Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science

Biodegradation of oil spills and fuel contaminated soils by *Streptomyces* isolates

A Thesis

Submitted to the College of Science/ Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

By
Nour Abdulateef Abdullah

B.Sc. Biotechnology/ College of Science/ Al-Nahrain University (2010)

Supervised by

Dr. Rehab S. Ramadha (Instructor)

Dr.Rebah N. Jabbar (Assit. Professor)

June,2013 Shaaban,1434

Acknowledgment

First of all praise to **Allah** the lord of the universe, peace be upon **Mohammed** the Messenger of **Allah** and upon his **Relatives**.

I am grateful to the Ministry of Higher education and Scientific Research for providing me the chance to complete this study.

First and foremost my thanks must go to my Supervisor Dr. Rehab S. Ramadha and Dr.Rebah N. Jabbar for their kindness and great support throughout my studies. I specially appreciate their warm and gentle approach to my supervison, as well as their immense patience during my work.

I am grateful to Mrs.Zainab Yaseen and Mrs. Ghydaa Hussain/Biotechnology research center for help and support.

Its pleasure to thank Mrs. Marwa, Mr.Ahmed and Mr.Aws Fadhil /Ministry of Science and Technology.

My Sincere thanks and appreciation go to my best friends for their understanding, endless love, encouragement and great friendship that help me to overcome difficulties in all hard times.

My special thanks to my family, and I am very grateful for their unreserved love and encouragement which inspired throughout my study.

Nour

Supervisor Certification

We, certify that this thesis entitled "**Biodegradation of oil spills and fuel contaminated soils by *Streptomyces* isolates**" was prepared by "**Nour Abdulateef Abdullah**" under our supervision at the College of Science/Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science / Biotechnology.

Signature:

Name: Dr.Rehab S. Ramadha

Scientific Degree: Instructor

Date: / / 2013

Signature:

Name: Dr. Rebah N. Jabbar

Scientific Degree: Assit Professor

Date: / / 2013

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

Signature:

Dr. Hameed M. Jasim

Scientific Degree: Professor

Title: Head of Biotechnology Department

Date: / / 2013

Committee Certification

We, the examining committee certify that we have read this thesis entitled **" Biodegradation of oil spills and fuel contaminated soils by *Streptomyces* isolates"** and examined the student **"Nour Abdulateef Abdullah"** in its contents and that in our opinion, it is accepted for the Degree of Master of Science / Biotechnology.

Signature:
Name: Dr. Abdulwahid B. Al-Shaibani
Scientific Degree: Professor
Date: / / 2013
(Chairman)

Signature:
Name: Dr. Hayfa H. Hassani
Scientific Degree: Professor
Date: / / 2013

Signature:
Name: Dr. Nuha J. Kandala
Scientific Degree: Assit Professor
Date: / / 2013

Signature:
Name: Dr. Rehab S. Ramadha
Scientific Degree: Instructor
Date: / / 2013
(Member & Supervisor)

Signature:
Name: Dr. Rebah N. Jabbar
Scientific Degree: Assit Professor
Date: / / 2013
(Member &Supervisor)

I, hereby certify upon the decision of the examining committee.

Signature:
Name: Dr. Khulood W. Abbood
Scientific Degree: Professor
Title: Dean of College of Science

Summary

Five soil samples (contaminated with oil spills for many years) were collected from different places in Baghdad. From these samples, fourteen isolates were obtained as a preliminary degraders for hydrocarbons. The isolates were recultured in the mineral salt medium supplemented with crude oil and diesel, separately, to ensure their degradation ability. Despite that with 5 isolates S7, S8, S11, S12, and S14 gave good degradation activity, but isolates S7 and S14 were the most efficient due to their highest , degradation ability for crude oil and diesel as a sole source of carbon.

The two isolates were identified depending on their morphological, cultural, and biochemical characteristics. Results showed that they were belonged to *Streptomyces* by their ability to use various pure sources of hydrocarbons. Isolate S7 was superior growth comparing with S14 isolate in (Hexadecane, Tetrahydrofuran, Anthracene, and Naphthalene).

Optimum conditions for degradation of crude oil and diesel by S7 and S14 isolates were investigated. By growing them in the modified yeast extract malt extract media in different range of pHs, temperatures, agitation, and salt concentrations with addition of 1% crude oil and diesel , separately, after incubation for 10 days.

Isolation and purification of biosurfactants produced by *Streptomyces* using acid precipitation method yielded (0.5, 1) g/L of biosurfactant from S7 and S14 isolates, respectively. The biochemical detection tests and Fourier Transformed Infrared spectroscopy analysis indicated the lipopeptide nature of biosurfactants.

Gas chromatography analysis for liquid-liquid extraction of residual crude oil and diesel showed significant differences in the composition at the end of the experiment. Most of long and complex carbon chains were broken in to simpler types, and disappearance of some compounds was observed during the process.

When the plasmid profile for S7 and S14 isolates were studied, results showed that these isolates harbored small plasmid DNA bands. In order to study the role of its plasmid in degradation of hydrocarbons, curing experiment was performed by using low pH curing method. Results in this regard the plasmid DNA has no role in the biodegradation since both isolates ability in degradation of the hydrocarbon.

List of contents

Chapter Two: Materials and Methods

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Mean
µg/ml	microgram/ milliliter
DMSO	Dimethyl sulfoxide
DNA	Deoxyribo nucleonic acid
E_{24}	Emulsification Index in 24 hour
FTIR	Fourier Transformed Infrared spectroscopy
GC	Gas chromatography
IR	infrared
ISP	International Streptomyces project
mN/m	Milli Newton / meter
OD	Optical density
SDS	Sodium Dodecyl Sulphate
TBE	Tris – Borate – EDTA

TLC	Thin layer chromatography
v/cm	Volt / centimeter
v/v	Volume per volume
w/v	Weight per volume
YEME	Yeast extract malt extract
µl	Micro liter

Chapter One

Introduction
and
Literatures Review

1.Introduction and literature review

1.1.Introduction

A large number of ecosystems have been changed by the growing influence of human activity. Past analysis of reported oil spills indicated that most of the oil comes from tankers, barges and other vessels as well from land pipeline spills. It is necessary to select an appropriate clean-up method (Hinchee and Kitte, 1995).

Petroleum recovered from different reservoirs varies widely in compositional and physical properties. The composition of particular petroleum product ranges from the very low molecular weight hydrocarbons to the very high. Petroleum is a complex mixture of hydrocarbons and other organic compounds, including some organometallic-constituents. Hydrocarbons can be classified generally into three major groups including: aliphatic and aromatic hydrocarbons, upon their chemical structure. Aliphatic substances are with saturated or unsaturated straight (C_1-C_{40}) or branched (C_6-C_8) chains, while aromatic compounds are unsaturated having ring structures. Physically, the crude petroleum has light oil fractions with high volatility such as gasoline and kerosene, and heavy oil fractions like tars and residual fuel oil(Al-Hadhrami *et al.,* 1995; Anton *et al*., 2000).

