to biodegradation, as he noticed that some hydrocarbon compounds resist degradation, some of them degrade easily and others that are not degradable, which increases the risk of their presence in the soil.

1.2.3 : Composition of oil

Petroleum is a dark viscous liquid consisting mainly of a complex mixture of organic compounds consisting of aromatic and aliphatic hydrocarbons in addition to some mineral organic components as shown in figure (1-1) (Hamsavathani *et al*., 2015). Mancera-López *et al*. (2008) Found that the petroleum hydrocarbons as a complex mixture TPH included 21% of polycyclic aromatic hydrocarbons and 40% of aliphatic hydrocarbons. Polycyclic aromatic compounds are harmful chemicals consisting of two or more benzene rings incorporated in a cluster, linear, or angular arrangement (Juckpench *et al*., 2012). Kumar *et al*. (2011) classify it depending on the number of rings that contain small aromatic compounds and large aromatic compounds, where the latter contains more than six aromatic rings, while the small is composed of six intense aromatic rings and these are more soluble and dissolvable than the large rings.

As for aliphatic hydrocarbons, they are composed of hydrogen and carbon, which can be cyclic, branched, linear, saturated or unsaturated. There are many types of aliphatic hydrocarbons such as alkanes, alkynes and alkenes (Mahjoubi *et al*., 2018). Al-Taee *et al*. (2017) indicated that they are among the basic ingredients of crude oil, alkanes are one of the most abundant and common ingredients in crude oil. Aliphatic hydrocarbons are divided into four groups depending on their molecular weight which are gaseous alkanes, aliphatic hydrocarbons with a high molecular weight consisting of more than 28 carbon atoms, as for 17-28 carbon atoms, they are medium molecular weight. Finally, aliphatic hydrocarbons with low molecular weight containing 8-16 Carbon atom (Erdogan *et al*., 2012).

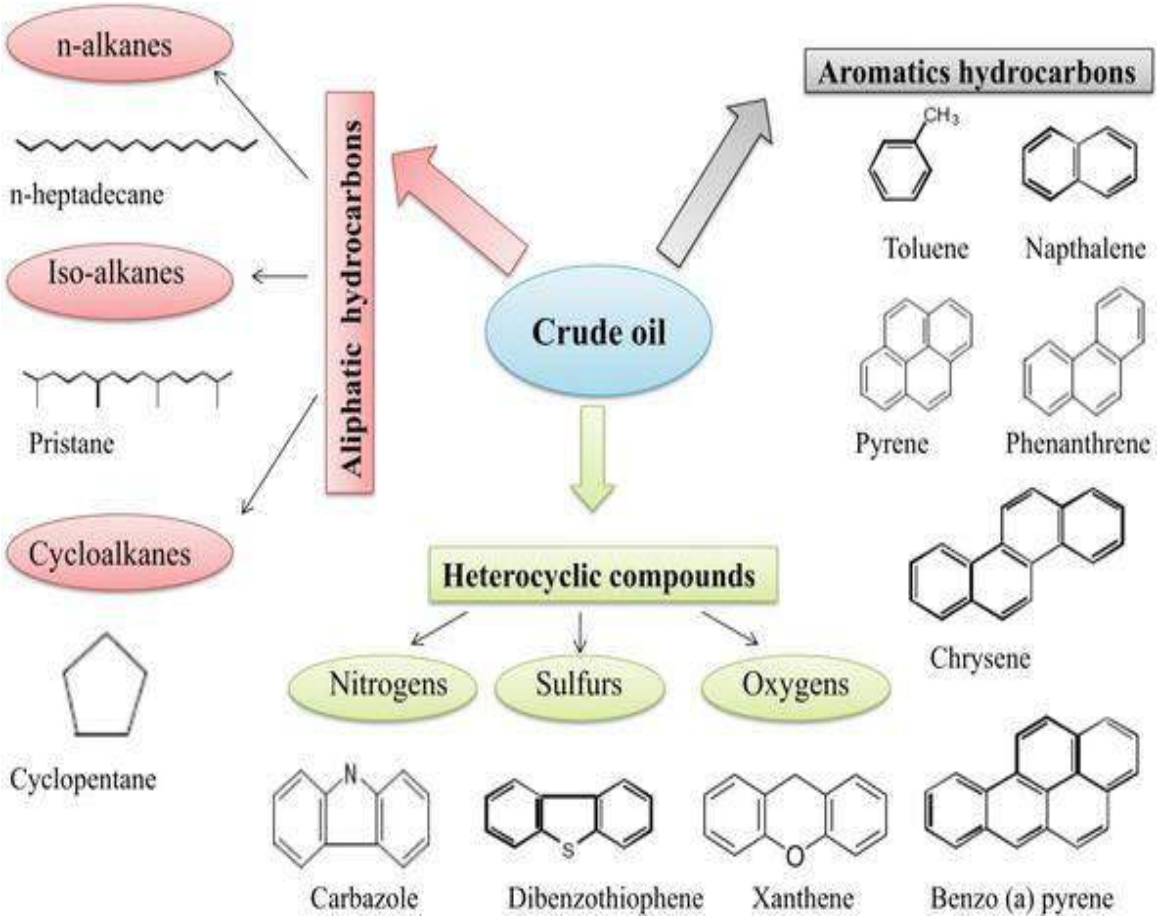


Figure (1-1): Different classes of hydrocarbon (Mahjoubi *et al*., 2018).

1.2.4 : Impact of oil pollution on living organisms :

Oil spills or leaks affect the soil, causing significant damage to the ecosystem (Han *et al.*, 2016). Because petroleum hydrocarbons are toxic to living organisms (Eze *et al.*, 2014). Ikuesan (2017) instructed that contamination of the soil with oil leads to sterility of the soil and consequently changes in its composition, its microbiological and physicochemical properties, which causes delayed growth of plants. As a result of the loss of soil fertility and its ability to penetrate and retain water (Moorthi *et al*., 2008). These impacts resulting from the oil spill lead to a decrease in agricultural productivity, which results in negative impacts on people's lives in economic terms (Chorom *et al*., 2010).

It was noted that many human risks are related to the spread of hydrocarbons, in fact it was reported by Tormoehlen *et al*. (2014) that acute exposure to hydrocarbons generates many diseases including dermatitis, arrhythmia, acidosis and encephalopathy. As for what concerns the carcinogenic effects by some types of petroleum hydrocarbons were specific, but it was observed during the studies that it increases in working people associated with petroleum, including liver, stomach, bladder and lung cancer, in addition to noticing some neurological and reproductive effects (Latif *et al*., 2010 ; Chandra *et al*., 2013). Aromatic hydrocarbons as a result of their carcinogenic activity have been included in the lists of priority pollutants in the Environmental Protection Agency and the European Union (Luch, 2005). Human exposure to them occurs in three ways, such as skin contact, inhalation and consumption of contaminated foods, which constitute a rate ranging between 88-98% of pollution methods and this shows that the main source of human exposure to these pollutants is the diet (Rey-Salgueiro *et al*., 2008).

As for the effect of aliphatic hydrocarbons, their presence in the soil results in oily spots that limit the exchange of nutrients and oxygen in the soil (Wasmund *et al*., 2009 ; Militon *et al.*, 2010). It may also affect the human nervous system, causing loss of consciousness, headache, dizziness, temporary limb paralysis, limb numbness and fatigue (Adgate *et al.*, 2014). Perelo (2010) indicated that applying the optimal treatment to oiled sites will reduce the risks of pollutants to the environment and human health.

1.2.5: Fate of oil pollution in environment :

Petroleum hydrocarbons, upon entry into the environment are subject to a variety of processes represented by chemical, physical, a biotic, and biological processes, all of it called by weathering factors through interaction with microorganisms and metabolic pathways (Hassanshahian and Cappello, 2013 ; Abdel-Shafy and Mansour, 2016). Weathering processes include optical oxidation, dissolution, evaporation, emulsification, dispersion, diffusion and biodegradation (Jain *et al.*, 2011). Mishra and Kumar (2015) and Esbaugh *et al.* (2016) Indicated that the level at which the different hydrocarbon components deteriorate

depends largely on weathering factors on the chemical and physical properties of hydrocarbons, their nature and composition.

The biodegradation process is the best among other physical and chemical weathering processes, because the latter is very expensive due to the cost of drilling and transporting pollutants in large quantities outside the site for treatment (Erdogan *et al.,* 2012). As for the biodegradation process, it is cost-effective in addition to being environmentally friendly, effective and appropriate in removing pollutants and cleaning the environment from hydrocarbons (Borah and Yadav, 2016). Varjani and Upasani (2016) mentioned the biodegradation process was considered one of the main weathering processes that depended on the availability of bacteria and its activity in addition to its dependence on the type of hydrocarbons and their components. This process indicates that the pollutants are destroyed, removed, or analyzed into less harmful or harmless substances, because they reduce the migration of oil pollutants, their transmission and spread to non-polluted places (Liu *et al*., 2019). Through this process, the risk they pose to the environment is reduced, but without changing the toxicity of pollutants (Yuniati, 2018).

1.2.6 : Distribution of bacteria in soil :

Bacteria possesses the ability to grow easily in a wide range of environmental conditions, in addition to having a very interesting metabolic capacity that greatly assisted in the widespread distribution of bacteria in the biosphere and this is extremely important to benefit from the nutritional diversity of bacteria in the biological decomposition of pollutants for the purpose of obtaining energy and biomass production in the biodegradation process (Tang *et al.,* 2007 ; Abatenh *et al.,* 2017). Hammad *et al.* (2015) Note the wide spread of bacteria in the soil when discovering many types of bacteria that have the ability to use soil hydrocarbon contaminated as a source of carbon and energy, But despite this widespread of bacteria in the soil. Soil properties are known to have a strong influence on the underground microbial communities, notable soil pH, nutrient availability like organic carbon and nitrogen, as well as soil texture. The ability to biodegrade varies from one environment to another

due to the fact that some environments are more sensitive than others to the effects of oil pollution and that these differences are mainly calculated based on the different requirements of the analyzed bacteria (Anyanwu *et al*., 2011).

Societies exposed to hydrocarbons have become adapted to genetic changes and selective fertilization, which leads to increased levels of bacteria that have the ability to analyze hydrocarbons, as well as increased bacterial plasmids that symbolize hydrocarbon genes (Quartrini *et al.*, 2008). The main reason for the presence of bacteria everywhere is due to their small size in relation to their large surface area and their possession of an active biomass, which is larger than any other group of living organisms, which gives them the ability to compete with other living organisms for dissolved compounds (Kirchman, 2008 ; Alexopoulos *et al.*, 2013). Spini *et al.* (2018) Was able to isolate many optional anaerobic, facultative anaerobic and aerobic bacteria in oil spill sites and soil contaminated with hydrocarbons using molecular and culture-based technology. In addition to relying on high and low temperatures, the base and acidic environment, as well as relying on high concentrations of pressure and salinity (Tang *et al.*, 2012 ; Scheduler *et al.*, 2014).

1.2.7 : Common of biodegradable bacterial species

Many bacterial species found in oil-contaminated soil have been studied and discovered, which have adapted to the surrounding environmental conditions to be able degraded oil and thus remove it from the soil and treat it, from this species such as *Burkolderia fungorum, Ralstonia mannitolilytica, Alcaligenes aquatilis, Variovorax paradoxus, Acidovorax delafieldii ,Moraxella* osolensis*, Acinetobacter calcoaceticus, Pseudomonas vancouverensis, Ochrobactrum pseudintermedium, Gordonia polyisoprenivorans, Mycobacterium vanbaalenii, Mycobacterium gilvum, Klebsiella pneumoniae* (Darmawan *et al.*, 2015).

Some researchers have relied on the biochemical test by Vitek 2 system and on molecular diagnostics by 16S r DNA sequencing to diagnose and identify bacterial species from oil-contaminated soil, such as *Ochrobactrum sp., Sphingomonas thalophilum, Stenotrophomonas*

maltophila, Pseudomonas aeruginosa, Pseudomonas putida, P*seudomonas geniculata, Pseudomonas hibiscicola* (Varjani *et al.*, 2015).

There were many studies on the bacterial strains capable of biodegradation of oil, as some studies dealt with the study of bacteria in general, and other research was limited to studying the role of one or two species of bacteria and their ability to degraded petroleum, where Khalifa (2017) discovered the role of *Kocuria sediminis* bacteria while Parhamfar *et al.* (2018) studied the role of both *Alcanivorax* and *Idiomarina* bacteria , as well as Godini *et al.* (2019) was able to isolate seven bacterial species and studied their ability to degrade petroleum, these species are *Stenotrophomonas maltophilia, Thermomonas koreensis, Achromobacter pulmonis, Pseudomonas stutzeri, Achromobacter sp., Azospirillum brasilense, Brevibacillus brevis.*

Solomon *et al.* (2018) was able to isolate 26 types of bacteria from oil-contaminated soil, *Pseudomonas sp., Micrococcus sp., Corynebacterium sp., Staphylococcus sp., Achromobacter sp., Klebsiella sp., Serratia sp., Arthobacter sp., Bacillus sp., Proteus sp., Lactobacter sp., Citrobacter sp., Alcaligenes sp., Acentobacter sp., Flavobacterium sp., Nocardia sp., Mycobacterium sp., Aquitalea sp., Shewanella sp., Halomonas sp., Brevundimonas sp., Rhodococcus sp., Sphingobacterium sp., Erwinia sp., Azospirillum sp., Gordonia sp.*

There are several studies about biodegradation by bacteria in different province in Iraq. Shlimon *et al*. (2020) study microbial community composition in crude oil from the Kurdistan region of Iraq, while Abd-Alridha in his work (2014) isolated nine polycyclic aromatic hydrocarbons PAHs degrading bacteria from oil field contaminated soil samples at Basrah city and automobile workshop sites at Babylon city. *Pseudomonas aeruginosa* and *Bacillus* sp. were isolated from fule station in Hilla city by Al-Alaq *et al*. (2016) which showed the ability to degrade crude oil.

Enterobacter cloacae, Staphylococcus aureus, Sphingomonas paucimobilis and Pantoae sp. were isolated from oil contaminated soil samples at Al-Dura oil refinery and the local generators in Baghdad city by Mohammed *et al*. (2017).

1.2.8: Role of bacteria in biodegradation :

Soil remediation requires the availability of various mechanical and chemical methods that include soil washing, solvent extraction, soil vapor extraction, air avoidance, stabilization and hardening, packaging, pumping and treatment technology and other techniques for repairing contaminated sites (Varjani, 2017). Despite the multiplicity of techniques for treating pollutants, the process of biodegradation using bacteria is the most effective way to fully mineralize pollutants and eliminate their danger and toxicity (McGenity *et al*., 2012). On the other hand, the hydrocarbon decomposition process is a relatively complicated process by bacteria, where it first takes the hydrocarbons and then converts them from inactive molecules to more active molecules through metabolism (Abbasian *et al*., 2015).

Geetha *et al*. (2013) Indicated that petroleum hydrocarbons can be decomposed by many microorganisms including bacteria, fungi and yeast, though bacteria have the most important role the central role in hydrocarbon decomposition. The bacteria have a specialized metabolic capacity to break down the hydrocarbons in the polluted soil into nutrients and organic matter for use as a single source of carbon and energy, in addition to their abundance and great ability to analyze hydrocarbons (Sivagamasundari and Jeyakumar, 2018). Ghosal *et al*. (2016) and Jiao *et al*. (2016) emphasized the occurrence of the natural decomposition process naturally by bacteria already present in the contaminated environment, in addition to the possibility of using the microbial pollination method to increase the abundance of bacteria and accelerate the process of biodegradation, because the survival of the pollutants or their decomposition depends on the factors available, including growth and presence of bacteria Within the polluted area and the extent of the decomposition of petroleum substances, as well as the factors surrounding the polluted area, the availability of nutrients that increase the ability of bacteria to analyze the pollutants present in the soil and other influencing factors. Khan *et al*. (2018) indicated that the speed and quantity of hydrocarbon compounds in petroleum depend on factors that differ in their proportions, types and nature, and also the degree of toxicity of hydrocarbons has a role in their decomposition.

1.2.9: The Role of bacterial Enzymes in biodegradation :

Bacteria possesses an enormous ability to analyze oil biologically is considered one of the most prevalent microorganisms prevalent in this field, because it contains various enzyme genes that enabled it to consume and break down oil hydrocarbons dynamically (Karigar and Rao, 2011). One of these enzyme is catechol 2,3-dioxygenase and alkane hydroxylase such as monooxygenase, which is one of the main enzymes possessed by many species of bacteria that have role in the analysis of aromatic and aliphatic hydrocarbons, respectively (Parthipan *et al.*, 2017a ; Muthukamalam *et al.*, 2017). In addition to owning bacteria, special decomposition enzymes such as hydroxylases and oxygenase are add (Atlas and Philp, 2005). Also, the honrizontal transfer of genes is one of the main mechanisms in bacteria responsible for improving the biodegradation process of hydrocarbons (Shahi *et al.*, 2017). Kumar *et al.* (2011) indicated that for bioremediation to be effective, bacteria must attack contaminated hydrocarbons in an enzymatic fashion to dispose of them by converting them into harmless products. Zeyaullah *et al.* (2009) explained that the decomposition of pollutants enzymatically depends on three things, namely the production of the enzyme in sufficient quantities and the ability of the enzyme to stimulate a reaction to the decomposition and the arrival of the compound to the enzymes, because the ability of bacteria to analyzed polluted hydrocarbons depends on the contact of these pollutants with the enzyme or a series of enzyme.

1.2.10 : Mechanism of microbial degradation:

The biodegradation mechanism of petroleum hydrocarbons is mostly done through enzyme- specific decomposition mechanisms that involve anaerobic decomposition mechanisms (in the absence of oxygen) and aerobic decomposition mechanisms (with oxygen) (Das and Chandran, 2010 ; Hu *et al.*, 2016 ; Varjani and Upasani, 2016). Anaerobic bacteria, in the absence of oxygen, are not able to degrade the organic matter, especially those are buried under the surface of the earth (Sherry *et al.*, 2013). However, anaerobic bacteria have the ability to carry out the process of decomposition through several chemical reactions, including the reduction reactions represented by reducing nitrates to nitrogen (N_2) as well as reducing sulfates to sulfide gas (H_2S) (Rabus *et al.*, 2016). As for the presence of oxygen, the bacteria analyze and break down organic

matter through aerobic decomposition , or the so-called aerobic breath, which the rate of biodegradation is faster compared to anaerobic analysis (Cao *et al.*, 2009).

Oxygen consists of two types of enzymes that belong to the group of oxidizing enzymes, which are monooxygenases and dioxygenases enzyme, depending on the number of oxygen atoms required for oxidation to occur in aerobic biodegradation as shown in figure (1-2) (karigar and Rao, 2011). Arora *et al.* (2010) indicated that these oxygenases enzymes transport oxygen and use with nicotinamide adenine dinucleotide phosphate, nicotinamide adenine dinucleotide or flavinadenine dinucleotide for oxidation and break down organic compounds through the adhesion of enzyme to these compounds that lead to increased susceptibility to reaction and dissolution in water. Yadav *et al.* (2018) explain that monooxygenases can work in the biodegradation process as biostimulants that have stereotoxic selectivity on different substrates, as well as have high selectivity for the reagion. Therefore, it can be used to stimulate various reactions, the most important of biodegradation and biotransformation of aromatic and aliphatic hydrocarbon compounds (Arora *et al.*, 2010). The final product of the aliphatic and aromatic compounds decomposition process in aerobic degradation water and CO_2 (Moneke and Nwangwu, 2011).

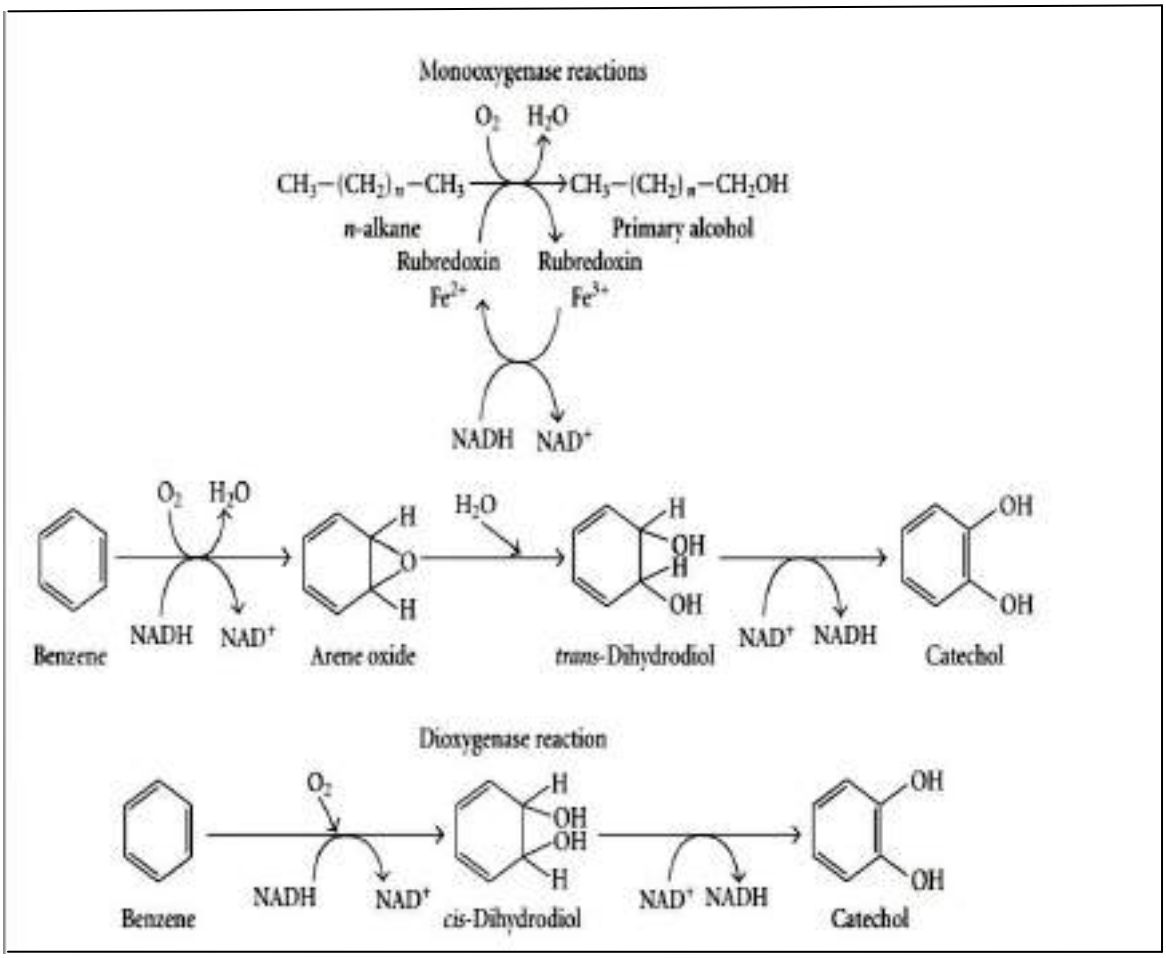


Figure (1-2): Enzymatic reactions involved in the processes of hydrocarbons degradation (Das and Chandran, 2011).

1.2.10.1 : Aerobic microbial degradation of aromatic hydrocarbon :

Polycyclic aromatic hydrocarbons are pollutants everywhere in the atmospheres (Chen *et al.,* 2013). It has a wide spread in the various ecosystems that contribute to the continued presence of these compounds in the environment (Kim *et al.,* 2013). It is considered one of the most dangerous environmental pollutants in the soil and its derivatives resulting from incomplete combustion of organic materials from human activities that carry the ability to stay for a long time while resisting degradation (Geetha *et al.,* 2013 ; Farzadki *et al.,* 2014). The fate of their presence in the environment is directly related to the biological and

abiotic processes represented by oxidation, bioaccumulation and degradation by bacteria (Zeng *et al.,* 2010).

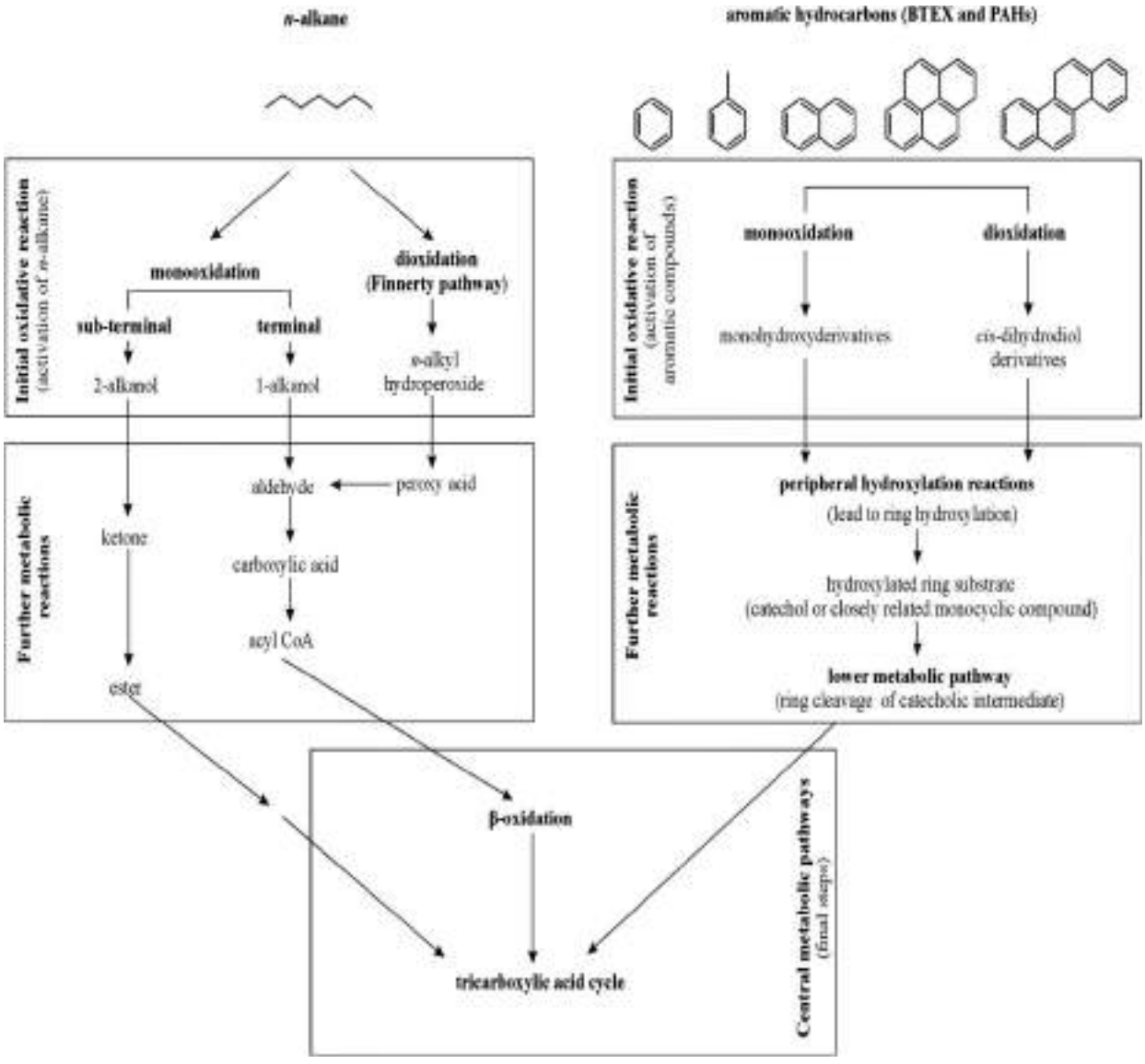
The biodegradation of aromatic compounds is accomplished by dividing the benzene ring by means of an intracellular enzymatic reaction as shown in figure (1-3) (Mohite *et al.,* 2011). The aromatic ring is hydroxylated via oxygenase enzymes which form acis-dihydrodiol, which transforms to adiol intermediate via adehydrogenase (Kweon *et al.,* 2010). The ortho-cleavage or meta-cleavage pathways then use oxygenase enzymes to destroy the aromatic ring and produced aughter products(i.e., catechols, which later transform to intermediates of the citric acid cycle) (Peng *et al.,* 2008) . Studies have proven that aromatic compounds containing two or three rings are degradable through bacteria through monoxygenase and dioxygenase enzymes that create a cyclic fission that reduces complexity in the catechols and makes them more exposed and easier to consume (Ezikpe *et al.,* 2010).

1.2.10.2: Aerobic microbial degradation of aliphatic hydrocarbon

Alkanes are an essential portion of aliphatic crude oil components (Whale *et al.,* 2018). It is one of the most abundant components in crude oil and is the first compound to decompose (Mahjoubi *et al.,* 2018). In the event of an oil spill, aliphatic alkanes of short-chain generally fly faster than the original petroleum. However, these compounds may also spread to solid surfaces and enter sandy mud sediments, where they continue to exert a toxic effect on the ecosystem (Martinez-Gomes *et al.,* 2010). Compounds of greater chain lengths (C20-C40) are more stable in soil and do not volatilize easily and difficult to degrade due to their low solubility in water, their biological availability and structure (Shao and Wang, 2013). McGenity *et al.* (2012) Show that the long chain alkanes are completely dissolved in aerobic conditions.

Biological degradation of aliphatic hydrocarbons occurs when the carbon backbone of the pollutants is split or a functional substitution occurs by electron loss to the molecular oxygen (Truskewycz *et al.,* 2019). The first steps of the biodegradation process begin with the help of dioxygenase and monooxygenase enzymes and the process occurs under aerobic conditions by adding the oxygen atom to the sub-terminal or

terminal carbon and this aliphatic compounds are converted into primary and secondary alcohol through converging pathways (Imron and Titah, 2018). After that, these products enter the peripheral metabolism pathway in the bacterial cell and have an oxidation process through the C-C bonds are broken step by step resulting in smaller components entering via β-oxidation in to the primary metabolism of the bacterial cell as shown in figure (1-3) (Moreno and Rojo, 2017).



Figure(1-3) : Aerobic pathways of aliphatic and aromatic hydrocarbons degradation by bacteria (Sierra-Garica and Oliverira, 2013).

1.2.11: Mechanism of uptake hydrocarbon by bacteria :

Many bacteria are very effective in breaking down and oxidizing various organic compounds into simpler, more stable end products (Atalia *et al.*, 2015). The bacteria first take the hydrocarbons and then convert them from inactive molecules into more active forms of metabolism, Although bacteria break down each group of hydrocarbons through specific enzymes, finally remains the same products (Ladygina *et al.*, 2006). Brown *et al.* (2017) explain that the main factor in the biological effectiveness of biodegradation of hydrocarbons as a whole is the bioavailability or amount of hydrocarbons that can be accessed by bacteria that requires physical contact between these hydrocarbons and bacteria for the biodegradation process.

Some bacteria show a chemical reception by detect pollutants and move towards them, the bacteria in the soil work first by identifying the oil and its components by emulsions and vital factors, then they adhere to themselves and use the hydrocarbons in the oil as a source of carbon and energy (Thapa *et al.*, 2012 ; Hua and Wang, 2014). Tzintzun-Camacho *et al.* (2012) found three different mechanisms through bacteria take or absorb hydrocarbons, which are: dissolved hydrocarbons in the aqueous phase, absorption of hydrocarbon drops by direct contact with cells, use of biological agents to absorb dissolved hydrocarbon drops.

Chikere *et al.* (2011) instruct to mention the main reason that limits the ability of bacteria reach to the hydrocarbons that are generally present in the aqueous phase to dissolve it these petroleum hydrocarbons are hydrophobic and bacteria can overcome this by producing vital factors. Bacteria can reach the long and medium chain alkanes by sticking to the hydrocarbon drops, aided by the production of vital substances (Rojo, 2009). Stroud *et al.* (2007) indicated that the bacterium *Acinetobacter* produces a bioemulsion or biogenic agents to allow the cellular contact of the bacteria with hydrocarbons hydrophobic during the biodegradation process.

1.2.12 : Impact Factors on bacterial biodegradation

1.2.12.1 : Biotic Factor:

Competition between microorganisms over limited carbon sources, as well as their exposure to predation by devoured and primitive, and the occurrence of conflicting interactions between microorganisms these factors all affect on the process of biodegradation of hydrocarbons in addition to the rate of decomposition of hydrocarbons contaminated with the soil depends on the concentration of these pollutants and the amount of catalyst present (Madhavi and Mohini, 2012). The microbial strains that prevail in the polluted soil are the ones that have the ability to survive in the presence of these pollutants and use them as a source of metabolism and growth, as it depends on determining the rate of decomposition of the contaminated hydrocarbons in the soil through the number of the organisms analyzed so as to note the occurrence of a low rate of decomposition of hydrocarbons in the event of a decrease in the number of bacteria in the soil the opposite occurs if there are sufficient numbers of active bacteria and it is possible through a dynamic increase that means pollinating the soil through suitable strains of decomposing pollutants to overcome the deficiency of microbes(Perelo, 2010 ; Ghosal *et al.*, 2016 ; Abatenh *et al.*, 2017).

1.2.12.2 : Abiotic factor :

1.2.12.2.1 : Nutrient :

Nutrients are required to obtain the microbial use of hydrocarbons in order to stimulate the growth and activity of bacteria (Singh *et al*., 2014). One of the most important of these nutrients that must be provided is carbon in addition to other nutrients that bacteria need, such as nitrogen and phosphorus, to effectively decompose hydrocarbons (Coulon *et al.*, 2012). The oil spill often causes nutrients to be closed and inaccessible to bacteria and is required in order to generate the building blocks for new microbial cells, in addition to supporting the proper performance of all structural and metabolic processes of cells (Bento *et al.*, 2005). Koshlaf and Ball (2017) note that the presence of nutrients such as nitrogen and phosphorous in excessive quantities in the soil can negatively affect the biodegradation of hydrocarbons, which leads to the inhibition of the

decomposition activity of hydrocarbons by bacteria. Brown *et al.* (2017) showed that the important control element in soil hydrocarbon decomposition is the availability of inorganic nutrients, especially phosphorus and nitrogen, which have an important role in stimulating the rates of biodegradation of hydrocarbons in the soil.

1.2.12.2.2 : Oxygen

Oxygen is an important factor for breathing the aerobic bacteria that it requires most, while some bacteria do not need oxygen depending on their requirements, which facilitates the rate of biodegradation in a better way (Macaulay, 2014). Sihag *et al.* (2014) Indicated that the metabolism of hydrocarbons can be enhanced in most cases by the presence of oxygen, but the biodegradation is carried out in anaerobic and aerobic conditions. In aerobic conditions, the biodegradation process is faster than in anaerobic conditions (Wang *et al.*, 2016 ; Al-Hawash *et al.*, 2018).

1.2.12.2.3 : Temperature :

The temperature is one of the most important physical factors in the formation of hydrocarbons and determining the survival of bacteria (Das and Chandran, 2011). The increase in temperature increases the solubility of hydrocarbons, as well as reduces the viscosity of the oil, accelerates the spread of hydrophobic pollutants and enhances the rates of hydrocarbon decomposition (Zekri and Chaalal, 2005) . Conversely, if the temperature decreases, this leads to a delay in the occurrence of the biodegradation process (Si-Zhong *et al.*, 2009).The optimum biodegradation temperature for oil is from 30 to 37 ^{o} C for isolated bacteria, and the highest rates of biodegradation of oil in the soil have been obtained at a temperature ranging from 30 to 40 ^{o} C (Aleer et al., 2011 ; Sihag *et al*., 2014 ; Ibrahim, 2016).

1.2.12.2.4 : pH

The pH is the most important factor affecting the growth and activity of bacteria in the soil where metabolism is affected as well as the decomposition of pollutants and their removal depending on the degree of pH of the compounds, whether they are alkaline, acidic or neutral (Asira, 2013). Low and high pH values showed an effect on the biodegradation process of petroleum pollutants, even if the change in pH values was a slight changes (Wang *et al.*, 2011). Because some enzymes produced by bacteria to conduct the process of biodegradation work at a specific pH value (Hasan *et al.*, 2016 ; Kurniawan *et al.*, 2018). Al-Hawash *et al.* (2018) showed the most bacteria prefer growth in alkaline to natural pH.

1.2.12.2.5 : Salinity

Salinity is an important factor for the activity of many neighborhoods. The salinity affects the growth and diversity of microbes, which has a major impact on the biodegradation process (Qin *et al.*, 2012). The high concentrations of salt create a selective pressure that makes the conditions inappropriate for many bacteria due to the nutrient shutdown and results in reduced availability of organic compounds and a change in osmotic pressure leading to decreased solubility and the occurrence of so-called salting (Martin and Perixoto, 2012 ; Fathepure, 2014). Gao *et al.* (2015) refer to the decrease in the vital treatment rate due to a decrease in microbial respiration.

Sharma *et al.* (2019) pointed out that the presence of sodium chloride with high concentrations in the soil negatively affects the deterioration of the crude oil because it stops the activity of the major enzymes present in the microbial system as well as osmotic shock events in some bacteria, which causes the inhibition of the biological structure of large molecules, plasma dissolution and inhibition of many physiological processes.

CHAPTER TWO

MATERIALS

&

METHODS

2. Materials and Methods

2.1 :Materials

2.1.1 : Equipments and Instruments

Equipments and Instruments used in the present study which are summarized in following table (2-1).

Table (2-1): The equipments and instruments that used with their producing companies and countries.

NO.	Equipments and Instruments	Company /origin
1	Autoclave	Hirayama/ Japan
2	Biosafety cabinet	Lab Tech/France
3	Burner	Indiamart/India
4	Colony counter	Boeco/Germany
5	Cooling Centrifuge	Eppendroff/Germany
6	Distillator	GFR/ Germany
7	Electrophoresis apparatus	Consort/Belgium
8	Gas chromatography	Agilent technologic/USA
9	Gel Documentation	Vilber lourmat/France
10	Incubator	Human Lab/Korea
11	Light Microscope	Olymps/Japan
12	Magnetic stirrer	Heidolph/ Germany
13	Microwave	Shownic/Korea
14	Oven	Memmert/ Germany
15	Refrigerator	Vistal/Poland
16	Sensitive Balance	Sartorius/ Germany
17	Shaker incubator	Zenith Lab/China
18	Soxhlet	Sai Enterprises/India
19	Spectrophotometer	Shimadzu/India
20	Thermal cycler apparatus	Prime /UK
21	Thermometer	Indiamart/India
22	Thimble	Merck / Germany
23	U.V- Transilluminator	ELETTROFOR/Italy
24	Vitek 2 compact system	Biomerieux/ France
25	Vortex mixture	Medilab/Korea
26	Water path	Memmert/ Germany
27	Beakers	Iso Lab/ Germany
28	Brown glass (vials)	Indiamart/India
29	Cylinder	Iso Lab / Germany
30	Disposable Petri dishes	Al-Hani company/Lebenon

31	Eppendrof tubes	Bio neer /South Korea
32	Flask (250 , 500 , 1000)	Iso Lab/ Germany
33	Funnel glass	Iso Lab/ Germany
34	Gloves	Broche/Malaysia
35	Micropipettes	DragonMED/China
36	pH meter	Jenway Germany
37	Screw cap	Citoglas/China
38	Slides and cover slides	Superestar/India
39	Standard wire loop	John Bolten/England
40	Test tube	AFCO-Dispo/Jordan
41	Tips	Sterellin Ltd./UK

2.1.2 : Chemical and Biological materials

The chemical and biological materials which used in the present study are listed in table (2-2) .

Table (2-2): Chemical and Biological materials used in the present study.

NO.	Materials	Company /origin
1	Absolute Ethanol	Scharlau / Spain
2	Agarose gel	Biobasic / Canada
3	Alumina (Al_2O_3)	Himedia /India
4	Ammonium Sulfate $(NH_4)_2SO_4$	BDH/ UK
5	Anhydrous Sodium Sulphate (Na_2SO_4)	Himedia /India
6	Benzen	Alpha Chemika /India
7	Calcium Chloride ($CaCl_2$)	Oxford labchem / India
8	Chloroform	Alpha Chemika /India
9	DNA ladder (100 bp)	Bioneer / South Korea
10	Ethidium bromide	Promega / USA
11	Ferric Chloride ($FeCl_3$)	BDH /UK
12	Glass Wool	Merck / Germany
13	Hydrogen Chloride (HCl)	Oxford labchem / India
14	Loading Dye	Biobasic / Canada
15	Magnesium Solfate ($MgSO_4$)	Oxford labchem / India
16	Methanol	Chem-Lab/Belgium
17	Misan Regular Crude Oil	Misan oil company

18	n-hexan	Scharlab/Spain
19	Nuclease-Free water	Promega / USA
20	Potassium Hydroxide (KOH)	Oxford labchem / India
21	Potassium Nitrate (KNO_3)	Oxford labchem / India
22	Potassium Phosphate Monobasic (KH_2PO_4)	BDH/ UK
23	Silica gel (SiO2)	Himedia /India
24	Sodium Hydroxide (NaOH)	Oxford labchem / India
25	50X TBE (Tris- Boric acid-EDTA)	Biobasic / Canada

2.1.3 : Culture Media

The culture media which used in the present study are listed in the table (2-3).

Table (2-3) : Culture media which used in the present study

NO.	Media	Company (origin)
1	Blood agar	Acumedia lab
2	MacConkey agar	Himedia(India)
3	Mineral salt medium (MSM)	It was prepared in the laboratory
4	Nutreint agar	Himedia(India)

2.1.4 : The Kits :

The kits which used in the present study are listed in the table (2-4).

Table (2-4) : The kits which used in the present study

NO.	Kits type	Purpose	Company(Origin)
1	AccuPower® PCR PreMix	For amplified primer by PCR	Bioneer/South Korea
2	Gram stain	Differentiation of Microorganisms	Himedia /India
3	Presto™ Mini g DNA Bacteria	Extraction bacterial genomic DNA	Geneaid／Taiwan
4	Vitek 2-GB Kit	Identification of Gram-Positive bacteria	BioMerieux/France
5	Vitek 2-GN Kit	Identification of Gram-Negative bacteria	BioMerieux/France

2.1.5 : The primers

The primers prepared by (Bioneer, South Korea company) which used in the present study according to Dixit *et al*. (2018) for universal primer and Al-Deeb and Malkawi (2009) for *Pseudomonas* sp. are summarized in table (2-5) .

Table (2-5) : The primers which used in the present study.

NO.	Bacteria	Primer target sequence	Primer sequence (5'→3')
1	All bacteria (universal)	16S rDNA gene sequence	27F 5'-AGAGTTTGATCCTGGCT CAG-3' 1492R 5'-TACGGGTACCTTGTTACGA CTT-3'
2	*Pseudomonas* sp.	16S rDNA gene sequence	F 5'-CTACGGGAGGCAGC AGTGG-3' R 5'-TCGGTAACGTCAAAACAGCAA AGT-3'

2.2 : Methods

The general steps for research are shown in figure (2-1) .

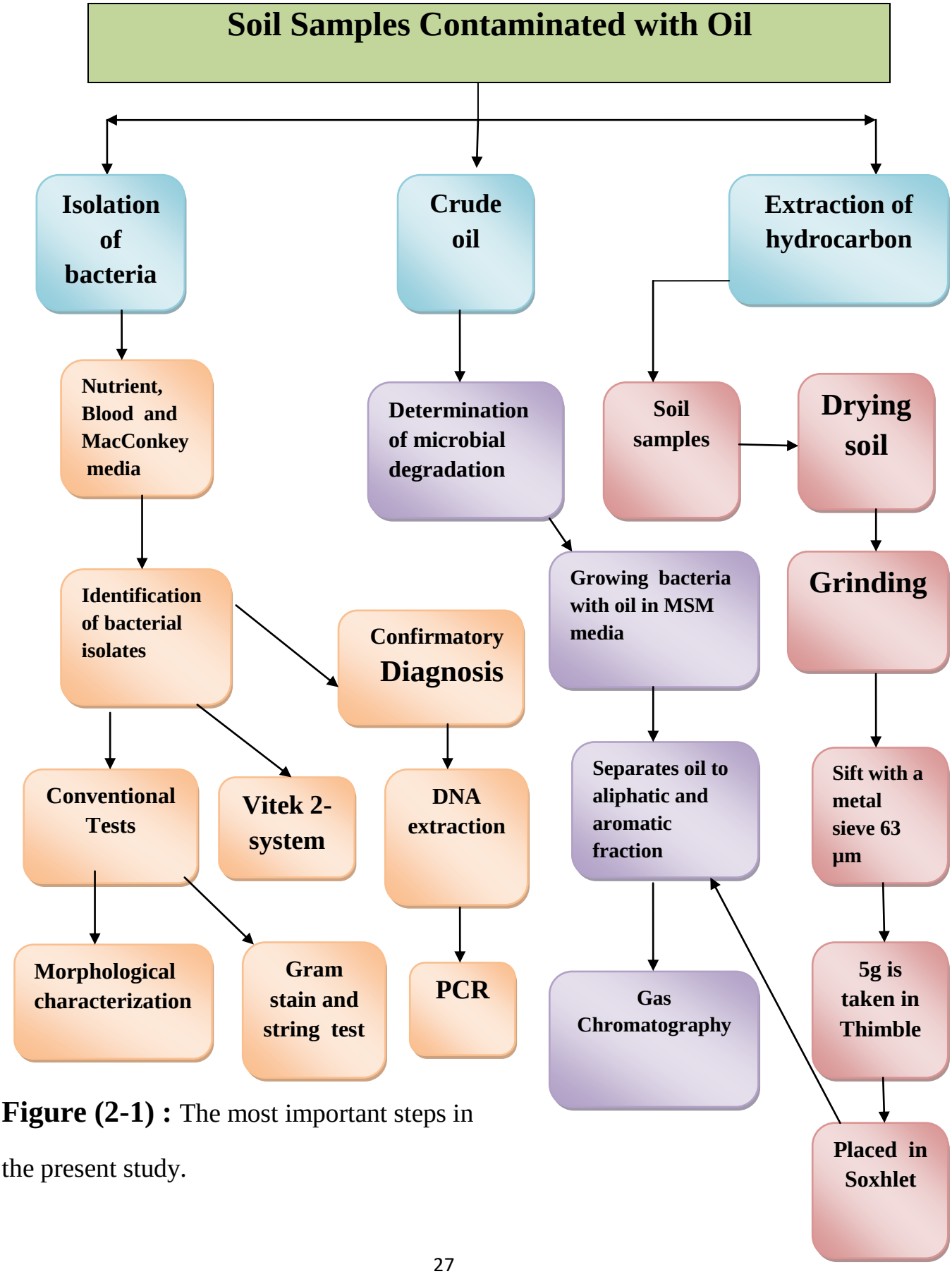


Figure (2-1) : The most important steps in the present study.

2.2.1 : Samples collection :

In this study, oil contaminated soil samples were collected randomly from three different regions in Misan governorate, which contained oil fields and refineries for many years as shown in figure (2-2). These regions were as follows :

1-Site "A" : Misan Oil Company / Bazerkan Refinery.

2- Site "B" : South Oil Company Refinery / Misan.

3- Site "C" : PetroChina Company / Al-Kahlaa Oil Fields

Three replicates of soil were taken for each site mentioned above. The amount of soil taken ranged from 100-200 g at a depth of 5-20 cm under surface. The samples were collected with sterilized plastic cans and transferred to the laboratory for isolation and diagnosis of bacteria that are biodegradable to the petroleum. The sampling were collected during November (2019).

2.2.2 : Crude oil :

Crude oil samples were obtained from Bazerkan Refinery station. Oil placed in sterilized plastic bottles with a capacity of 150 ml were used to collect samples.

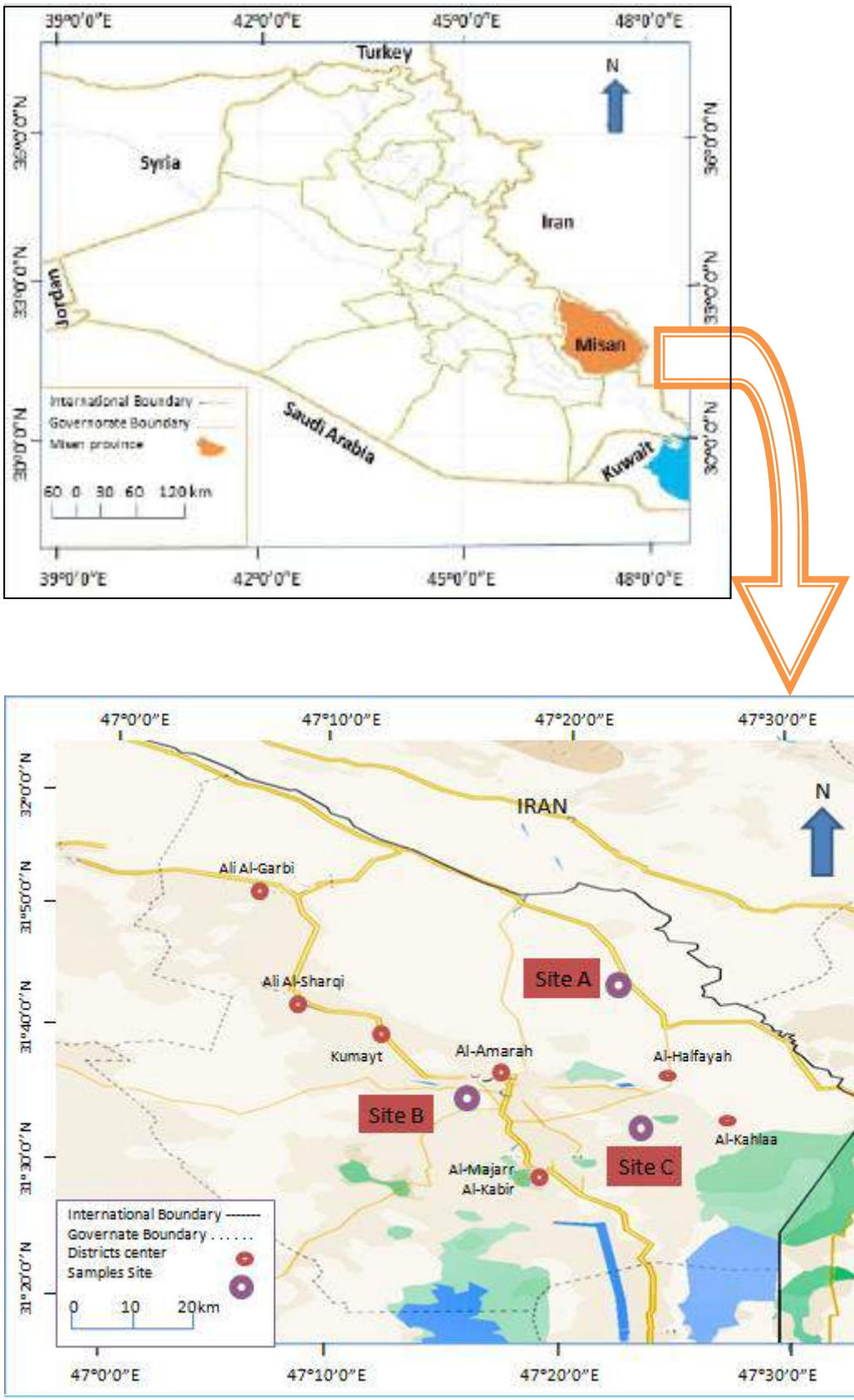


Figure (2-2) : A Map showing the sites of samples collection in Misan province. (A) Bazerkan Refinery . (B) South Oil Company. (C) PetroChina Company .

2.2.3 : Measuring of Soil Properties :

2.2.3.1 : Soil Color :

Soil color were determined depended on the morphological properties of oil contaminated soil.

2.2.3.2 : Soil Temperature :

During the sampling period, the temperature was measured for oil contaminated soil by placing the thermometer compass inside the soil at the same depth from which the soil samples was taken, which is from 5-20 cm and the degree was recorded.

2.2.3.3 : Soil pH :

Soil pH was measured inside the laboratory after bringing samples from the above mentioned sites by using a pH meter, according by kissel *et al*. (2009) was used as follows:

Ten gram of soil were weight and put in a flask 250 ml containing 100 ml of distilled water. The mixture was mixed until the soil was completely dissolved in the water, then the pH was measured and the degree was recorded.

2.2.4 : Extraction of Hydrocarbons from Contaminated Soil :

2.2.4.1 : Preparation of Soil Samples :

The soil samples were dried by leaving them exposed to air for 3 days. A metal sieve of size (63 μm) was used to remove the coarse materials, then placed in a clean glass vials to be ready for analysis (Talal ,2008).

2.2.4.2 : Extraction of Hydrocarbon compounds:

The procedure of Goutex and Saliot (1980) was used for the extraction of hydrocarbon compounds from the soil samples as follow:

1- Five grams from the soil was weigh and put in a cellulose thimble.

2- The Soxhlet apparetus was prepared and put the thimble containing the soil sample in it and add 200 ml of methanol: benzene (1: 1 v/v) for 24-36 hours.

3- At the end of the period, the saponified of the extract was made by using KOH (4 N) for two hours until the extract stabilized and formed two layers. The unsaponified layer was taken and it contains hydrocarbons.

4- The unsaponified layer was placed in a chromatographic column containing a layer of glass wool at the bottom and above it a layer of silica (100-200 mesh) topped by a layer of alumina (100-200 mesh) .

5- Fifty millilter of n-hexane was added to isolate the aliphatic fraction , and then added to the column 30 ml of benzene to isolate the aromatic fraction.

6- Then, the aliphatic and aromatic fractions was placed separately in the dark vials to be ready for analysis by Gas Chromatography.

2.2.5 : Preparation of Culture media and Solutions :

2.2.5.1 : Mineral Salt Medium :

The medium was prepared according to Malatova (2005) it was used to determine the susceptibility of the isolated bacteria to the consumption of hydrocarbons. This medium consists of the following salts: (1g) KH_2PO_4 , (1g) $(NH_4)_2$ SO_4 , (1g) KNO_3 , (0.2g) $MgSO_4$, (0.02g) $CaCl_2$ and (0.05g) $FeCl_3$. These salts were dissolved in a liter of distilled water and adjusted the pH to 7, then sterilized with an autoclave and used in an experiment to measure the quantitative loss of crude oil.

2.2.5.2 : Nutrient agar Medium :

prepared by dissolving (28 g) of nutrient agar in a liter of distilled water, then sterilized in the Autoclave at a temperature of 121º C for 20 minutes. The medium was used for the primary isolation and studying the cultural and phenotypic properties of the isolated bacteria.

2.2.5. 3 : Blood agar Medium :

The medium was prepared according to the instructions of the manufacturer, then sterilized in the Autoclave at a temperature of 121º C for 20minutes, and left to cool down, then 5% human blood was added to

it by Mix well. This medium was used to detect the ability of bacterial isolates to produce the hemolysis enzyme.

2.2.5. 4 : MacConkey agar Medium :

The medium was prepared according to the instructions of the manufacturer, then sterilized in the Autoclave at a temperature of 121º C for 20 minutes, This medium was used for the detection of lactose-fermented bacteria.

2.2.5.5 : Tris Borate EDTA (TBE) buffer :

Preparation of 1X TBE buffer. The 1X TBE buffer was prepared from 50X TBE buffer (as stock solution) by adding 20 ml of this stock solution to 980 ml of distilled water (Sambrook and Rusell, 2001).

2.2.5. 6 : NaOH (0.1 %) :

The sodium hydroxide solution was prepared by dissolving 0.1 g of sodium hydroxide in 100 ml of sterile distilled water. This solution was used for the adjusting the pH of the culture medium natural to the base.

2.2.5. 7 : HCl (0.1 %) :

The hydrochloric acid solution was prepared by dissolving 0.1 ml of hydrochloride in 100 ml of sterile distilled water. This solution was used for the adjusting the pH degree of the culture medium neutral to acidic.

2.2.5. 8 : KOH (3 %) :

The potassium hydroxide solution was prepare by dissolving 3 g of potassium hydroxide in 100 ml of sterile distilled water. This solution was used for the string test.

2.2.6 : Isolation of bacteria from soil samples :

The procedure of Saadoun (2002) and Khan *et al.* (2006) was used for the Isolation of bacteria from soil samples :

One gram for each oil-contaminated soil samples was weigh and suspended in 100 ml of sterile distilled water in flask (250 ml), and the mixture was stirred manually until all the soil particles have dissolved in 15 minutes . Then the mixture was serially diluted in test tubes containing

9 ml of distilled water and the dilution was carried out by transferring 1 ml of the bacterial suspension to the first tube 10^{-1} , after mixing, one milliliter was taken from the same tube (10^{-1}) was transferred to the second tube 10^{-2} and thus the transfer process continued sequentially until reaching dilution 10^{-7}.

After completing the series dilution, 0.1 ml of the dilution 10^{-7} was taken and deployed by a L-shaped glass instrument on the surface of the nutrient agar. The plates were inverted and incubated at 37 º C for 24 hours.

After 24 hours many bacteria developed colonies have been observed on the nutrient agar were counted by colony counter. The CFU/ml was calculated as under :

CFU per ml = No. of colonies * dilution factor/ volume of inoculume

Finally, the bacterial isolates are pure by making several subcultures of the colonies, to be ready for morphological, biochemical and molecular tests that were necessary to identify the isolated bacteria.

2.2.7 : Identification of bacteria isolation :

2.2.7.1 : Conventional Tests

The growing colonies of bacterial species isolates were initially identified depending on:

2.2.7.1.1 : Morphology characterization of bacteria :

The morphological characteristics of the growing colonies of bacteria include color, size, form, elevation and margin of the colonies on ordinary enrichment, selective and differential media (Nutrient agar, Blood agar and MacConkey agar) (Goldmann and Lorrence, 2009).

2.2.7.1.2 : Gram Staining :

Gram stain were used to differentiate a shapes of bacterial isolates and distinguish between Gram positive and Gram negative.

2.2.7.1.3 : String Test :

A loopful of bacterial growth was emulsified on the surface of a glass slide in a suspension of 3% KOH (Suslow *et al*., 1982; Arthi *et al*., 2003).

2.2.7.2 : Identification by VITEK 2 System:

In present study the Vitek 2 system was used in order to confirm the diagnosis of bacterial isolates from oil-contaminate soil. This system required a bacterial suspension from the suspected bacteria, which placed in the inoculated tube and after that the suspension was transferred to the card, which is incubated in thermally controlled conditions. As a result of the metabolic activity of the bacteria the color changes in the card, and every 15 minutes are measured in interrupted form by light intensity. Then the information's were stored, analyzed and printed automatically (Pincus , 2006).

The steps are described in more detail as follows :

I- Preparation of bacterial suspension

A sufficient number of bacterial colonies are transferred by a sterile disposable loop from pure culture which grown on MacConkey agar and Nutrient agar and suspended in sterile saline solution (3 ml) in a clear plastic test tube. The density was checked after adjusted the turbidity at the range (0.5- 0.63).

II- Inoculation of identification card

An integrated vacuum apparatus was used for inoculated identification card was with bacterial suspension. In the special rack (cassette) was placed a test tube containing the bacterial suspension in the neighboring slot the identification card was placed, while inserted into the

corresponding suspension tube the transfer tube. The cassette can accommodate up to 10 tests or up to 15 tests. After that, the filled cassette was placed either transported automatically or manually into a vacuum chamber station. After the air was reintroduced and vacuum was applied into the station, the bacterial suspension was inserted and forced through the transfer tube into micro-channels which that filled all the test wells.

III- Card sealing and incubation

A mechanism was used to passed an inoculated card, which cuts off the transfer tube and locks the card before it is loaded into the carousel incubator. The carousel incubator can hold up to 30 or up to 60 cards. All card kinds were incubated on-line at (35.5 + 1.0^{o} C). Each card is transferred once every 15 minutes from the carousel incubator, transported for reaction readings to the optical system. Then returned to the carousel incubator until the next reading time. Data were collected during the entire incubation period at 15 minute intervals.

2.2.7.3 : Molecular Identification :

Molecular detection of some gene of bacteria isolated from oil-contaminated soil, Polymerase Chain Reaction (PCR) assay was performed .This method was implemented as in the following steps:

2.2.7.3.1 : Genomic DNA extraction:

Genomic DNA Mini Bacteria Kit was used to extract Genomic DNA from bacteria according to company's instructions, the bacterial culture has been inoculated in 10 ml of medium brain heart infusion broth and incubated at 37^{0} C at 24 hours in the incubator as follows :

Step 1: Sample Preparation :

• One milliliters of fresh culture was added to a 1.5ml microcentrifuge tube.

•The microcentrifuge tube was Centrifuged for 1 minute at 13,000 rpm. Then the supernatant was discard.

• One handered and eighty microlitiers of GT buffer by micropipette were dded, then the cell pellet was resuspend by the vortex.

•Twenty microlitiers of proteinase K (make sure distilled water added) was added and incubated for 10 minutes at 60^{o} C, the tubes were inverted during incubation every 3minutes.

Step 2: Lysis Step

∗Tow handred microlitiers of GB buffer was added to the sample and mixed for 10 seconds by vortex .

∗The tubes was incubated at 60^{o} C for at less 10 minutes to ensure the sample lysate is clear, the tubes was inverted every 3 minutes during incubation. At this time the Elution buffer was pre-heated (200µl per sample) to 70^{o} C (for step DNA Elution).

Step 3: DNA Binding

◊Tow hundred microliters of absolute ethanol was added to the sample lysate and mixed immediately by shaking vigorously.

◊Tow milliliters of GD column was placed in a collection tube.

◊ The mixture (including any insoluble precipitate) was transferred to GD column then the mixture was centrifuged for 2 minutes at 13,000 rpm.

◊Two milliliters of the collection tubes containing the flow-through were discard.

◊The GD column was placed in tow ml a new collection tube.

Step 4: Washing Step

®Forty hundred microliters of W1 Buffer was added to the GD column and then centrifuge for 30 seconds at 13,000 rpm then discard the flow-through, the GD column were placed back in tow ml collection tube.

®Six hundred microliters of wash buffer 600 (make sure ethanol was added) was added to the GD column, centrifuge for 30 second at 13,000 rpm then discard the flow through, the GD column were placed back in tow ml the collection tube .

®The columns matrix were centrifuge for 3 minutes at 13,000 rpm to dry.

®The dried GD column was transferred to 1.5ml a clean micro centrifuge tube.

Step 5:Elution

•The pre-heated Elution Buffer was added into center of the column matrix.

•The tubes were left for at least 3 minutes to allow Elution buffer to be completely absorbed.

• The tubes was placed in Centrifuge for 30 seconds at 13,000 rpm to elute the purified DNA.

•The DNA was Stored at 2-8º C.

2.2.7.3.2 : Detection of DNA content by Agarose Gel Electrophoresis :

The extraction of DNA have been carried out. Agarose gel was prepared according to Sambrook and Russell (2006) to confirm the integrity and presence of extracted DNA of bacterial isolates according to the protocol of gel electrophoresis which included the following steps:

1- One hundred of 1X TBE buffer was taken in a flask.

2- One gram of agarose powder was added to 100 ml of 1X TBE buffer.

3- The solution was heated until boiling by using a microwave until all the gel particles were dissolved.

4- Three microliters of ethidium bromide (0.5 µg/ml) was added to the agarose solution, and then stirred the agarose in order to get mixing.

5- The solution was left to cool at 50-60^{o} C.

6- The agarose solution was poured into the gel tray, after sealing the edges of the gel tray with a cellophane tape and fixing the comb from one edge in 1 cm away .

7- The agarose was left until solidify for 30 minutes at room temperature. After that the fixed comb was removed carefully and the gel tray was placed in the gel tank. Then the tank was filled with 1X TBE buffer, until the buffer arrived (3-5 mm) the surface of the gel.

8- Five microliters of DNA sample was transferred to Eppendrof tube and 2µl of loading dye was added to the tube and mixed well ,then the mixture was loaded into the wells in agarose gel. Electric current was allowed for 1hour at 80 volt. Finally, the bands were visualized at wave length 350 nm on a UV transiluminator.

2.2.7.3.3 : Preparation of primers solution :

The oligonucleotide primers were suspended by dissolving the lyophilized product after spinning down briefly with free-ionized water depending on manufacturer's instruction as stock suspension. Working primer tube was diluted with free-ionized water, the final picomoles depended on the procedure of each primer.

2.2.7.3.4: Master Mix .

The master mix components were mentioned in table (2-6)

Table (2-6): Master Mix (AccuPower®PCR PreMix) used in this study:

No.	Component	Reaction volume 25 µl reaction
1	Taq DNA polymerase	1U
2	Each: dNTPs (dATP, dCTP, dGTP, dTTP)	250 µM
3	Tris-HCL(PH 9.0)	10 mM
4	KCl	30 mM
5	MgCl2	1.5 mM
6	Sterilizer and tracking dye1	Trac

2.2.7.3.5 : Polymerase Chain Reaction protocol :

The protocol used according to the instructions of the manufacturer Bioneer. All components of PCR were assembled in PCR tube and mixed by cooling microcentrifuge for 10 sec at 850 rpm.

The steps were conducted are as following :

1- Primers and Template DNA were dissolved before usage.

2- Primers and Template DNA were added into the AccuPower®. Taq premix tubes as shown in table (2-7).

3- The lyophilized blue pellets were completely dissolved and spin down by using vortex.

4- The Eppendorf PCR tubes were placed in the thermocycler and the appropriate PCR cycle program parameter conditions with some modifications according to Xia *et al*. (2017) for universal primer and Al-Deeb and Malkawi (2009) for *Pseudomonas* sp. as shown in table (2-8) and (2-9).

Table (2-7) : The volume of mixture of PCR

No.	PCR Master mix	Volume (µl)
1	Free inonized water	9 µl
2	DNA template	7 µl
3	Forward primer	2 µl
4	Reverse primer	2 µl
5	Master Mix	5 µl
6	Final volume	25 µl

Table (2-8): PCR program of Universal primer

PCR step	Temperature (°C)	Time	Repeat
Inatial denaturation	95	5 min	1
Denaturation	95	30 s	30 cycle
Annealing	52	45 s	
Extension	72	1.5 min	
Final extention	72	10 min	1
Hold	4		-

Table (2-9): PCR program of *Pseudomonas* sp. primer

PCR step	Temperature (°C)	Time (min)	Repeat
Inatial denaturation	95	5 min	1
Denaturation	94	1 min	25 cycle
Annealing	55	1 min	
Extension	72	1 min	
Final extention	72	10 min	1
Hold	4		-

2.2.7.3.6: Agarose Gel Electrophoresis :

Agarose gel electrophoresis was preparing for universal primer by dissolving 1g in 100 ml TBE buffer (1X) and dissolving 1.5 g for *Pseudomonas* sp. primer. The solution was heated by microwave until boiling and all the gel particles were dissolved. After that, left to cool at 50°C and 3 µl of ethidium bromide was added to agarose and poured on preparing tray. The agarose was left to solidify at room temperature for 30 minutes. The comb was removed after hardening of agarose leaving wells (Sambrook and Russell, 2006). The first well was loaded with 4µl of DNA ladder (the standard molecular weight) , and each well is loaded with 3µl of DNA sample. TBE buffer (1X) was added to the electrophoresis tank, tray with agarose which had previously attended was immersed in electrophoresis tank. Electrophoreses run for 60 min at 80 volt, the gel was photographed by using a gel Documentation system (Mishra *et al.*, 2010).

2.2.8 : Growth of bacteria in different Temprature :

Different bacterial isolates were grown on nutrient agar. The dishes were incubated at different temperatures, including (24º C, 28º C, 32º C, 37º C 42º C and 46º C) for 24 h.

2.2.9 : Growth of bacteria in different pH :

Different bacterial isolates were grown on nutrient agar with different pH levels (4, 5, 6, 7, 8 and 9) at 37º C for 24 h.

The neutral pH of nutrient agar 7 changed to 8 and 9 by adding 0.1% NaOH .

The pH of nutrient agar was reduced to acidity value 4, 5 and 6 by adding 0.1% HCl .

2.2.10 : The ability of bacteria species for crude oil degradation:

The procedure of Obuekwe and Al-Zarban (1998) with some modifications by Al-wasify and Hamed (2014) was used to measure the ability of bacteria species to degradation crude oil :

1- The bacterial isolates were activated by cultured them on nutrient agar for 24 hours at 37° C .

2- The MSM medium was prepared as in paragraph (2.2.5.1), sterilized in the autoclave and then distributed on flasks with a capacity of 250 ml. One hundered milliliter of the MSM medium was placed in each flask.

3- A single bacterial colony was taken for each pure bacterial isolation grown at 37° C within 24 hours and suspended in a test tube containing 10 ml distilled sterile water for dilution 10^{-1} .

4- One milliliter of the bacterial suspended was added to the each flask contain on MSM medium and then 0.5 ml of crude oil was added.

5- All the flask was incubated in a shaker incubator at 37° C in different intervals including 7, 14 and 21 days for 121 rpm.

2.2.10.1 : Extraction petroleum hydrocarbons from MSM broth :

Petroleum hydrocarbons were extracted from MSM broth following the procedure of UNEP (1989) with some modifications by Al-Dossari (2008) as follow:

1- After each incubation period 100 ml of chloroform was added to the flask containing MSM medium, bacteria and crude oil.

2- The mixture was put in a separating funnel and left for some time until become stable and separate two layers, one layer at the bottom which containing oil hydrocarbons and aqueous layer on top, the lower layer is isolated and passed onto column chromatography.

3- The extract was passed through column chromatography provided with glass wool at the bottom then a small amount of anhydrous sodium sulphate was added to remove the excess of water and the chloroform extracts were collected in clean beaker and left until evaporation.

4- After evaporation, 50 ml of n-hexane was added to the clean beaker which contains the sample and passed through the column chromatography provided with glass wool at the bottom then placed silica gel (100-200 mesh) and 8 gm from alumina (100-200 mesh) is placed at the top to isolate the aliphatic fraction , then 30 ml of benzene were added to isolate the aromatic fraction these fractions were reduced to a suitable volume prior to analysis.

2.2.10.2 : Estimation of Optical Density :

Optical density was estimated by taking 5 ml of bacteria grown in MSM inside the flask before the separating process and measuring growth by spectrophotometer in terms of optical density with a wavelength of 620 nm (Amit and Rashmi, 2013).

2.2.10.3 : Measuring the percentage of degradation of crude oil

The degradation efficiency was calculated according to the equation which described by Oudot (1984) as follows :

Degradation rate % = Initial conc. of crude oil – Final conc. of crude oil / Initial conc. of crude oil x 100 %

2.2.11 : Methods of Short-term preservation

Nutrient agar was distributed in screw cap tubes in 20 ml quantities sterilized and left tilted to solidify. The inclined surface was inoculated with bacteria by shedding and incubation at 37º C for 24 h and stored at 4º C. The bacteria were transferred to a new medium each month to activate the isolates and avoid contamination (Collee *et al*., 1996).

CHAPTER THREE

RESULTS

&

DISCUSSION

3.Results and Discussion :

3.1 : Chemical and Physical Properties of Soil :

The soil used in the current study, which was collected from three sites from oil contaminated soil in Misan province, characterized by some of the properties that were observed and diagnosed such as color, temperature and pH as shown in table (3-1).

The current results showed varying degrees in color among soil samples where it graded from dark brown, black and brown for the three sites of the Bazerkan refinery (site A), South Oil Company (site B) and PetroChina Company (site C) respectively. From previous studies, increasing the concentration of petroleum hydrocarbons in the soil could alter the morphological properties of soil (Gong *et al*., 2008 ; Kisic *et al*., 2009).

The temperature measurement values were recorded 28^{o} C for both the site of Bazerkan refinery (site A) and PetroChina Company (site C) and 30^{o} C for the South Oil Company (site B). Increasing the temperature above the optimum will decrease growth, however, decreasing the temperature will not kill the organisms, but only slow down growth (Barcenas-Moreno *et al*., 2009). pH values ranged from 7.95, 7.43 and 7.98 for Bazerkan refinery (site A), South Oil company (site B) and PetroChina Company (site C) respectively. In agreement with our results, Hamamura *et al*. (2006) observed range of pH for soil contaminated with oil from neutral to slightly alkaline. Because the high acidity or alkalinity leads to toxic effects on living organisms in the soil in addition to the soil imbalance, as well as has an effect on the activity of bacteria (Fernaddez-Calvino and Baath, 2010).

Table (3-1) : Chemical and Physical properties of soil used in this study.

Characteristic of soil	Site A	Site B	Site C
color	Dark brown	Black	Brown
Temperature	28^{o} C	30^{o} C	28^{o} C
pH	7.95	7.43	7.98

3.2 : n-alkanes and PAHs in soil samples

The three soil samples were analyzed to identified and determined the petroleum hydrocarbons components, which were measured by the GC. The results of extraction of hydrocarbons components showed that the oil contaminated soil contain on two types of crude oil are n-alkanes and PAHs as shown in table (3-2) and (3-3), (Appendix 1,2 and 3 A and B).

The highest concentration rate was recorded in the soil of the South Oil Company (site B) was 66644.43 µg/gm d.w for n-alkanes and 4106.503µg/gm d.w for PAHs followed by a Bazerkan refinery (site A), which were 38445.39 µg/gm d.w for n-alkanes and 2953.512 µg/gm d.w for PAHs, As for the soil of PetroChina company (site C), they have been less concentration for n-alkanes hydrocarbons (14180.85 µg/gm d.w) and PAHs hydrocarbons (1361.24 µg/gm d.w).

Wang *et al*. (2009) concluded the reason for the difference in the concentration of pollutants in these soils is that pollution with crude oil leads to a significant increase in the total organic carbon contents due to the high concentration of total petroleum hydrocarbons resulting from the crude oil spill. Also, there are another reasons for the difference in the concentration of organic hydrocarbon materials in the soil is due to weathering factors and the ability of bacteria to biodegrade hydrocarbons and use them as a source of carbon and energy (Wang *et al*., 2010).

Table(3-2): Concentration of n-alkanes components in soil samples

Carbon numbers	Concentrations (µg/gm dry weigh)		
	Site A	Site B	Site C
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	0	0	0
C12	0	0	0
C13	14.06133	214.4547	0
C14	174.1133	1432.239	0
C15	773.024	3056.68	44.45807
C16	1548.398	4777.736	218.5553
C17	2894.128	4360.285	311.2558
C18	2424.324	3926.586	500.8723
C19	2205.91	3499.995	458.1413
C20	2850.761	5685.864	696.5796
C21	2244.232	2745.352	764.4968
C22	4315.973	3256.114	1931.294
C23	2943.681	3119.55	1202.698
C24	3545.554	2815.744	1237.947
C25	3049.516	3883.384	1411.766
C26	1828.538	4263.081	1247.588
C27	1609.502	3511.922	713.3418
C28	870.1335	2085.607	411.844
C29	1404.636	2678.358	728.2184
C30	957.3721	1886.302	448.5532
C31	818.4926	2213.16	446.5361
C32	931.7454	1299.549	369.4892
C33	618.6222	1455.95	434.3088
C34	158.6868	1186.821	119.4467
C35	52.41275	1203.378	288.3977
C36	65.47155	1066.303	103.0522
C37	88.73602	557.3349	53.59104
C38	57.36659	440.5857	38.42067
C39	0	22.09262	0
C40	0	0	0
ΣTPH	38445.39	66644.43	14180.85

Table(3-3): Concentration of PAHs components in soil samples.

Compounds name	Concentrations (µg/gm dry weigh)		
	Site A	Site B	Site C
Naphthalene	0	0	0
2-Methylnaphthalene	0	0	0
1-Methylnaphthalene	0	0	0
Acenaphthylene	84.05682	188.7809	0
Acenaphthene	64.08016	99.3141	16.24057
Fluorene	18.79195	51.61667	20.90197
Phenanthrene	69.61778	219.7251	30.42354
Anthracene	153.7817	245.1306	81.02906
Fluoranthene	331.8467	228.3201	126.4932
Pyrene	230.634	840.0592	341.0519
Benzo(a)anthracen	506.6761	154.5222	363.4944
Chrysene	184.5762	215.092	127.4479
Benzo(b)fluoranthene	623.3372	1073.55	73.91197
Benzo(k)fluoranthene	292.1468	495.2156	91.24673
Benzo(a)pyrene	289.7963	97.06339	54.5168
Indeno(1,2,3-c,d)pyrene	73.77786	182.9528	34.48176
Benzo(g,h,i)perylene	30.39291	15.16077	0
ΣPAHs	2953.512	4106.503	1361.24

3.3 : Isolation and Identification of bacteria :

Twenty-five bacterial strains were isolated from oil contaminated soil samples by using serial dilutions have been enumerated on the nutrient agar and it is easy to perform and very small number of organisms can be counted as CFU/ml (Obuekwe and Semple, 2013). The bacterial population isolated from oil contaminated soil had total aerobic counts ranging from ($1.5X10^4$ – $9.8X10^5$) CFU/ml as shown in table (3-4).These results are close to study of Al-Deeb and Malkawi (2009) which conducted to isolating bacteria from oil contaminated soil. The enumeration was the best method to study the bacterial strains that able to degrade hydrocarbons (Zhao *et al.*, 2009).

Table (3-4): Enumeration of bacterial colonies on nutrient agar plate by the aeroplate count.

Soil samples	Site code	CFU/ml of dilution
1	A1	2.8×10^4
2	A2	2.9×10^4
3	A3	9.6×10^5
4	B1	2.9×10^4
5	B2	1.75×10^5
6	B3	9.8×10^5
7	C1	3×10^4
8	C2	1.5×10^4
9	C3	2.5×10^4

Several subculture was made to obtained pure culture for identification bacteria (Figure 3-1). Gram stain was showed that Gram negative bacteria was most bacterial strains (74%) and a few was Gram positive (36%). This is proven by many studies that have observed that the percentage of Gram negative bacteria is more than that of Gram positive bacteria in oil contaminated soil (Hassanshahian *et al*., 2012 ; Mujahid *et al.*, 2015 ; Pranowo and Titah. 2016 ; Hashmat *et al.*, 2018). Hussen (2009) explained that the gram negative bacteria have a membrane that contains fats that enable them to obtain the largest amount of hydrocarbon compounds from the environment and then oxidize and exploit them as a source of carbon and energy. The forms of bacteria were ranged from cocci, bacilli and variable pleomorphic (Figure 3-2). The colony characteristic was also identified such as colony color, size, form, elevation and margin as shown in table (3-5).

Table (3-5) : Morphological characterization of bacterial strains.

Bacterial isolates	Gram stain	Bacterial shape	Colony color	Colony size	Colony form	Colony elevation	Colony margin
A11	+ve	Bacilli	White	Medium	Circular	Convex	Irregular
A12	+ve	Bacilli	White	Medium	Circular	Convex	Irregular
A13	+ve	Bacilli	White	Medium	Circular	Convex	Irregular
A21	-ve	Bacilli	Blue green	Medium	Irregular	Raised	Undulated
A22	-ve	Bacilli	Shiny white	Small	Circular	convex	Entire
A31	+ve	Cocci	Yellow	Small	Circular	Convex	Entire
A32	+ve	Bacilli	Opaque	Small	Circular	Low convex	Entire
A33	+ve	Bacilli	White	Large	Circular	Convex	Entire
A34	+ve	Bacilli	White	Medium	Circular	Convex	Irregular
B11	-ve	Bacilli	Cream	Medium	Circular	Convex	Entire
B12	-ve	Bacilli	Cream	Medium	Circular	Low convex	Entire
B13	-ve	Cocci	Cream	Medium	Circular	Convex	Entire
B21	-ve	Bacilli	Off-white	Large	Circular	Convex	Entire
B22	-ve	Cocci	Cream	Medium	Circular	Convex	Entire
B31	-ve	Bacilli	Cream	Medium	Circular	Low convex	Entire
B32	-ve	Bacilli	Off-white	Large	Circular	Convex	Entire
B33	+ve	Bacilli	White	Medium	Circular	Convex	Irregular
C11	+ve	Cocci	Yellow	Small	Circular	Convex	Entire
C12	-ve	Bacilli	Opaque buff	Large	Circular	Convex	Round
C13	-ve	Cocci	Cream	Medium	Circular	Convex	Entire
C21	-ve	Bacilli	Yellow	Large	Circular	Convex	Entire
C22	-ve	Cocci	Cream	Medium	Circular	Convex	Entire
C23	-ve	Cocci	Yellow	Medium	Circular	Raised	Entire
C31	-ve	Cocci	Cream	Medium	circular	Convex	Entire
C32	-ve	Cocci	Cream	Medium	Circular	Convex	Entire

Note : -ve : Gram negative , +ve : Gram positive

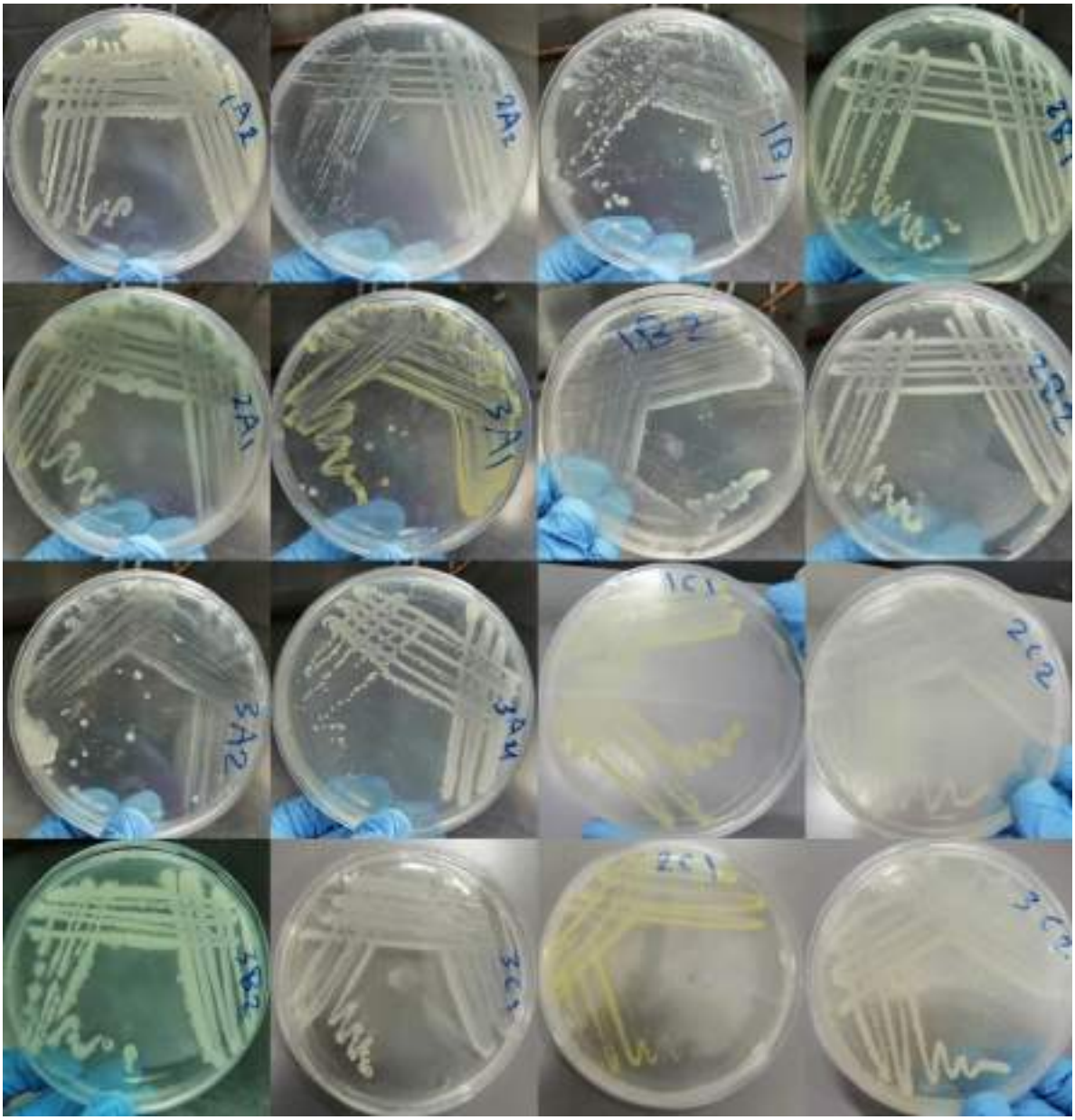


Figure (3-1): A pure culture of bacterial strians.