Most of the physicochemical methods use chemical agents, as well as their emulsion with oil cause toxicity to organisms. They produce another source of pollution and also increase the oil recovery cost. Additionally, abiotic losses due to evaporation of low molecular hydrocarbons, dispersion and photo oxidation (involves only aromatic compounds) play a major role in decontamination of the oil spill environments (Mills *et al*., 2003).

There is an increased interest in promoting biological methods in the process of cleaning oil-polluted sites. These methods are less expensive and do not introduce additional chemicals to the environment. Compared to physiochemical methods, bioremediation offers a very feasible alternative for an oil spill response. This technique is considered an effective technology for treatment of oil pollution (Sanchez *et al*., 2006).

The biological method has taken two directions one involves the provision of suitable condition like temperature, pH, and addition of nutrients (Stuart-keial *et al*., 1998). The other direction depends upon the genetic development of specific microbial strains especially the bacteria present in polluted area. These bacteria are efficient in their utilization of different hydrocarbon compounds and contain plasmids responsible for degradation of some hydrocarbons and different bacterial strains have been developed which contain different plasmids capable of degradation different hydrocarbons (Mitra, 1996; Hohnstock *et al*., 2000).

In many ecosystems there is already an adequate indigenous microbial community capable of extensive oil biodegradation, providing that environmental conditions are favorable for oil-degrading metabolic activity (Capelli *et al*., 2001). There are several advantages relying on indigenous microorganisms rather than adding microorganisms to degrade hydrocarbons. First, natural populations have developed through many years. These microorganisms are adapted for survival and proliferation in that environment. Secondly, the ability to utilize hydrocarbons is distributed among a diverse microbial population. This population occurs in natural ecosystems and either independently or in combination metabolizes various hydrocarbons. Many times, when the amount of microorganisms is sufficient in the contaminated environment, microbial seeding is not required (Kim *et al*., 2005).

Microorganisms are equipped with metabolic machinery to use petroleum products as a carbon and energy source. The metabolic pathways that hydrocarbon-degrading heterotrophs use can be either aerobic or anaerobic. Aerobic degradation usually proceeds more rapidly and is considered to be more effective than anaerobic degradation. One reason is that aerobic reactions require less free energy for initiation and yield more energy per reaction (Richard and Vogel, 1999).

Iraq is considered as one of the main productive and exporting countries of crude petroleum. Oil extracting fields, oil refineries, oil pipelines and many oil dependent industries are distributed throughout the country. As a result of unexpected accidents or misuse of oil products, the possibility of soil and water pollution with oil and its derivatives is considered of great importance, in addition to pollution by the refinery by-product. According to those mentioned above and because of the limited studies about the genetics of hydrocarbons biodegradation in Iraq, this study was aimed to:

1. Isolation and identification of bacteria that capable of degrading oil spills.
2. Screening the bacteria for their ability to degrade hydrocarbons and select the efficient isolate(s).

3. Determination of plasmid(s) involvement in hydrocarbons utilization.

1.2. literature review

1.2.1. Concept of Hydrocarbon Biodegradation

Biodegradation is the natural way of recycling wastes or breaking down the organic matter into nutrients that can be carried out by various microorganisms. Soil pollution by petroleum products is widely spread. Petroleum and mineral oil hydrocarbons are the most frequently occurring environmental contaminants. The biodegradation of petroleum products and natural waste is the modification or decomposition of the product by microbes to produce ultimately energy, CO_2 and water (Kannahi, and Thara, 2012)**.** The microbial utilization of hydrocarbons was highly dependent on the chemical nature of the compounds within the petroleum mixture and on environmental condition. However, not all organic compounds are readily mineralized. When the structural elements of such compounds are chemically very stable, have novel features not generally found in organic molecules of biological origin, are very toxic to microorganisms, or inhibit degradative enzymatic attack, then the compounds itself is said to be recalcitrant to many biological degradation (Atlas,1981; Alexander,1991).

1.2.2. *Streptomyces* in Industry and Nature

Actinomycetes are filamentous, branching bacteria with a fungal type of morphology. They are part of the microbial flora of most natural substrates. Numerous methods have been advocated to facilitate the isolation of actinomycetes and to separate them from their relatives. It is not difficult to isolate actinomycetes from an intimate mixture with fungi, since the physiological properties of these two groups of microorganisms are different. For example, strictly antifungal antibiotics, which do not affect the growth of actinomycetes, can be used successfully. It is more

difficult to separate actinomycetes from true bacteria. Actinomycete bacteria, including species of *Nocardia*, *Streptomyces*, and *Micromonospora*, as many as millionsper gram, are present in warm, dry soils (Martin and Focht 1977).

The increasing number of novel metabolites from Actinobacteria reported annually shows that habitats are a promising source of biotechnological commercially significant products (Battershill *et al*. 2005; Blunt *et al.* 2007). This is not surprising given the unique capacity of their terrestrial counterparts to produce new products, notably antibiotics in order to combat new and emerging diseases and antibiotic resistant pathogens (Taylor *et al*. 2001; Be´rdy, 2005; Strohl, 2005). Novel antibiotics from Actinobacteria currently include the anticancer metabolite salinosporamide A from a Salinispora strain (Fehling *et al*. 2003), the structurally unique marinomycins from *Marinophilus* strains (Jensen *et al*. 2005), abyssomicin C, a potent inhibitor of the para-aminobenzoic acid pathway, from a *Verrucosispora* strain (Riedlinger *et al.* 2004) and marinopyrroles from a *Streptomyces* strain (Hughes *et al*. 2008).

Streptomyces is the largest genus of actinobacteria and the type genus of the family streptomycetaceae have been described as showed in the Table(1.1).

As with the other actinobacteria, streptomycetes are typical aerobic gram positive soil bacteria developing hyphal networks and spores that resist to the unfavorable environment, and have genomes with high guanine and cytosine content. Found predominantly in soil and decaying vegetation, most streptomycetes produce spores, and are noted for their distinct "earthy" odor that results from production of a volatile metabolite, geosmin.

Table(1.1).*Streptomyces* scientific classification (Brenner *et al*, 2001).

Scientific classification	
Kingdom:	Bacteria
Class:	*Actinobacteria*
Subclass	*Actinobacteridae*
Order1:	*Actinomycetales*
Suborder.	*Streptomycineae*
Family1:	*Streptomycetaceae*
Genus1:	*Streptomyces*

Streptomycetes are characterized by a complex secondary metabolism (Madigan and Martinko, 2005). They produce over two-thirds of the clinically useful antibiotics of natural origin (e.g., neomycin, chloramphenicol) (Kieser *et al*.,2000). The now uncommonly used streptomycin takes its name directly from *Streptomyces*. In the soil they utilize organic polymers and they play an important role in waste removal and recycling of materials in nature (Chater and losik, 1997).Since they produce a high proportion of known antibiotics, many enzymes and other biologically active substances, they are industrially important, and therefore can be produced in large scale. *Streptomyces* are present in different kinds of soils and can adapt to extreme environmental conditions (e.g. hot deserts, salt-marsh areas, and the cold Alpine slopes). Their versatility allows them to live and propagate under unfavorable circumstances, such as in oil polluted soils. In contrast, although Streptomyces are saprophytic and known to play important roles in biotransformation and biodegradation in nature, the metabolic pathways have been little studied (Omura *et al*., 2001).