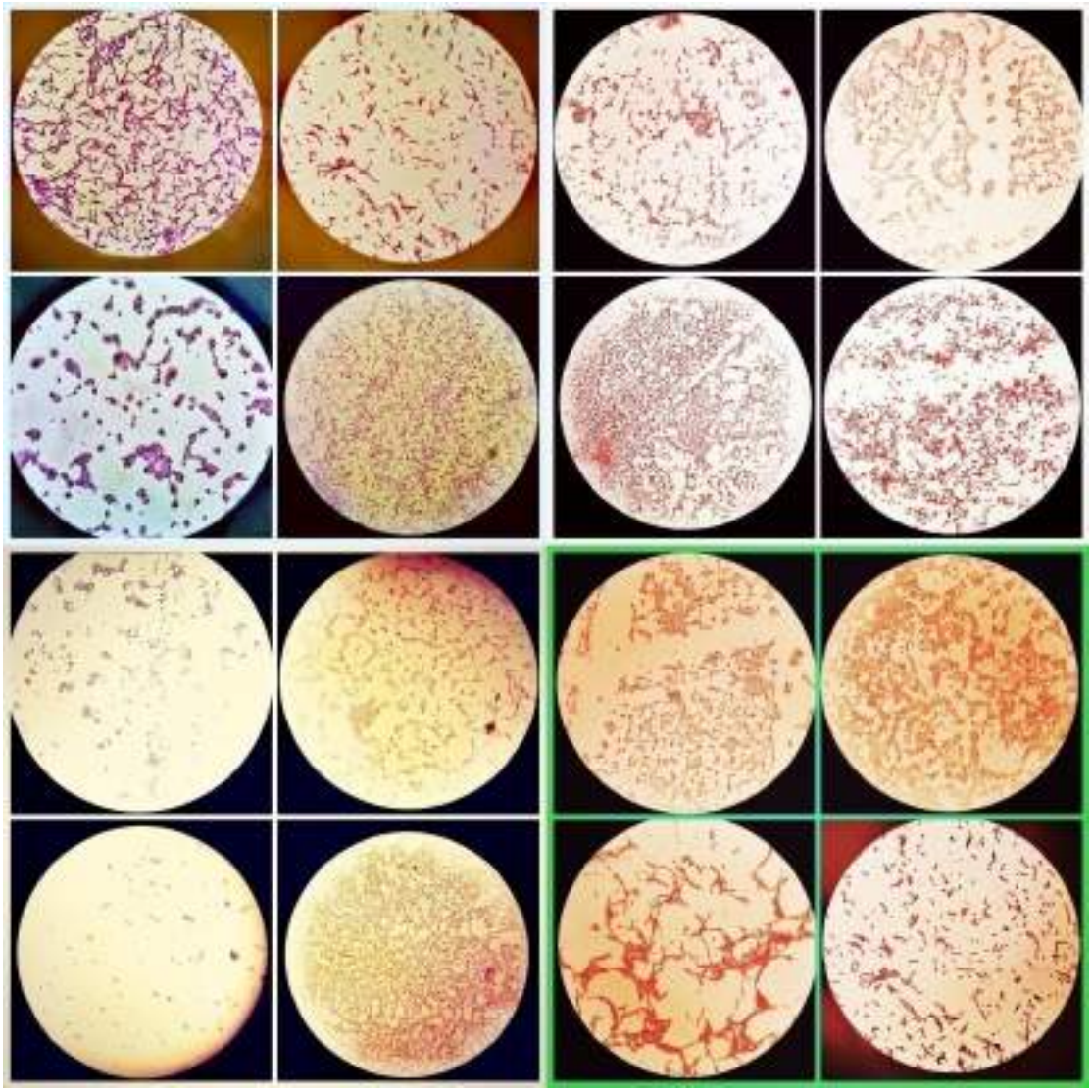


Figure (3-2): Gram stain of bacterial isolates, examined under light microscope with magnification power 1000x.

Table (3-6) illustrates the growth of bacterial isolation on Blood agar and MacConckey agar as well as the results of String test. All the bacterial strain have shown good growth on the Blood agar with different patterns of hemolysis as show in figure (3-3A). The gram positive bacteria didn't show any growth on the MacConkey agar while the gram negative bacteria were grow very well (Figure 3-3B). Furthermore these bacteria were interacted with the KOH in the String test as compared with the gram positive bacteria. As show in figure (3-4 C and D), as they become viscous and String out whereas gram positive bacteria were not affected, this corresponds to a study of (Dash and Payyappilli, 2016).

Table (3-6) : Bacterial strains growth on Blood agar and MacConkey agar and interaction with string test.

Bacteria isolaties	Blood agar	MacConkey agar	String test
A 11	+	-	-
A 12	+	-	-
A 13	+	-	-
A 21	+	+	+
A22	+	+	+
A31	+	-	-
A32	+	-	-
A33	+	-	-
A34	+	-	-
B11	+	+	+
B12	+	+	+
B13	+	+	+
B21	+	+	+
B22	+	+	+
B31	+	+	+
B32	+	+	+
B33	+	-	-
C11	+	-	-
C12	+	+	+
C13	+	+	+
C21	+	+	+
C22	+	+	+
C23	+	+	+
C31	+	+	+
C32	+	+	+

Note : (+) grow, (-) no grow. (+) interacted with string test, (-) no interacted.

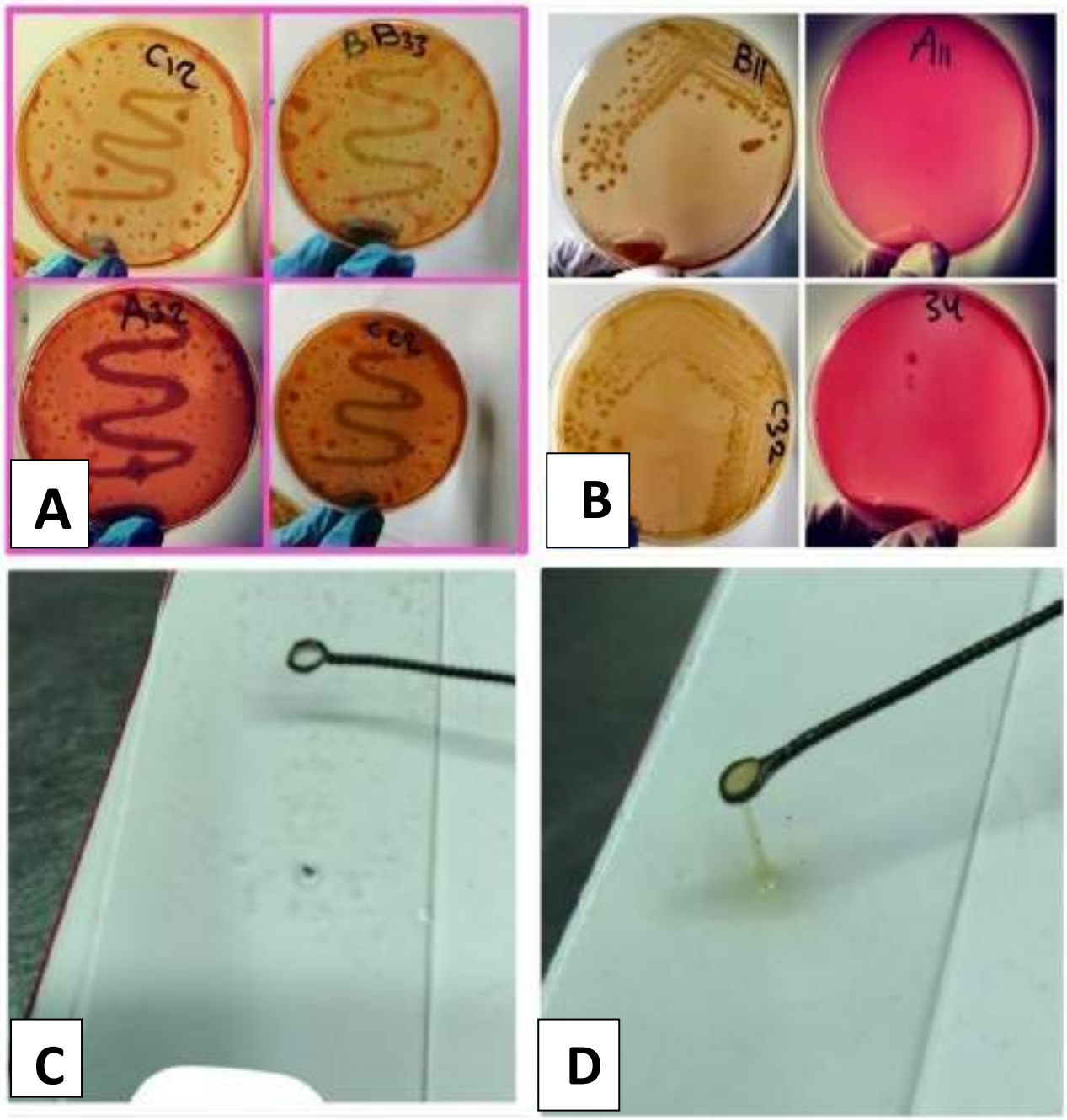


Figure (3-3) : (A) The growth of bacteria on the Blood agar. (B) the growth of bacteria on the MacConkey agar. (C) The negative string test. (D) The positive string test.

Table (3-7) represents results for biochemical and molecular identification. Based on the results of VITEK®2 Compact (Appendix 22) and 16S rDNA sequence homology studies (Appendix 23). The results of molecular identification were more consistent with the initial phenotypic diagnosis (Table 3-5) than the results of VITEK®2 Compact system, this in agreement with the study of Bellinaso *et al.* (2003) and Varjani *et al.* (2015).

Table (3-7) : Biochemical and Molecular identification of bacterial strains

Bacterial code	Vitek 2 system	16S rDNA sequence
A11	*Staphylococcus vitulinus*	*Bacillus safensis*
A12	*Staphylococcus lentus*	*Bacillus safensis*
A13	*Staphylococcus sciuri*	*Bacillus pumilus*
A21	*Pseudomonas aeruginosa*	*Pseudomonas sp.*
A22	*Ralstonia mannitolilytica*	*Brevundimonas sp.*
A31	*Kokuria kristinae*	*Arthrobacter luteolus*
A32	*Gemella bergeri*	*Bacillus sporothermodurans*
A33	*Granulicatella elegans*	*Bacillus safensis*
A34	*Staphylococcus lentus*	*Bacillus subtilis*
B11	*Pseudomonas putida*	*Pseudomonas putida*
B12	*Pantoea sp.*	*Undefined*
B13	*Acinetobacter haemolyticus*	*Acinetobacter sp.*
B21	*Sphingomonas paucimobilis*	*Undefined*
B22	*Acinetobacter haemolyticus*	*Acinetobacter baumannii*
B31	*Pantoea sp.*	*Sporosarcina luteola*
B32	*Sphingomonas paucimobilis*	*Novosphingobium subterraneum*
B33	*Staphylococcus lentus*	*Bacillus subtilis*
C11	*Kocuria kristinae*	*Undefined*
C12	*Aeromonas hydrophila*	*Aeromonas salmonicida*
C13	*Acinetobacter haemolyticus*	*Acinetobacter junii*
C21	*Sphingomonas paucimobilis*	*Sphingomonas paucimobilis*
C22	*Acinetobacter haemolyticus*	*Acinetobacter junii*
C23	*Aeromonas salmonicida*	*Arthrobacter luteolus*
C31	*Acinetobacter junii*	*Acinetobacter junii*
C32	*Acinetobacter haemolyticus*	*Acinetobacter sp.*

Pairwise Sequence Alignment of the Sequencing data were obtained by using NCBI-BLAST(Nucloutide BLAST) as shown in table (3-8). The bacterial universal primer pair specific to 16S rDNA gene fragment was used to identify bacterial isolates; positive results were recorded for all bacterial isolates with an amplification band corresponding to 1500 bp, confirming that all isolates were bacterial species (Figure 3-4). Furthermore, some bacterial isolates were identified using primer pair specific to 16S rDNA gene fragment specific for the genus *Pseudomonas* (Figure 3-5).

Table(3-8) : Bacterial identification based on 16S rDNA sequencing data.

Bacterial strains	Maximum score	Total score	Query coverage	E. valume	Identity Percentg	Accession No.
Bacillus safensis	1862	1862	94%	0.0	98.04%	MK501608.1
Bacillus safensis	1851	1851	98%	0.0	95.7%	JX475127.1
Bacillus pumilus	1853	1853	99%	0.0	95.71%	FJ763645.1
Pseudomonas sp.	97.1	97.1	3%	4e-15	90.67%	EF590133.1
Brevundimonas sp.	1962	1962	91%	0.0	99.27%	MK 729043.1
Arthrobacter luteolus	1951	1951	98%	0.0	98.38%	KX783591.1
Bacillus sporothermodurans	1247	1247	98%	0.0	87.45%	EN 430991.1
Bacillus safensis	1199	1199	74%	0.0	93.18%	MK746245.1
Bacillus subtilis	717	717	35%	0.0	95.75%	KR 999939.1
Pseudomonas putida	1982	1982	99%	0.0	98.07%	KT 984874.1
Acinetobacter sp.	1205	1205	59%	0.0	95.73%	KX622562.1
Acinetobacter baumannii	1991	1991	98%	0.0	98.25%	KJ958271.1
Sporosarcina luteola	1881	1881	96%	0.0	96.97%	MN589774.1
Novosphingobium subterraneum	1951	1951	98%	0.0	98.3%	KJ573537.1
Bacillus subtilis	1086	1086	48%	0.0	97.49%	KY820912.1
Aeromonas salmonicida	1980	1980	98%	0.0	98.15%	MF445389.1
Acinetobacter junii	2056	2056	98%	0.0	99.82%	MK418695.1
Sphingomonas paucimobilis	327	327	16%	6e-85	98.4%	MK829514.1
Acinetobacter junii	2049	2049	98%	0.0	100%	MK418695.1
Arthrobacter luteolus	1722	1722	89%	0.0	96.81%	JX649224.1
Acinetobacter junii	462	462	78%	2e-125	77.02%	MG 551868.1
Acinetobacter sp.	2013	2013	99%	0.0	99.37%	KX989239.1

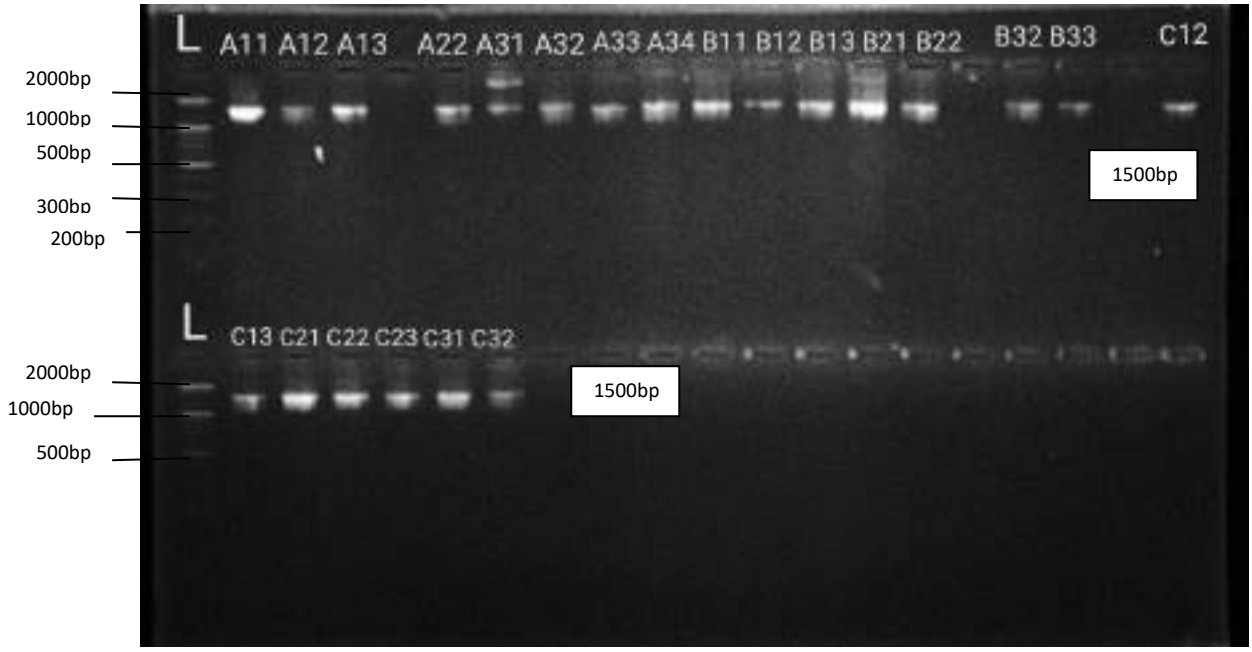


Figure (3-5) : Ethedium bromide stained gel electrophoreses of the 16s rRNA gene of bacterial strains, lane (L) represents the molecular ladder (100bp) and lanes (A11-C32) represents positive PCR product size (1500 bp) of universal primer.

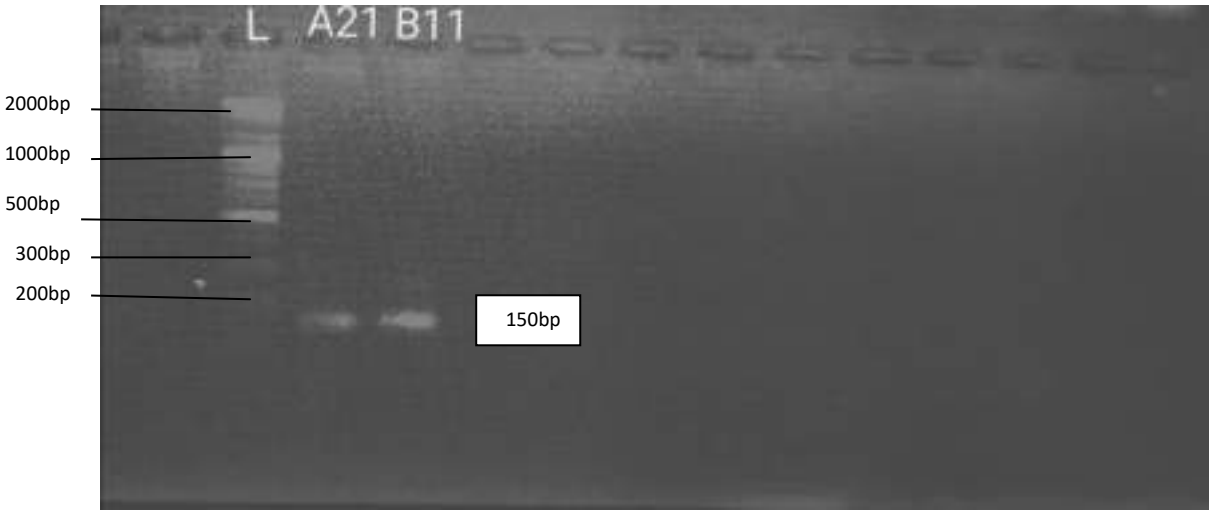


Figure (3-6) : Ethedium bromide stained gel electrophoreses of the 16s rRNA gene of bacterial strains, lane (L) represents the molecular ladder (100bp) and lanes (A21 and B11) represents positive PCR product size (150 bp) of *Pseudomonas* sp. primer.

The current study was able to recorded twelve bacterial strains in GenBank data belong different accession number as shown in table (3-9)

Table (3-9) : Recorded of bacterial strains in GenBank

Bacterial strains	Title in GenBank	Accession number
Bacillus safensis	*Bacillus safensis* strain zsh-2020 16S ribosomal RNA gene, partial sequence	MW130721.1
Bacillus pumilus	*Bacillus pumilus* strian zsh-2020 16S ribosomal RNA gene, partial sequence	MW136775.1
Bacillus subtilis subsp. subtilis	*Bacillus subtilis* subsp. subtilis strian zsh-2020 16S ribosomal RNA gene, partial sequence	MW139244.1
Pseudomonas putida	*Pseudomonas putida* strian zsh-2020 16S ribosomal RNA gene, partial sequence	MW130256.1
Brevundimonas sp.	*Brevundimonas sp*.strian zsh-2020 16S ribosomal RNA gene, partial sequence	MW131456.1
Sporosarcina luteola	*Sporosarcina luteola* strian szh-2020 16S ribosomal RNA gene, partial sequence	MW130449.1
Arthrobacter luteolus	*Arthrobacter luteolus* strian zsh-2020 16S ribosomal RNA gene, partial sequence	MW130288.1
Acinetobacter baumannii	*Acinetobacter baumannii* strian zsh-2020 16S ribosomal RNA gene, partial sequence	MW130446.1
Novosphingobium subterraneum	*Novosphingobium subterraneum* strian zsh-2020 16S ribosomal RNA gene, partial sequence	MW131506.1
Sphingomonas paucimobilis	*Sphingomonas paucimobilis* strian szh-2020 16S ribosomal RNA gene, partial sequence	MW138104.1
Aeromonas salmonicida	*Aeromonas salmonicida* strian szh-2020 16S ribosomal RNA gene, partial sequence	MW138105.1
Acinetobacter junii	*Acinetobacter junii* strian hsz-2020 16S ribosomal RNA gene, partial sequence	MW130253.1

3.4 : The growth of bacterial strains at different Temperature and pH

The sixteen bacterial strains (thirteen bacterial strains were identified by molecular testes and three bacterial strains were identified according to Vitek-2 system) were grew on the nutrient agar with a different degrees of pH ranged from (4-9) and different temperature ranged from (24-46) ^{o} C . The bacterial strains were showed its best growth at pH 7, which symbolized **(+++)** while at 6, 8 and 9 degrees of growth were ranged from best growth (+++), good growth (++), weak growth(+) and some of bacteria was showed no growth (-). The results showed that all bacterial strains were unable to grow at 4 and 5 degrees of pH (Table (3-10) and Figure(3-6).

At pH 6, the best growth results were recorded for *Sphingomonas paucimobilis* and *Novosphingobium subterraneum* bacteria. As for *Bacillus safensis, Bacillus sporothermodurans, Pseudomonas aeruginosa, Brevundimonas sp., Pantoea sp. and Sporosrcina luteola*, they showed good growth, while *Bacillus subtilis, Bacillus pumilus, Arthrobacter luteolus , Kocuria kristinae and Acinetobacter junii* exhibited weak growth. The rest of the isolates represented by *Pseudomonas putida, Aeromonas salmonicida* and *Acinetobacter baumannii* did not show any growth at this degree of pH.

Arthrobacter luteolus, *Sphingomonas paucimobilis*, *Novosphingobium subterraneum* and *Kocuria kristinae* were showed a best growth at pH 8 and 9, while *Sporosrcina luteola* and *Aeromonas salmonicida* were showed a best growth at pH 8 but at pH 9 were showed a good growth. Only *Pantoea sp.* was showed a good growth at pH 8 and 9 while *Brevundimonas sp.* was showed a good growth at pH 8 whereas it was unable to grew at pH 9 .

Some bacteria were showed a good growth at pH 8 but its growth were a weak at pH 9 these bacteria were *Pseudomonas aeruginosa Pseudomonas putida, Acinetobacter junii* and *Acinetobacter baumannii.*

Bacillus sp. did not able to grow at pH 8 and 9. The growth of bacterial isolates in the basic medium is better compared to the acidic medium, as most isolates recorded good growth at pH 8-9, while they were unable to grow at pH 4-6 or weak growth at pH 6. Bacteria are generally neutrophiles, they grow best at neutral pH close to 7 (optimum growth pH), Growth occurs slowly or not at all below the minimum growth pH and above the maximum growth pH (Jin and Kirk, 2018) as shown in table (3-10). Yan *et al*. (2013) indicated that the bacterial strains were isolated from oil contaminated soil have adapted to grow at a pH closely from pH of soil environment. This is a good agreement with the fact that the soil is normally with a neutral to alkaline (Mbachu *et al*., 2020).

Table (3-10): The bacterial strains growth on different range of pH

Bacterial isolaties	pH4	pH5	pH6	pH7	pH8	pH9
Bacillus safensis	-	-	++	+++	-	-
Bacillus subtilis	-	-	+	+++	-	-
Bacillus pumilus	-	-	+	+++	-	-
Bacillus sporothermodurans	-	-	++	+++	-	-
Pseudomonas aeruginosa	-	-	++	+++	++	+
Brevundimonas sp.	-	-	++	+++	++	-
Arthrobacter luteolus	-	-	+	+++	+++	+++
Pseudomonas putida	-	-	-	+++	++	+
Pantoea sp.	-	-	++	+++	++	++
Sphingomonas paciumobilis	-	-	+++	+++	+++	+++
Novosphingobium subterraneum	-	-	+++	+++	+++	+++
Sporosarcina luteola	-	-	++	+++	+++	++
Kocuria kristinae	-	-	+	+++	+++	+++
Aeromonas salmonicida	-	-	-	+++	+++	++
Acinetobacter junii	-	-	+	+++	++	+
Acinetobacter baumannii	-	-	-	+++	++	+

Note: (+++) best growth, (++) good growth, (+)weak growth, (-) no growth.

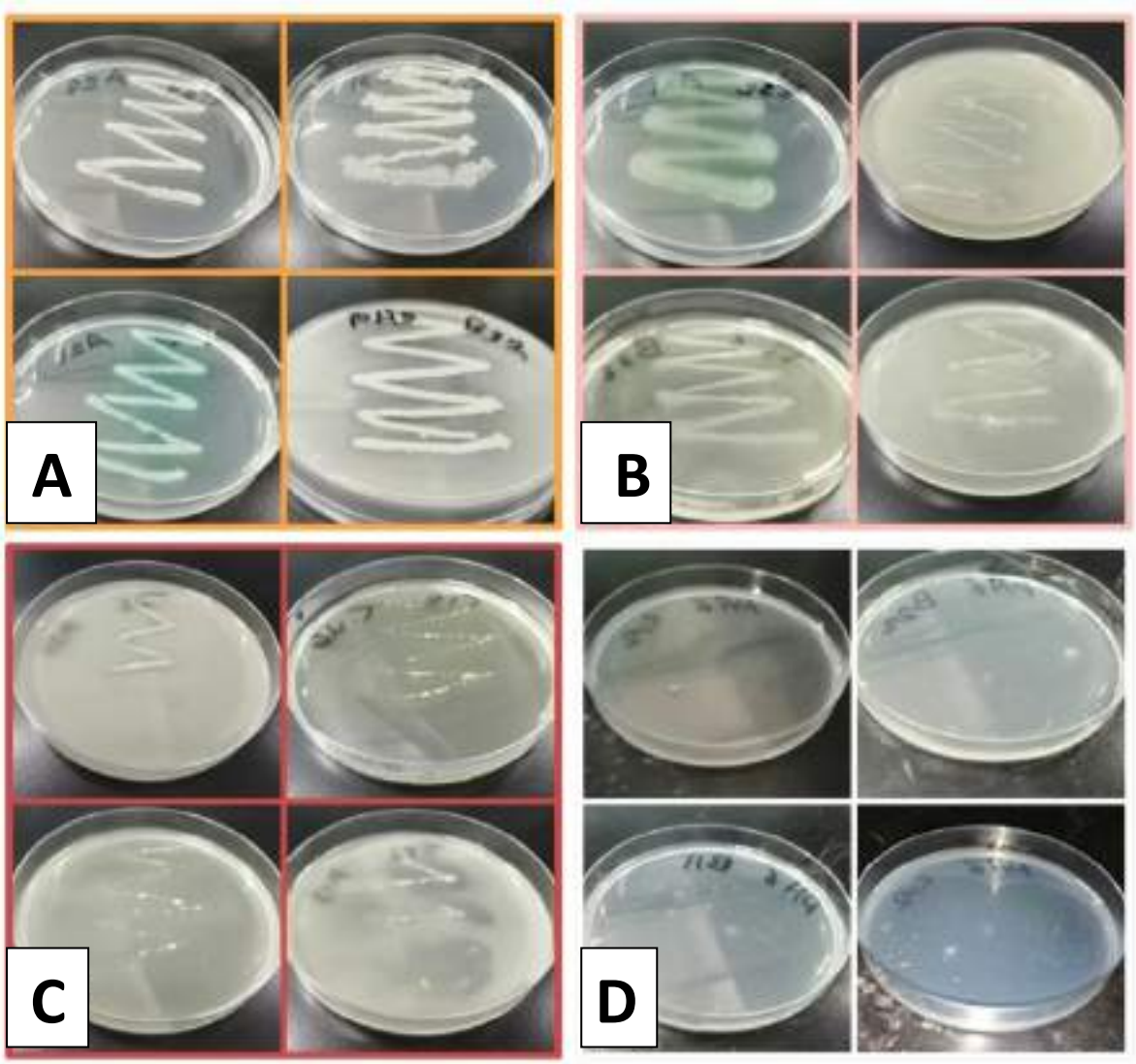


Figure (3-6) : The ability of bacterial strains growth on different range of pH. (A) refer to the best growth, (B) refer to the good growth, (C) refer to the weak growth and (D) refer to inability to grow.

Table (3-11) and figure (3-7) show the growth of bacterial strains in different range of temperatures (24, 28, 32, 37, 42 and 46)^{o} C, all bacterial strains show a best growth (+++) at 24^{o} C 28^{o} C and 37^{o} C except *Bacillus safensis* which showed good growth (++) at 24^{o} C, whereas at 32^{o} C the bacterial strains showed uneven growth. *Bacillus sporothermodurans, Pseudomonas aeruginosa, Brevundimonas sp. and Novosphingobium subterraneum* were showed a best growth (+++) at 32^{o} C , while *Bacillus sp., Arthrobacter luteolus, Sphingomonas paucimobilis, Sporosarcina luteola, Kocuria kristinae, Aeromonas salmonicida and Acinetobacter sp*. were showed good growth (++) at 32^{o}

C followed by *Bacillus pumilus, Pseudomonas putida and Pantoea* sp. were showed a weak growth (+).

The bacteria showed weak growth and the inability to grow at high temperatures at 42° C and 46° C. All *Bacillus* sp. were showed a weak growth at 42 ° C except *Bacillus sporothermodurans* was showed inability to grow at same degree of temperatures, whereas all *Bacillus* sp. were showed inability to growth at 46° C. Only *Pseudomonas aeruginosa* and *Novosphingobium subterraneum* have the best growth at 42° C and a good growth at 46° C followed by *Brevundemonas sp., Sphingomonas paucimobilis, Kocuria kristinae and Acinetobacter junii* which showed a good growth at 42° C and a weak growth at 46° C. On the contrary, *Aeromonas salmonicida* showed weak growth at both degrees of temperatures. As for *Arthrobacter luteolus, Pseudomonas putida, Pantoea sp., Sporosarcina luteola and Acinetobacter baumannii* showed weak growth at 42° C and were unable to grow at 46° C.

This is consistent with fact that most bacteria do not grow well at temperatures much higher than 37° C (Irshaid and Jacob, 2015).Our findings are similar to previous investigations which reported that the temperatures have exhibit similar effects on growth rates of various bacterial species (Andreoni and Gianfreda, 2007; Higashioka *et al*., 2011 ; Alrumman *et al*., 2015).

Table (3-11): The bacterial strains growth on different range of temperatures.

Bacterial isolaties	T24	T 28	T32	T37	T42	T46
Bacillus safensis	++	+++	++	+++	+	-
Bacillus subtilis	+++	+++	++	+++	+	-
Bacillus pumilus	+++	+++	+	+++	+	-
Bacillus sporothermondurans	+++	+++	+++	+++	-	-
Pseudomonas aeruginosa	+++	+++	+++	+++	+++	++
Brevundimonas sp.	+++	+++	+++	+++	++	+
Arthrobacter luteolus	+++	+++	++	+++	+	-
Pseudomonas putida	+++	+++	+	+++	+	-
Pantoea sp.	+++	+++	+	+++	+	-
Sphingomonas paciumobilis	+++	+++	++	+++	++	+
Novosphingobium subterraneum	+++	+++	+++	+++	+++	++
Sporosarcina luteola	+++	+++	++	+++	+	-

Kocuria kristinae	+++	+++	++	+++	++	+
Aeromonas salmonicida	+++	+++	++	+++	+	+
Acinetobacter junii	+++	+++	++	+++	++	+
Acintobacter baumannii	+++	+++	++	+++	+	-

Note: (+++) best grwoth, (++) good growth, (+) weak growth, (-) no growth.

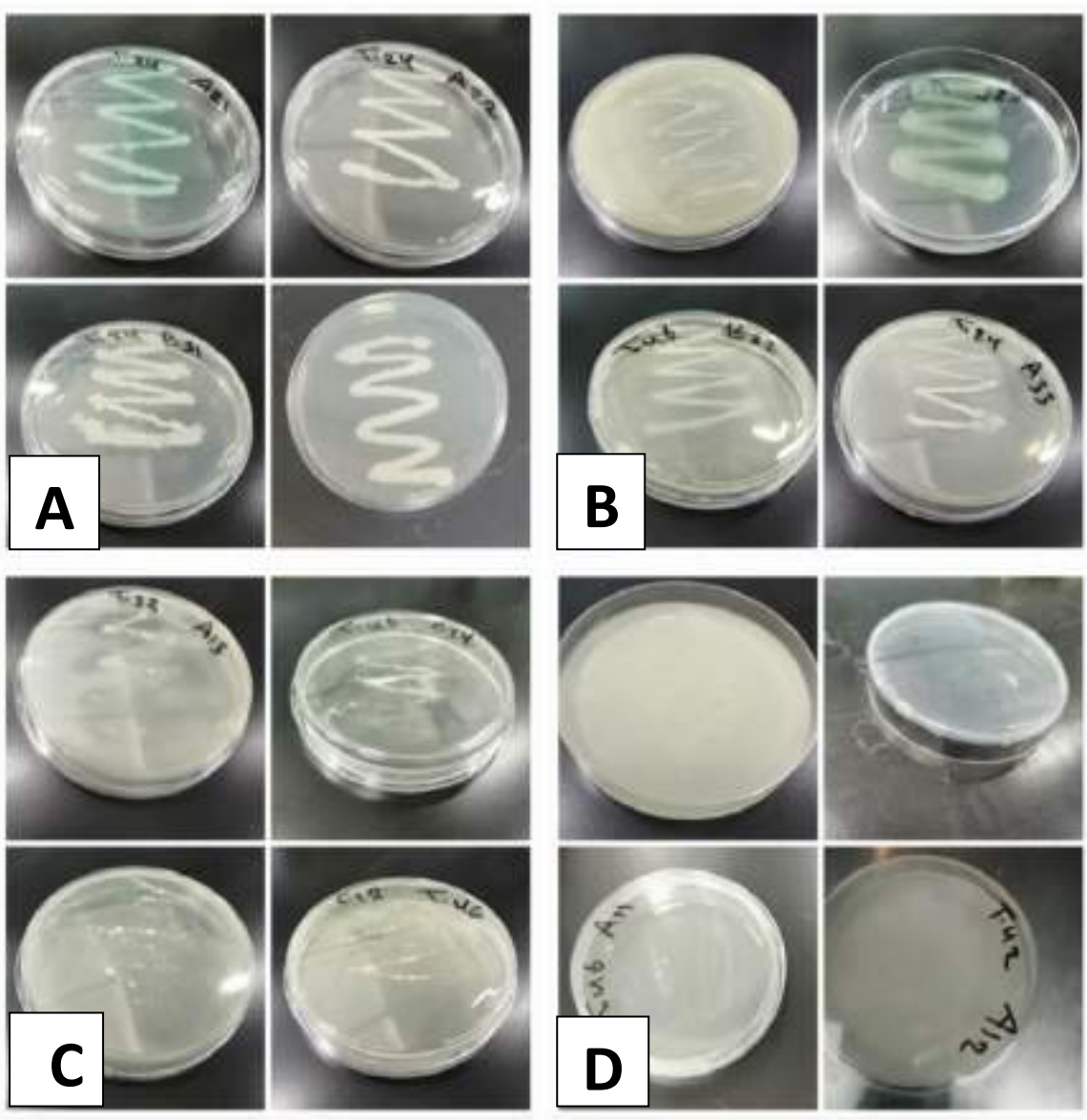


Figure (3-7): The ability of bacterial strains to grow in different range of temperatures. (A) refer to the best growth. (B) refer to the good growth. (C) refer to the weak growth. (D) refer to the inability to growth.

3.5 : The optical density (OD) of the bacterial strains growth in MSM with Crude oil

After the bacterial strains were identified, sixteen out of twenty-five bacterial isolates grew on mineral salt medium supplemented with 0.5 % crude oil, indicating that the bacterial isolates used the hydrocarbons of the crude oil as a sources of carbon and energy. Compared with the negative control which composed from mineral salt with crude oil, none of the bacterial isolates grew on the media.

The growth of isolates were detected by measuring the optical density (OD) with the spectrophotometer at a wavelength of 620 nm, an increase in intensity of growth was observed at 7, 14 and 21 days of incubation periods, the amount of increase in bacterial growth was recorded compared to the control medium as it is shown in table (3-12). The highest values of OD (102.9) were recorded for *Bacillus subtilis, Pseudomonas aeruginosa and Aeromonas salmonicida* an increase in the growth rate was recorded during the last incubation period (21 days) while the OD values for the same isolates were recorded during the 14 days of incubation period (1 01.8, 101.8 and 101.2) and (100.8, 99.8 and 98.9) during the 7 days for *Bacillus subtilis, Pseudomonas aeruginosa and Aeromonas salmonicida* respectively, followed by the *Pantoia* sp. which recorded values (100.4, 101.9 and 102.8) for the three incubation periods (7, 14 and 21) days respectively. While *Brevundimonas sp., Arthrobacter luteolus and Acinetobacter junii* recorded the same value during the third incubation period (102.7), but during the second period, the three isolates showed a difference in the intensity of growth. *Arthrobacter luteolus* showed the highest growth value (101.5), followed by *Brevundimonas* sp. (101.4) and 101.3 for *Acinetobacter junii.* In the first incubation period the same values of OD were recorded for *Brevundimonas sp.* and *Arthrobacter luteolus* (99.5) and *Acinetobacter junii* was recorded (99.2).

On the other hand, the results showed that both *Kocuria kristina* and *Acinetobacter baumannii* showed the same growth value, their values of OD were recorded as (97.7, 101.7 and 102.5) during the first, second and third incubation period respectively. On the contrary, *Pseudomonas putida* and *Sphingomonas paucimobilis* and *Novosphingubium*

subterraneum showed similar growth values with a slight difference in OD (98.3, 100.8 and 102.1) for *Pseudomonas putida*, (98.3, 100.7 and 102.3) for *Sphingomonas paucimobilis* and for *Novosphingubium subterraneum* (98.5, 100.5 and 102.7). As for *Bacillus sp.* isolates they showed a variable density of growth, the highest growth density was recorded for *Bacillus subtilis* (100.8, 101.8,102.9), followed by *Bacillus pumilus* (99.3, 101.5, 102.3) and then *Bacillus safensis* (96.8, 101.2, 102.5), as for *Bacillus sporothermodurans*, it recorded the lowest growth density (96.4, 100.4, 101.8). *Sporosarcina luteola* showed a growth density (99.4, 101.3, 102.6) at OD_{620} . The bacterial strains could grew rapidly on crude oil because it was capable of metabolizing hydrocarbons and used it as a sole source of carbon and energy (Markandey and Rajvaidya, 2004). Bacteria are capable and diverse of utilizing contaminants as energy and carbon source to survive in natural environment (Singh and Lin, 2010).

Table (3-12) : The optical density (OD_{620}) of bacterial strains in MSM suplemented with crude oil.

Incubation peroids Optical density (OD_{620}) of bacteria in MSM with crude oil			
Bacterial isolaties	7 days	14 days	21 days
Control	94.2	94.7	94.3
Bacillus safensis	96.8	101.2	102.5
Bacillus subtilis	100.8	101.8	102.9
Bacillus pumilus	99.3	101.5	102.3
Bacillus sporothermodurans	96.4	100.4	101.8
Pseudomonas aeruginosa	99.8	101.8	102.9
Brevundimonas sp.	99.5	101.4	102.7
Arthrobacter luteolus	99.5	101.5	102.7
Pseudomonas putida	98.3	100.8	102.1
Pantoea sp.	100.4	101.9	102.8
Sphingomonas paucimobilis	98.3	100.7	102.3
Novosphingobium subterraneum	98.5	100.5	102.7
Sporosarcina luteola	99.4	101.3	102.6
Kocuria kristinae	97.7	101.7	102.5
Aeromonas salmonicida	98.9	101.2	102.9
Acinetobacter junii	99.2	101.3	102.7
Acinetobacter baumannii	97.7	101.7	102.5

3.6 : Biodegradation of crude oil by bacterial strains

To examine the ability of bacterial strains to degrade hydrocarbons (aliphatic and aromatic fractions), GC analysis of the control (Appendix 4A and B) which only crude oil (0.5%) showed that it was a mixture of different aliphatic fractions including low molecular weight (C7-C16), medium molecular weight (C17-C28) and high molecular weight (C29-C40) and different aromatic fractions including low molecular weight (Naphthalene, 2-Methylnaphthalene, 1-Methylnaphthalene, Acenaphthyene, Acenaphthene, Fluorene, Phenanthrene, Anthracene) and high molecular weight (Fluoranthene, Pyrene, Benzo(a)anthracene, Chrysene, Benzo(b)fluoranthene ,Benzo (k)fluoranthene ,Benzo (a) pyrene, Indeno(1,2,3-c,d) pyrene , Benzo(g,h,i)perylene). These corresponds to findings of Simon *et al*. (2010) and Glover (2012).

The medium molecular weight (MMW) n-alkanes hydrocarbons concentration more than another compounds of n-alkanes (low molecular weight (LMW) and high molecular weight (HMW), also concentration of this three types of n-alkanes turn out to more concentration than PAHs concentration (LMW and HMW) as showed in GC analyzing of control sample of crude oil (Figure 3-8). Table (3-13A and B).

Hassanshahian *et al*. (2012) and Huang *et al*. (2013) proved that crude oil consists mainly of aliphatic fraction (n-alkanes) as organic pollutants, and this is consistent with the current results, as it appeared that crude oil consists of aliphatic fraction with a higher concentration of aromatic components (PAHs) according to GC analysis.

Table(3-13A) : The concentrations of each component of crude oil with the total concentration of each group of aliphatic fractions (n-alkanes).

Carbon numbers	Concentrations (µg/gm dry weight)
C7	0
C8	0
C9	0
C10	0
C11	42.10774
C12	2512.75
C13	8389.68
C14	12865.01
C15	13499.24
C16	18533.54
Σ LMW	55842.33
C17	10287.55
C18	8478.72
C19	16500.56
C20	7300.349
C21	5847.324
C22	5708.591
C23	5405.177
C24	4412.427
C25	6543.87
C26	6864.507
C27	5835.931
C28	5967.423
Σ MMW	89152.43
C29	7277.658
C30	3205.703
C31	3899.895
C32	2401.409
C33	3465.241
C34	2517.359
C35	2565.269
C36	2043.603
C37	1911.294
C38	1844.376
C39	452.2588
C40	0
Σ HMW	31584.07

Table(3-13B) :The concentrations of each component of crude oil with the total concentration of each group of aromatic fractions (PAHs).

Compounds name	Concentration (µg/gm dry weight)
Naphthalene	74.32799
2-Methylnaphthalene	2270.463
1-Methylnaphthalene	658.4242
Acenaphthylene	1813.479
Acenaphthene	1014.953
Fluorene	813.2189
Phenanthrene	1031.528
Anthracene	1310.902
Σ LMW	8987.296
Fluoranthene	671.1723
Pyrene	2466.833
Benzo(a)anthracene	648.9231
Chrysene	436.2029
Benzo(b)fluoranthene	1867.228
Benzo(k)fluoranthene	2351.404
Benzo(a)pyrene	383.1854
Indeno(1,2,3-cd)pyrene	3719.327
Benzo(g,h,i)perylene	520.6151
Σ HMW	13064.89

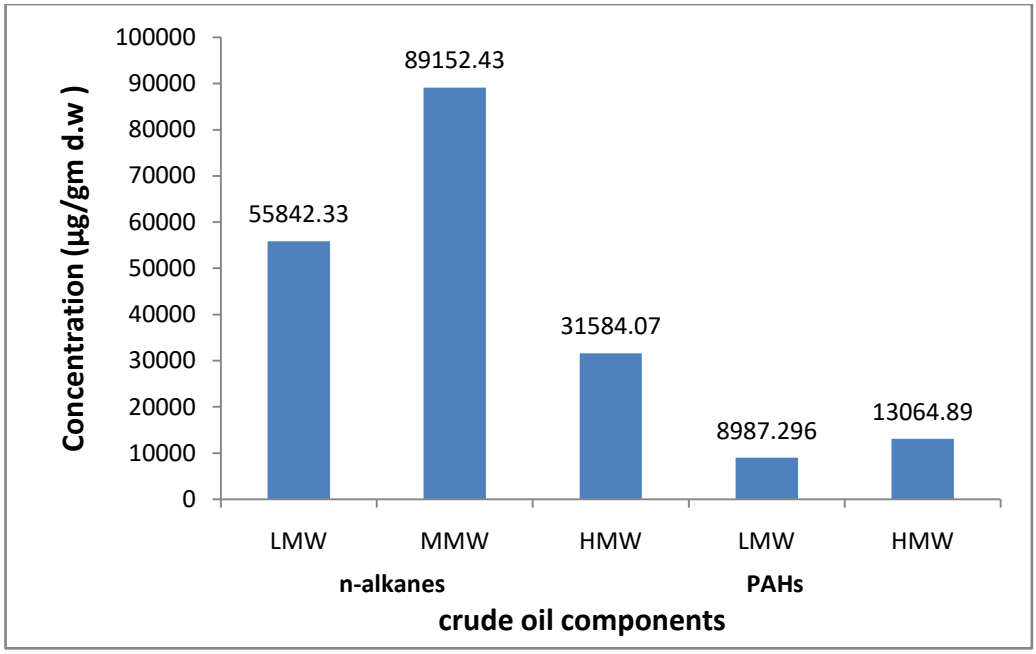


Figure (3-8): The concentration of crude oil components in control sample.

After the crude oil was exposed to bacterial degradation individually during the weekly incubation periods as shown in figure (3-9), (Appendix 5A and B), the results were observed that degradation of low molecular weight compounds was occurred during the first week from the experiment, some of the compounds were completely degraded while the higher molecular weight ones were gradually degraded during the incubation periods. These results coincide with the fact that the bacteria first attack the lower molecular weight compounds while attacking the intermediate and upper compounds later during the incubation (Koolivand *et al*., 2019). When n-alkanes (C7–C40) and PAHs compounds were tested as the sole carbon sources for bacterial strains, growth was observed in all cases. The strains grew obviously and rapidly with n-alkanes including C7-C20 and PAHs including low molecular weight (LMW), while with the medium molecular weight (MMW) and high molecular weight (HMW) for both aliphatic and aromatic it grew a bit slower as shown in table (3-14) and (3-15). Every bacterial strains has a different capability to degrade crude oil depending on its condition and metabolism and the crude oil concentration (Bhuvaneswar *et al*., 2012).

Table (3-14) : Initial concentration of n-alkanes in control sample and final concentration of n-alkanes in incubation periods.

Bacterial isolates	Initial conc. of crude oil	Incubation periods Final conc. of crude oil		
	n-alkanes	7 days	14 days	21 days
Bacillus safensis	176578.8	112553.89	57437.41	41462.36
Bacillus subtilis	176578.8	50117.14	36670.35	29315.58
Bacillus pumilus	176578.8	97243.66	49863.493	44943.466
Bacillus sporothermodurans	176578.8	75043.34	50502.14	38996.56
Pseudomonas aeruginosa	176578.8	53872.42	41336.28	27076.02
Brevundimonas sp.	176578.8	66924.95	41960.5	27453.85
Arthrobacter luteolus	176578.8	82254.68	46979.81	39259.23
Pseudomonas putida	176578.8	65332.48	40416	27866.31
Pantoea sp.	176578.8	62454.55	41732.61	24190.1
Sphingomonas paucimobilis	176578.8	100460.7	63535.68	35969.0996
Novosphingobium subterraneum	176578.8	52329.42	41524.86	38524.26
Sporosarcina luteola	176578.8	53722.91	44890.65	22459.34
Kocuria kristinae	176578.8	49817.45	44031.43	30555.377
Aeromonas salmonicida	176578.8	55449.52	53225.11	38866.21
Acinetobacter junii	176578.8	61497.26	54771.14	49610.38
Acinetobacter baumannii	176578.8	55873.52	37118.49	19089.94

Table (3-15) : Initial concentration of PAHs in control sample and final concentration of PAHs in incubation periods.

Bacterial isolates	Initial conc. of crude oil	Incubation periods Final conc. of crude oil		
	PAHs	7 days	14 days	21 days
Bacillus safensis	22052.19	10371.681	8790.137	4292. 724
Bacillus subtilis	22052.19	7999.188	3225.037	3087.517
Bacillus pumilus	22052.19	8613.132	4691.711	2796.8178
Bacillus sporothermodurans	22052.19	8425.776	5561.902	4773.321
Pseudomonas aeruginosa	22052.19	7403.143	3459.102	1999.376
Brevundimonas sp.	22052.19	12280.928	4444.136	1943.037
Arthrobacter luteolus	22052.19	13160.501	5799.242	2939.549
Pseudomonas putida	22052.19	10619.813	4313.872	2459.296
Pantoea sp.	22052.19	5639.43	4651.91	2636.332
Sphingomonas paucimobilis	22052.19	9592.69	3748.860	1455.733
Novosphingobium subterraneum	22052.19	12443.23	3579.964	1763.328
Sporosarcina luteola	22052.19	7696.0748	4245.1452	3126.5542
Kocuria kristinae	22052.19	6711.381	3033.55	2694.034
Aeromonas salmonicida	22052.19	6183.951	5746.033	2743.42
Acinetobacter junii	22052.19	6573.444	5623.879	3256.855
Acinetobacter baumannii	22052.19	7198.099	4816.113	4094.101

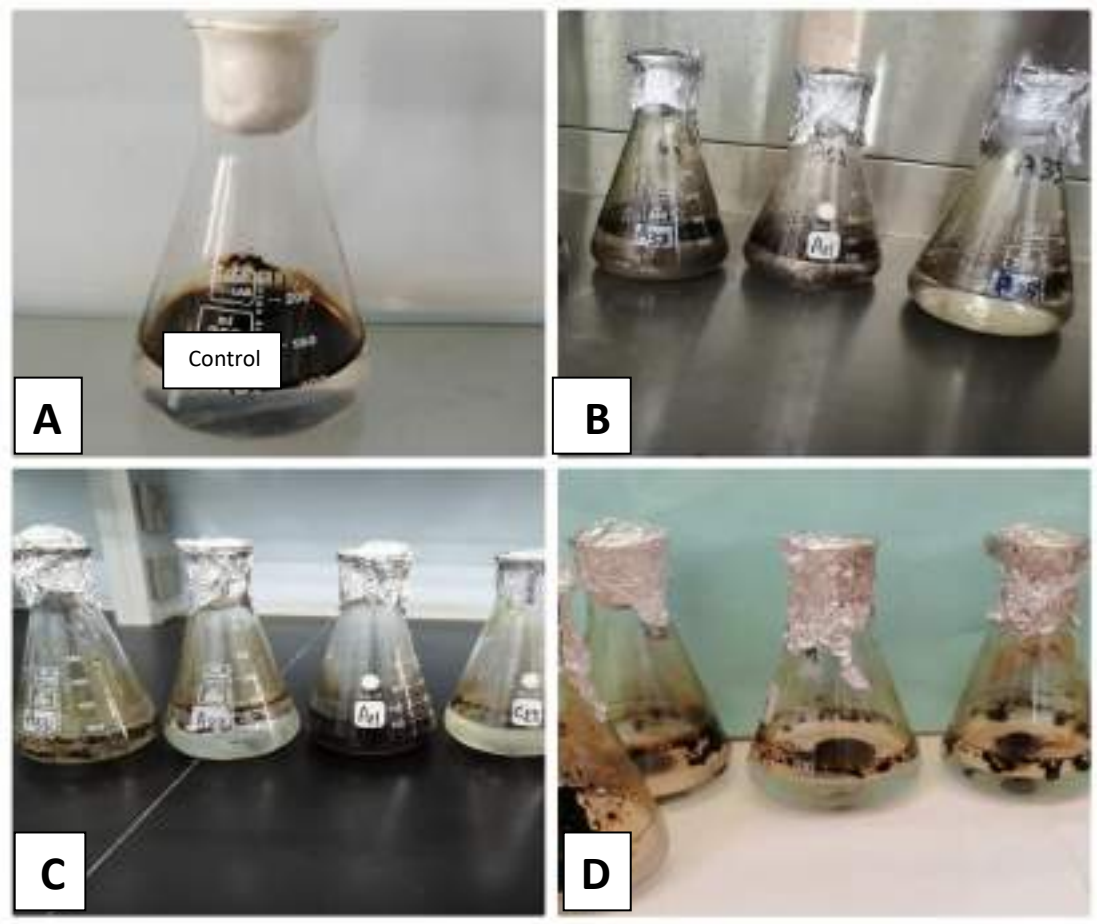
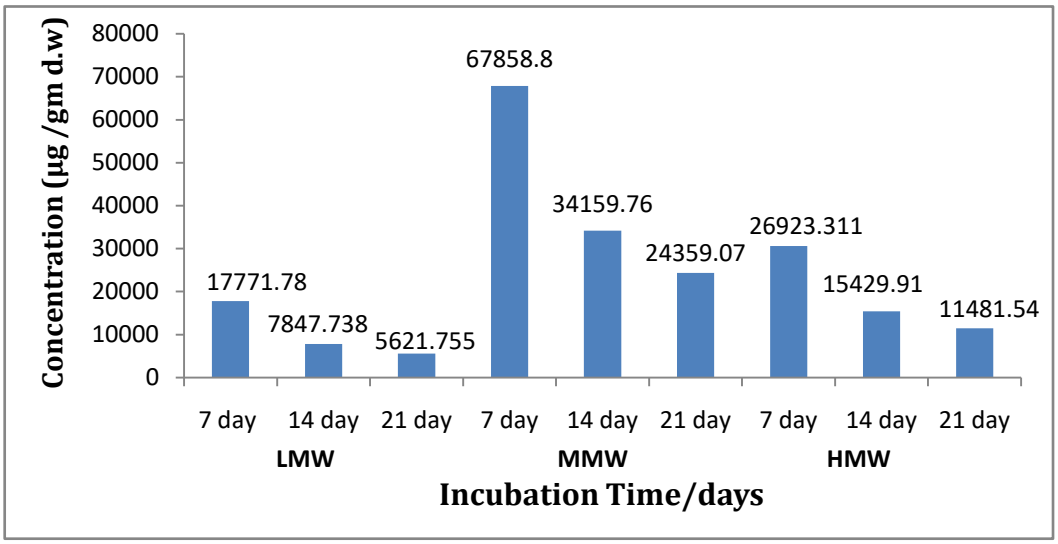


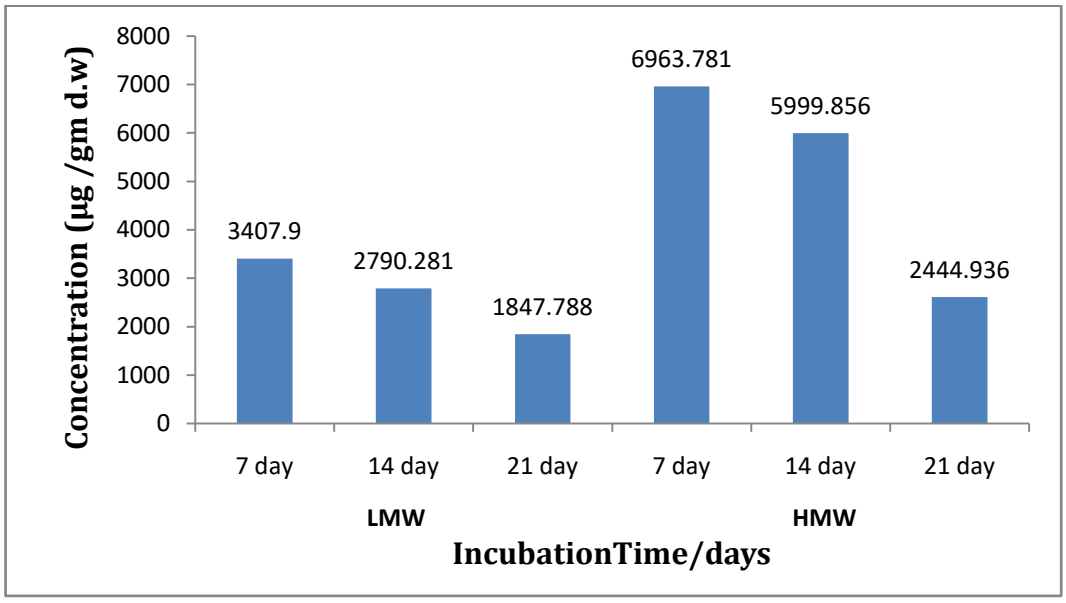
Figure (3-9):Degradation of crude oil by bacterial isolates in three incubation periods. (A) refer to the control sample. (B) refer to the 7 days of incubation. (C) refer to the 14 days of incubation. (D) refer to 21 days of incubation.

3.6.1: Biodegradation by *Bacillus safensis*

As shown in figure (3-10A), (Appendix 6A) *Bacillus safensis* degradation was 17771.78, 7847.738 and 5621.755 µg/gm d.w for the LMW of n-alkanes hydrocarbons firstly and followed by MMW where their degradation were 67858.8, 34159.76 and 24359.07 µg/gm d.w and the HMW were 29623.311, 15429.91 and 11481.54 µg/gm d.w degrade later in the final incubation period (21 days) according to its concentration in control sample of crude oil (Figure 3-8). In addition, the PAHs compounds were also gradually degraded, starting from LMW in the first week of the incubation (3407.9, 2790.281 and 1847.788) µg/gm d.w followed by HMW (6963.781, 5999.856 and 2444.936) µg/gm d.w in the later weeks of the incubation periods as showed in figure (3-10B), (Appendix 6B).



(A)



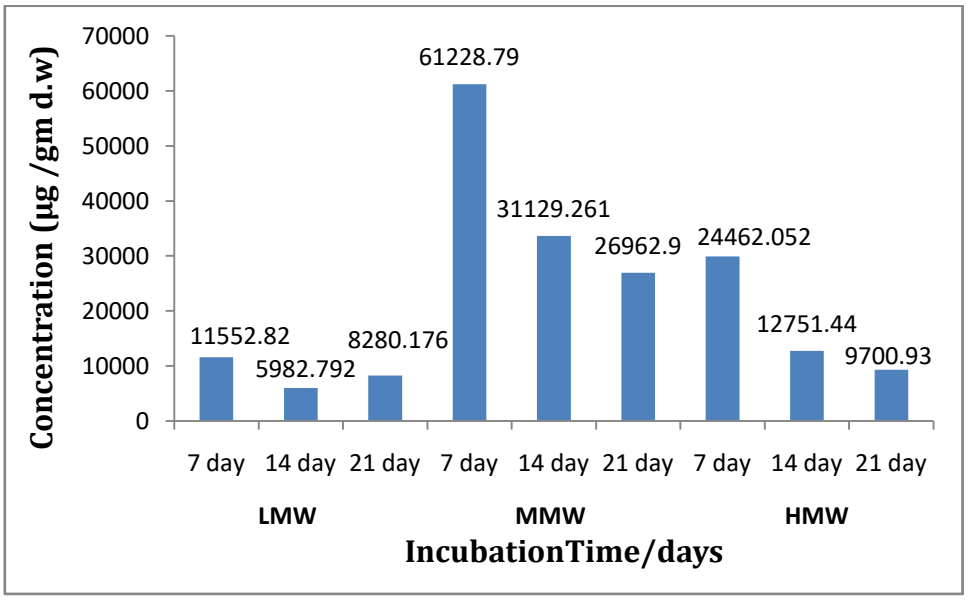
(B)

Figure (3-10): Biodegradation of crude oil by *Bacillus safensis*. (A) n-alkanes. (B) PAHs .

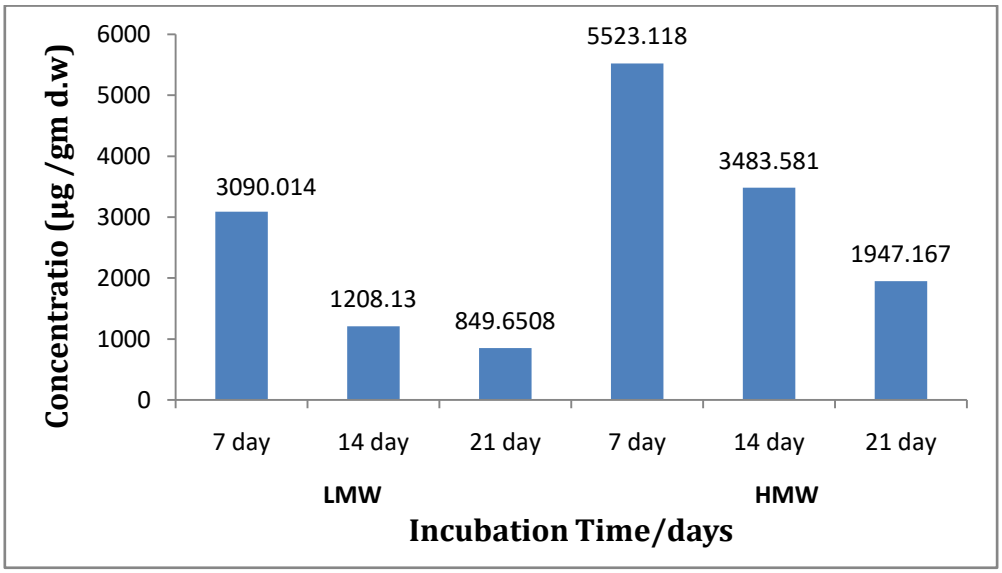
3.6.2: Biodegradation by *Bacillus pumilus*

Bacillus pumilus is another type of bacterial strains that revealed its capacity to degrade both type of hydrocarbons in crude oil including n-alkanes where the concentrations of degradation were 11552.82, 5982.792 and 8280.176 µg/gm d.w for LMW hydrocarbons, 61228.79, 31129.261 and 26962.9µg/gm d.w for MMW and 24462.052, 12751.44

and 9700.93 µg/gm d.w for HMW as shown in figure (3-11A), (Appendix 7A). additionally to the degradation of LMW PAHs hydrocarbons were 3090.014, 1208.13 and 849.6508 µg/gm d.w and 5523.118, 3483.581 and 1947.167 µg/gm d.w for HMW as shown in figure (3-10B), (Appendix 7B).



(A)

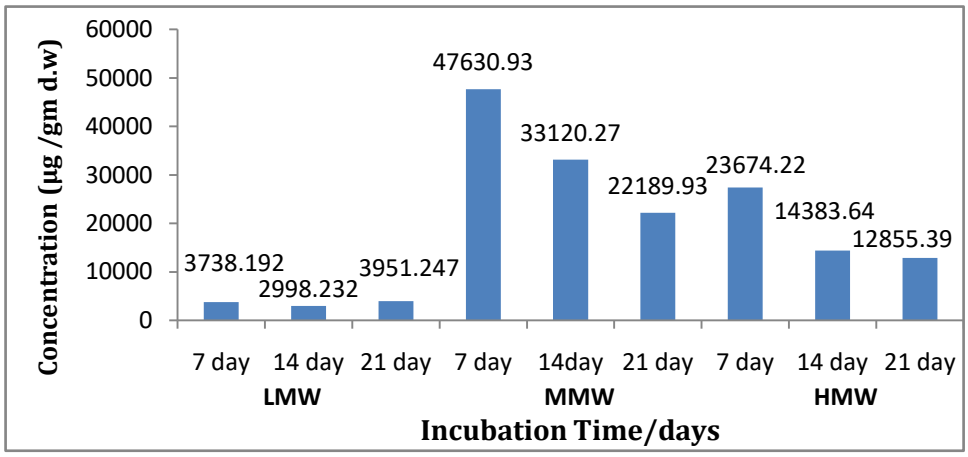


(B)

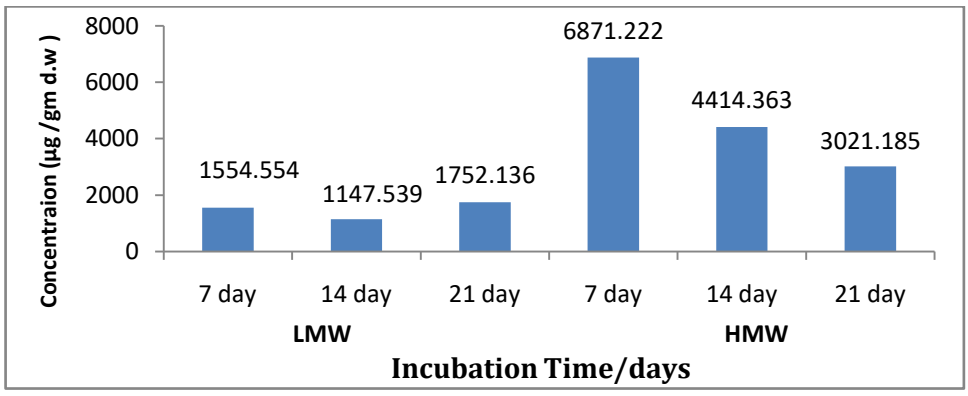
Figure (3-11): Biodegradation of crude oil by *Bacillus pumilus*. (A) n-alkanes. (B) PAHs.

3.6. 3: Biodegradation by *Bacillus sporothermodurans*

As seen in figure (3-12A), (Appendix 8A) which illustrate the biodegradation of crude oil by *Bacillus sporothermodurans* where the LMW of n-alkanes hydrocarbons completely degraded such 3738.192, 2998.232 and 3951.247 µg/gm d.w while others compounds remain at low concentrations such as MMW (47630.93, 33120.27 and 22189.93) µg/gm d.w while the concentrations of HMW were 23674.22, 14383.64 and 12855.39 µg/gm d.w. Also this bacteria shown its ability to degrade PAHs hydrocarbons gradually from LMW where their degradation were 1554.554, 1147.539 and 1752.136 µg/gm d.w to HMW (6871.222, 4414.363 and 3021.185) µg/gm d.w as shown in figure (3-12B), (Appendix 8B) in different incubation periods (7, 14 and 21 days).



(A)

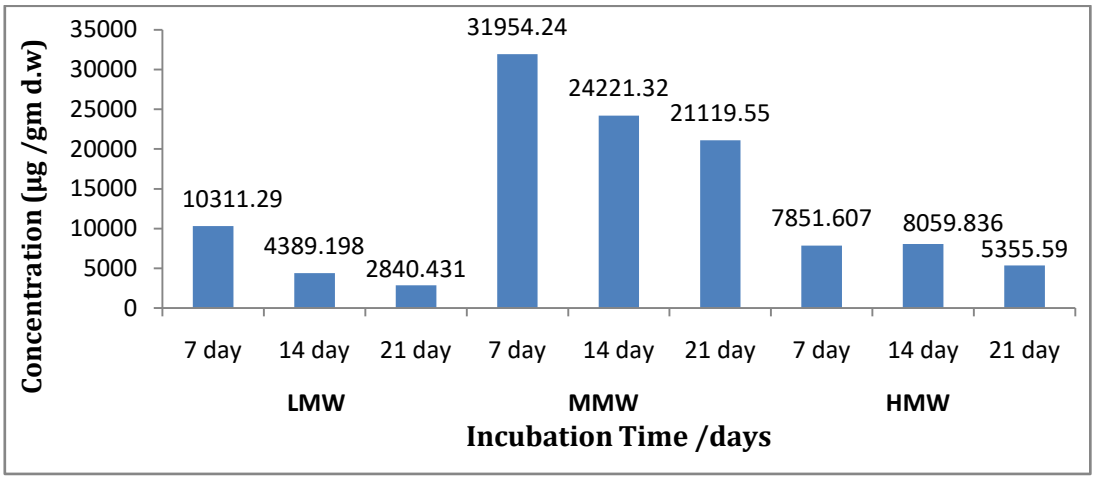


(B)

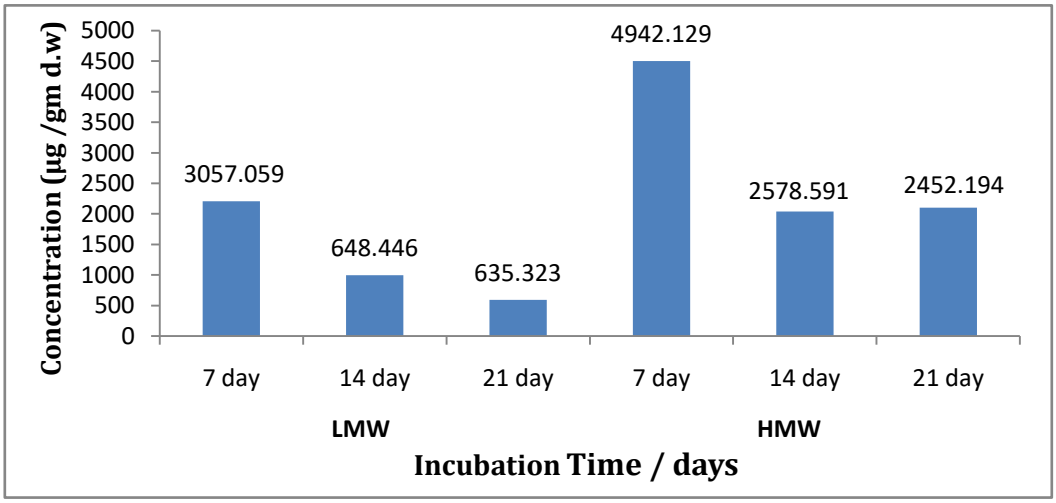
Figure(3-12): Biodegradation of crude oil by *Bacillus sporothermodurans*. (A) n-alkanes . (B) PAHs.

3.6.4 : Biodegradation by *Bacillus subtilis*

According to figure (3-13 A and B), (Appendix 9A and B) *Bacillus subtilis* was showed its ability to degrade n-alkanes hydrocarbons components in all form through three incubation periods, which were 10311.29, 4389.198 and 2840.431µg/gm d.w for LMW, whereas 31954.42, 24221.32 and 21119.55µg/gm d.w for MMW and 7851.607, 8059.836 and 5355.59 µg/gm d.w for HMW at (7, 14 and 21) days respectively. As for PAHs hydrocarbons were gradually degraded from LMW (3057.059, 648.446 and 635.323) µg/gm d.w to HMW (4942.129, 2578.591 and 2452.194) µg/gm d.w at (7, 14 and 21) days of incubation periods respectively.



(A)



(B)

Figure(3-13): Biodegradation of crude oil by *Bacillus subtilis*.

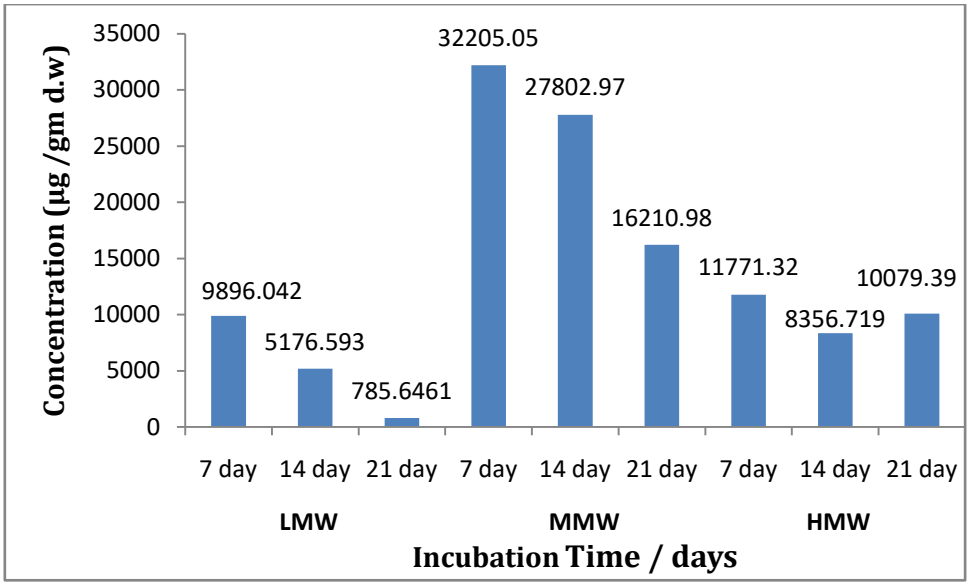
(A) n-alkanes. (B) PAHs.

El-Sheshtawy and his team (2013) isolated Bacillus sp. and showed its biodegradation capacity in MSM containing crude oil as a sole source of carbon and energy. In accordance with results of the Lily *et al*. (2009) and Gupta (2012) *Bacillus subtilis* have been reported to have the potentials to utilise several compounds of n-alkanes and PAHs hydrocarbons as sole source of carbon and energy.

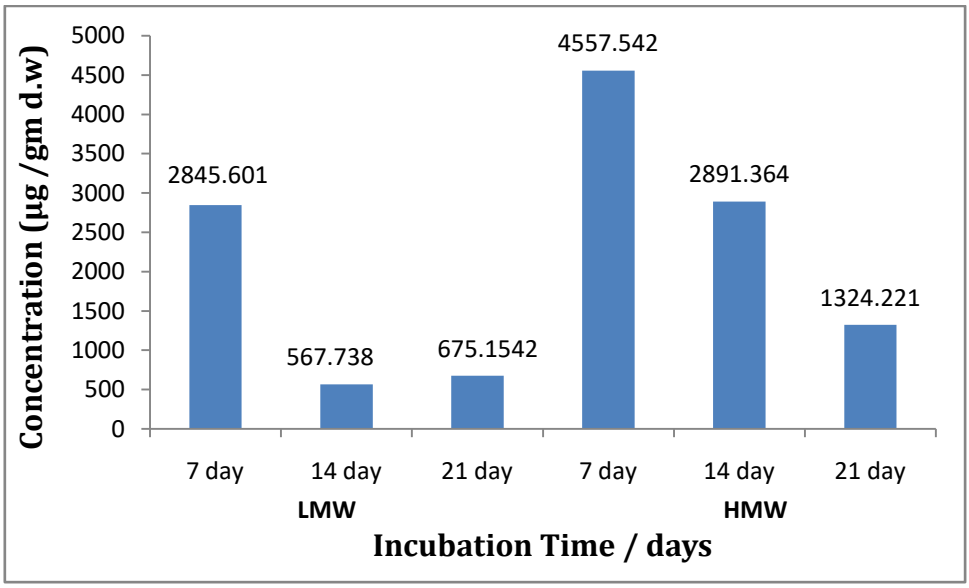
Bacillus species are more tolerant high levels of oils due to their resistant endospores. They are known to possess a more competent and active oil degrading enzymes than other biodegraders (Darsa *et al*., 2014). Degradation of oil by these microbial consortia shows that they have specialized co-metabolic capacities (Bisht *et al*., 2014).

3.6.5 : Biodegradation by *Pseudomonas aeruginosa*

As turn out from GC analyzing to n-alkanes hydrocarbons (Appendix 10A) and PAHs hydrocarbons fractions (Appendix 10B), *Pseudomonas aeruginosa* was able to degrade almost concentration of LMW and HMW sequentially for both types of crude oil, n-alkans as seen in figure (3-14A), which degraded to concentrations of 9896.042, 32205.05 and 11771.32µg/gm d.w at the first incubation periods (7 days) for LMW, MMW and HMW respectively, while at second and third incubation periods (14 and 21) days the concentration of n-alkanes were 5176.593, 27802.97 and 8356.719 µg/gm d.w and 785.6461, 16210.89 and 10079.39µg/gm d.w for LMW, MMW and HMW respectively. As concerning with PAHs as shown in figure (3-14B). LMW were firstly degraded 2845.601, 567.738 and 675.1542 µg/gm d.w and later degraded 4557.542, 2891.364 and 1324.221 µg/gm d.w for HMW at the three incubation periods.



(A)



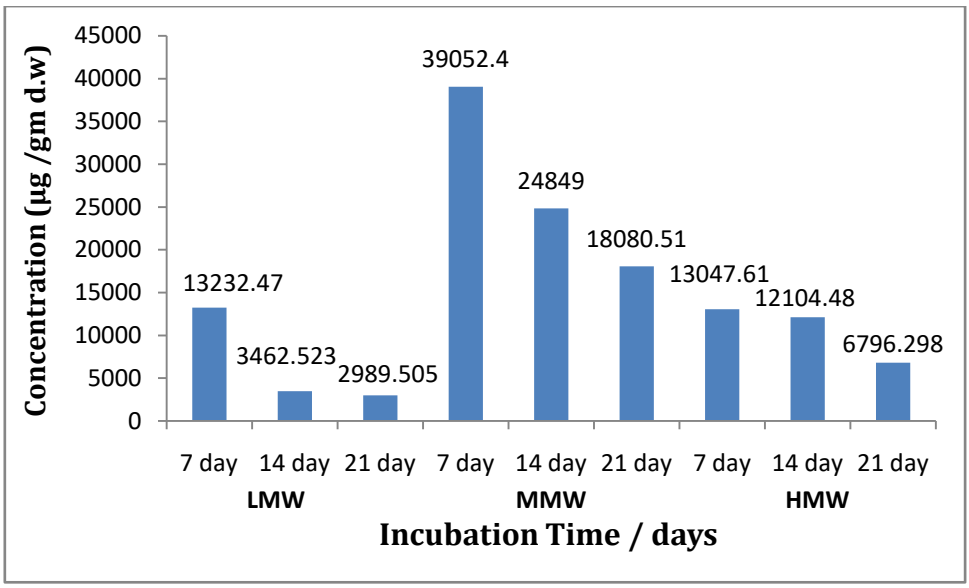
(B)

Figure(3-14): Biodegradation of crude oil by *Pseudomonas aeruginosa*. (A) n-alkanes. (B) PAHs.

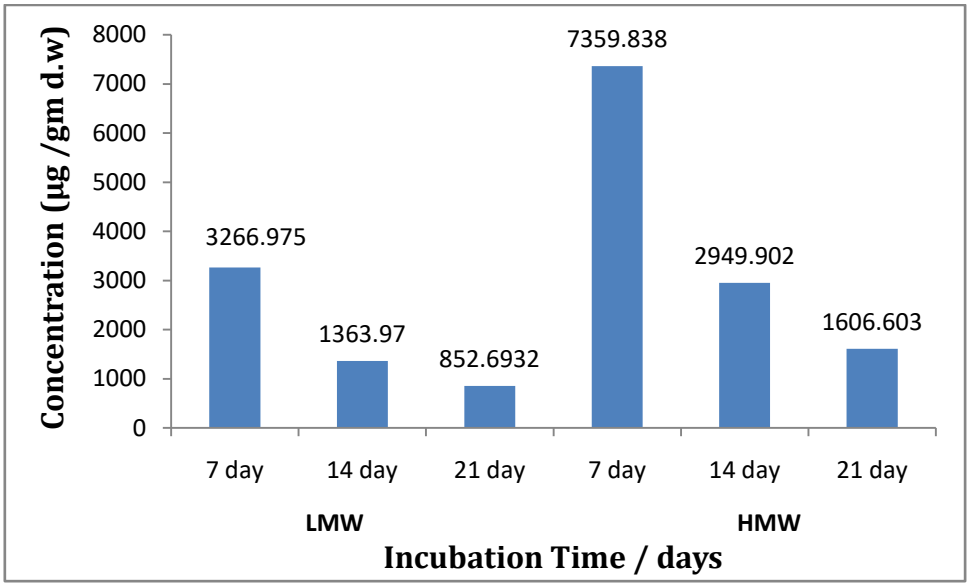
3.6.6 : Biodegradation by *Pseudomonas putida*

The results of bacterial GC analyzing (Appendix 11A and B) applying a pure bacterial culture of *Pseudomonas putida* exhibited that after 21 days it is possible to degrade LMW and HMW of n-alkanes and PAHs hydrocarbons, as displayed in figure (3-15 A and B). It is apparent from the obtained results that the application of this bacterial culture is suitable

for the degradation of LMW (13232.47, 3462.523 and 2989.505) µg/gm d.w, MMW (39052.4, 24849 and 18080.51) µg/gm d.w and HMW (13047, 12104 and 6796.298) µg/gm d.w. In addation to degradation of PAHs were 3266.957, 1363.97 and 852.6932µg /gm d.w for LMW and 7359.838, 2949.902 and 1606.603 µg /gm d.w for HMW at the three incubation periods.