Hydrocarbon degrading bacteria and fungi are widely distributed in marine, freshwater and soil habitats. The most important (based on frequency of isolation) genera of hydrocarbon utilizing bacteria aquatic and soil environments were: *pseudomonas, arthrobacter, achromobacter, micrococcus, corynebacterium, acinetobacter, nocardia, alcaligenes, Streptomyces, vibrio* and *flavobacterium* (Atlas and Bartha, 1973).

The ability to isolate high numbers of certain oil degrading microorganisms from oil polluted environment is commonly taken as evidence that these microorganisms are the active degraders of that environment (Okerentugba and Ezeronye, 2003).

In the last 20 years much has been published about the ability of microorganisms to utilize hydrocarbons, especially n-alkanes as carbon and energy sources. These microorganisms belong to *Pseudomonas sp*. (Naas *et al*., 2009), *Alcaligenes sp*. (Weissenfels *et al.,* 1990), *Mycobacterium sp*. (Pagnout *et al*., 2007) and *Rhodococcus sp*. (Martinkova *et al*., 2009).

One of the most important characteristics of hydrocarbon degrading bacteria is the ability of emulsifying hydrocarbons in solution by producing surface active agents such as biosurfactants. Biosurfactants are directly involved in the process of hydrocarbon removal from the environment through increased bioavailability and subsequentbiodegradation of the hydrocarbons by direct cell contact (Hommel, 1990; Banat *et al*., 2000; Ganesh, and Lin, 2009). Barabas *et al.* (2001) have reported three *Streptomyces* strains (*Streptomyces griseoflavus, Streptomyces parvus* and *Streptomyces plicatus*) from the Kuwait Burgan oil field with the ability to utilize n-hexadecane, n-octadecane, kerosene and crude oil as sole carbon and energy sources.

Fritsche and Hofrichter (2000) mentioned the essential characteristics of aerobic microorganisms degradingorganic pollutants are:

- Metabolic processes for optimizing the contact between the microbial cells and the organic pollutants. The chemicals must be accessible to the organisms having biodegrading activities. For example, hydrocarbons are water insoluble and their degradation requires the production of biosurfactants.
- The initial intracellular attack of organic pollutants is an oxidative process, the activation and incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases.
- Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, e.g., the tricarboxylic acid cycle.
- Biosynthesis of cell biomass from the central precursor metabolites, e.g., acetyl-CoA, succinate, pyruvate. Sugars required for various biosynthesis and growth must be synthesized by gluconeogenesis.

1.2.3. The composition of crude petroleum

The crude petroleum involved the hydrocarbon compounds , which consider the main part of petroleum compounds, oxidized completely to H_2O and CO_2 (Bartha, 1986). According to their ability to be degraded, it divided to:

Alkanes hydrocarbon chains may be straight, branched, cyclic chains. The short chains alkanes (C_5-C_{10}) was consider inhibiter for some degrader organisms, because of these compounds behaved as solvent lead to destroy the microbial membrane structure and also volatilization, while the degradation of alkanes have medium length chains (C_{10}-C_{20}) was more faster. Waxes was more than C_{20} have low degradation. The straight chains alkanes (C_{17}-C_{18}) degraded faster than branch one, because of the

branch chains make it more resistance to biodegradation and low availability to oxidative enzymes (Watanabe,2001).

Poly aromatic hydrocarbons was a group of compounds contained one or more aromatic rings. The complication of biodegradation increase when the number of aromatic rings increase. Poly aromatic hydrocarbons was found as soil and marine main pollutant, because their toxicity and carcinogenic effect on organisms (Johnsen *et al*., 2005). In general the Poly aromatic hydrocarbons biodegradation represented three function:

- Partial or complete degradation of these compounds by assimilative biodegradation to produce carbon and energy for degraders.
- Intracellular detoxification to make them more soluble in water. The cellular oxidation and hydroxylation of the aromatic compounds considered the first step of breaking up the aromatic ring for microbial assimilation of carbon(Creniglia, 1984).
- Co-metabolism which accomplished by mixture of microorganisms , their enzymes competed to oxidized specific compound (Jain *et al*., 2005).

Asphaltic compound formed a few amount of non-hydrocarbon compounds which contained the (N,S and O).

All these compounds have different average of degradation in the environments. The biodegradation depending on the length and branch carbon chain and the number of aromatic rings of these compounds. Addition to their solubility and toxicity (Bartha, 1986; Atlas, 1991).

1.2.4.Factors Affecting Biodegradation Process:

A number of environmental factors affecting the biodegradation process can be summarized as follow:

1.2.4.1.pH

In general the optimum pH for microbial biodegradation is slightly above 7 and increase when pH increase to 8 (Hambrick *et al*.,1980). The change of pH lead to change the acidity inside/outside the microbial cell in which influence on enzymatic system and effecting on reaction speed because of change the ionic state of substrates or reaction products. Occurrence of pH change effected on the interaction between substrate and enzymes (Bartha,1986).

1.2.4.2.Temperature

Merino and Bucala (2007) reported that increase in temperature made the biological membranes to have more fluid due to increased vibrational activity to the fatty acid chains in the phospholipids bilayer. The increase in the rate of fluidity helps in increasing the rate of substance uptake from a cell's surrounding medium (in the case of thermophile bacteria). Biodegradation of hydrocarbon has been shown to occur over a wide range of temperature, though, in general optimum degradation occurs in the (25- 40◦C) temperature range (Al-Maghrabi *at el*.,1994).

1.2.4.3.Agitation

The agitation proved oxygen which consider the important factor in aerobic biodegradation because of work as electro accepter and associate with the enzymatic inducing reactions. Most studies have shown aerobic biodegradation of hydrocarbon was more efficient than anaerobic process. So enough amount of oxygen should provide for the microorganisms because of the few amount of it limited the

biodegradation process. Bartha, 1986 indicated the first attack of many hydrocarbon occurred by oxygenase system, in the absence of oxygen, nitrogen and sulfate work as electro accepter for partial oxidation of intermediate hydrocarbon compounds (Anton *et al*., 2000).

1.2.4.4.Salt Concentration

The activity of many microorganisms was high at low concentrations of salt, comparing with high salt concentrations which cause reduction in biodegradation (Leahy and Colwell, 1990). The variation of salt concentrations effect on the permeability of microbial cell well reflected on their ability to metabolize hydrocarbon compounds (Ward and Brock, 1978). Margesin and Schinner (2001) found the increase of salt concentration lead to lower the solubility of poly aromatic hydrocarbon such as benzene.