(A)



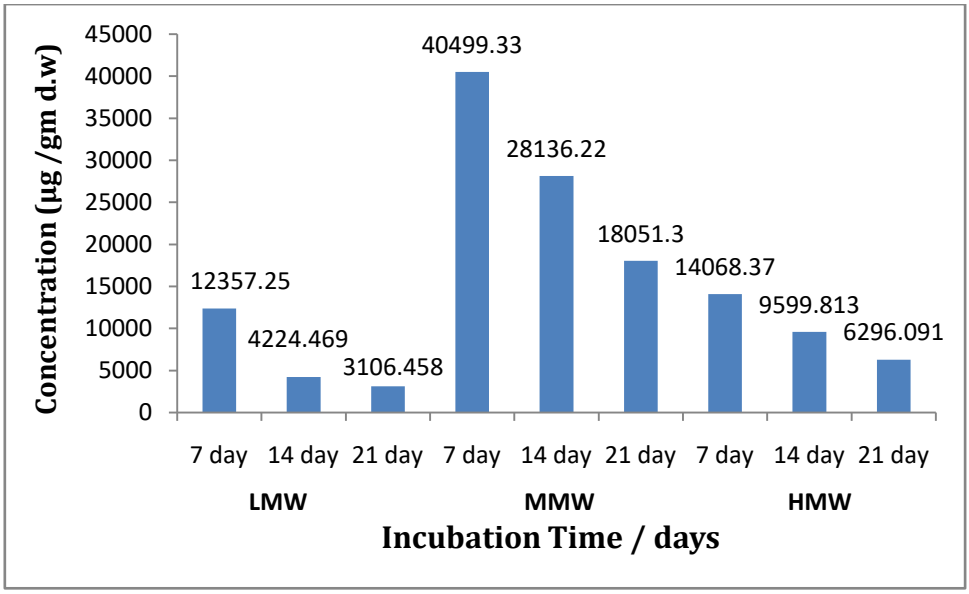
(B)

Figure(3-15) : Biodegradation of crude oil by *Pseudomonas putida*. (A) n-alkanes. (B) PAHs.

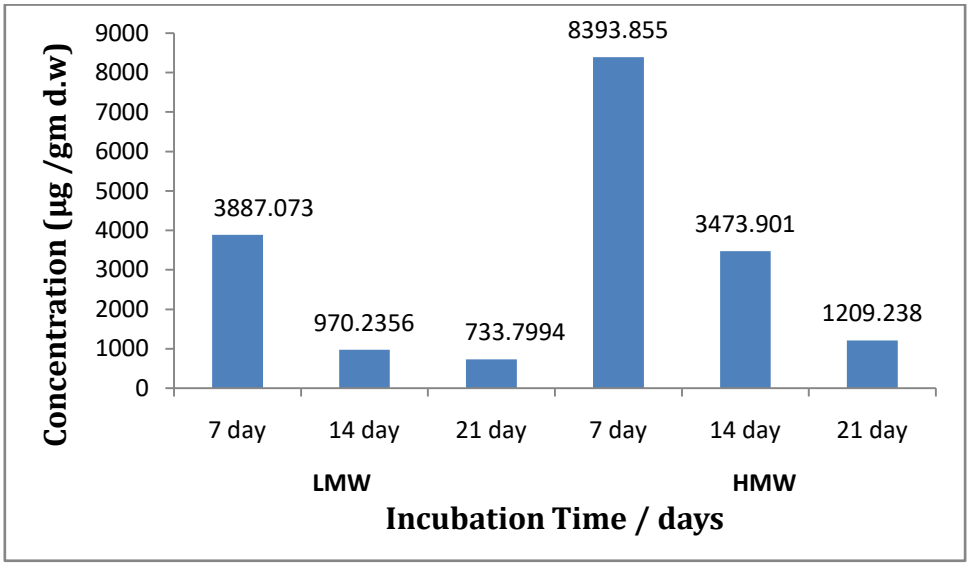
Pseudomonas sp. has the ability to degrade and remove n-alkanes and other PAHs hydrocarbons, because these bacteria has normally involves the enzymatic activity to degradation almost crude oil compounds (Sarang *et al*., 2013 ; Safiyanu *et al*., 2015).

3.6.7 : Biodegradation by *Brevundemonas sp.*

GC analysis of all hydrocarbon components in the oil sample after 7, 14 and 21 days of biodegradation by *Brevundemonas sp.* is shown in figure (3-16 A and B), (Appendix 12A and B). *Brevundemonas sp.* can significantly degrade LMW (12357.25, 4224.469 and 3106.458) µg /gm d.w, MMW (40499.33, 28136.22 and 18051.3) µg /gm d.w and HMW (14068.37, 9599.813 and 6296.091) µg /gm d.w for n-alkanes and PAHs hydrocarbons where the concentrations of degradation were 3887.073, 970.2356 and 733.7994 µg /gm d.w for LMW and 8393.855, 3473.901 and 1209.238 µg /gm d.w for HMW. This bacteria has the potential to be used as the single microbe for biodegradation of soil contaminated by crude oil. These findings were in agreement with our findings, Basuki (2017), found that *Brevundemonas sp.* was able to degrade most all types of the hydrocarbons within the oil for 14 days.



(A)



(B)

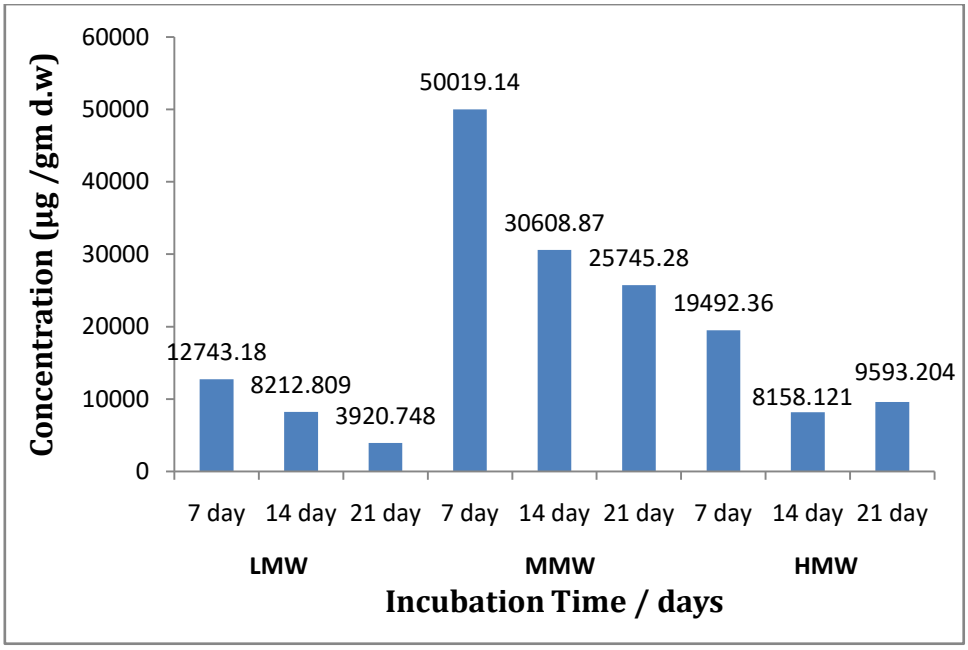
Figure (3-16) : Biodegradation of crude oil by *Brevundemonas sp.*

(A) n-alkanes. (B) PAHs.

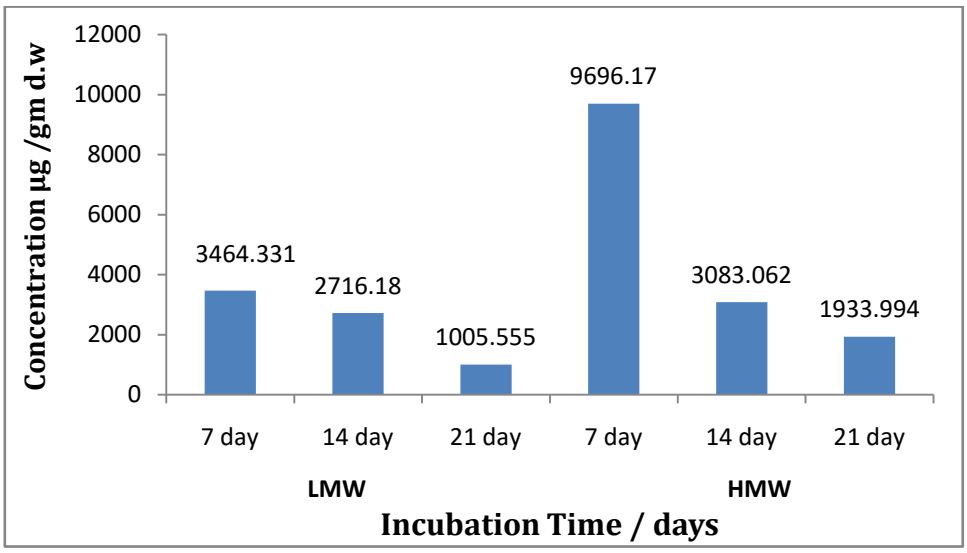
3.6.8 : Biodegradation by *Arthrobacter luteolus*

As shown in figure(3-17 A and B), (Appendix 13A and B) *Arthrobacter luteolus* was able to degrade n-alkanes for LMW (12743.18, 8212.809 and 3920.748) µg/gm d.w, 50019.14, 30608.87 and 25745.28 µg/gm d.w for MMW and 19492.36, 8158.121 and 9593.204 µg/gm d.w for HMW. As for PAHs were gradually degraded from LMW (3464.331, 2716.18 and 1005.555) µg/gm d.w to HMW (9696.17, 3083.062 and 1933.994) µg/gm d.w at one, two and three weeks of incubation periods respectively. *Arthrobacter luteolus* is one of the types of bacteria able to biodegrade oil and use it as a source of carbon and energy. *Arthrobacter* species have been isolated from soil contaminated with n-alkanes and PAHs hydrocarbons, they are extremely tolerant and resistant to most metals and other toxic substances, but able to degrade some hydrocarbons to its less toxic form (Unell, 2008).

Arthrobacter species in conjunction with several other strains use their vast co-metabolic activities to degrade pollutants in a synergistic relationship, using their target contaminant as their sole source of carbon and energy (Sahoo et al., 2011 ; kuce et al., 2015). Cameotra and Makkar (2010) reported that *Arthrobacter* sp., which were also identified in this study have specific adhesion mechanisms and produce extracellular emulsifying agents for contact with water-insoluble hydrocarbons.



(A)



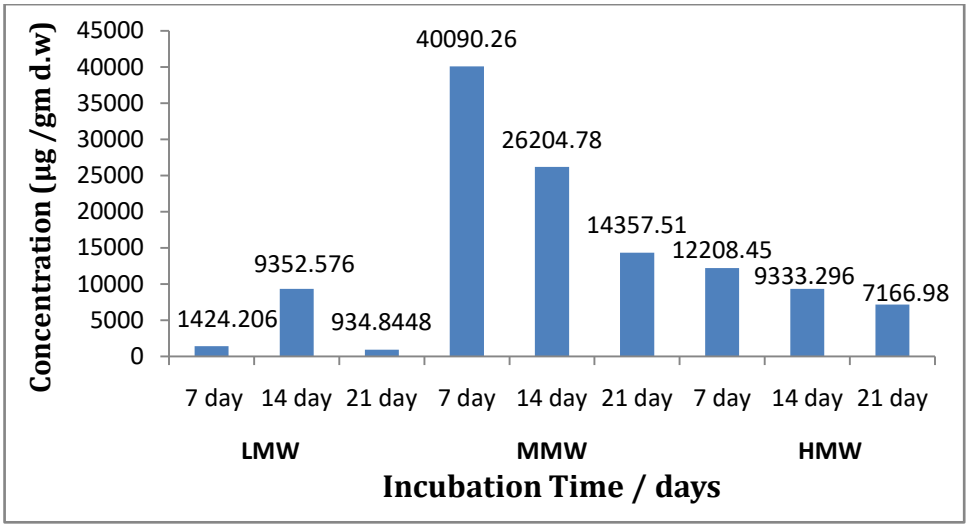
(B)

Figure(3-17) : Biodegradation of crude oil by *Arthrobacter luteolus*. (A) n-alkanes. (B) PAHs.

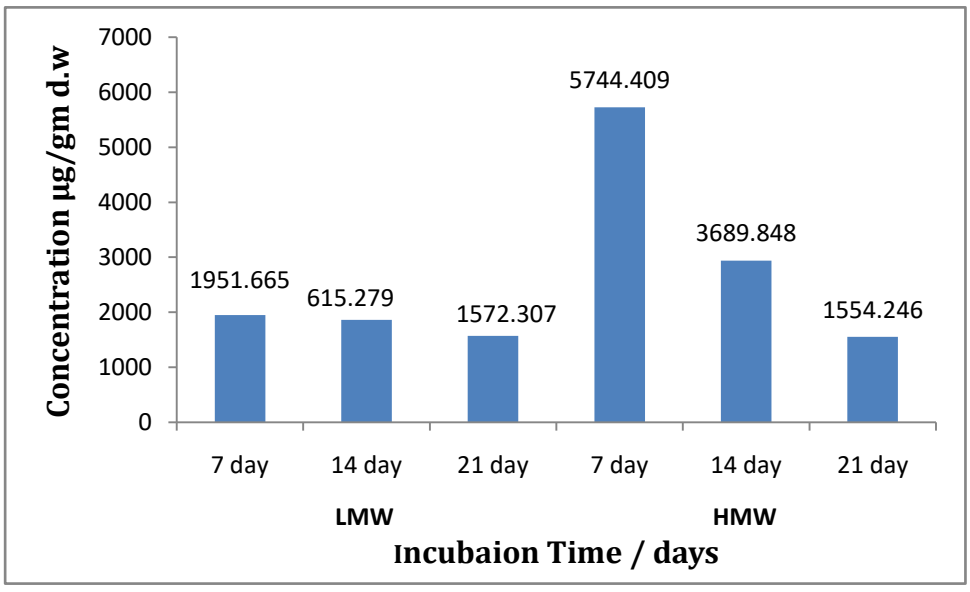
3.6.9: Biodegradation by *Sporosarcinia luteola*

Sporosarcinia luteola was a type of bacteria that has a capacity to degrading crude oil components in all forms, which exhibited its ability to degradation after 7 day of incubation periods and it continued to a later weeks as shown in figure (3-18 A and B) (Appendix 14A and B). n-alkanes concentration were decreasing from LMW (1424.206, 9352.576

and 934.8448) µg/gm d.w to MMW (40090.26, 26204.78 and 14357.51) µg/gm d.w then to HMW (12208.45, 9333.296 and 7166.98) µg/gm d.w. PAHs were also degraded from LMW (1951.665, 615.297 and 1572.3074) µg/gm d.w to HMW (5744.409, 3689.848 and 1554.246) µg/gm d.w. Ran *et al*. (2016) found that the degradation mechanism may be as a result of a bacterium induced enzymatic reaction. .



(A)

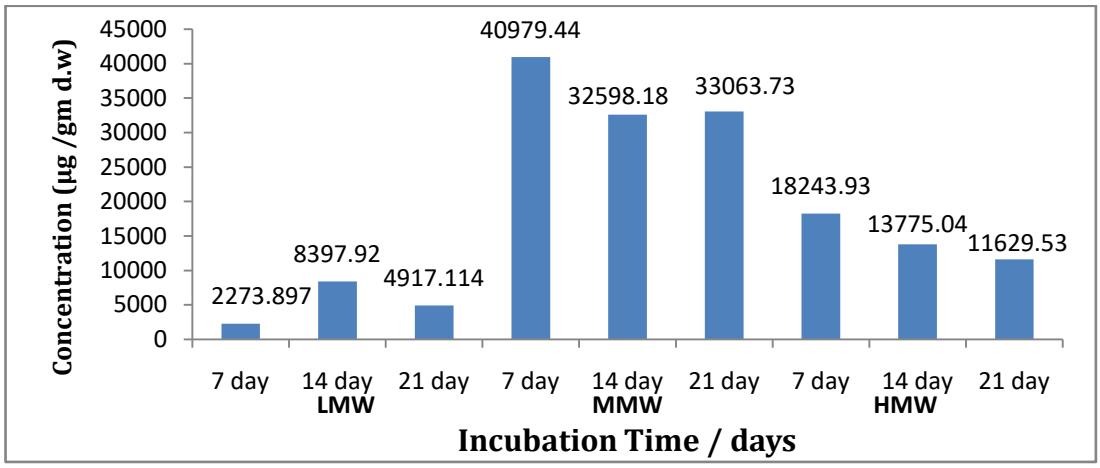


(B)

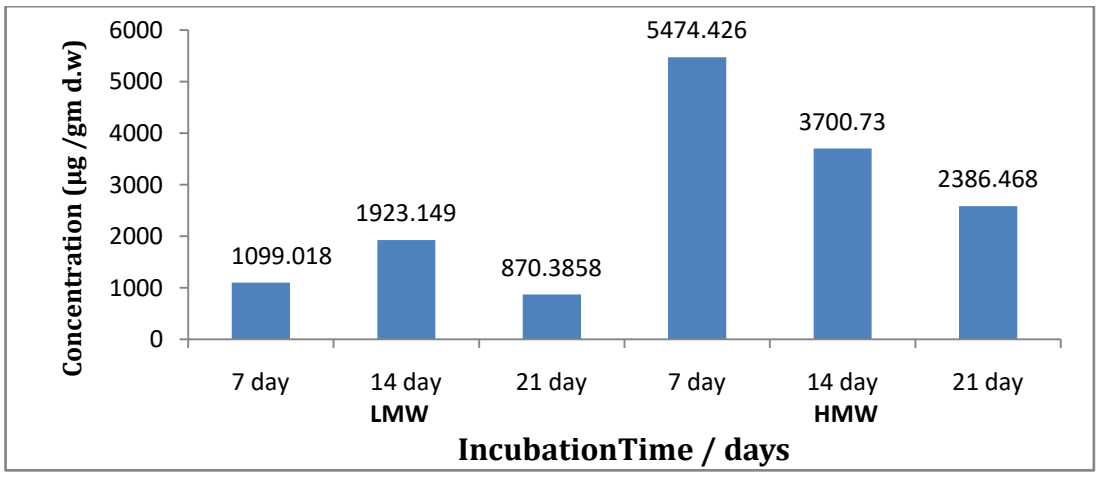
Figure(3-18) : Biodegradation of crude oil by *Sporosarcinia luteola*. (A) n-alkanes. (B) PAHs.

3.6.10: Biodegradation by *Acinetobacter junii*

According to the Figure (3-19 A and B), (Appendix 15A and B) *Acinetobacter junii* preferentially degraded almost the aromatic fraction hydrocarbons compared to aliphatic fraction compounds (C7–C40) gradually from LMW and HMW which are usually much more difficult to degrade within the 21 days duration of the experiment. n-alkanes were degraded from LMW (2273.897 8397.92 4917.114) µg/gm d.w to MMW (40979.44 , 32598.18 and 33063.73) µg/gm d.w and then degraded to HMW (18243.93, 13775.041 and 1629.53) µg/gm d.w. PAHs were degraded from LMW (1099.018, 1923.149 and 870.3858) µg/gm d.w to HMW (5474.426, 3700.73 and 2386.468) µg/gm d.w.



(A)

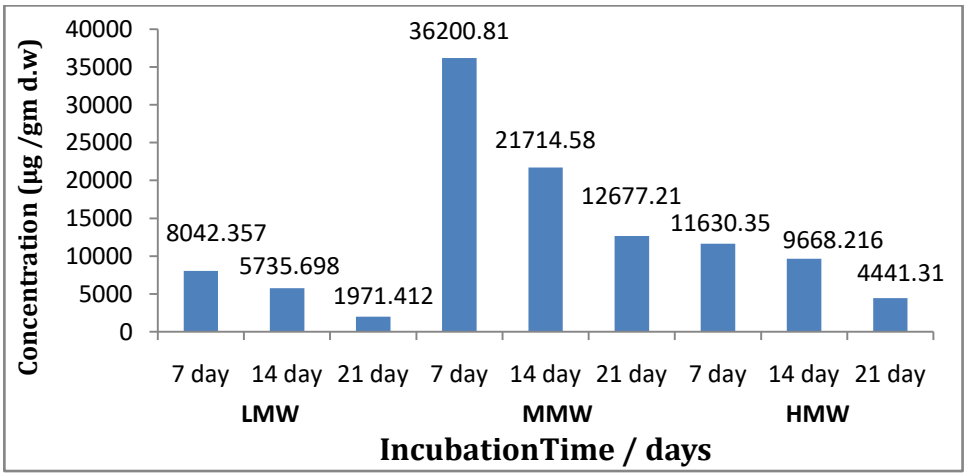


(B)

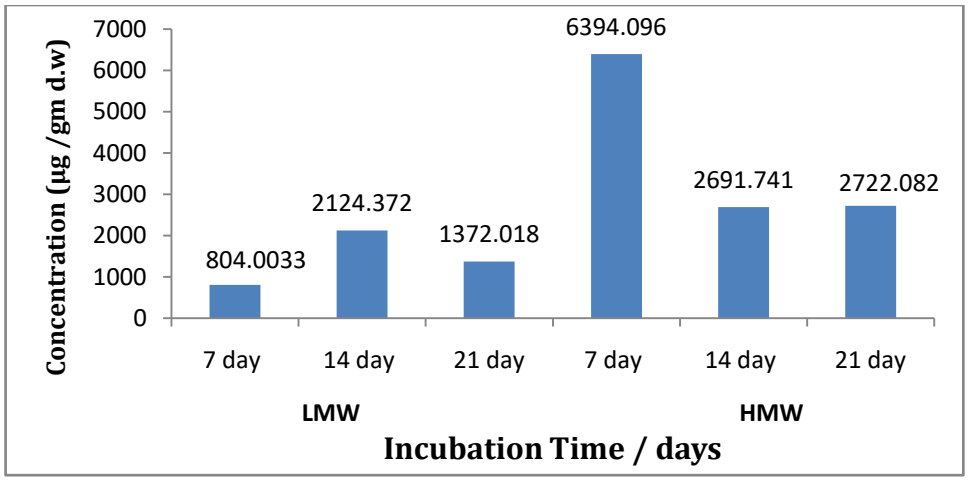
Figure(3-19) : Biodegradation of crude oil by *Acinetobacter junii*. (A) n-alkanes. (B) PAHs.

3.6.11: Biodegradation by *Acinetobacter baumannii*

Acinetobacter baumannii were also able to degrade crude oil hydrocarbons, it is degraded n-alkanes compounds (C7–C40) gradually from LMW were 8042.357, 5735.698 and 1971.412µg /gm d.w followed by MMW were 36200.81, 21714.58 and 12677.21 µg /gm d.w and HMW were 11630.35, 9668.216 and 4441.31 µg /gm d.w. The degradation of PAHs compounds were 804.0033, 2124.372 and 1372.018µg /gm d.w of LMW and 6394.096, 2691.741 and 2722.08µg /gm d.w of HMW within the 21 days duration of the experiment (figure, 3-20 A and B), (Appendix 16A and B).



(A)



(B)

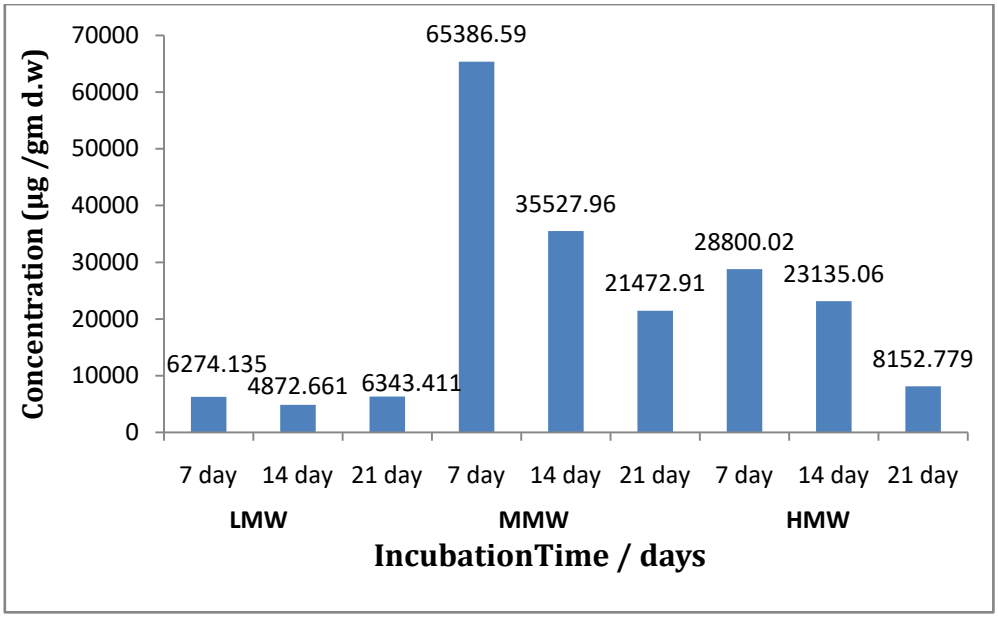
Figure (3-20) : Biodegradation of crude oil by *Acinetobacter baumannii*. (A) n-alkanes. (B) PAHs.

Other studies showed that *Acinetobacter* sp. isolated from hydrocarbon-contaminated soil sites have been reported to have a good ability to grow on crude oil, findings of the current study were in agreement with the results of Saimmai *et al*. (2012) and Huang *et al.* (2013). *Acinetobacter sp.* was able to grow well on media containing crude oil as a sole carbon source because of their ability to produce biosurfactants , and emulsifying these non-hydrophilic hydrocarbons. Luo and his team (2013) showed that PAH-degrading bacteria can produce extracellular biosurfactants to solubilize hydrocarbons into the aqueous medium and enhance the bioavailability for degradation. *Acinetobacter* sp. which is able to utilize n-alkanes and PAHs in the current study, was also found among the PAH degrading isolates retrieved from surface microlayer in an estuarine system (Coelho *et al*., 2010).

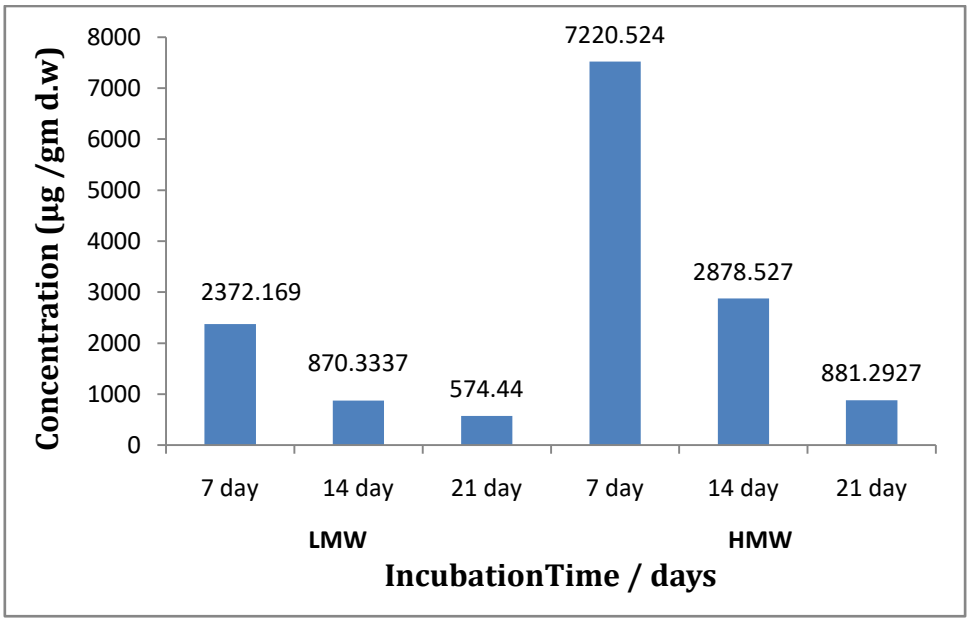
3.6.12 : Biodegradation by *Sphingomonas paucimobilis*

Sphingomonas paucimobilis was capable of actively degrading all types of hydrocarbons compounds present in the crude oil of 0.5% concentrations (Figure 3-21A and B), (Appendix 17A and B) begin from the seven days of incubation and it continued during the other days of incubation through two and three weeks. n-alkanes were degraded 6274.135, 4872.661 and 6343.411µg/gm d.w for LMW , 65386.59, 35527.96 and 21472.91 µg/gm d.w for MMW and 28800.02, 23135.06 and 8152.779 µg/gm d.w for HMW. In addition to the degradation of PAHs, 2372.169, 870.3337 and 574.44 µg/gm d.w for LMW and 7220.524, 2878.527 and 881.2927 µg/gm d.w for HMW.

The results of the present study were in agreement with Al-Taee and his team. (2017) who reported the ability of *Sphingomonas paucimobilis* to degrade n-alkanes compounds as illustrated in figure (3-21A). Also, *Sphingomonas paucimobilis* has shown a high ability to degrade PAHs hydrocarbons from crude oil as shown in figure (3-21B). Based on these findings, *Sphingomonas paucimobilis* is considered as an excellent agent in biodegradation soil polluted with both hydrocarbons types as reported by Barth (2003) who found that *Sphingomonas paucimobilis* bacteria was typical in hydrocarbon degradation .



(A)



(B)

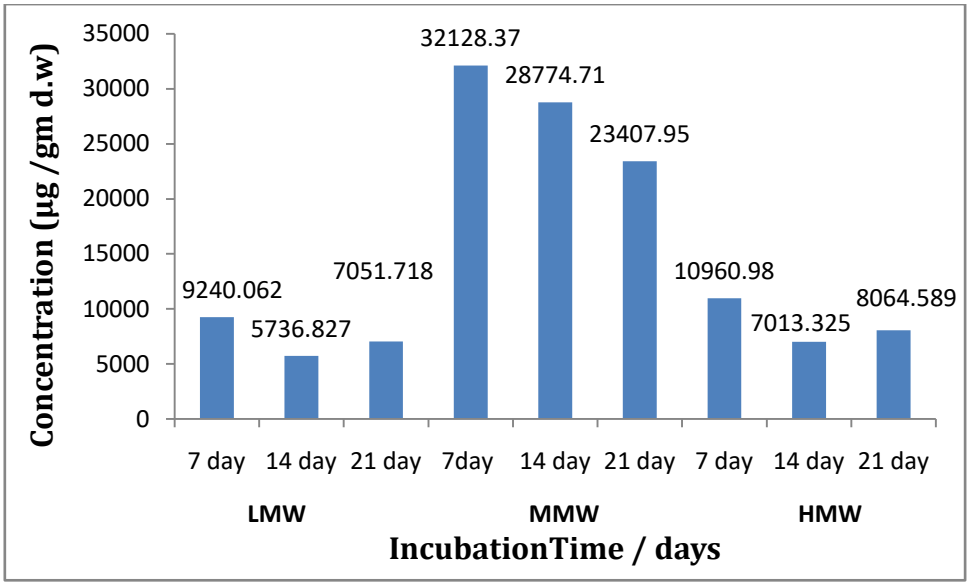
Figure (3-21) : Biodegradation of crude oil by *Sphingomonas paucimobilis*. (A) n-alkanes. (B) PAHs.

3.6.13 : Biodegradation by *Novosphingobium subterraneum*

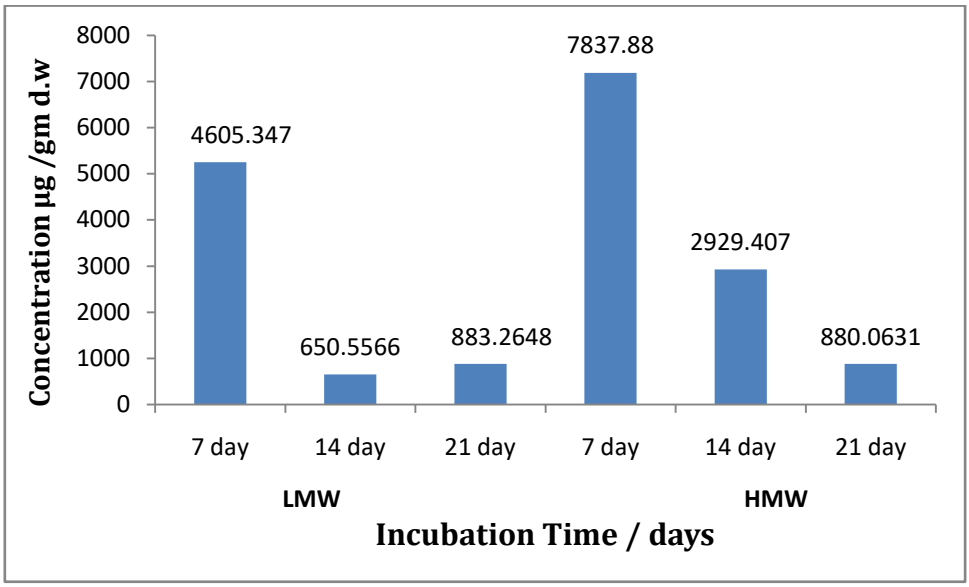
As shown in the current results the degradation of n-alkanes compounds by *Novosphingobium subterraneum* through three incubation periods (7, 14 and 21 days) started from LMW where degradation were 9240.062, 5736.827and 7051.718µg/gm d.w to MMW (32128.37, 28774.71 and 23407.95) µg/gm d.w and HMW were degraded in the end of periods (10960.98, 7013.325 and 8064.589) µg/gm d.w (Figure (3-22A), (Appendix 18A) . On the other hand, the degradation of PAHs compounds also started from LMW (4605.347, 650.5566 and 883.2648) µg/gm d.w to HMW (7837.88, 2929.407 and 880.0631) µg/gm dry weight (Figure 3-22B), (Appendix 18B) in the same periods of incubations of *Novosphingobium subterraneum* in compared with control concentration.

The decreasing in the concentration of crude oil compounds as show in figure (3-22 A and B) due to the abilty of *Novosphingobium subterraneum* to use the crude oil as a carbon source. Sohn *et al*.(2004) and Liu *et al.* (2005) considering *Novosphingubium subterraneum* one of the most important bacteria which used to treatment and removal oil from soil because its high ability to degrade oil.

The ability of strain *Novosphingobium subterraneum* to spontaneously form biofilm on several surfaces could allow the adhesion of cells to soil, Moreover, its ability to form emulsions reduces the need to use detergents which are often required in bioremediation treatments and its capacity to encapsulate oil drops and to preferentially remove the crude oil components may avoid the dispersion of toxic hydrocarbons components in the environment (Notomista *et al*., 2011).



(A)

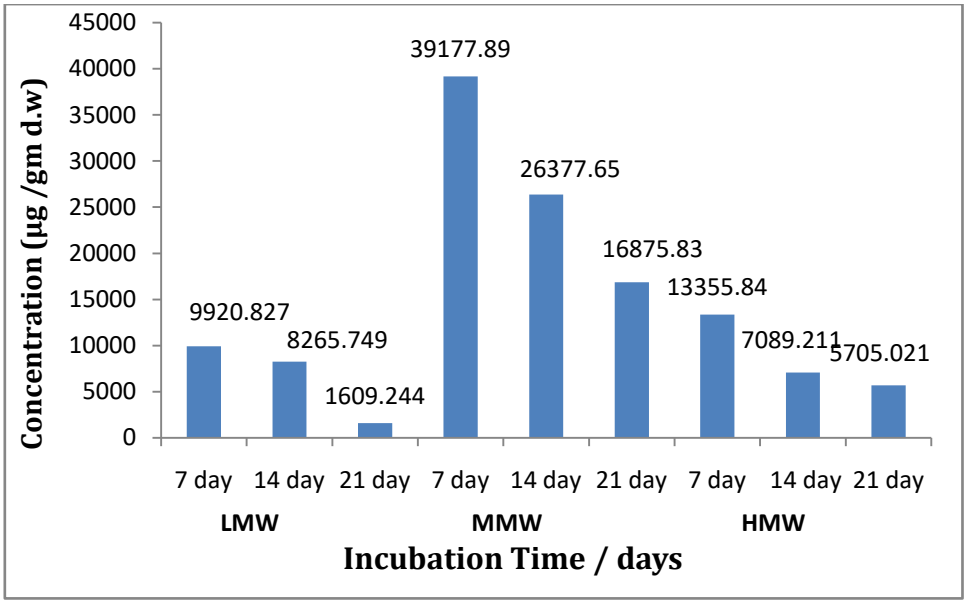


(B)

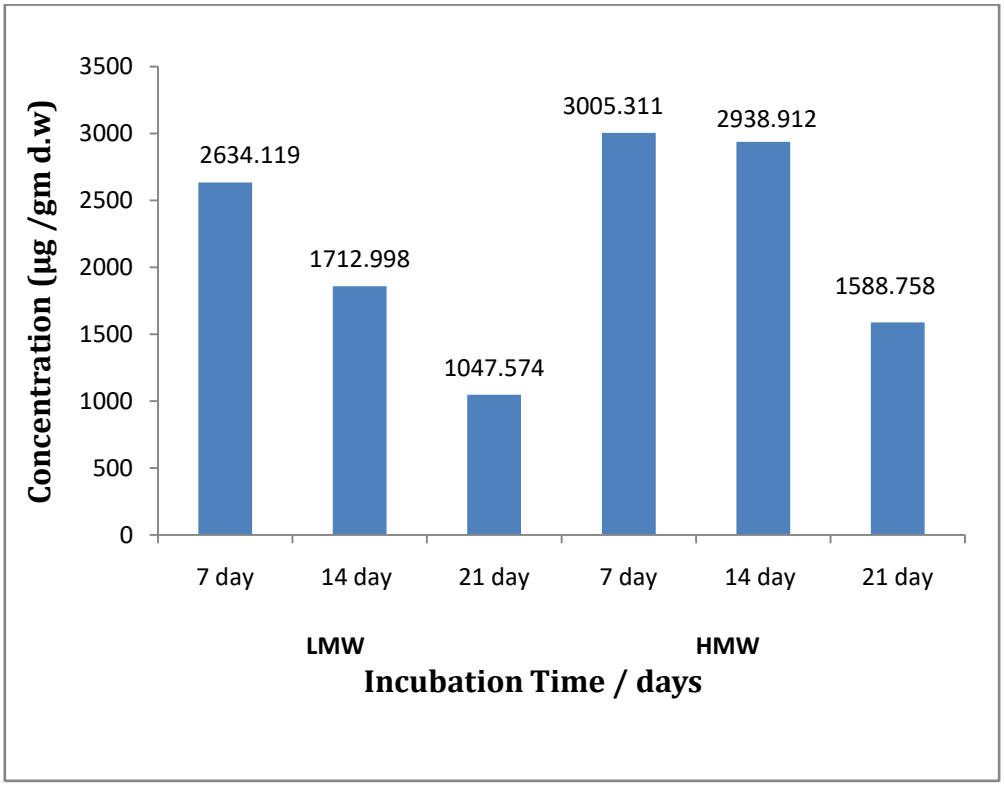
Figure (3-22) : Biodegradation of crude oil by *Novosphingobium subterraneum*. (A) n-alkanes. (B)PAHs.

3.6. 14 : Biodegradation by *Pantoea sp.*

Figure (3-23A and B), (Appendix 19A and B) showed the degradation of hydrocarbons compounds by the bacterium *Pantoea sp.* , the results showed the ability of *Pantoea sp*. to degrade n-alkanes starting from LMW where the degradation were 9920.827, 8265.749 and 1609.244 µg /gm d.w followed by MMW (39177.89, 263774.65 and 16875.83) µg /gm d.w and HMW (13355.84, 7089.211 and 5705.021) µg /gm d.w. Also it is able to degrade PAHs compounds (2634.119, 1712.998 and 1047.574) µg /gm d.w for LMW and 3005.311, 2938.912 and 1588.758 µg /gm d.w for HMW. In the first week the results showed that the LMW compounds were degraded firstly, MMW and HMW were degraded later days after two and three weeks. The same conclusion was achieved by Mohammed *et al*. (2017). The results obtained by Vasileva-Tonkova and Gesheva (2007) showed increased cell hydrophobicity and enhanced both the surface tension lowering capacity and the emulsifying potential of biosurfactants produced by *Pantoea* sp. when grown on crude oil.