1.2.5. Surfactants and Biosurfactants

Surfactants are amphiphilic molecules consisting of ahydrophilic and a hydrophobic domain. The non-polar, hydrophobic part is frequently a hydrocarbon chain. The polar component appears in many variations (Banat *et al*., 2000)**.** This suggest that it has a certain affinity for both polar and nonpolar solvents. Depending on the number and nature of the polar and nonpolar groups present, the amphiphilic may be predominantly "hydrophilic" (water-loving),"lipophilic"(oil-loving) or responsibly well balance between these two extremes. Surfactants are surface active agents that reduce the surface tension. At the air-water interface, the lipophilic chains are directed upward in to the air, oil-water interface, they are associated with the oil phase. In order for the amphiphilic to be concentrated at the interface, it must be balanced with the proper amount of water and oil-soluble groups. If the molecules is to hydrophilic, it

remains within the body of the aqueous phase and exerts no effect at interface. Likewise, if it is too lipophilic, it dissolved completely in the oil phase and little appears at the interface (Lakshmipathy *et al*., 2010). Most of the Chemical surfactants available are petroleum derivatives which are highly toxic and non-degradable. In an effort to overcome these disadvantages microorganisms were investigated for the production of biosurfactant molecules with low toxicity and biodegradability (Seghal *et al*., 2009).

Biosurfactants are a specialized class of surfactants that are produced by bacteria, yeast and fungi as extracellular or membrane-associated surface active compounds called biosurfactants. The term biosurfactant refers to any types of compounds produced by microorganisms with surface active or emulsifying properties. The term "bioemulsifiers" has also been used to describe surface active agent forming emulsion(when one liquid phase is dispersed as microscopic droplets in another liquid continuous phase). Microbial surfactants or biosurfactant molecules are surface active agents synthesized by microbial cells and help in reducing the surface tension of media (Fiechter, 1992; Gandhimathi *et al*., 2009). Biosurfactants are both biodegradable, which is a positive ecological aspect and non-toxic or less toxic than chemical surfactants. They occur naturally in soil, which makes them acceptable from a social and ecological point of view. According to Rosenberg and Ron (2001) biosurfactants are divided into low molecular weight compounds such as glycolipids or lipopeptide and high molecular weight compounds such as polysaccharides, proteins, lipopolysaccharides or lipoproteins.

Several therapeutic and biomedical importance including antibacterial, antifungal, antimycoplasmic, inhibit fibrin clotformation, antitumoral and hemolytic agents are recorded for surface active microorganisms (Cameotra and Makkar, 2004; Rodrigues *et al*., 2006).

1.2.6.Utilization of Hydrocarbon Compounds

1.2.6.1.Uptake Mechanisms of Hydrocarbon by Microbial cell

The mechanisms of uptake hydrocarbon compounds or the way to introduce it to specific sit inside the cell to get their energy, depend on the main factors : type and physical state of a hydrocarbon and also on the type of hydrocarbon degradation organisms.

1.2.6.1.1. Uptake of Dissolved Hydrocarbons

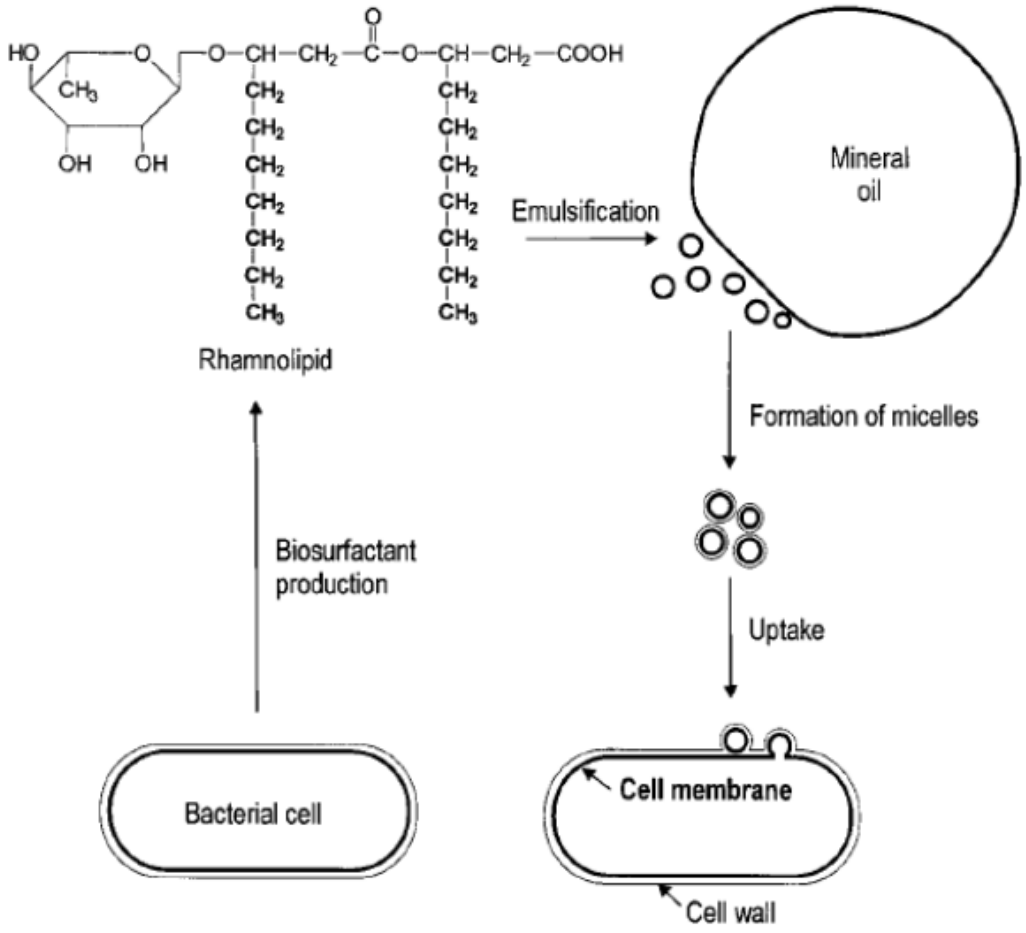
Consider a limit mechanisms because depending on the microbial growth on soluble hydrocarbon only. The soluble hydrocarbon was define a dispersed hydrocarbon. These compounds spreading as Submicron droplets, so uptake inside the microbial cell as soluble form. This type of mechanisms involved short chains alkane such as the n-decane (C_{10}). According to the low solubility of many hydrocarbon, it was not express the real ability of microbial biodegradation (Gibson, 1984;Barab´as *et al*.,2001).

1.2.6.1.2. Direct Contact Mechanisms

In this mechanisms the uptake of hydrocarbon compounds and transfer it through the microbial membrane according to the direct contact between the microbial cell surface and hydrocarbon droplets. Harowitz *et al*., (1975) and Zhang and Miller (1994) indicated that the microbial cell adhering itself to the hydrocarbon droplet, so the carbon chain become apart with microbial phospholipid membrane. This mechanisms depend on the affinity of hydrocarbon to contact with microbial hydrophobic cell surface and on the nature of microbial membrane.

1.2.6.1.3. Emulsification Mechanisms

The biodegradation of many hydrocarbon was difficult because of the low solubility in water. These low solubility compound be more accessible to microorganisms when the last was able to produce biosurfactant as shown in the figure (1.1) (Barkay *et al*., 1999; Fritsche and Hofrichter, 2000; Plaza *et al*., 2005).

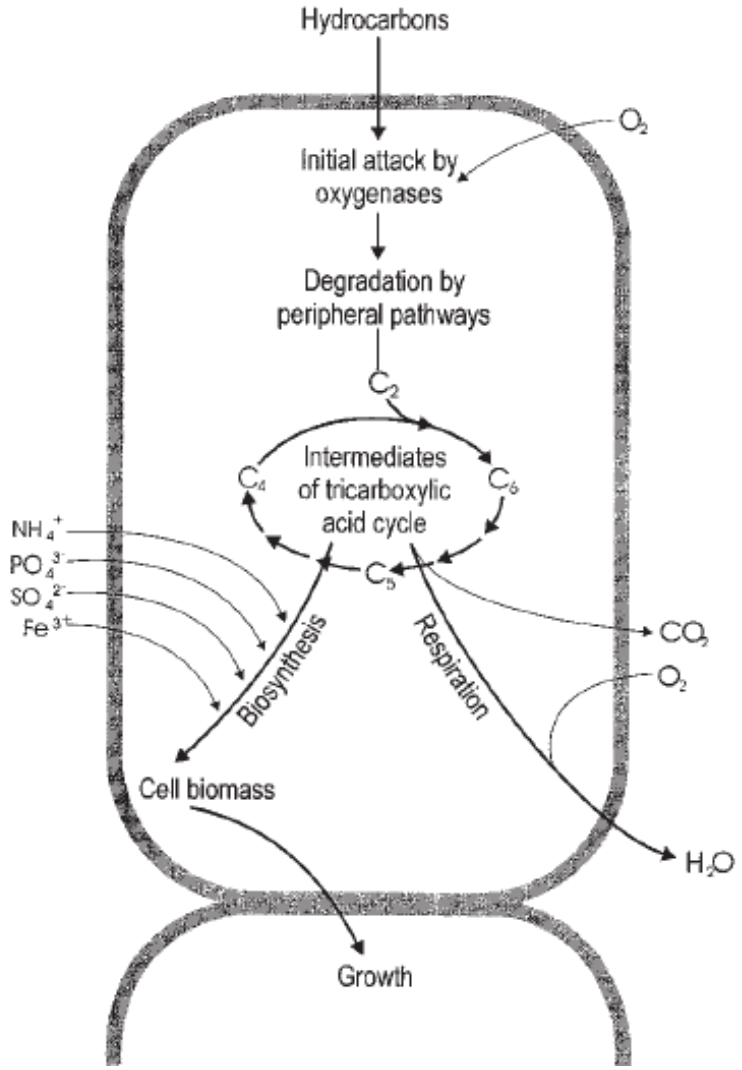


Figures(1.1). Involvement of biosurfactants in the uptake of hydrocarbons (Fritsche and Hofrichter, 2000).

1.2.6.2. Mechanisms for Microbial Oxidation of Hydrocarbons

The biodegradation of hydrocarbons as a sole source of carbon and energy is attempted by a series of catabolic reactions that are catalyzed by a group of enzymes yield in the end of the reaction energy, water and CO_2. Hydrocarbon degradative organisms differ in their oxidation ability

(Clarke,1984; Singh *et al*., 2012).The figure(1.2) simplify biodegradation process illustrated hydrocarbon utilization.



Figures (1.2). Main principle of aerobic degradation of hydrocarbons (Singh *et al*., 2012).

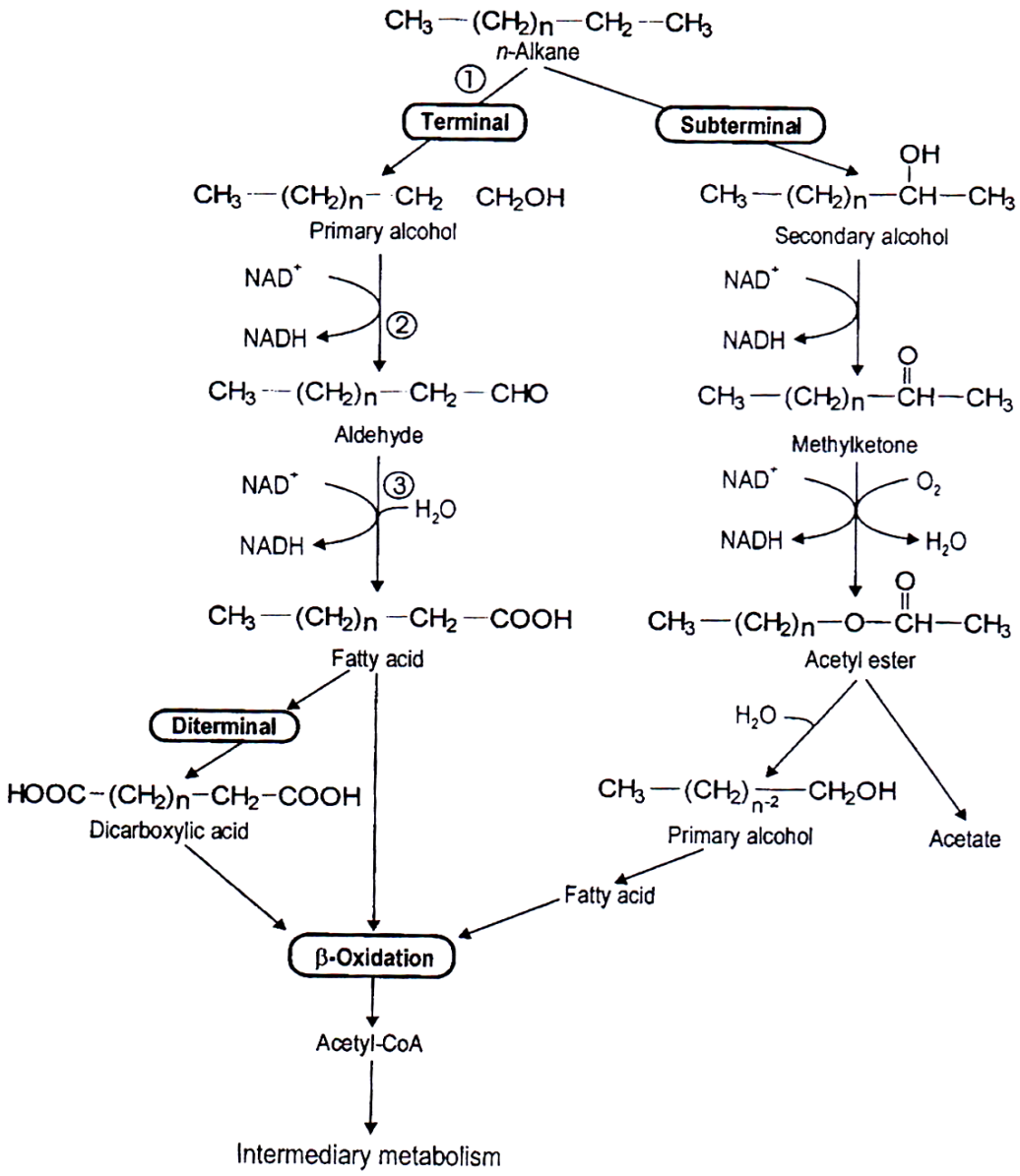
1.2.6.2.1. Oxidation of Aliphatic Hydrocarbons