(A)



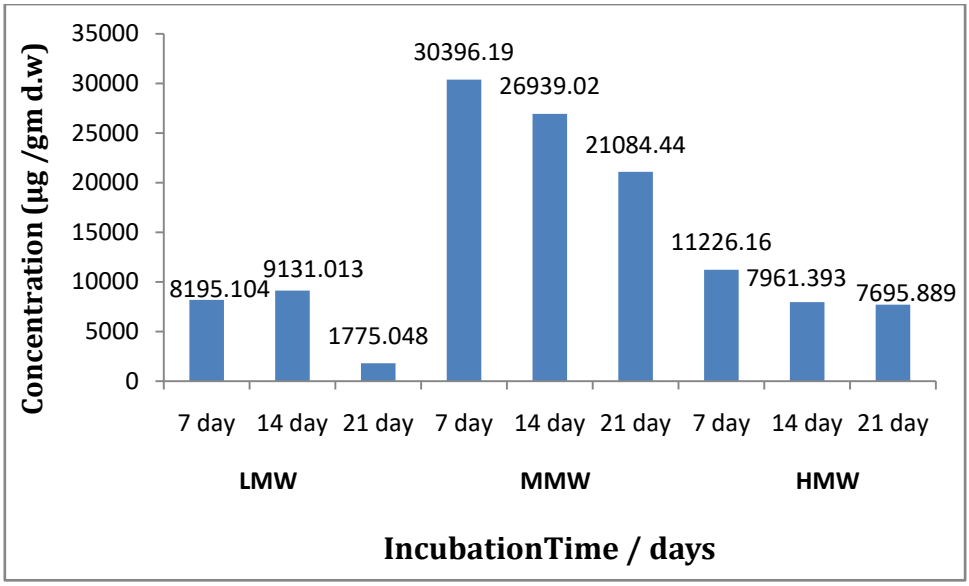
(B)

Figure(3-23) : Biodegradation of crude oil by *Pantoea sp*. (A) n-alkanes. (B) PAHs.

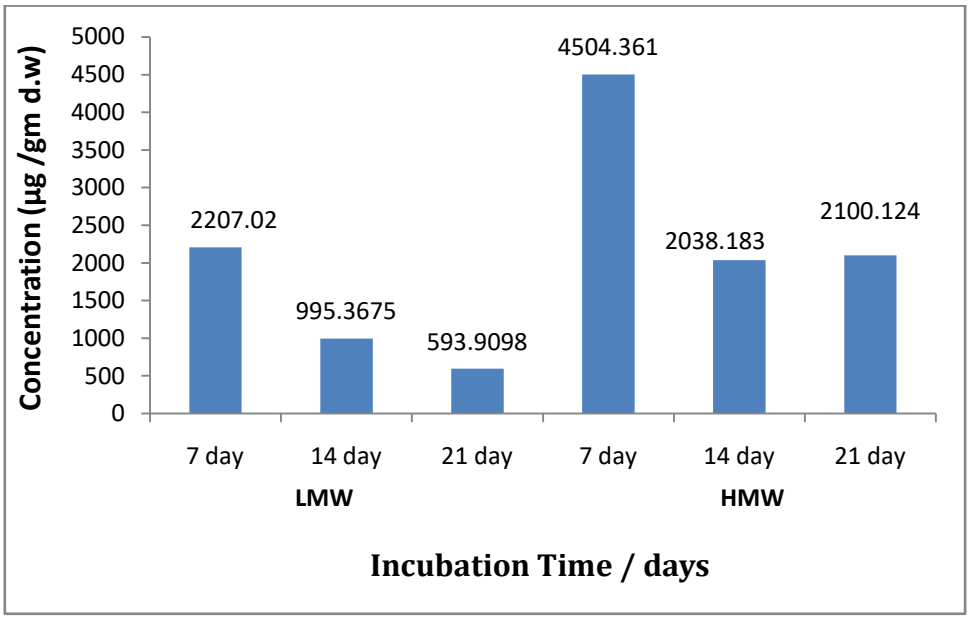
3.6.15 : Biodegradation by *Kocuria kristina*

PAHs and n-alkanes were degraded by *Kocuria kristina* in according to molecular weight for each compounds. n-alkanes were degraded from LMW (8195.104, 9109.382 and 1775.048) µg /gm d.w to MMW (30396.19, 26939.02 and 21084.44) µg /gm d.w and at the end of incubation periods HMW were degraded 11226.16, 7961.393 and 7695.889 µg /gm d.w as shown in figure (3-24A), (Appendix 20A). PAHs were degraded also from LMW in the first incubation periods (2207.02, 995.3675 and 593.9098) µg /gm d.w and at the end of incubation periods HMW were degraded 4504.361, 2038.183 and 2100.124µg /gm d.w as shown in figure (3-24B), (Appendix 20B).

As shown in figure (3-24 A and B) the decreasing in the n-alkanes and PAHs concentration due to the ability of *Kocuria kristina* in biodegradation, the present results are in accordance with many researchers who documented that *Kocuria kristina* is one of the bacterial type that capable to biodegrading of crude oil and showed the role of *Kocuria sp.* in biodegradation of hydrocarbons and used it as a sole sources of carbon and energy (Mariano *et al*., 2007 ; Tumaikina *et al*., 2008 ; Esmaeil *et al*., 2009). Matvyeyeva *et al*. (2014) reported that the ability of *Kocuria sp.* to degraded crude oil compounds due to production of biosurfactants. Sarafin *et al*. (2014) also have highlighted that efficiency of *Kocuria sp.* in degradation of crude oil by producing biosurfactants which facilitate enzyme attack via increasing water solubility.



(A)



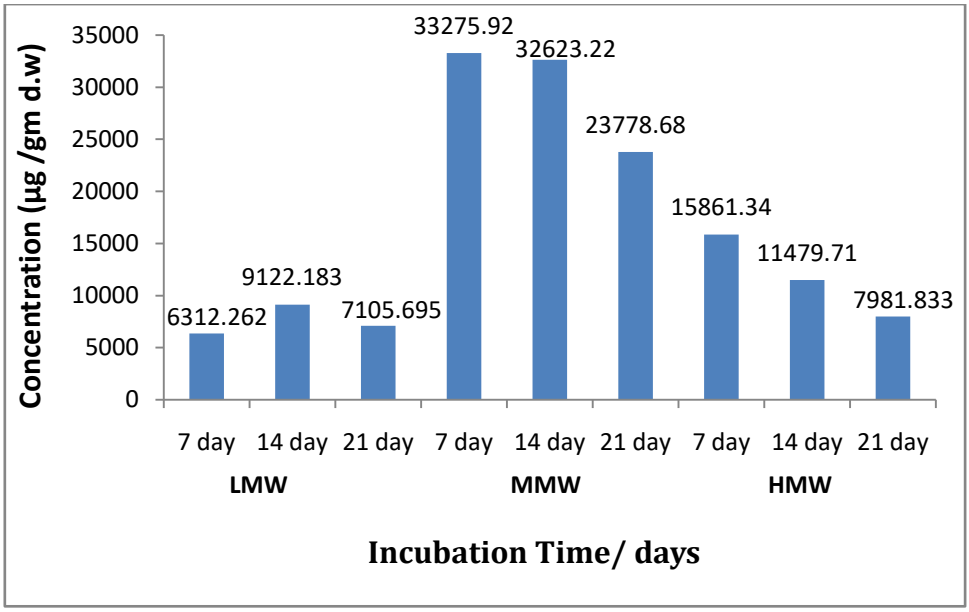
(B)

Figure (3-24) : Biodegradation of crude oil by *Kocuria kristina*.

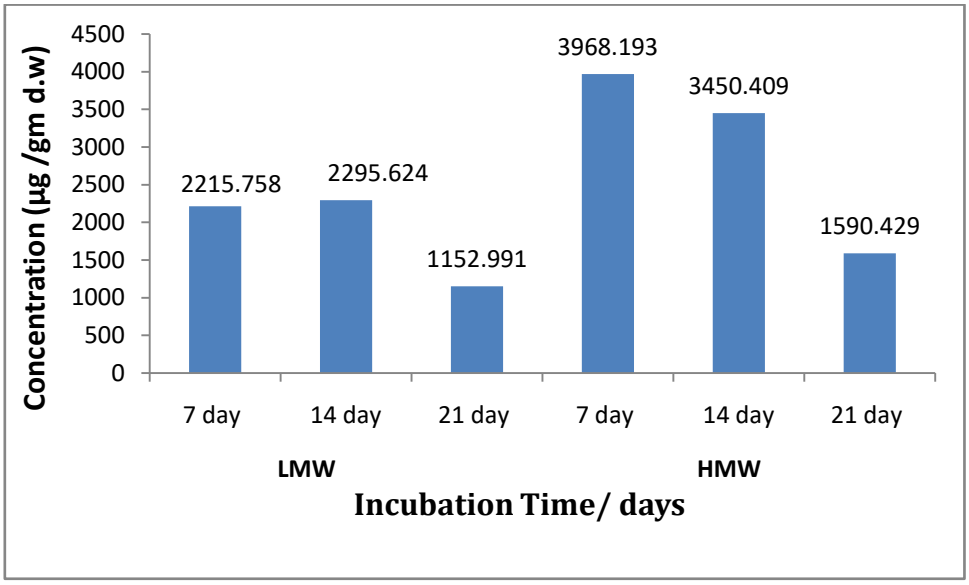
(A) n-alkanes. (B) PAHs.

3.6.16 : Biodegradation by *Aeromonas salmonicida*

As illustrated in figure (3-25 A and B), (Appendix 21A and B) *Aeromonas salmonicida* was also able to grow in both type of crude oil including n-alkanes were gradually degraded from LMW (6312.262, 9122.183 and 7105.695) µg /gm d.w to MMW (33275.92, 32623.22 and 23778.68) µg /gm d.w and then HMW degraded were 15861.34, 11479.71 and 7981.833 µg /gm d.w. LMW of PAHs compounds were degraded 2215.758, 2295.624 and 1152.991 µg /gm d.w and HMW (3968.193, 3450.409 and 1590.429) µg /gm d.w along the three incubation period (7, 14 and 21 days). The GC analyzing was showed *Aeromonas salmonicida* was capable to degrade n-alkanes and PAHs hydrocarbons depending on the molecular weight to begin with LMW and ended with HMW. There are several studies showed the role of *Aeromonas sp.* to degrade crude oil and used it as a sole source of carbon and energy (Mrozik *et al*., 2003 ; Kaczorek *et al*., 2010 ; Obiakalaije *et al*., 2015). Ilori *et al.* (2005) showed that the ability of *Aeromonas salmonicida* to degrade crude oil due to produced the biosurfactant that could emulsify hydrocarbons.



(A)



(B)

Figure(3-25) : Biodegradation of crude oil by *Aeromonas salmonicida*. (A) n-alkanes. (B) PAHs.

3.7 :The Percentages of total concentration of crude oil degradation by bacterial strains

The biodegradation percentage of crude oil by the sixteen isolates after 7, 14 and 21 days of incubations at 37^{o} C in a mixing speed of 121 rpm was demonstrated in table (3-14). The results showed that the maximum degradation rate was achieved through 21 days of incubation of bacteria with 0.5% of crude oil in MSM up to 93.39%, 92%, 91.18% and 90.93% for PAHs compounds and 79.63%, 78.18%, 84.45% and 84.66% for n-alkanes compounds by *Sphingomonas paucimobilis, Novosphingobium subterraneum, Brevundomonas sp.* and *Pseudomonas aeruginosa* respectively. This results were broadly in line with the results of AlKanany *et al*. (2017) who found that *Sphingomonas paucimobilis* has high degradation percentage (97.39%), also Jaafar (2019) suggested that *Sphingomonas paucimobilis* can potentially be a safe biological treatment strategy to remediate soil polluted with hydrocarbons in crude oil extraction sites. Whereas Basuki in his work (2017) found the high ability of degradation was occurred by *Brevundomonas sp.*, while *Pseudmonas aeruginosa* was reported by Darsa and Thatheyus (2014) and *Novosphingobium subterraneum* by Notomista *et al*. (2011). This observation are consistent with Al-Wasify and Hamed (2014) who proved that after three weeks of incubation, the bacteria degrade up to 88.5% of crude oil. *Bacillus sp., Arthrobacter luteolus, Pseudomonas putida and Aeromonas salmonicida* also showed a high degradation percentage ranged from 78.35-88.84 % for PAHs compounds and 77.98 % - 83.39% for n-alkanes compound , a similar pattern of results were obtained by Farid (2012) when study oil degrading bacteria were subjected to different periods of incubations.

On the other hands, *Acinetobacter* sp. showed their ability to degrade crude oil. *Acinetobacter junii* have high ability to degrade PAHs hydrocarbons (85.23%) than *Acinetobacter baumannii* (81.43%), while with n-alkanes hydrocarbon *Acinetobacter baumannii* showed high percentage of degradation (89.18%) than *Acinetobacter junii* (71.9%). Mishra *et al*. (2004) reported that n-alkanes compounds were degraded by *Acinetobacter baumannii* .

Pantoea sp. , Kocuria kristinae and Sporosarcina luteola were showed convergent rates of crude oil degradation with the difference in the degradation of the components of crude oil. *Pantoea sp. and Kucoria kristinae* was able to degrade PAHs hydrocarbons with high percentage (88.04%) for *Pantoea* sp. and (87.78%) for *Kocuria kristinae* than n-alkanes hydrocarbons (86.3%) for *Pantoea* sp. and (82.69%) for *Kocuria kristinae*. On the contrary, *Soprosarcina luteola* was able to degrade n-alkanes hydrocarbons with high percentage (87.28%) than PAHs hydrocarbon (85.82 %). In fact, the biodegradation of crude oil was required to complete mechanisms and this not available in one organism because the hydrocarbon mixture varies markedly in the volatility, solubility, tendency to biodegradable and the certain enzymes cannot be gained in a single organism (Mohammed *et al*., 2017).

Through the results shown in table (3-16), we note that the percentage of crude oil degradation increases with the increase of the incubation periods. We also note that aromatic compounds have higher rates of degradation during the incubation periods.

Degradation of oil by these bacterial strains shows that they have specialized co-metabolic capacities (Nzila, 2013). In oil polluted environments, specialized bacterial strains are abundant because of their adaptation ability to pollutants (Azubuike *et al*., 2016). It has also been showed that these bacteria are the most predominant microorganism among others in situ or ex situ biodegradation processes, indicating that they are the main agents responsible for the degradation of oil, but every organism has its own level of biodegradation (Jyothi *et al*., 2012). The use of these native bacteria in contaminated environment could prove a more environmentally friendly approached to biodegradation (Ite and semele, 2012). The efficiency of local bacterial strains in hydrocarbon degradability was found to be substantially higher than that of the introduced strains (Wu *et al*., 2013).

Table (3-16) : Percentage of total concentration of crude oil degradation by bacterial isolates.

Incubation periods % degradation of crude oil components						
Bacterial isolates	**7 days**		**14 days**		**21 days**	
	n-alkanes	PAHs	n-alkanes	PAHs	n-alkanes	PAHs
Bacillus safensis	36.25%	52.69%	67.47%	60.13%	76.51%	80.85%
Bacillus subtilis	71.61%	63.27%	79.23%	85.37%	83.39%	85.99%
Bacillus pumilus	44.92%	60.94%	70.33%	78.72%	74.75%	87.31%
Bacillus sporothermodurans	57.5%	61.79%	71.39%	74.77%	77.91%	78.35%
Pseudomonas aeruginosa	69.49%	66.42%	76.59%	84.31%	84.66%	90.93%
Brevundimonas sp.	62.09%	44.30%	76.23%	79.84%	84.45%	91.18%
Arthrobacter luteolus	53.41%	40.32%	73.39%	73.70%	77.76%	86.67%
Pseudomonas putida	63%	51.84%	77.11%	80.43%	84.21%	88.84%
Pantoea sp.	64.63%	74.42%	76.36%	78.9%	86.3%	88.04%
Sphingomonas paucimobilis	43.1%	56.5%	64.01%	83%	79.63%	93.39%
Novosphingobium subterraneum	70.36%	43.57%	76.48%	83.76%	78.18%	92%
Sporosarcina luteola	69.57%	65.1%	74.57%	80.74%	87.28%	85.82%
Kocuria kristinae	71.78%	69.56%	75.06%	86.24%	82.69%	87.78%
Aeromonas salmonicida	68.57%	71.95%	69.85%	73.94%	77.98%	87.55%
Acinetobacter junii	65.17%	70.19%	68.98%	74.49%	71.9%	85.23%
Acinetobacter baumannii	68.35%	67.35%	78.97%	78.16%	89.18%	81.43%

CONCLUSIONS

&

RECOMMENDATIONS

Conclusions

1- Temperature and pH of the soil studied are affected by concentration of petroleum hydrocarbons.

2- The soil collected from oil contaminated sites contain a wide variety of hydrocarbon degrading bacteria.

3- Biodegradation is the main natural mechanism that can clean up the petroleum hydrocarbon pollutants from the environment. The process uses microscopic organisms (primarily bacteria) that live in soil and feeds on petroleum hydrocarbon.

4- The bacterial isolates were showed their ability to grow at temperature ranged from 24-42 ^{o} C , while a few bacterial strains (*Pseudomonas aeruginosa, Brevundimonas sp., Sphingomonas paucimobilis, Novosphingobium subterraneum, Kocuria kristina, Aeromonas salmonicida and Acinetobacter junii*) were also able to grow at 46^{o} C. The optimum growth of these bacterial isolates were at 24^{o} C, 28^{o} C and 37^{o} C, except *Brevundemonas sp.* and *Bacillus sporothermodurans* were also showed optimum growth at 32^{o} C, as well as *Pseudomonas aeruginosa* and *Novosphingobium subterraneum* were also showed optimum growth at 32^{o} C and 42^{o} C.

5- The ability of bacterial strains to degrade all compounds of crude oil gradually starting from LMW at the first weekly incubation periods (7 days) followed by degradation of HMW at the end periods of incubation (14, 21 days).

6- The increasing in the optical density for bacterial growth that measured by spectrophotometer at OD_{620} were indicated to the ability of bacteria to using the hydrocarbons in crude oil as sole carbon and energy sources.

7-The highest percentage of n-alkanes degradation within 21 days of the incubation period by bacteria *Acinetobacter baumannii* (89.18%) and the highest percentage of degradation of PAHs during the 21 days of the incubation period by bacteria *Sphingomonas paucimobilis* (93.39%).

8- The results went toward recording biodegradable bacterial strains might occur for the 1st time in Iraq which in turn might 1st reported in GenBank.

Recommendations

1-Must be studying the number of influencing degradation factors which identify to reduce the toxicity of oil contamination in the environment by removing, degrading or transforming contaminants. Therefore, a successful bioremediation treatment requires understanding of those factors.

2- Study the role of other living organisms in the biodegradation of crude oil, such as fungi.

3- Isolation and diagnosis of bacteria from other oil-contaminated sites such as gas stations and studying their ability to biodegrade of organic pollutants from the environment.

4- Determining the susceptibility of bacteria to degradation of crude oil at concentrations higher than 0.5 % such as 1 % and 2%.

5- Study the role of isolates bacteria in the biodegradation and bioremediation process on other materials such as plastics, heavy metals and pesticides.

6-Isolation and identification of bacteria from aquatic ecosystem contaminated with oil pollution in Marshes southern Iraq.

7- Study of the mechanisms used by bacteria in the biodegradation process of petroleum hydrocarbons.

8- Study the role of isolates bacteria for bioremediation in field.

9- Study the effect of seasons on biodegradation.

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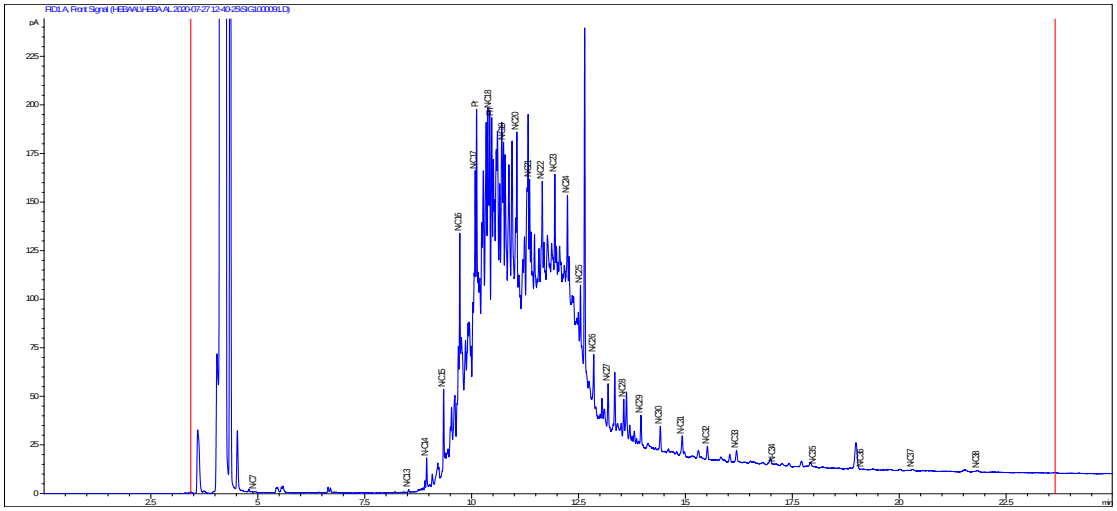
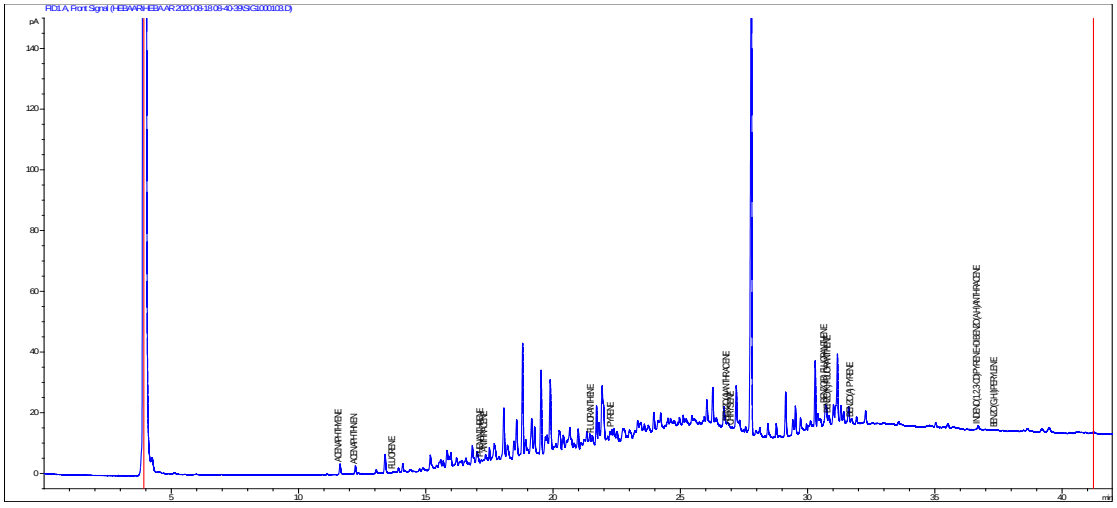
APPENDICES

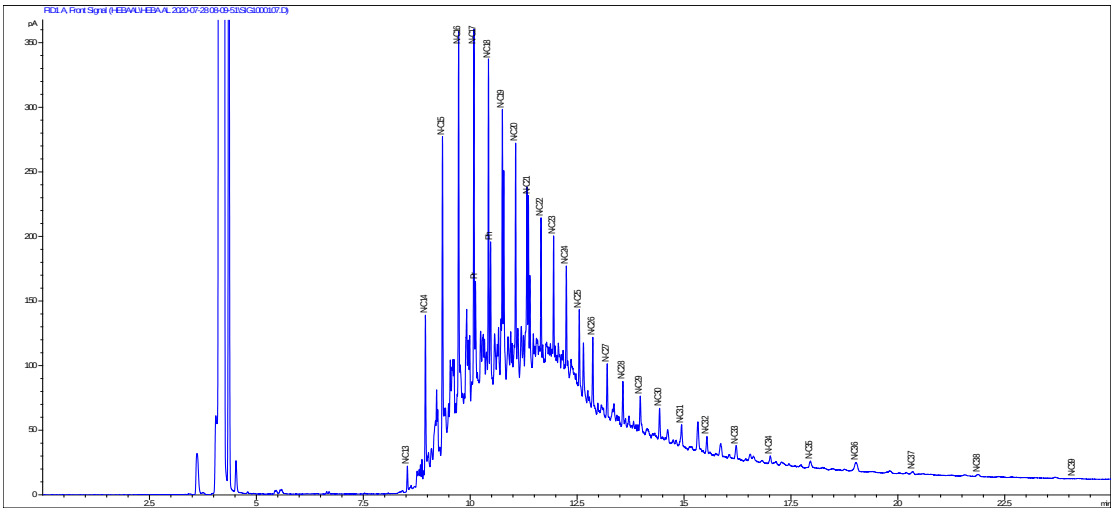
Appendix (1A) **:** Gas Chromatography for n-alkanes of soil sample from Bazerkan refinery



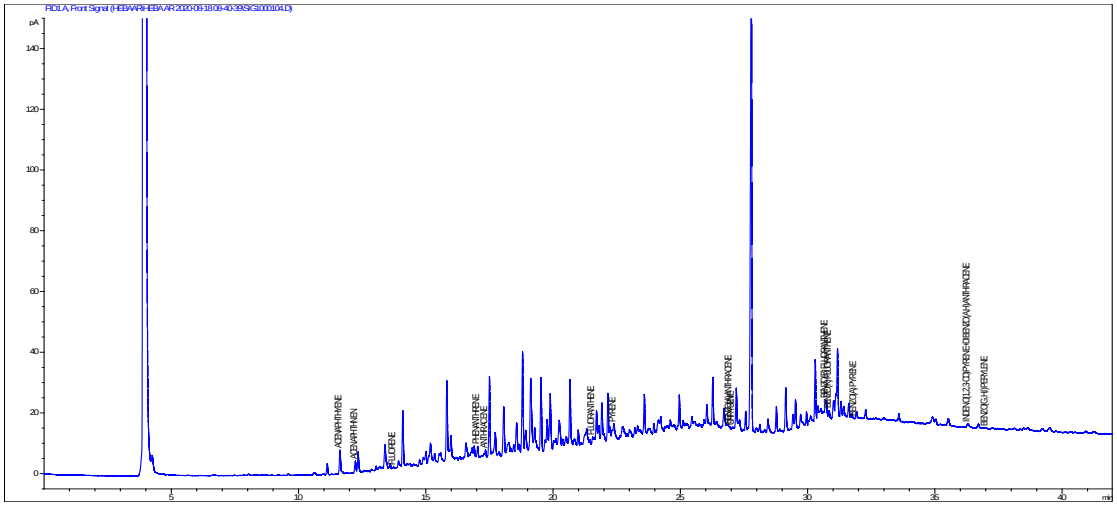
Appendix (1B) : Gas Chromatography for PAHs of soil sample from Bazerkan refinery.



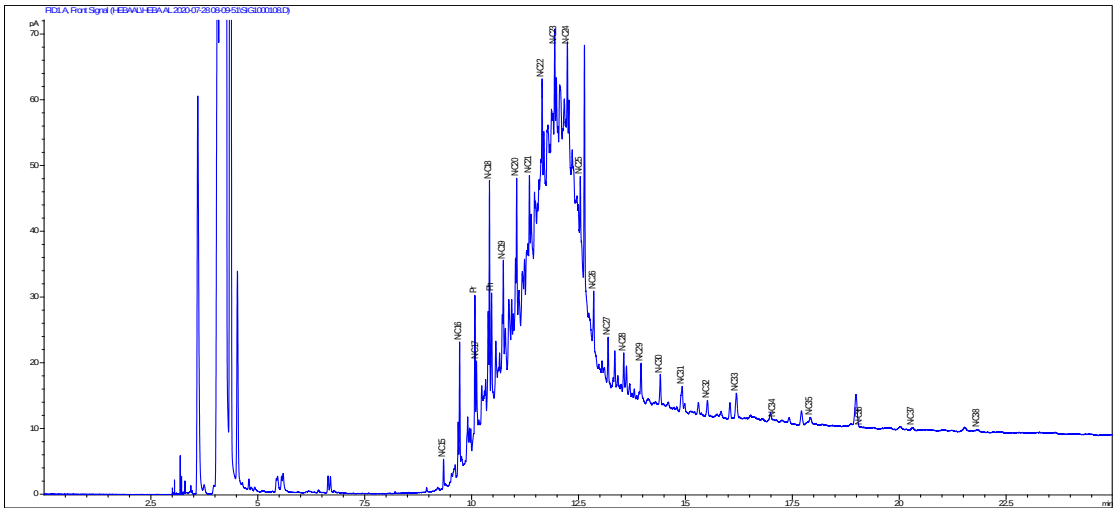
Appendix (2A): Gas chromatography for n-alkanes of soil sample from South Oil Company.



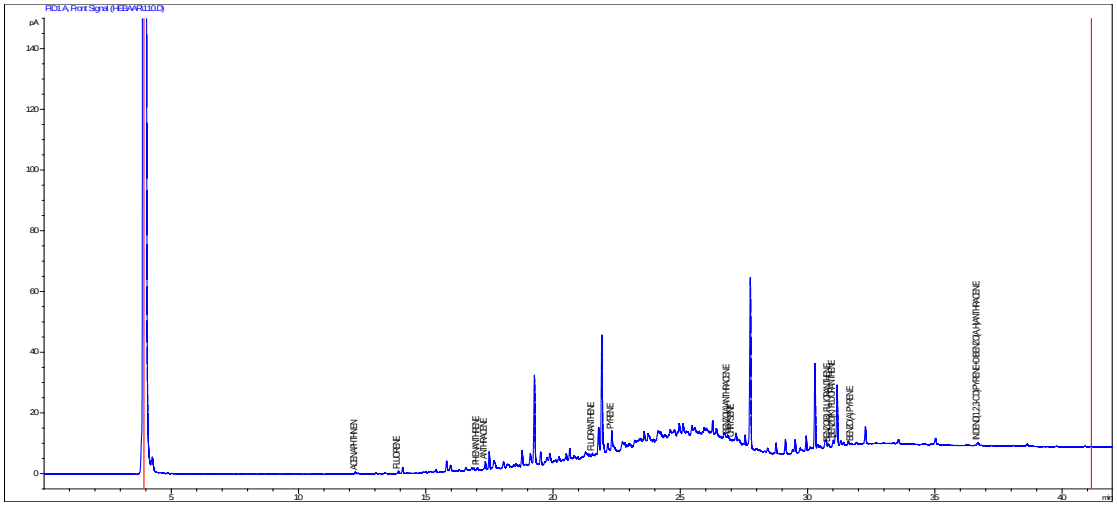
Appendix (2B) : Gas Chromatography for PAHs of soil sample from South Oil Company.



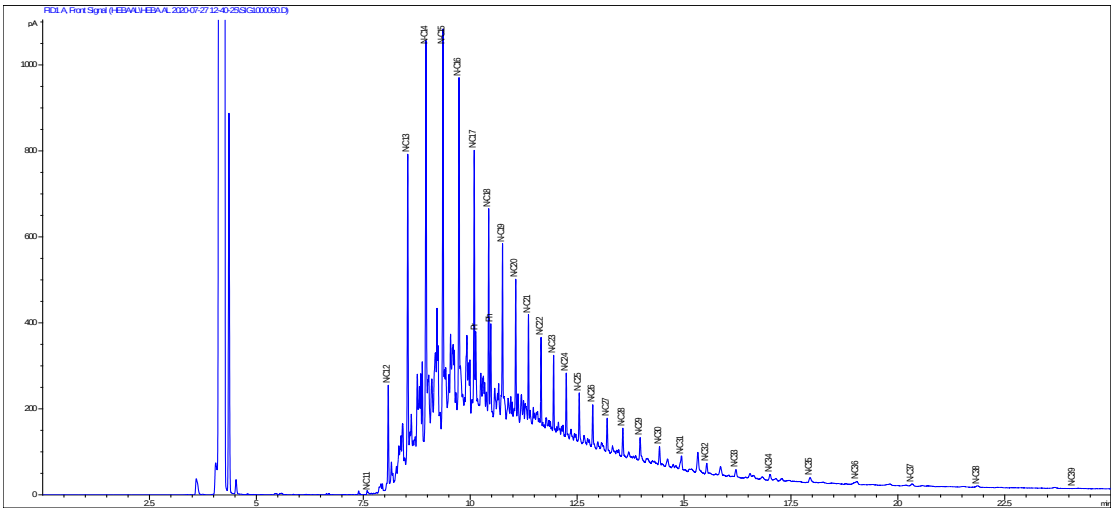
Appendix (3A) : Gas Chromatography for n-alkanes of soil sample from PetroChina Company.



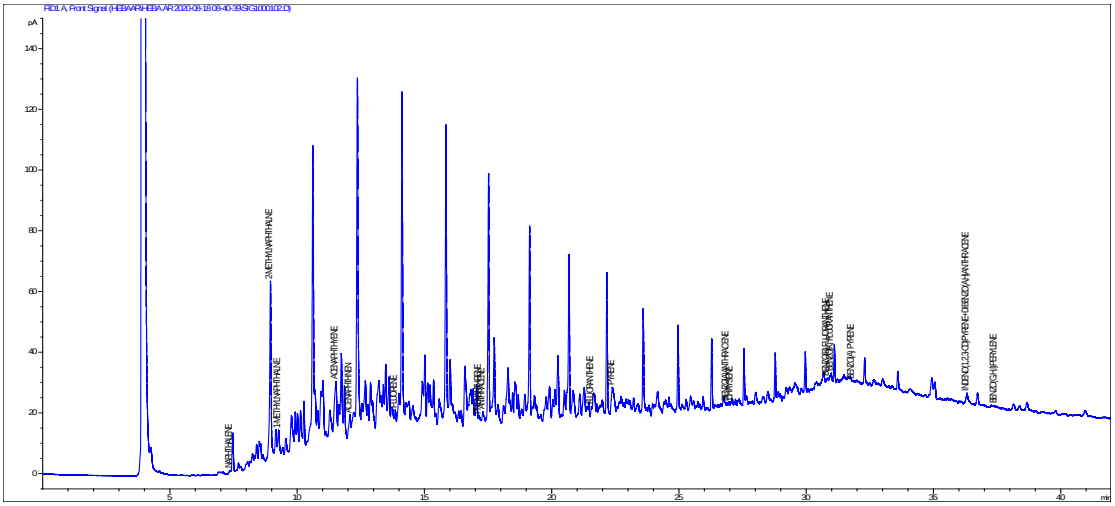
Appendix (3B) : Gas Chromatography for PAHs of soil sample from PetroChina Company.



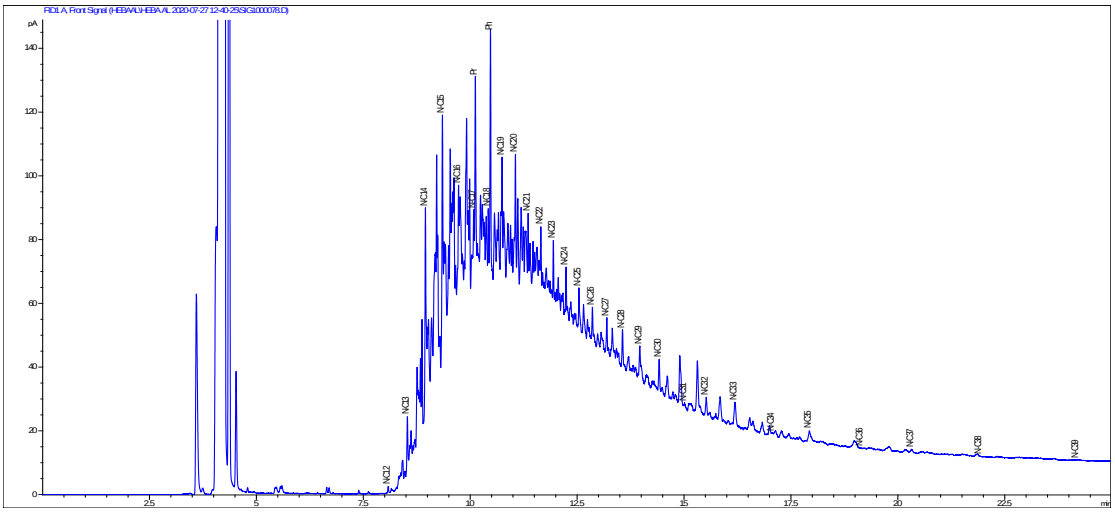
Appendix (4A): Gas Chromatography for n-alkanes of control sample of crude oil

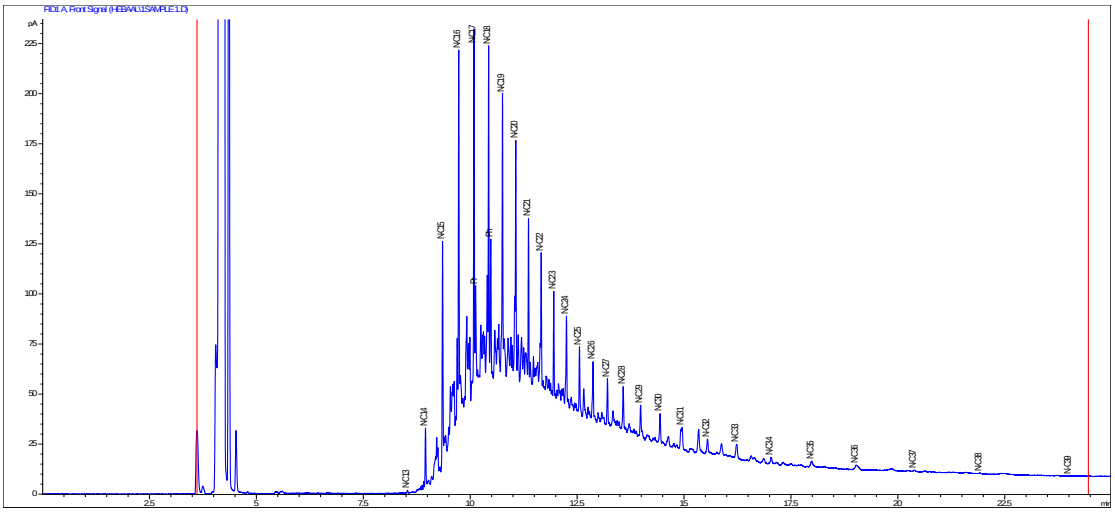


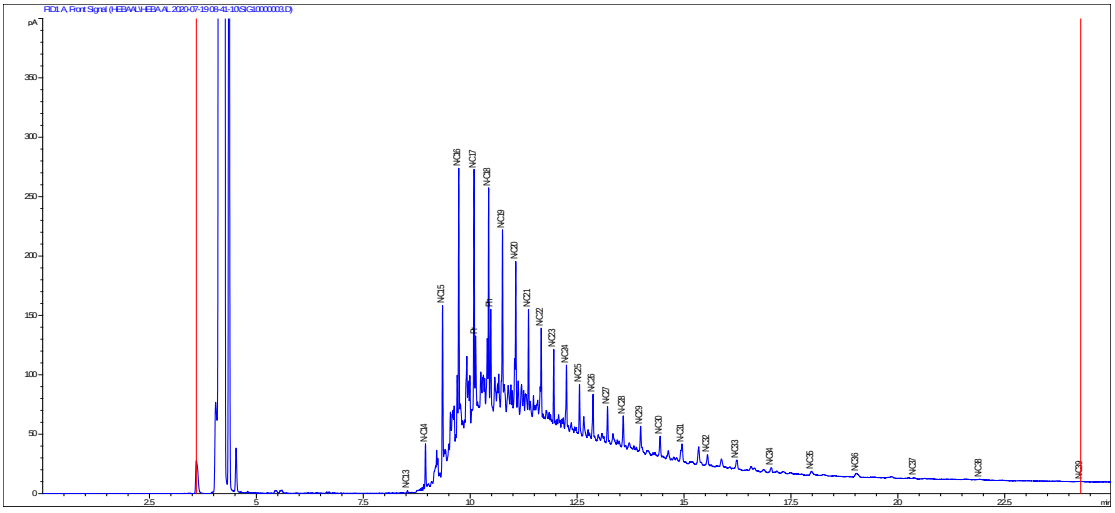
Appendix (4B) : Gas Chromatography for PAHs of control sample of crude oil

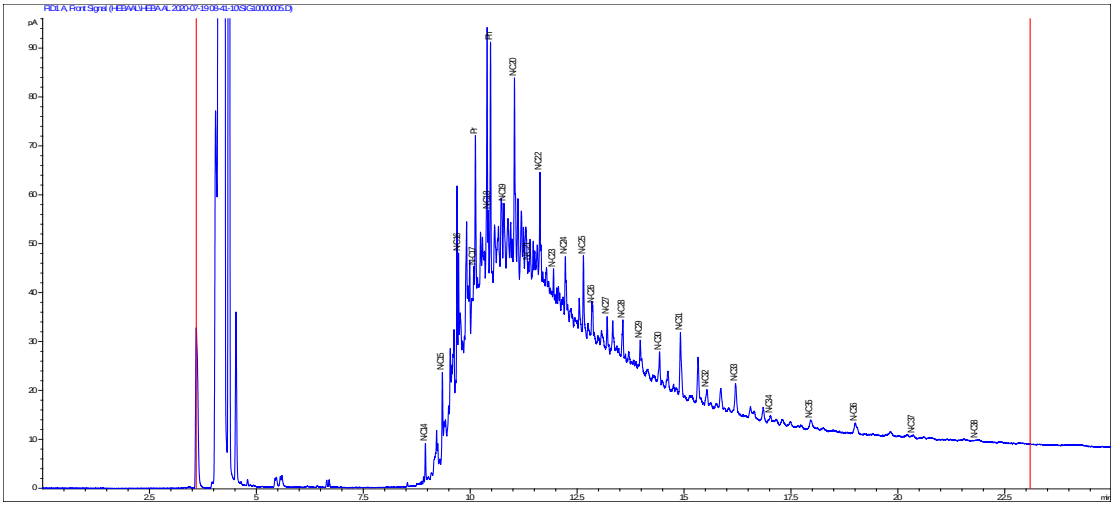


Appendix (5A) : Gas Chromatography for degradation of n-alkanes by bacterial isolates.