The aerobic initial attack of aliphatic hydrocarbons requires molecular oxygen. Figure (1.3) shows both types of enzymatic reactions involved in these processes. It depends on the nature of the substrate and the enzymatic equipment of the involved microorganisms, what kind of enzymatic reaction is realized. Oxidation of alkanes is classified as being

terminal or sub-terminal**.** Both terminal and sub-terminal oxidation can co-exist in some microorganisms (Meintanis *et al*., 2006; Singh *et al*., 2012).

The Monoterminal Oxidation is the main pathway. It proceeds via the formation of the corresponding alcohol, aldehyde, and fatty acid. β-oxidation of the fatty acids results in the formation of acetyl-CoA. *n*-Alkanes with an uneven number of carbon atoms are degraded to propionyl-CoA, which is in turn carboxylated to methylmalonyl-CoA and further converted to succinyl-CoA. Fatty acids of a physiological chain length may be directly incorporated into membrane lipids, but the majority of degradation products is introduced into the tricarboxylic acid cycle (Rehm and Reiff, 1981;Coon2005).

Sub-terminal Oxidation of n-alkanes has been used by some microorganisms. The oxygen is inserted on a carbon atom within the chain instead of its end. The product generated a secondary alcohol which is converted to the corresponding ketone, and then oxidized by a monooxygenase to render an ester. The ester is hydrolyzed by an esterase, generating an alcohol and a fatty acid and then metabolized further byβ-oxidation (Britton, 1984; Kotani *et al*. 2007; Singh *et al.,* 2012).



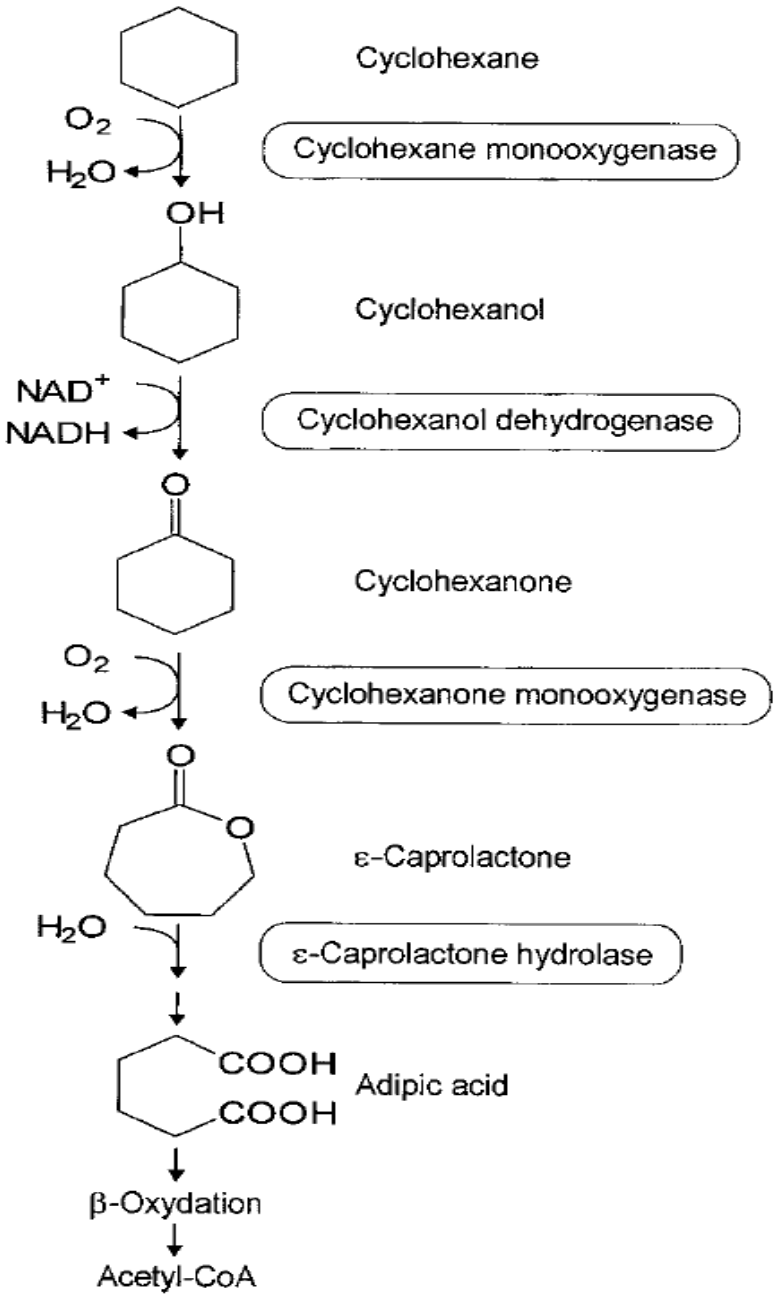
Figures(1.3). Peripheral pathways of alkane degradation. The main pathway is the terminal oxidation to fatty acids catalyzed by (1)*n*-alkane monoxygenase,(2) alcohol dehydrogenase and (3) aldehyde dehydrogenase (Singh *et al*., 2012).

1.2.6.2.2. Oxidation of Alicyclic Hydrocarbons

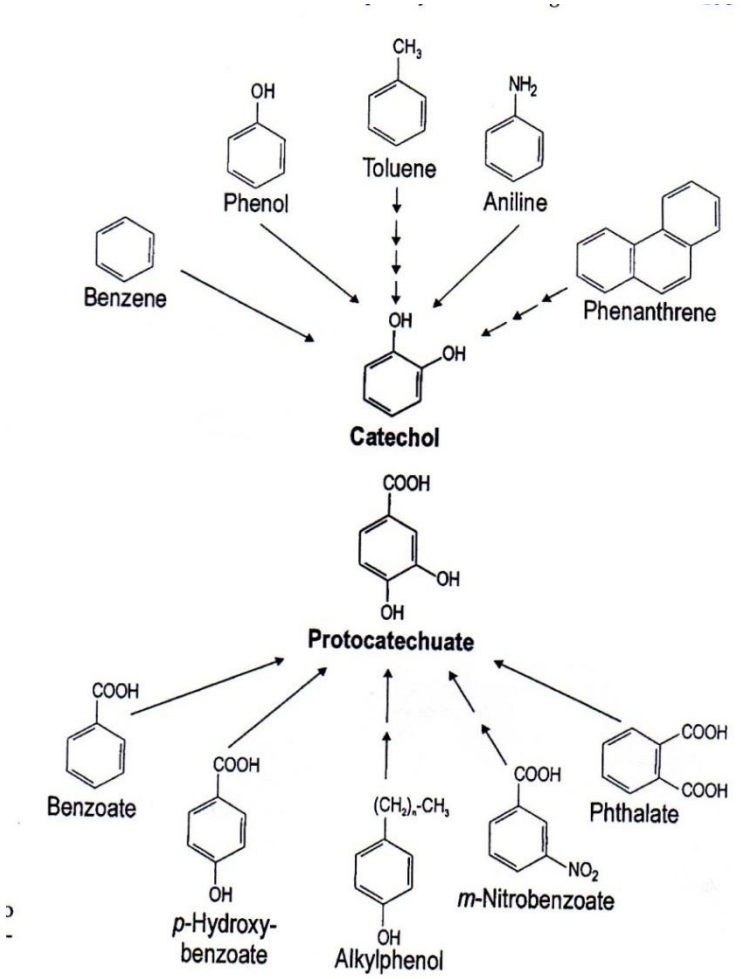
Alicyclic Hydrocarbons representing minor components of mineral oil but they widely distributed in the biosphere, and the potential for environmental pollution. Alicyclic Hydrocarbons are relatively resistant to microbial attack and toxic, but have a high degree of volatilization that aids in their removal . The absence of an exposed terminal methyl group complicates the primary attack. Hydroxylation by monoxygenase leads to an alicyclic alcohol. Dehydrogenation leads to the ketone. The hydroxyl group is oxidized in sequence, to an aldehyde and carboxyl group. The resulting dicarboxylic acid is further metabolized byβ-oxidation. The mechanism of cyclohexane degradation is shown in figure (1.4) (Atlas,1981;Towell *et al*., 2011; Fritsche and Hofrichter, 2000).

1.2.6.2.3.Oxidation of poly Aromatic Hydrocarbons

The oxidation of poly aromatic hydrocarbons is more complicated than aliphatic compounds, because it requires energy to cleave the poly aromatic ring, as well as enzymes that participate in poly aromatic hydrocarbon oxidation differing from those off aliphatic hydrocarbon oxidation. A diverse groups of poly aromatic hydrocarbon oxide to intermediates of the central metabolisms (catechol and protocatechuate) as shown in the figure (1.5).Oxidation firstly involved add hydroxyl group (Hydroxylation) to produce diol compound, breaking the ring lead to form (Hydroxymuconic- semialdehyde or Cis-muconate), and when the alkyl group in aromatic ring are present its first oxidize. The resulting products of breaking aromatic ring usually (catechol and protocatechuate) then entered one of the pathway (meta cleavage or ortho cleavage) to complete degradation as shown in the figures (1.6) (Atlas, 1981; Wallnofer and Engelhardt, 1984; Fritsche and Hofrichter, 2000).



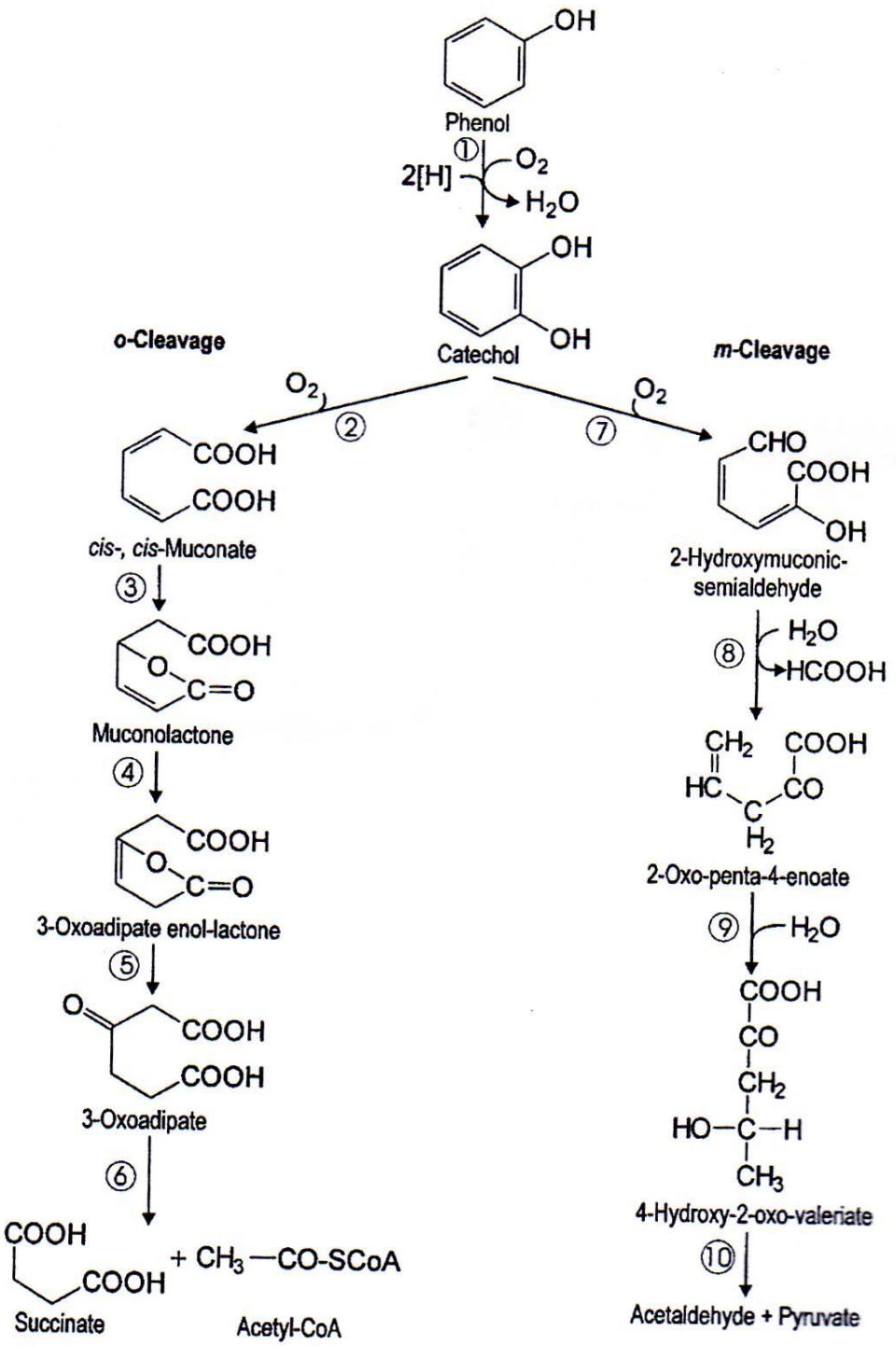
Figures(1.4). Peripheric metabolic pathway of cycloaliphatic compounds (Fritsche and Hofrichter, 2000).