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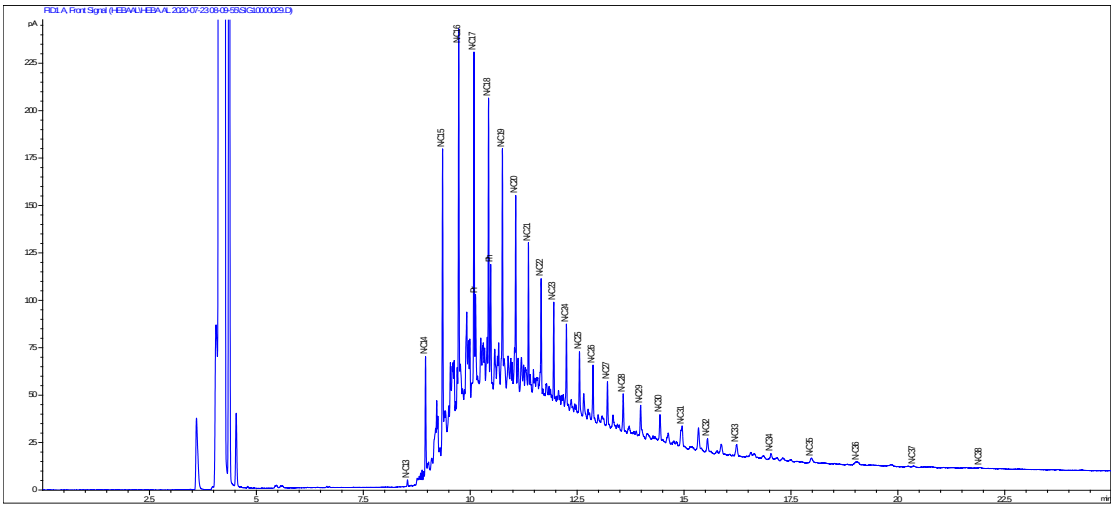
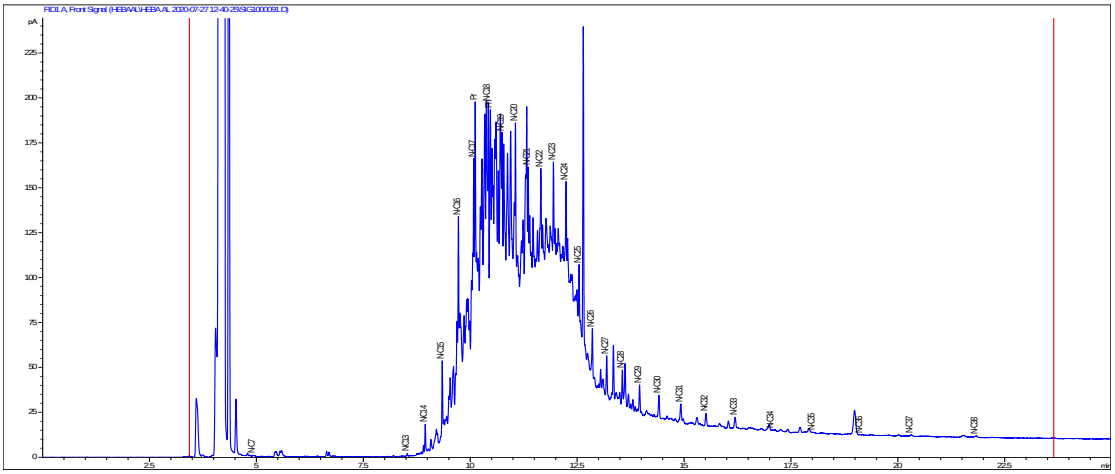
pA
min


FID1 A, Front Signal (HEBAUHEBA-AL 2020-07-23 09-09-59\SIGS0000029.D)
pA
min


FID1 A, Front Signal (HEBAUHEBA-AL 2020-07-27 12-40-28\SIGS0000081.D)
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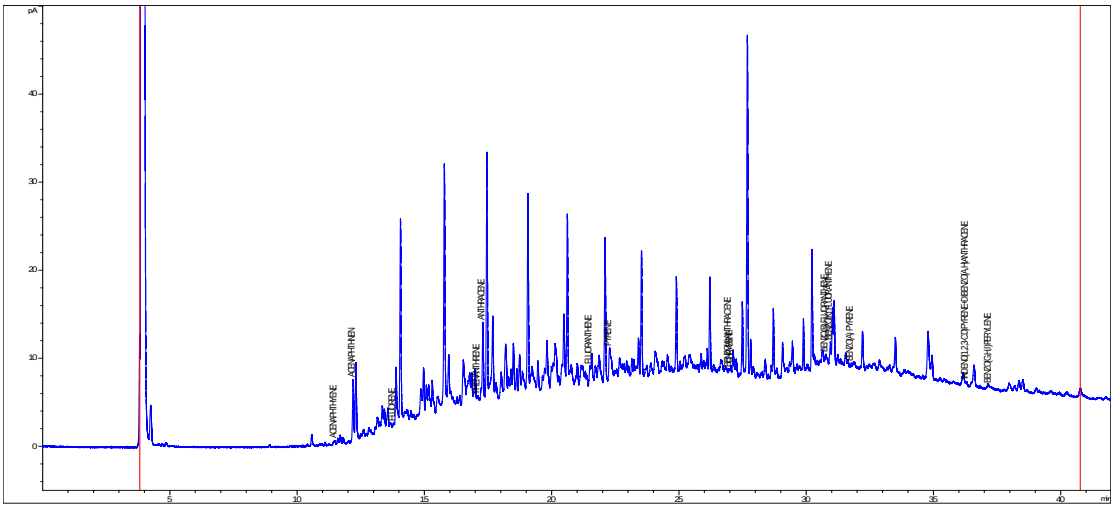
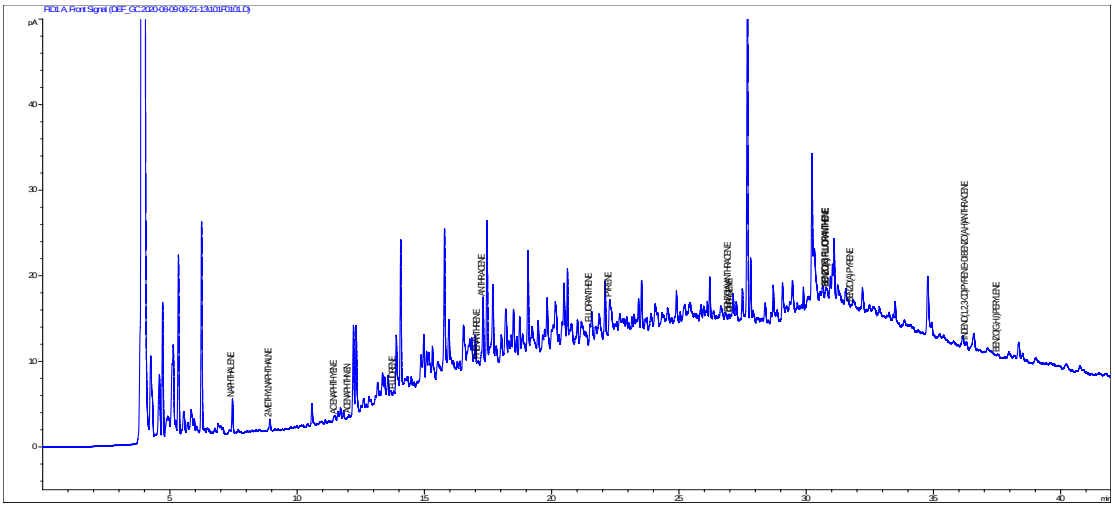
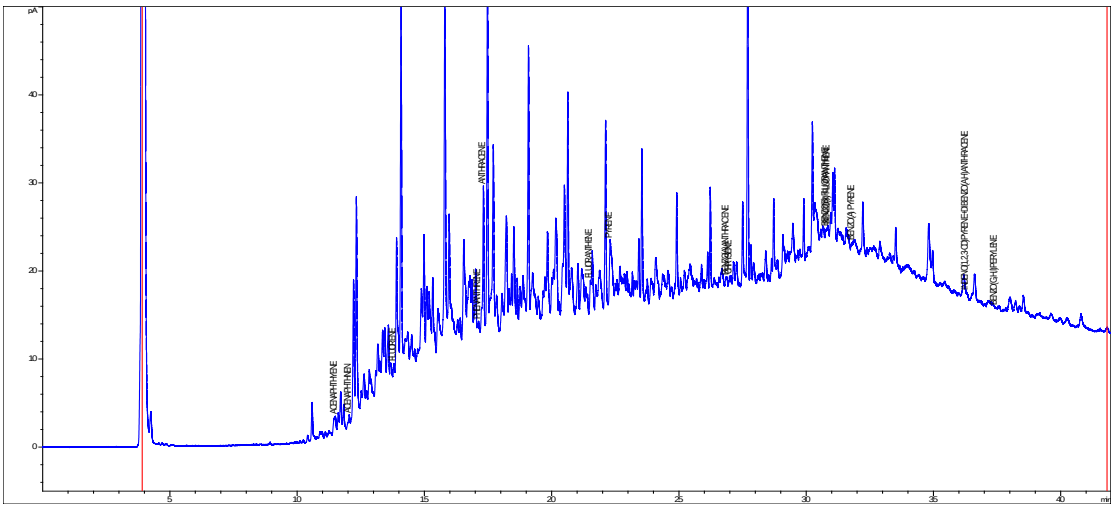
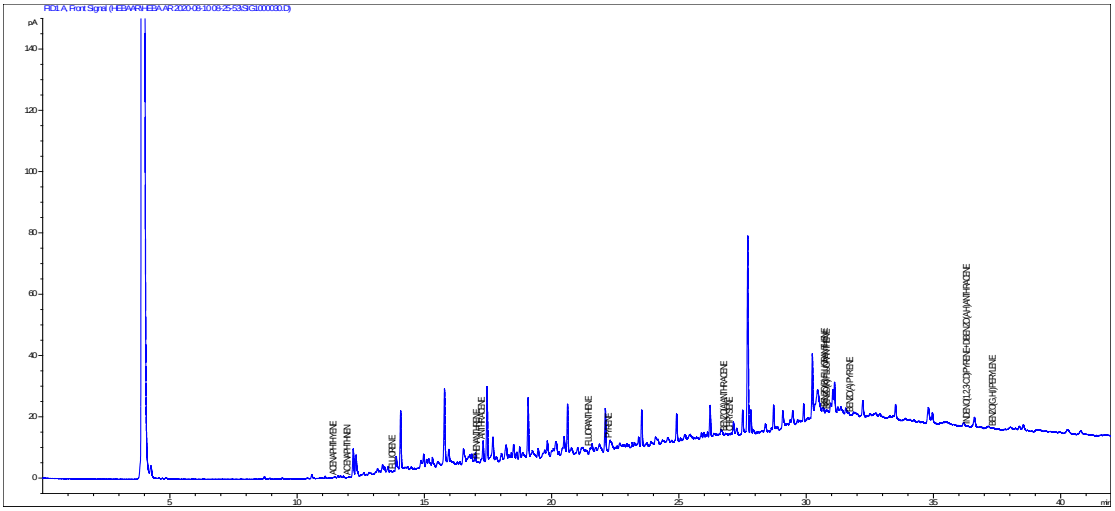
Appendix (5B) : Gas Chromatography for degradation of PAHs by bacterial isolates.





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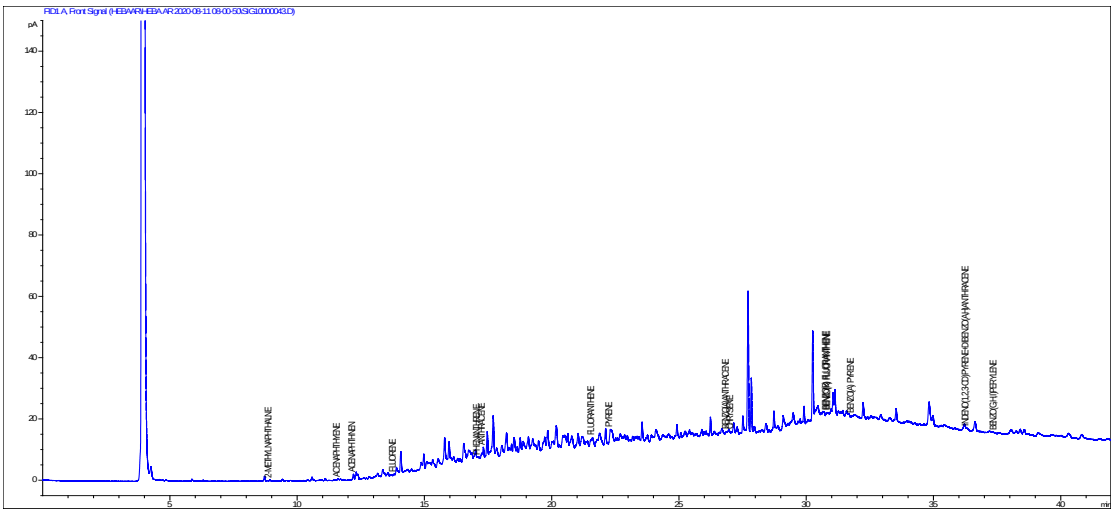
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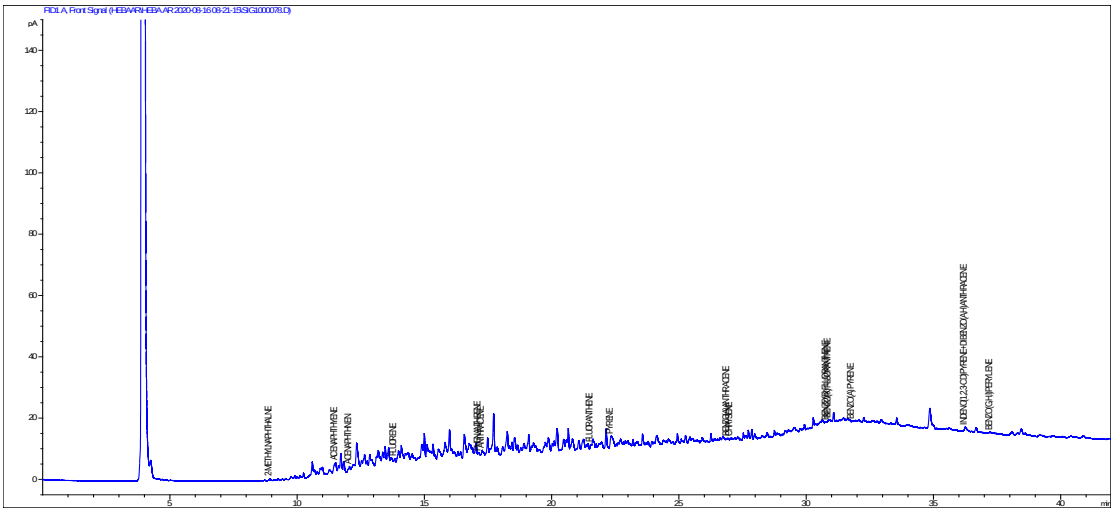

pA
min

Appendix (6A): Biodegradation of n-alkanes hydrocarbons by *Bacillus safensis.*

Carbon numbers	Incubation periods		
	7 days	14 days	21 days
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	0	12.71401	13.51999
C12	14.3729	0	0
C13	162.2391	120.5578	72.03715
C14	2340.047	1198.145	834.1855
C15	6093.63	2668.794	1857.734
C16	9131.121	3847.527	2844.278
Σ LMW	17771.78	7847.738	5621.755
C17	7657.216	3581.542	2561.061
C18	6507.118	3169.232	2289.896
C19	8456.636	4289.738	3110.042
C20	5809.341	4296.145	2155.235
C21	4579.587	2129.794	1727.852
C22	4681.205	2219.678	1694.989
C23	3262.738	1994.283	1642.667
C24	3788.281	2892.581	1369.862
C25	5540.252	2661.412	2120.722
C26	6518.363	1849.51	2347.752
C27	5132.295	2501.648	2018.949
C28	5925.773	2574.198	1320.038
Σ MMW	67858.8	34159.76	24359.07
C29	6684.536	2158.086	2589.822
C30	3060.975	1397.581	1146.472
C31	2534.157	1179.54	1556.546
C32	2172.013	1243.996	1028.231
C33	3282.267	1889.332	1288.567
C34	1999.332	1496.419	173.8307
C35	2302.544	1799.97	1334.24
C36	1640.523	1065.837	575.0359
C37	1526.303	944.1063	471.7444
C38	1324.004	1680.008	1153.245
C39	396.6571	424.2684	314.5766
C40	0	0	0
Σ HMW	26923.311	15429.91	11481.54

Appendix (6B) : Biodegradation of PAHs hydrocarbons by *Bacillus* safensis.

Compounds names	Incubation periods		
	7 day	14 day	21 day
Naphthalene	0	0	0
2-Methylnaphthalene	38.94936	103.5299	89.34653
1-Methylnaphthalene	0	19.37698	14.80518
Acenaphthylene	589.1782	771.7563	461.4301
Acenaphthene	387.5498	471.5846	241.4134
Fluorene	554.076	590.5566	273.0201
Phenanthrene	605.4592	356.6208	328.7754
Anthracene	1232.688	476.8559	438.997
Σ LMW	3407.9	2790.281	1847.788
Fluoranthene	633.4014	599.6821	210.2704
Pyrene	2378.876	2057.849	756.8912
Benzo(a)anthracene	630.676	548.5926	204.8342
Chrysene	337.518	496.7742	152.1605
Benzo(b)fluoranthene	853.5314	534.0392	298.7954
Benzo(k)fluoranthene	734.5699	614.1396	71.13838
Benzo(a)pyrene	311.4223	138.107	141.8734
Indeno(1,2,3-cd)pyrene	752.0285	867.8006	573.3764
Benzo(g,h,i)perylene	331.7578	142.5124	35.59982
Σ HMW	6963.781	5999.856	2444.936

Appendix (7A) :Biodegradation of n-alkanes hydrocarbons by *Bacillus pumilus.*

Carbon numbers	Incubation periods		
	7day	14day	21 day
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	18.95493	0	13.74665
C12	0	0	0
C13	122.7175	49.02227	368.8175
C14	259.5905	665.8581	1806.685
C15	4298.226	1815.733	2784.37
C16	6853.329	3418.265	3306.557
ΣLMW	11552.82	5982.792	8280.176
C17	6312.155	280.906	2881.854
C18	5189.223	2300.023	2404.141
C19	6875.673	3897.746	3196.708
C20	6852.094	4401.71	3206.932
C21	3687.981	1998.038	1743.989
C22	5611.604	2215.167	1845.063
C23	3383.841	2052.86	1605.028
C24	3882.9	3730.019	2439.374
C25	4502.526	2768.018	2027.426
C26	4886.356	3566.783	2377.595
C27	4773.932	1909.423	1954.751
C28	5270.504	2008.568	1279.987
Σ MMW	61228.79	31129.261	26962.9
C29	4420.183	2004.259	2327.718
C30	3144.166	1613.475	1335.6
C31	2408.654	2027.972	1405.172
C32	1684.229	1397.915	903.0029
C33	3180.789	1607.033	1360.64
C34	1595.917	1244.726	630.4055
C35	2558.415	1150.449	647.1869
C36	2011.545	499.0959	511.4374
C37	1577.121	629.43	542.6817
C38	1700.329	498.7674	37.0859
C39	180.7042	78.32191	0
C40	0	0	0
Σ HMW	24462.03	12751.44	9700.93

Appendix (7B) : Biodegradation of PAHs hydrocarbons by *Bacillus pumilus*.

Compounds name	Incubation periods		
	7 days	14 days	21 days
Naphthalene	0	0	0
2-Methylnaphthalene	61.72457	0	22.26632
1-Methylnaphthalene	0	0	0
Acenaphthylene	573.9076	81.62626	155.572
Acenaphthene	344.3582	76.38239	91.94379
Fluorene	474.1013	183.6105	141.442
Phenanthrene	652.6858	341.2425	245.1762
Anthracene	983.2364	525.2679	193.2505
ΣLMW	3090.014	1208.13	849.6508
Fluoranthene	475.7163	808.9569	153.346
Pyrene	1627.767	926.2801	559.4447
Benzo(a)anthracene	523.7752	73.3553	116.9475
Chrysene	415.2038	144.125	109.9953
Benzo(b)fluoranthene	530.7169	379.6666	296.1869
Benzo(k)fluoranthene	572.3386	522.6375	513.7077
Benzo(a)pyrene	322.2041	89.27044	87.79708
Indeno(1,2,3-c,d)pyrene	903.3161	416.5152	81.50084
Benzo(g,h,i)perylene	152.081	122.7756	28.24163
ΣHMW	5523.118	3483.581	1947.167

Appendix (8A) : Biodegradation of n-alkanes hydrocarbons by *Bacillus sporothermodurans.*

Carbon numbers	Incubation periods		
	7day	14day	21 day
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	18.89193	0	0
C12	13.579	0	0
C13	68.46154	18.57685	56.98701
C14	40.31429	18.72159	576.1183
C15	1051.155	775.7299	1339.824
C16	2537.048	2185.204	1978.318
Σ LMW	3738.192	2998.232	3951.247
C17	3779.928	4011.64	2725.282
C18	2992.891	3053.736	1741.115
C19	2847.552	4265.568	2536.59
C20	3804.674	2729.859	2685.813
C21	2454.373	2369	1314.475
C22	3065.278	3649.234	1405.131
C23	3482.371	2025.862	1384.954
C24	4237.712	2388.954	1648.992
C25	5309.932	2392.005	2080.879
C26	6750.575	1922.965	1342.389
C27	5435.849	2439.169	1310.703
C28	3469.792	1872.273	2013.607
Σ MMW	47630.93	33120.27	22189.93
C29	4449.08	2857.341	2563.025
C30	2691.25	1474.653	1243.318
C31	3732.312	2894.004	1668.736
C32	2167.571	1286.41	1180.677
C33	2521.814	2202.31	1396.11
C34	2006.105	917.0241	171.9073
C35	2508.592	1205.349	1343.254
C36	1929.303	847.8458	1708.715
C37	1025.345	459.1645	785.0076
C38	600.4479	239.536	479.9186
C39	42.40441	0	314.717
C40	0	0	0
Σ HMW	23674.22	14383.64	12855.39

Appendix (8B) : Biodegradation of PAHs hydrocarbons by *Bacillus sporothermodurans.*

Compounds names	Incubation periods		
	7 days	14 days	21 days
Naphthalene	0	0	0
2-Methylnaphthalene	25.35346	21.41564	85.55653
1-Methylnaphthalene	0	0	17.88399
Acenaphthylene	76.50149	19.65376	425.3009
Acenaphthene	73.42211	67.37415	234.9379
Fluorene	187.6981	111.1099	286.6185
Phenanthrene	522.8103	492.3459	374.4801
Anthracene	668.7685	435.6393	327.3583
Σ LMW	1554.554	1147.539	1752.136
Fluoranthene	572.0125	448.073	240.3125
Pyrene	1840.21	1693.815	843.9347
Benzo(a)anthracene	511.2902	201.191	145.2829
Chrysene	402.1235	246.0935	139.3939
Benzo(b)fluoranthene	1222.16	654.9417	331.1005
Benzo(k)fluoranthene	768.578	295.6066	612.0554
Benzo(a)pyrene	312.015	310.4261	273.0454
Indeno(1,2,3-c,d)pyrene	737.6767	437.906	406.865
BENZO(g,h,i)perylene	505.0526	126.2115	29.19502
Σ HMW	6871.222	4414.363	3021.185

Appendix (9A): Biodegradation of n-alkanes hydrocarbons by *Bacillus subtilis*

Carbon names	Incubation periods		
	7 days	14 days	21 days
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	10.99024	0	0
C12	16.31998	0	0
C13	528.589	15.46126	13.8516
C14	2286.509	325.971	207.5785
C15	3390.352	1349.951	946.9414
C16	4078.533	2697.815	1672.059
Σ LMW	10311.29	4389.198	2840.431
C17	3516.918	2516.735	2621.306
C18	2936.909	2215.456	1807.312
C19	3830.504	2897.328	2454.042
C20	3745.68	2879.028	2633.731
C21	2029.454	1572.571	1346.329
C22	2002.646	2438.38	2121.234
C23	1840.79	1427.263	1667.763
C24	2914.569	1577.641	1373.97
C25	2352.93	1752.78	1378.483
C26	2762.856	2161.45	1773.321
C27	2080.701	1598.755	951.3613
C28	1940.285	1183.935	990.7024
Σ MMW	31954.24	24221.32	21119.55
C29	2146.15	2165.082	1723.561
C30	1132.873	907.059	847.4204
C31	680.8231	1043.321	1153.016
C32	1057.417	747.3342	682.9976
C33	1139.476	1127.266	489.0609
C34	150.2679	675.6382	43.83372
C35	785.7682	822.6358	231.677
C36	587.8014	240.9188	108.7519
C37	122.9783	232.7583	40.06912
C38	48.05221	97.82233	35.20231
C39	0	0	0
C40	0	0	0
Σ HMW	7851.607	8059.836	5355.59

Appendix (9B) : Biodegradation of PAHs hydrocarbons by *Bacillus subtilis*

.

Compounds names	Incubation periods		
	7 days	14 days	21 days
Naphthalene	0	70.41312	0
2-Methylnaphthalene	277.8326	30.18211	0
1-Methylnaphthalene	109.7882	0	0
Acenaphthylene	785.6997	34.85971	34.97731
Acenaphthene	465.4994	14.25153	21.97651
Fluorene	482.3414	65.25236	125.6611
Phenanthrene	152.3969	122.8365	224.4858
Anthracene	783.5016	310.6509	228.2224
Σ LMW	3057.0598	648.446	635.323
Fluorantheren	375.3204	311.0963	215.4058
Pyrene	1283.139	576.8857	369.0713
Benzo(a)anthracenen	258.9956	150.9979	369.3028
Chrysene	339.4386	200.5373	168.004
Benzo(b)fluoranthene	625.4847	753.3302	706.0134
Benzo(k)fluoranthene	1087.459	382.8002	474.2959
Benzo(a)pyrene	60.90322	156.4593	80.90169
Indeno(1,2,3-c,d)pyrene	528.0768	44.48414	51.34728
Benzo(g,h,i)perylene	383.3121	16.31521	17.85192
ΣHMW	4942.129	2578.591	2452.194

Appendix(10A) :Biodegradation of n-alkanes hydrocarbons by *Pseudomonas aeruginosa*.

Carbon numbers	Incubation periods		
	7 days	14 days	21 days
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	15.29135	0	0
C12	0	0	0
C13	376.9302	35.12586	13.26643
C14	2016.264	662.1664	38.03717
C15	3313.711	1896.463	145.5765
C16	4173.845	2582.838	588.766
Σ LMW	9896.042	5176.593	785.6461
C17	3440.442	2872.954	1562.696
C18	2981.753	2349.889	1013.734
C19	3860.226	3103.206	1032.348
C20	3797.9	3183.555	1254.136
C21	2097.502	1720.106	835.1477
C22	2133.748	2620.154	1222.663
C23	1963.1	1590.594	1243.472
C24	2123.616	2450.46	1543.407
C25	2765.289	1974.792	2110.641
C26	2986.916	2293.324	1363.935
C27	1672.817	1764.012	1916.165
C28	2381.744	1879.927	1112.635
ΣMMW	32205.05	27802.97	16210.98
C29	2781.94	2297.76	2561.574
C30	1518.924	1229.401	1148.511
C31	1142.074	1117.663	1591.281
C32	1169.302	867.563	944.3306
C33	1444.258	737.5885	1056.686
C34	261.7724	727.0459	759.0658
C35	1416.693	509.575	834.8736
C36	911.5742	549.5199	772.0936
C37	1124.784	206.5417	270.597
C38	0	114.0614	140.3797
C39	0	0	0
C40	0	0	0
ΣHMW	11771.32	8356.719	10079.39

Appendix(10B) : Biodegradation of PAHs hydrocarbons by *Pseudomonas aeruginosa.*

Compounds name	Incubation periods		
	7 days	14 days	21 days
Naphthalene	0	0	0
2-Methylnaphthalene	203.3462	14.00391	20.66486
1-Methylnaphthalene	56.86105	0	0
Acenaphthylene	744.4759	15.22516	142.4965
Acenaphthene	423.4976	54.55818	69.92247
Fluorene	445.7298	40.0905	113.3354
Phenanthrene	513.2789	199.6499	112.9917
Anthracene	458.4112	244.2103	215.7434
ΣLMW	2845.601	567.738	675.1542
Fluoranthene	303.3786	571.6678	103.8144
Pyrene	1241.274	729.9814	359.1697
Benzo(a)anthracenen	236.2051	375.0213	97.78978
Chrysene	253.8814	239.3737	69.91335
Benzo(b)fluoranthene	671.9478	416.6801	63.58744
Benzo(k)fluoranthene	907.0752	326.042	438.1562
Benzo(a)pyrene	289.2884	180.2039	114.286
Indeno(1,2,3-c,d)pyrene	636.4382	20.00693	59.26915
Benzo(g,h,i)perylene	18.05364	32.3868	18.23536
Σ HMW	4557.542	2891.364	1324.221

Appendix(11A) : Biodegradation of n-alkanes hydrocarbons by *Pseudomonas putida*.

Carbon numbers	Incubation periods		
	7 days	14 days	21 days
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	14.07022	12.75193	0
C12	27.93237	0	0
C13	784.8893	24.31924	14.61698
C14	3030.23	292.8676	273.2912
C15	4309.382	1217.283	1069.417
C16	5065.969	1915.301	1632.18
Σ LMW	13232.47	3462.523	2989.505
C17	4348.249	2198.265	1890.999
C18	3527.329	1842.201	1642.862
C19	4620.655	2917.968	2504.029
C20	4591.516	1799.158	1443.653
C21	2527.607	1529.148	1164.133
C22	2545.231	2951.149	1021.947
C23	2275.151	1595.784	1092.096
C24	2494.044	1881.74	1365.335
C25	3113.817	2281.593	1666.775
C26	3415.748	2883.821	2104.374
C27	2819.457	1439.122	1027.072
C28	2773.598	1529.048	1157.233
ΣMMW	39052.4	24849	18080.51
C29	3465.37	2998.907	1969.575
C30	1546.03	1693.969	937.4974
C31	1155.419	1765.068	618.5627
C32	1314.78	1186.025	732.6648
C33	2483.402	1178.005	1189.238
C34	168.5141	921.2094	568.3826
C35	1113.58	1037.905	595.8737
C36	1052.849	846.7847	59.22937
C37	610.9042	336.6133	39.34354
C38	106.2204	139.9995	59.75996
C39	30.53714	0	26.17104
C40	0	0	0
Σ HMW	13047.61	12104.48	6796.298

AppeniIx (11B): Biodegradation of PAHs hydrocarbons by *Pseudomonas putida.*

Compounds names	Incubation periods		
	7days	14 days	21 days
Naphthalene	0	0	0
2-Methylnaphthalene	161.9592	20.33634	0
1-Methylnaphthalene	61.94451	0	0
Acenaphthylene	641.8873	45.40455	19.82736
Acenaphthnen	411.936	25.20624	455.6155
Fluorene	446.9002	125.1736	27.18297
Phenanthrene	639.3509	333.0923	53.69844
Anthracene	902.997	814.7565	296.369
Σ LMW	3266.975	1363.97	852.6932
Fluoranthene	504.6314	300.5591	124.8652
Pyrene	1764.905	972.9434	363.9113
Benzo(a)anthracene	603.0359	234.3139	90.80893
Chrysene	353.3636	272.7347	312.5899
Benzo(b)fluoranthene	525.9974	544.4015	120.9202
Benzo(k)fluoranthene	346.0711	328.0361	246.1466
Benzo(a)pyrene	327.5544	219.7396	282.4468
Indeno(1,2,3-c,d)pyrene	2524.591	30.53874	37.32867
Benzo(g,h,i)perylene	509.6892	46.63468	27.58494
Σ HMW	7359.838	2949.902	1606.603

Appendix(12A) :Biodegradation of n-alkanes hydrocarbons by *Brevundemonas sp.*

Carbon numbers	Incubation periods		
	7 days	14 days	21 days
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	20.23093	0	0
C12	13.16912	0	0
C13	525.7901	18.78593	14.1892
C14	2678.5	55.39511	346.6462
C15	4130.183	1556.829	1164.711
C16	4989.38	2593.459	1580.911
ΣLMW	12357.25	4224.469	3106.458
C17	4201.801	3005.569	1806.67
C18	3240.969	2690.709	1591.99
C19	4611.217	3500.246	2257.102
C20	4849.629	3518.16	2442.996
C21	2383.868	1916.542	1096.638
C22	2512.401	2762.237	2035.254
C23	2392.258	1640.563	1016.778
C24	2825.097	1873.945	1241.692
C25	3288.037	1889.426	1555.764
C26	3796.015	2187.351	1045.765
C27	3206.88	1755.999	945.9417
C28	3191.162	1395.472	1014.714
ΣMMW	40499.33	28136.22	18051.3
C29	3426.109	2359.862	1634.244
C30	1631.351	1107.461	830.7474
C31	1034.368	1765.557	1070.849
C32	1291.35	833.4818	578.0338
C33	2567.623	981.848	777.3297
C34	1256.767	741.643	475.9137
C35	1032.81	695.2112	460.2851
C36	1203.059	669.2705	390.723
C37	523.5287	321.2509	77.96444
C38	72.31685	124.2275	0
C39	29.08314	0	0
C40	0	0	0
Σ HMW	14068.37	9599.813	6296.091

Appendix (12B) :Biodegradation of PAHs hydrocarbons by *Brevundemonas sp.*

Compounds names	Incubation periods		
	7days	14 days	21 days
Naphthalene	0	0	0
2-Methylnaphthalene	68.00052	0	0
1-Methylnaphthalene	20.75623	0	0
Acenaphthylene	661.8567	0	37.12894
Acenaphthene	445.8114	78.61678	16.33532
Fluorene	557.0876	46.83361	78.36304
Phenanthrene	823.8536	229.9365	147.4514
Anthracene	1309.707	614.8487	454.5207
Σ LMW	3887.073	970.2356	733.7994
Fluoranthene	603.6118	210.9939	195.0361
Pyrene	2458.145	721.4655	459.6984
Benzo(a)anthracene	646.7467	352.6523	28.3697
Chrysene	374.0351	208.9025	27.66872
Benzo(b)fluoranthene	685.1533	356.6074	215.5198
Benzo(k)fluoranthene	682.8523	522.3412	185.0798
Benzo(a)pyrene	346.8038	269.3194	53.53491
Indeno(1,2,3-c,d)pyrene	2181.756	639.4299	28.59072
Benzo(g,h,i)perylene	414.7508	192.1886	15.73983
Σ HMW	8393.855	3473.901	1209.238

Appendix (13A):Biodegradation of n-alkanes hydrocarbons by *Arthrobacter luteolus* .

Carbon numbers	Incubation periods		
	7 days	14 days	21 days
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	11.83055	0	0
C12	0	0	0
C13	168.7117	165.9654	13.53397
C14	1932.06	1486.192	310.4768
C15	4433.251	2880.54	1295.239
C16	6197.325	3657.466	2301.498
ΣLMW	12743.18	8212.809	3920.748
C17	5566.756	3038.462	2810.187
C18	4925.457	2761.739	2520.98
C19	6367.276	3920.113	3399.322
C20	4234.213	3961.836	2254.824
C21	3523.363	2088.773	1859.092
C22	3411.551	2080.201	2986.069
C23	3171.823	1932.017	1610.197
C24	2662.564	2099.525	1871.722
C25	4103.599	2672.936	1990.444
C26	4421.252	2847.537	1619.397
C27	3799.551	1612.388	1332.8
C28	3831.739	1593.347	1490.246
Σ MMW	50019.14	30608.87	25745.28
C29	4453.821	2675.48	1726.368
C30	2030.952	1346.6	1456.907
C31	2772.35	806.8457	736.6369
C32	1663.53	949.9106	1157.578
C33	2433.031	1106.063	1194.356
C34	1580.173	590.9187	837.9273
C35	1871.761	370.6223	779.7977
C36	930.7362	172.4679	954.1069
C37	1371.452	72.81403	467.319
C38	182.3662	66.39873	253.0674
C39	202.1885	0	29.14049
C40	0	0	0
Σ HMW	19492.36	8158.121	9593.204

Appendix (13B): Biodegradation of PAHs hydrocarbons by *Arthrobacter luteolus*

Compounds names	Incubation periods		
	7 days	14 days	21 days
Naphthalene	0	0	0
2-Methylnaphthalene	117.4335	84.34824	0
1-Methylnaphthalene	38.32378	16.2268	0
Acenaphthylene	709.9604	581.2403	20.99203
Acenaphthene	483.9882	339.2475	176.7522
Fluorene	574.0713	413.8271	62.87103
Phenanthrene	630.3775	475.3224	204.3432
Anthracene	910.1768	805.9681	540.5967
Σ LMW	3464.331	2716.18	1005.555
Fluoranthene	609.5383	413.1118	204.7966
Pyrene	2306.301	1318.987	629.083
Benzo(a)anthracene	464.4493	192.0005	153.9568
Chrysene	415.5788	222.4103	142.8762
Benzo(b)fluoranthene	1492.146	473.5837	383.0226
Benzo(k)fluoranthene	378.1574	146.8348	251.0921
Benzo(a)pyrene	291.0394	260.2729	106.4619
Indeno(1,2,3-c,d)pyrene	3293.706	39.04737	46.87954
Benzo(g,h,i)perylene	445.2505	16.81444	15.82488
Σ HMW	9696.17	3083.062	1933.994

Appendix (14A) :Biodegradation of n-alkanes hydrocarbons by *Sporosarcina luteola*.

Carbons number	Incubation period		
	7 days	14 days	21 days
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	12.38579	0	0
C12	0	53.87433	0
C13	18.13739	755.5731	0
C14	51.52139	2321.906	83.68927
C15	194.7328	2925.532	302.6931
C16	1147.428	3274.247	548.4625
Σ LMW	1424.206	9352.576	934.8448
C17	3112.362	2762.325	1393.295
C18	3186.719	2257.613	593.6681
C19	4681.186	3048.076	1678.718
C20	4963.432	3098.184	2240.714
C21	2770.525	1666.198	527.088
C22	4089.144	1730.61	1912.387
C23	2435.13	1582.109	815.1097
C24	3755.079	1835.654	1155.405
C25	2980.942	2308.014	1639.013
C26	3448.446	2440.864	474.1361
C27	2803.746	1455.951	915.8403
C28	1863.549	2019.187	1012.141
Σ MMW	40090.26	26204.78	14357.51
C29	3459.887	2193.892	717.0394
C30	1656.941	1223.801	964.4125
C31	1007.665	900.7475	1120.624
C32	1104.041	1892.915	653.4518
C33	1194.714	888.532	1337.028
C34	1033.253	705.3774	597.5731
C35	1466.999	966.1455	784.7747
C36	791.0342	267.601	552.5884
C37	311.9909	220.419	201.7626
C38	159.9695	73.86549	237.7259
C39	21.9553	0	0
C40	0	0	0
ΣHMW	12208.45	9333.296	7166.98

Appendix (14B) :Biodegradation of PAHs hydrocarbons by *Sporosarcina luteola*

Compounds names	Incubation periods		
	7days	14days	21 days
Naphthalene	0	0	0
2-Methylnaphthalene	24.32372	16.84086	0
1-Methylnaphthalene	0	0	0
Acenaphthylene	273.0596	0	29.98611
Acenaphthene	210.7281	31.40906	533.8913
Fluorene	366.1372	16.93551	72.40919
Phenanthrene	645.7249	260.1451	118.7157
Anthracene	431.6919	289.9667	817.3051
Σ MW	1951.6654	615.2972	1572.3074
Fluoranthene	605.5564	323.8753	275.8426
Pyrene	1841.924	1146.593	348.016
Benzo(a)anthracene	641.5517	272.9728	57.78496
Chrysene	414.5679	301.012	117.3734
Benzo(b)fluoranthene	879.159	407.2902	143.7106
Benzo(k)fluoranthene	509.0228	620.8921	309.8494
Benzo(a)pyrene	333.9171	348.0117	264.9546
Indeno(1,2,3-c,d)pyrene	224.4785	203.4167	15.96896
Benzo(g,h,i)perylene	274.232	65.78433	20.47633
Σ HMW	5744.4094	3689.848	1554.2468

Appendix (15A) :Biodegradation of n-alkanes hydrocarbons by *Acinetobacter junii*.

Carbon numbers	Incubation periods		
	7day	14day	21 day
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	0	13.00135	0
C12	0	0	0
C13	25.11444	188.2284	18.56268
C14	68.69903	1491.129	389.4757
C15	395.3674	2836.122	1645.733
C16	1784.716	3849.985	2863.343
Σ LMW	2273.897	8397.92	4917.114
C17	3853.553	3244.902	3438.218
C18	3433.78	2691.147	2918.206
C19	4714.581	3774.203	3990.522
C20	4749.719	3958.334	2554.765
C21	2737.88	2062.082	2115.825
C22	3873.599	2141.916	3498.666
C23	2455.397	2099.909	1885.747
C24	3690.768	2398.811	3238.067
C25	3042.824	2994.631	2195.245
C26	3520.864	1912.323	3059.392
C27	2775.27	2689.058	2550.214
C28	2131.202	2630.861	1618.867
Σ MMW	40979.44	32598.18	33063.73
C29	3511.282	3425.905	2762.228
C30	1791.976	1524.771	1505.175
C31	2121.968	366.8997	2205.664
C32	1608.096	1231.675	1342.784
C33	1909.401	2449.665	1083.635
C34	1165.477	1188.805	750.664
C35	1378.164	1269.04	852.7257
C36	1735.476	1130.297	692.9712
C37	1308.314	720.1629	338.5267
C38	1465.699	405.8111	66.98888
C39	248.0773	62.01372	28.16463
C40	0	0	0
Σ HMW	18243.93	13775.04	11629.53

Appendix (15B) :Biodegradation of PAHs hydrocarbons by *Acinetobacter junii*

Compounds names	Incubation periods		
	7 days	14 days	21 days
Naphthalene	0	0	0
2-Methylnaphthalene	0	29.95048	0
1-Methylnaphthalene	0	0	0
Acenaphthylene	39.36137	262.4976	24.13378
Acenaphthnen	37.66451	165.6066	15.42821
Fluorene	90.96922	286.1631	78.18625
Phenanthrene	266.0527	463.2853	157.2285
Anthracene	664.9707	715.6459	595.409
Σ LMW	1099.018	1923.149	870.3858
Fluoranthene	346.3882	345.8321	373.2835
Pyrene	1051.809	1185.834	647.7444
Benzo(a)anthracene	585.17	263.2493	179.8896
Chrysene	410.6837	257.1746	143.1453
Benzo(b)fluoranthene	961.7945	493.4187	406.3779
Benzo(k)fluoranthene	680.1743	800.4163	326.599
Benzo(a)pyrene	250.8187	211.3465	193.8396
Indeno(1,2,3-c,d)pyrene	741.3462	94.25854	59.17143
Benzo(g,h,i)perylene	446.2415	49.20366	56.41813
Σ HMW	5474.426	3700.73	2386.4693

Appendix (16A) :Biodegradation of n-alkanes hydrocarbons by *Acinetobacter baumannii*

Carbon numbers	Incubation periods		
	7 days	14 days	21 days
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	18.71771	0	0
C12	0	22.11078	0
C13	243.929	321.6493	0
C14	278.255	1541.896	154.982
C15	3229.231	1848.889	677.0798
C16	4272.225	2001.154	1139.351
Σ LMW	8042.357	5735.698	1971.412
C17	5020.043	1589.894	1323.631
C18	3212.041	1201.604	1126.292
C19	4219.244	2640.701	1746.461
C20	4211.842	3121.381	1019.537
C21	2348.797	1314.163	822.5083
C22	2381.172	1421.015	743.959
C23	2207.841	1484.482	816.9307
C24	2363.238	1829.102	1033.124
C25	2496.93	2058.139	1161.783
C26	3381.761	1458.334	909.7182
C27	1895.87	1508.337	1152.414
C28	2462.031	2087.43	820.8549
Σ MMW	36200.81	21714.58	12677.21
C29	3358.881	2767.624	892.178
C30	1367.455	1098.138	904.0108
C31	945.5943	1655.976	785.8778
C32	1414.104	951.6536	553.7989
C33	1358.505	1081.061	695.9131
C34	1016.086	328.1711	317.213
C35	851.6135	1026.787	152.274
C36	897.415	119.7474	93.6981
C37	347.5451	504.4325	28.01347
C38	73.1481	93.39443	18.33269
C39	0	41.23136	0
C40	0	0	0
Σ HMW	11630.35	9668.216	4441.31

Appendix (16B) :Biodegradation of PAHs hydrocarbons by *Acinetobacter baumannii*

Compounds names	Incubation periods		
	7 days	14 days	21 days
Naphthalene	0	0	34.69995
2-Methylnaphthalene	15.3236	56.12128	88.19287
1-Methylnaphthalene	0	0	0
Acenaphthylene	0	415.9657	62.38938
Acenaphthene	37.32772	256.4535	56.80006
Fluorene	46.89867	335.0139	119.6827
Phenanthrene	317.6405	405.7292	308.2147
Anthracene	386.8128	655.0885	702.0386
Σ LMW	804.0033	2124.372	1372.018
Fluoranthene	479.8778	346.7934	310.4938
Pyrene	1703.092	1053.178	928.4114
Benzo(a)anthracene	619.0826	260.2703	384.0692
Chrysene	402.858	173.7239	243.9449
Benzo(b)fluoranthene	972.0794	237.6772	119.272
Benzo(k)fluoranthene	665.9245	256.4901	328.8751
Benzo(a)pyrene	318.8953	294.1468	91.52897
Indeno(1,2,3-c,d)pyrene	661.2682	54.06694	180.3824
Benzo(g,h,i)perylene	517.0179	15.39424	135.1045
Σ HMW	6394.096	2691.741	2722.082

Appendix (17A) : Biodegradation of n-alkanes hydrocarbons by *Sphingomonas paucimobilis*.

Carbon numbers	Incubation periods		
	7 days	14 days	21 days
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	14.29282	0	0
C12	17.06468	11.45878	0
C13	58.59999	63.68763	236.594492
C14	50.7681	601.8389	1371.88826
C15	1901.58	1782.308	2098.21351
C16	4231.83	2413.368	2636.71477
Σ LMW	6274.135	4872.661	6343.411
C17	7203.33	3251.161	2230.29896
C18	5136.869	2252.464	1926.97981
C19	7172.46	2093.987	2698.77605
C20	7220.49	6178.194	2780.99439
C21	4008.734	1644.96	1467.77916
C22	5429.8	1978.681	1482.49701
C23	5160.259	2411.791	1398.66956
C24	4404.994	1585.901	1865.53872
C25	4655.012	3969.403	1975.39622
C26	5799.539	3270.234	1288.23917
C27	4659.511	3820.646	1133.4845
C28	3135.589	3070.54	1224.25648
Σ MMW	65386.59	35527.96	21472.91
C29	5579.216	4345.331	2055.04822
C30	3137.061	3025.777	948.342495
C31	3348.885	2986.566	1267.98028
C32	2349.374	1915.959	745.726223
C33	3040.225	2097.246	821.504679
C34	2100.12	1551.898	598.641099
C35	1697.413	2266.393	701.354997
C36	1303.179	1854.503	453.574806
C37	1904.148	1010.441	301.713548
C38	1825.187	1464.989	615.953
C39	225.2135	221.79712	37.0950745
C40	0	0	0
Σ HMW	28800.02	23135.06	8152.779

Appendix (17B) :Biodegradation of PAHs hydrocarbon by *Sphingomonas paucimobilis*

Compounds names	Incubation periods		
	7 days	14 days	21 days
Naphthalene	0	0	0
2-Methylnaphthalene	30.36562	0	26.30358
1-Methylnaphthalene	0	0	0
Acenaphthylene	265.3338	21.9727225	144.9109
Acenaphthene	219.245	332.68418	68.50899
Fluorene	381.4065	60.838561	97.62072
Phenanthrene	696.6908	141.768261	88.90679
Anthracene	779.1271	313.070024	148.1891
Σ LMW	2372.169	870.3337	574.44
Fluoranthene	600.4208	253.836548	50.72658
Pyrene	1873.822	603.801121	233.7885
Benzo(A)anthaclene	616.9381	204.370733	31.57364
Chrysene	385.8777	124.590072	24.90105
Benzo(B)fluoranthene	796.4252	488.494474	94.46821
Benzo(K)fluoranthene	802.0071	413.400149	268.6197
Benzo(A)pyrene	334.0545	49.8519631	82.98457
Indeno(1,2,3-c,d)pyrene	1302.45	711.707351	71.34686
BENZO(G,H,I)perylene	508.5287	28.4743744	22.88365
Σ HMW	7220.524	2878.527	881.2927

Appendix (18A) :Biodegradation of n-alkanes hydrocarbons by *Novosphingobium subterraneum*

Carbon numbers	Incubation periods		
	7 days	14 days	21 days
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	11.87037	0	0
C12	11.5409	0	0
C13	177.7015	0	0
C14	1588.863	61.50394	223.034
C15	3218.996	32.20392	1415.935
C16	4231.09	1919.528	2421.558
Σ LMW	9240.062	5736.827	7051.718
C17	3942.563	3723.591	2991.191
C18	3189.471	3303.487	2658.686
C19	4215.491	2802.075	2227.004
C20	2848.478	4130.1	2822.768
C21	2195.564	2712.567	2007.614
C22	2177.764	2078.077	1593.167
C23	1975.892	3169.948	1591.303
C24	1702.13	1776.476	1483.717
C25	2848.609	1941.661	1977.11
C26	2987.158	1877.776	2078.675
C27	1703.769	2066.709	2116.567
C28	2341.485	1827.672	1160.053
Σ MMW	32128.37	28774.71	23407.95
C29	3142.235	1088.16	1691.288
C30	1345.599	698.2205	2063.599
C31	960.9849	529.2301	884.4488
C32	1109.323	2264.358	1805.548
C33	1559.836	420.5718	699.6884
C34	100.8445	1698.717	733.1034
C35	1266.612	342.4304	532.2704
C36	478.8429	545.6795	590.3129
C37	493.2761	271.8322	692.1355
C38	451.1546	155.7236	31.99417
C39	52.27378	86.56179	31.48775
C40	0	0	0
Σ HMW	10960.98	7013.325	8064.589

Appendix (18B) :Biodegradation of PAHs hydrocarbons by *Novosphingobium subterraneum*

Compounds names	Incubation periods		
	7 days	14 days	21 days
Naphthalene	0	0	0
2-Methylnaphthalene	110.8751	15.26084	23.04953
1-Methylnaphthalene	47.20014	0	0
Acenaphthylene	915.8675	16.54442	218.2527
Acenaphthene	644.3762	48.80149	107.7879
Fluorene	787.9885	36.33273	139.2255
Phenanthrene	1006.371	269.5197	134.5877
Anthracene	1092.669	264.0975	260.3615
Σ LMW	4605.347	650.5566	883.2648
Fluoranthene	523.9855	301.6354	79.98257
Pyrene	405.7505	388.5031	347.3949
Benzo(a)anthaclene	571.8574	427.5206	38.4459
Chrysene	347.8525	232.4127	22.49372
Benzo(b)fluoranthene	607.9786	491.2921	44.41668
Benzo(k)fluoranthene	1159.866	437.13	156.6451
Benzo(a)pyrene	352.2182	204.8367	61.73938
Indeno(1,2,3-c,d)pyrene	3628.741	389.3078	97.84081
BENZO(g,h,i)perylene	239.6297	56.76886	31.10407
Σ HMW	7837.88	2929.407	880.0631

Appendix (19A) :Biodegradation of n-alkanes hydrocarbons by *Pantoea sp.*

Carbon numbers	Incubation periods		
	7day	14day	21 day
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	11.25508	0	0
C12	0	13.80902	0
C13	193.9129	407.0737	0
C14	1639.951	1775.633	163.1062
C15	3359.956	2706.822	613.9574
C16	4706.856	3362.411	832.18
Σ LMW	9920.827	8265.749	1609.244
C17	4379.022	3014.853	1921.413
C18	3601.409	2519.435	979.2283
C19	4726.23	3318.335	2147.533
C20	4653.981	3299.322	1244.588
C21	2527.294	1826.695	1013.867
C22	2528.384	1823.637	922.6871
C23	2295.843	1730.892	1065.02
C24	2488.581	1823.626	1376.461
C25	3147.849	2106	1522.685
C26	3492.057	1509.443	2048.219
C27	2754.693	1954.14	1507.578
C28	2582.55	1451.274	1126.553
Σ MMW	39177.89	26377.65	16875.83
C29	3471.339	1658.87	2014.951
C30	1500.038	1055.449	888.5196
C31	2076.655	1523.446	379.4793
C32	1288.415	914.1579	721.6206
C33	1514.202	878.0851	1078.019
C34	808.8515	152.2899	307.1113
C35	1065.95	131.7425	138.955
C36	827.0885	460.6288	120.9365
C37	448.0787	293.2327	34.3034
C38	355.2174	21.3081	21.12542
C39	0	0	0
C40	0	0	0
Σ HMW	13355.84	7089.211	5705.021

Appendix (19B) :Biodegradation of PAHs hydrocarbons by *Pantoea sp*.

Compounds names	Incubation periods		
	7 days	14 days	21 days
Naphthalene	15.36829	0	0
2-Methylnaphthalene	280.7137	0	0
1-Methylnaphthalene	86.68755	34.82272	0
Acenaphthylene	704.8252	477.3985	24.31979
Acenaphthene	376.2346	260.4464	442.1881
Fluorene	391.0145	301.8674	53.47034
Phenanthrene	64.93823	111.4098	133.4566
Anthracene	714.3366	527.0532	394.139
Σ LMW	2634.119	1712.998	1047.574
Fluorantheren	344.7455	269.4642	221.7324
Pyrene	464.2328	876.095	418.3143
Benzo(a)anthracene	343.2589	226.6934	99.42572
Chrysene	253.9361	188.2161	83.10783
Benzo(b)fluorantheren	307.0956	467.6372	335.0408
Benzo(k)fluorantheren	848.9611	320.6372	250.2766
Benzo(a) pyrene	207.7439	288.3942	147.8115
Indeno(1,2,3-c,d) pyrene	107.3599	279.855	15.709
Benzo(g,h,i)perylene	127.977	21.92005	17.3402
Σ HMW	3005.311	2938.912	1588.758

Appendix (20A) : Biodegradation of n-alkanes hydrocarbons by *Kocuria kristinae*.

Carbons numbers	Incubation periods		
	7day	14day	21 day
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	10.88277	11.49663	0
C12	0	24.45179	0
C13	152.9804	605.9513	0
C14	1408.79	2163.012	58.19319
C15	2789.889	2937.11	473.4645
C16	3832.561	3367.36	1243.39
Σ LMW	8195.104	9109.382	1775.048
C17	3508.656	2912.173	2429.09
C18	2985.007	2362.595	1731.668
C19	3902.739	3169.751	2620.099
C20	2721.846	3189.016	1619.683
C21	2144.307	1685.983	1375.472
C22	2116.874	1701.069	2396.059
C23	1979.311	1574.063	1263.346
C24	1659.749	1800.672	2294.177
C25	2670.253	1988.436	1808.898
C26	2883.371	2465.224	1250.158
C27	1641.183	2078.517	1069.101
C28	2182.887	2011.523	1226.691
Σ MMW	30396.19	26939.02	21084.44
C29	2770.517	2453.468	1877.558
C30	1353.626	1043.602	1085.245
C31	1603.52	740.2681	957.2063
C32	954.2481	915.2792	932.3314
C33	1487.997	1024.205	742.1163
C34	856.1265	255.9973	743.4445
C35	928.3785	848.0826	743.4445
C36	927.834	375.9193	642.3318
C37	104.1341	90.42561	302.8772
C38	173.9561	185.9551	290.8477
C39	65.82297	28.1903	121.9316
C40	0	0	0
Σ HMW	11226.16	7961.393	7695.889

Appendix (20B) :Biodegradation of PAHs hydrocarbons by *Kocuria kristinae*

Compounds names	Incubation periods		
	7 days	14 days	21 days
Naphthalene	0	0	0
2-Methylnaphthalene	72.01601	46.45687	0
1-methylnaphthalene	20.02876	0	0
Acenaphthylene	470.9555	229.8228	57.88887
Acenaphthene	282.7693	96.64594	35.88749
Fluorene	343.3509	138.1226	117.2336
Phenanthrene	427.0357	171.347	187.5709
Anthracene	590.8635	312.9723	195.329
Σ LMW	2207.02	995.3675	593.9098
Fluoranthene	309.2644	171.2089	168.572
Pyrene	1057.75	678.5498	662.0395
Benzo(a)anthracene	274.1273	135.1881	190.3412
Chrysene	216.717	119.4446	123.501
Benzo(b)fluoranthene	513.0865	268.8945	525.3348
Benzo(k)fluoranthene	908.9019	319.5227	344.4106
Benzo(a)pyrene	332.43	234.2811	49.74231
Indeno(1,2,3-c,d)pyrene	666.0483	60.87777	16.99538
Benzo(g,h,i)perylene	226.0353	50.2152	19.18736
ΣHMW	4504.361	2038.183	2100.124

Appendix (21A): Biodegradation of n-alkanes hydrocarbons by *Aeromonas salmonicida*

Carbon numbers	Incubation periods		
	7day	14day	21 day
C7	0	0	0
C8	0	0	0
C9	0	0	0
C10	0	0	0
C11	0	13.64795	0
C12	0	0	0
C13	27.54041	330.8546	184.6134
C14	492.1781	1776.083	1366.465
C15	1885.687	3020.676	2427.935
C16	3906.978	3980.922	3126.681
Σ LMW	6312.262	9122.183	7105.695
C17	3548.061	3542.933	2828.827
C18	2870.366	3003.484	2418.18
C19	4425.701	3944.101	3160.134
C20	2819.022	3895.214	2204.846
C21	2244.967	2137.511	1768.648
C22	2429.566	2122.024	1766.653
C23	2337.437	1918.535	1619.419
C24	2705.441	3011.847	1367.438
C25	3204.168	2491.542	2170.713
C26	2394.861	1694.399	1364.681
C27	2123.292	2490.31	1266.031
C28	2173.04	2371.323	1843.115
Σ MMW	33275.92	32623.22	23778.68
C29	3370.717	2525.828	2024.975
C30	1743.772	1245.226	970.027
C31	2476.909	1939.081	1301.243
C32	1916.752	1273.191	980.2847
C33	2053.869	943.3736	1211.205
C34	1084.651	457.5799	196.6186
C35	1348.711	1503.239	672.6712
C36	1197.247	169.764	549.4085
C37	509.0191	1019.513	39.19821
C38	133.7342	198.5456	36.20231
C39	25.95638	204.3662	0
C40	0	0	0
Σ HMW	15861.34	11479.71	7981.833

Appendix (21B): Biodegradation of PAHs hydrocarbons by *Aeromonas salmonicida.*

Compounds names	Incubation periods		
	7 days	14 days	21 days
Naphthalene	0	0	0
2-Methylnaphthalene	0	121.2382	28.66799
1-Methylnaphthalene	0	34.31126	0
Acenaphthylene	122.7302	484.2015	256.1747
Acenaphthene	99.31995	284.6144	146.6158
Fluorene	252.1618	325.0832	190.5252
Phenanthrene	522.3934	413.2714	207.8838
Anthracene	1219.153	632.9038	323.1234
Σ LMW	2215.758	2295.624	1152.991
Fluoranthene	427.0773	366.7136	135.3306
Pyrene	1340.044	701.2923	537.7771
Benzo(a)anthacene	296.5024	309.578	53.23227
Chrysene	402.6668	281.2117	117.3693
Benzo(b)fluoranthene	540.929	403.2286	117.827
Benzo(k)fluoranthene	529.829	206.1679	188.3718
Benzo(a)pyrene	234.8163	245.6091	251.4927
Indeno(1,2,3-c,d)pyrene	176.8679	775.5545	172.2772
Benzo(g,h,i)perylene	19.45994	161.0531	16.75114
Σ HMW	3968.193	3450.409	1590.429

Appendix (22): Biochemical tests of bacterial isolates by Vitek-2 system

Isolates numbers	Bionumber	probability
A11	030200001463431	87%
A12	100000000443431	97%
A13	120200001463431	86%
A21	0043051003500250	99%
A22	424360130350001	99%
A31	451132140000010	89%
A32	040002000040001	88%
A33	055430300000000	93%
A34	110002020663431	95%
B11	0003011111500352	95%
B12	4201730450000000	98%
B13	0040000101400100	95%
B21	4001600000200000	95%
B22	0040002101400100	96%
B31	060173007000410	90%
B32	4011200040200210	87%
B33	110002021663431	95%
C11	070230302241010	92%
C12	0025617550400231	97%
C13	0040002101400140	96%
C21	1220100010600000	97%
C22	0040000101400140	96%
C23	1000001100000200	93%
C31	0040000101500102	97%
C32	0040000101400100	95%

Organism Quantity
Selected Organism : Acinetobacter junii

Comments:	

Identification Information	Analysis Time:	10.25 hours	Status:	Final
Selected Organism	97% Probability Bionumber:	Acinetobacter junii 0040000101500102		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	+	13	dGLU	-	14	GGT	-	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	-	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	-	39	5KG	-
40	ILATk	-	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlpA	-	47	OOC	-	48	LDC	-	53	tHSa	+	56	CMT	-	57	BGUR	-
58	O129R	-	59	GGAA	-	61	IMLTa	(-)	62	ELLM	-	64	ILATa	+			

Organism Quantity
Selected Organism : Kocuria kristinae

Comments:	

Identification Information	Analysis Time:	5.00 hours	Status:	Final
Selected Organism	92% Probability Bionumber:	Kocuria kristinae 070230302241010		
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	+	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	+	19	PHOS	-
20	LeuA	+	23	ProA	+	24	BGURr	-	25	AGAL	-	26	PyrA	-	27	BGUR	-
28	AlaA	+	29	TyrA	+	30	dSOR	-	31	URE	-	32	POLYB	-	37	dGAL	-
38	dRIB	-	39	ILATk	+	42	LAC	-	44	NAG	-	45	dMAL	(+)	46	BACI	-
47	NOVO	-	50	NC6.5	-	52	dMAN	+	53	dMNE	+	54	MBdG	-	56	PUL	-
57	dRAF	-	58	O129R	-	59	SAL	-	60	SAC	+	62	dTRE	-	63	ADH2s	-
64	OPTO	-															

Organism Quantity:
Selected Organism : Pantoea spp

Comments:	

Identification Information	Analysis Time:	4.75 hours	Status:	Final
Selected Organism	98% Probability Bionumber:	Pantoea spp 4201730450000000		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	-
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	-	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	(-)	29	TyrA	-	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	ILATk	-	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	-	57	BGUR	-
58	O129R	-	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Comments:	

Identification Information	Analysis Time:	7.00 hours	Status:	Final
Selected Organism	99% Probability Bionumber:	Pseudomonas aeruginosa 0043051003500250		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	+	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	+	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	+	62	ELLM	-	64	ILATa	-			

Organism Quantity:
Selected Organism : Sphingomonas paucimobilis

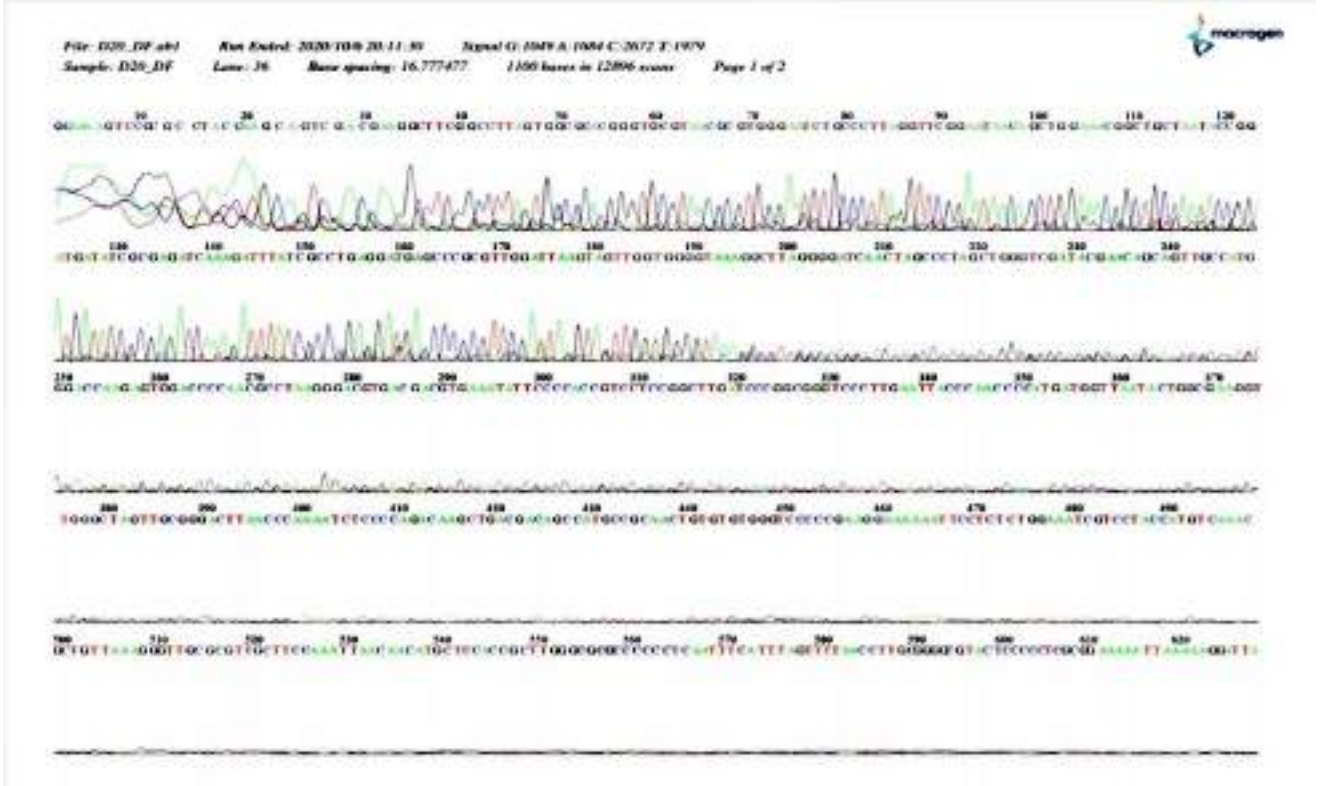
Comments:	

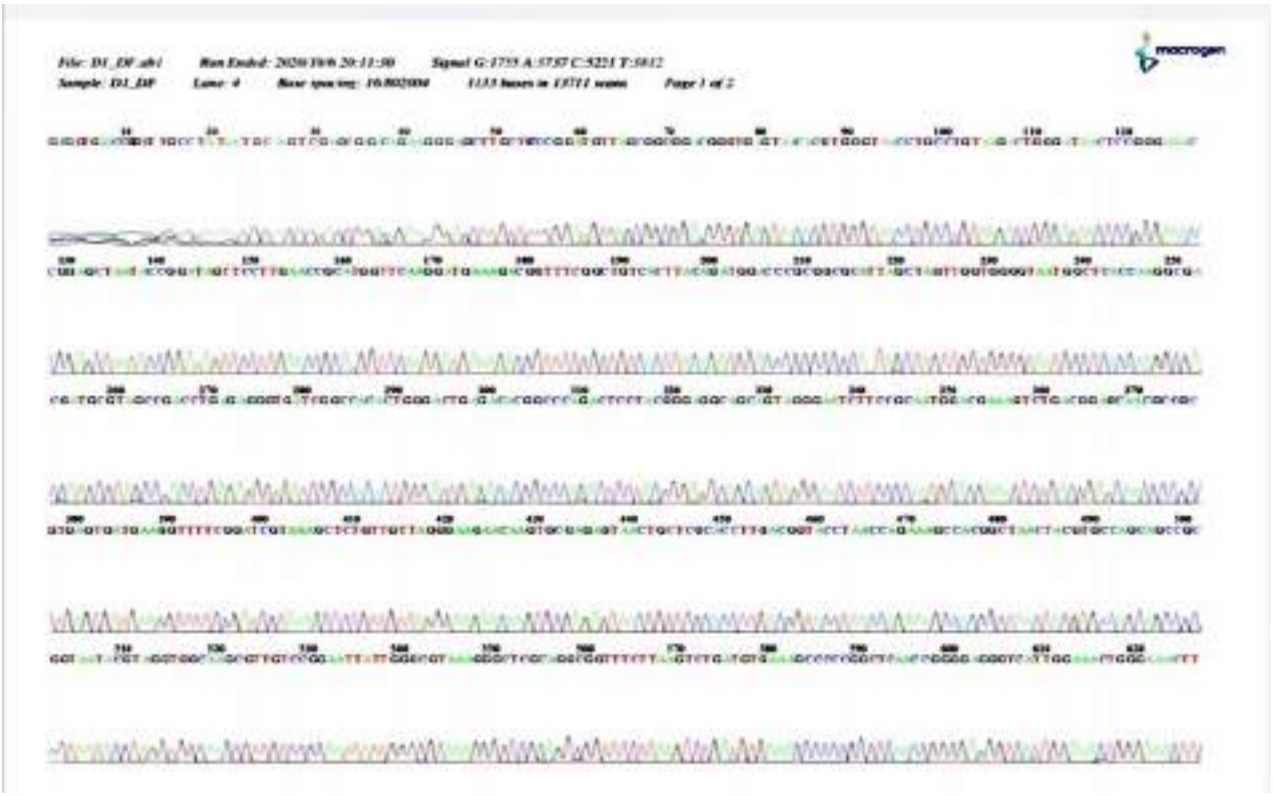
Identification Information	Analysis Time:	5.00 hours	Status:	Final
Selected Organism	97% Probability	Sphingomonas paucimobilis		
	Bionumber:	1220190010600000		
ID Analysis Messages				

Biochemical Details

2	APPA	+	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	+	9	BGAL	-
10	H2S	-	11	BNAG	+	12	AGLTp	-	13	dGLU	-	14	GGT	-	15	OFF	-
17	BGLU	+	18	dMAL	-	19	dMAN	-	20	dMNE	-	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	-
33	SAC	+	34	dTAG	-	35	dTRE	-	36	CIT	-	37	MNT	-	39	5KG	-
40	ILATk	-	41	AGLU	+	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	-	57	BGUR	-
58	O129R	-	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Appendix (23) : DNA sequencing






File: D10_DR.ab1 Run Finish: 2020/10/6 20:11:30 Signal G:1327 A:1575 C:3003 T:2247
Sample: D10_DR Lane: 46 Base spacing: 16.630934 1169 bases in 18015 scans Page 1 of 2

الخلاصة

جمعت عينات التربة الملوثة بالنفط من ثلاثة مواقع مختلفة في محافظة ميسان شملت كلا من مصفى بازركان وشركة نفط الجنوب و شركة النفط الصينية خلال شهر تشرين الثاني من عام 2019 . تم تحديد بعض الخصائص الكيميائية والفيزيائية لعينات التربة الملوثه بالنفط مثل درجة اللون ودرجة الحرارة والدالة الحامضية pH فضلا عن تقدير تراكيز المركبات الهيدروكربونية الالفاتية (الالكانات) والمركبات الاروماتية (الهيدروكاربونات الاروماتية متعددة الحلقات) .

أظهرت النتائج وجود اختلاف في درجات اللون والتي تراوحت من البني الى الاسود وكذلك سجلت اختلافا في درجات الحرارة اذ تراوحت من 28- 30 م^{o} أما فيما يخص قياس الدالة الحامضية pH فتراوحت من 7.43 - 7.98 لكل من تربة شركة النفط الصينية ومصفى بازركان و شركة نفط الجنوب على التوالي.

بينت الدراسة ايضا وجود اختلاف في تراكيز المكونات الهيدروكربونية في التربة الملوثة بالنفط والتي تم تحليلها بواسطة كروماتوكرافيا الغاز حيث تبين انها تحتوي مزيجا من الالكانات والهيدروكربونات الاروماتيه متعددة الحلقات. سجلت النتائج معدل تركيز الالكانات في هذة الترب الملوثة والذي تراوح من 14180.85 الى 66644.43 ميكروغرام / غرام وزن جاف أما الهيدروكربونات الاروماتية متعددة الحلقات تراوح تركيزها من 1361.24 الى 4106.503 مايكر غرام/ غرام وزن جاف .

تم عزل وتشخيص وتحديد السلالات البكتيرية القادرة على تحلل النفط في عينات التربة الملوثة، أذ شخصت 25 عزلة بكتيرية على المستويات الكيموحيوية عن طريق الاختبارات البكتريولوجية الاعتيادية والقياسية وتم تأكيد التشخيص بواسطة جهاز الفايتك- 2 والاختبارات الجزيئية .تم اختبار 16 عزلة بكتيرية من أصل خمس وعشرين عزلة للتحقق من قدرتها على تحلل النفط الخام.

استخدمت الفحوصات الجزيئية ممثله بتفاعل سلسلة انزيم البلمرة (PCR) لتشخيص العزلات البكتيرية اذ أستخدم بادئ عام لتضخيم جين 16s rDNA جزئيا والذي أعطى ناتج جين 1500bp وبادئ عام لتضخيم جين 16s rDNA جزئيا لجرثومة *Pseudomonas sp.* وناتج جين 150bp ودرست تتابعاتها في المركز الوطني لمعلومات التقانات الحيوية (NCBI) بأستخدام أداة بحث المحاذاة المحلية الأساسية (BLAST) اذ بينت النتائج تشخيص ثلاث عشرة عزلة بكتيرية ، أثنتا عشرة منها بأستخدام البادئ العام الاول وه ي (*Bacillus subtilis*، *Bacillus safensis*، *Bacillus pumilus*، *Bacillus sporothermodurans*، *Sphingomonas paucimobilis*، *Novosphingobium subterraneum*، *Brevundimonas sp.*، *Arthrobacter luteolus*، *Sporosarcina luteola*، *Acinetobacter junii*، *Acinetobacter baumannii*، *Aeromonas salmonicida*) وعزلة واحدة (*Pseudomonas putida*) بأستخدام البادئ العام الثاني كما تم تسجيل اثنتا عشرة عزلة بكتيرية في بنك الجينات وتحت اعداد انظمام مختلفة (Fj763645.1

,Ks783591 ,EF590133.1 ,Mk 729043.1 ,KF751673.1 ,Mk501608.1
Kj958271.1 ,Kx622562.1 ،Kr999939.1 ,Mk774b245.1 ،En430991.1
,Mt730013.1 ,Ky820912.1 ,Kj573537.1 ,Mn589774.1 ،Kt984874.1
و Mg 551868.1 ,Jx6499224.1 ,MK418695.1, Mk829514.1 ،MK418695.1
Kx989239.1) والتي قد تكون لأول مرة في العراق. أما العزلات الثلاث المتبقي ة (*Pseudomonas aeruginosa*، *Kocuria kristinae* و *Pantoea sp.*) فشخصت بالاعتماد على التشخيص المظهري وجهاز الفايتك-2.

أظهرت النتائج وبالأعتماد على طرق التشخيص المتعددة لعزل وتحديد الاجناس البكتيري ة الموجودة في التربة الملوثة بالنفط أن البكتريا السالبة لصبغة كرام هي الأكثر تواجدا في هذه الترب الملوثة .

بينت الدراسة أن الظروف المثلى لنمو البكتيريا القادرة على تحلل النفط مثل الدالة الحامضية pH تراوحت من (6-9). اظهرت العزلات البكتيرية قدرتها على النمو في درجات حرارة تراوحت بين 24-42 °م ، بينما كانت بعض السلالات البكتيرية (*Pseudomonas aeruginosa*، *Brevundimonas sp.*، *Sphingomonas paucimobilis*، *Kocuria Kristina*، *Acinetobacter junii*، *Aeromonas salmonicida* ، *Novosphingobium subterraneum*) قادرة على النمو عند 46 °م ،كان النمو الأمثل لهذه العزلات البكتيرية عند 24 °م و 28 °م و 37 °م، باستثناء *Bacillus sporothermodurans* و *Brevundemonas sp.* التي أظهرت نموًا مثاليًا عند 32 م° كما أظهرت *Pseudomonas aeruginosa* و *Novosphingobium subterraneum* نموًا مثاليًا عند 32 °م و 42 °م .

زرعت جميع العزلات البكتيرية (16 عزلة) على وسط الملح المعدني (MSM) المضاف له 0.5% من النفط الخام في ثلاث فترات حضانة (7، 14 و 21) يوما اذ أظهرت جميع العزلات تحللا حيويا موجبا للنفط الخام المتكون من الالكانات و الهيدروكاربونات الاروماتية متعددة الحلقات مختلفة الوزن الجزيئي . وكذلك تشير الزيادة الحاصلة في الكثافة الضوئية لنمو البكتريا والمقاسة بجهاز المطياف الضوئي عند OD_{620} الى قدرة العزلات على استخدام الهيدروكربونات الموجودة في النفط الخام كمصدر وحيد للكربون والطاقة .

بينت الدراسة قدرة العزلات المستخدمة على تحليل جميع مكونات النفط الخام بصورة تدريجية ابتداء من التراكيز منخفظة الوزن الجزيئي خلال فترات الحضن الاسبوعيه الاولى (7 يوم) ثم يليها تحلل التراكيز مرتفعة الوزن الجزيئي في نهاية فترة الحضن لمدة 14 و 21 يوم .

ظهرت نتائج كروماتوغرافيا الغاز لتحلل الالكانات بواسطة 16 عزله بكتيرية كما يلي :

Bacillus safensis تمكنت من تحليل الالكانات بنسبة 36.25 %، 67.47 % و 76.51% ، *Bacillus subtilis* (71.61%، 79.23% و 83.39%)، *Bacillus pumilus* (44.92%، 71.76% و 74.54%)، *Bacillus sporothermodurans* (57.5% ، 71.39% و 77.91%) ، *Pseudomonas aeruginosa* (69.49%، 76.59% و 84.66%) ،

Arthrobacter luteolus ، (%84.45 و %76.23 ،%62.09) *Brevundimonas sp*
(%53.41 ،%73.39 و %77.76)، *Pseudomonas putida* (63% ،%77.11 و
%84.21)، . *Pantoea sp* (64.63% ،%76.36 و 86.3%)، *Sphingomonas*
paucimobilis (43.1% ،%64.01 و 79.63%)، *Novosphingobium*
subterraneum (70.36% ،%76.48 و 78.18%)، *Sporosarcina luteola*
(69.57%،%74.57 و 87.28%)، *Kocuria kristinae* (71.78% ،%75.06 و
82.69%)، *Aeromonas salmonicida* (68.57% ،%69.85 و 77.98%)،
Acinetobacter junii (65.17% ،%68.98 و 71.9%) و *Acinetobacter*
baumannii (68.35% ،%78.97 و 89.18%)، نسب التحلل هذه للالكانات خلال فترات الحضن الاسبوعيه (7، 14 و 21) يوما على التوالي.

أما نتائج كروماتوغرافيا الغاز لتحلل الهيدروكاربونات الاروماتيۃ متعددة الحلقات بواسطة 16 عزلة بكتيرية ايضا ظهرت كما يلي:

Bacillus safensis تمكنت من تحلل الهيدروكاربونات الاروماتية متعددة الحلقات بنسبة (52.96% ، 60.13% و 80.85%)، *Bacillus subtilis* (63.27% ،%85.37 و85.99%)، *Bacillus pumilus* (60.94% ،%78.72 و 87.31%)، *Bacillus*
sporothermodurans (61.79% ،%74.77 و 78.35%)، *Pseudomonas*
aeruginosa (66.42% ،%84.31 و 90.93%)، . *Brevundimonas sp* (44.30% ،
79.84% و 91.18%)، *Arthrobacter luteolus* (40.32% ،73.70% و 86.67%)،
Pseudomonas putida (51.84% ،80.43% و 88.84%)، *Pantoea sp* (74.42% ،
78.9% و 88.04%)، *Sphingomonas paucimobilis* (56.5% ،83% و 93.39%)،
Novosphingobium subterraneum (43.57% ،83.76% و 92%)،
Sporosarcina luteola (65.1% ،80.74% و 85.82%)، *Kocuria kristinae*
(69.65% ،86.24% و 87.78%)، *Aeromonas salmonicida* (71.95% ،73.94%
و 87.55%)، *Acinetobacter junii* (70.19% ،74.49% و 85.23%) و
Acinetobacter baumannii (67.35% ،78.16% و 81.43%)، نسب التحلل هذه للهيدروكابونات الارماتية متعددة الحلقات خلال فترات الحضن الاسبوعيه (7، 14 و 21) يوما على التوالي.

واخيرا، بينت هذه الدراسة أن أعلى نسبة لتحلل الالكانات في الفترة الاخيرة من الحضن (21 يوم) كانت بواسطة *Acinetobacter baumannii* (89.18%) أما أعلى نسبة لتحلل الهيدروكربونات الاروماتية متعددة الحلقات كانت بواسطة *Sphingomonas paucimobilis* (93.39%).

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة ميسان

كلية العلوم

تشخيص البكتريا المحللة حيويا للبترول والمعزولة من ترب ملوثة في محافظة ميسان / العراق

رسالة مقدمة الى

مجلس كلية العلوم / جامعة ميسان وهي جزء من

متطلبات نيل شهادة الماجستير في علوم الحياة

من قبل

هبه نعيم ضيغم

بكالوريوس علوم حياة / جامعة ميسان

(2017)

بأشراف

أ.د. زاهد سعدون عزيز

أ.م.د. صالح حسن جازع

شعبان 1442 هـ

أذار2021 م