Figures(1.5). Degradation of a broad spectrum of aromatic natural and xenobiotic compounds into two central intermediates: catechol and protocatechuate (Fritsche and Hofrichter, 2000).

1.2.6.3. Role of Plasmids in Biodegradation

Microorganisms are known to degrade hydrocarbon compounds, as well as synthetic hydrocarbon derivatives. The genes for the degradation of hydrocarbon can be borne on either chromosome or plasmids. It was found that hydrocarbon degradative bacteria exhibit a higher incidence of plasmid DNA in hydrocarbon contaminated environments (Devereux and Sizemore ,1982).



Figures(1.6). The two alternative pathways of aerobic degradation of aromatic compounds: *ortho*- and *meta*-cleavage,(1) phenol monoxygenase,(2) catechol 1,2-dioxygenase,(3) muconatelactonizing enzyme, (4) muconolactoneisomerase, (5) oxoadipateenol-lactone hydrolase,(6) oxoadipatesuccinyl- CoA transferase,(7) catechol 2,3-dioxygenase, (8) hydroxymuconicsemialdehyde hydrolase, (9) 2-oxopent-4- enoic acid hydrolase, (10) 4-hydroxy-2- oxovaleratealdolase (Fritsche and Hofrichter, 2000)**.**

Plasmids are extra chromosomal elements that replicate independently of the chromosome and regulate their one replication. Plasmids play a significant role in bacterial adaptation and evaluation; they provide gene products that can benefit the bacterium under certain conditions (synder and champness, 1997).

Degradative plasmids represented a group of naturally occurring plasmids that have been distributed in a wide range of bacterial species. The degradative plasmids govern the metabolisms of a diverse group of aliphatic compounds , poly aromatic hydrocarbons and product of their oxidative metabolism. Degradative plasmids encode a complete degradative pathway or partial degradative steps, other microorganisms may possess plasmids that code for the rest of the pathway. The number of isolated degradative plasmids increase, which enable the bacteria to degraded hydrocarbon compounds and industrial pollutants and convert them to less harmful for the environments. Degradative plasmids are generally transmissible or conjugative plasmids, with high molecular weights (may reach 500kb) enough for loading biodegradation and conjugative genes. Plasmids may encode in complete catabolic pathways, therefore the degradation of hydrocarbon as a sole source of carbon and energy may require complementation of the plasmid genes by host chromosomal genes to link the plasmid pathway with energy yielding metabolisms, especially in poly aromatic hydrocarbons and xenobiotics (Lindow *et al*.,1989). In *Streptomyces*, two types of these elements were identified; the first is ccc DNA form with various copy numbers, ranging from 6 – 100 Kb, whereas the second type is the giant linear form that may range 50 – 350 Kb or more in some species. The importance of these plasmids may come from their involvement in conjugation and antibiotic production (Al- Gafari, 2004; Chater and Hopwood, 1993).

Chapter Two

Materials and Methods

2.Materials and Methods

2.1. Materials

2.1.1. Equipment and Apparatus

The following equipment and apparatus were used in this study:

Equipment or Apparatus	Company (origin)
Autoclave	Tomy (Japan)
Compound light microscope	Olympus (Japan)
Cooled incubator	Sanyo (Japan)
Cooling centrifuge	Hettich (Germany)
Electric oven	Sanyo(Japan)
Electrophoresis unit	Major Science
Fourier transformed infrared spectroscopy	Shimadzu (Japan)
Gas chromatography	Shimadzu (Japan)
Hot plate and magnetic stirrer	Gallen kamp (England)
Laminar air flow hood	Sanyo (Japan)
Microcentrifuge	Hermile (Germany)
pH meter	Martini (Germany)
Sensitive balance	Denver (USA)
Shaking incubator	Hirayama (Japan)
Spectrophotometer	Cecil (England)
Tensiometer	KSV (Finland)

UV-transilluminator	Viberlourmat (USA)
Vortex	Scientific works (USA)
Water bath	Grant (England)
Water distillator	GFL (Spain)

2.1.2. Chemicals and Biological Materials

The following chemical and biological materials were used throughout the study:

Material	Company (origin)
Arabinose, Iodine, Bromophenol blue, HCl, K_2HPO_4, Proline, Soluble Starch, Aniline, Raffinose, Bromocresol purple, Diethyl ether, Casamino acids, Glucose, H_2SO_4, Histidin, Inositol, Ammonium hydroxide, dichloromethane, D-xylose, Dextrose, Phenol red, Yeast extract, KH_2PO_4, Sucrose, SDS, Rhaminose, Peptone, Mannitol, NaCl, petroleum ether ,diethyl ether, acetic acid, alpha- naphthol	BDH (England)

Ammonium sulfate $(NH_4)_2SO_4$,,Calcium Chloride Hexahydrate ($CaCl_2.2H_2O$), $FeCl_3.6H_2O$, Hydrogen peroxide (H_2O_2),Potassium nitrate (KNO_3), $MgSO_4.7H_2O$,Disodium hydrogen phosphate (Na_2HPO_4), Sodium hydroxide (NaOH), Manganese Chloride Tetrahydrate ($MnCl_{2.4}H_2O$), Magnesium Chloride Hexahydrate ($MgCl_2.6H_2O$), Potassium sulfate (K_2SO_4), Toluene, Ferrous Sulfate Heptahydrate ($FeSO_4.7H_2O$), Calcium carbonate ($CaCO_3$), Manganese Sulfate Heptahydrate ($MnSo_4.2H_2O$),Copper(II) sulfate($CuSO_4$. $5H_2O$), Hexan, Urea ($ZnSO_4.7H_2O$),	Fluka (Switzerland)
Agar, Casein hydrolysate, Casein –peptone, Malt extract	Oxoid(England)
Glycerol, Ethanol, Sulphric, Methanol Chloroform, N-Buthanol, Ethyl acetate	Analar (England)
Ehidium bromide, Agarose, Lysozyme, Tris base	Sigma (USA)

2.1.3. Media

2.1.3.1. *Streptomyces* Isolation and Identification Media

2.1.3.1.1.Soil Extract Glucose Yeast Extract Agar (Shirling and Gottlieb, 1966)

Component	Quantity (g/l)
Soil Extract	250ml
Yeast Extract	1
Glucose	2
Agar	20
D.W	1

To prepared the Soil Extract Glucose Yeast Extract Agar medium, the components were dissolved in D.W. and sterilized by autoclave. After cooling 45 ºC add the soil extract solution and sterilized antibiotics (supplemented with 100 ug/ml of cycloheximide to preclude the growth of fungi).

2.1.3.1.2. International *Streptomyces* Project (ISP) Medium (Shirling and Gottlieb, 1966)

Component	Quantity (g/l)
Yeast extract	4
Malt extract	10
Dextrose	4
Agar	20

pH was adjusted to 7.0 and sterilized by autoclaving.

2.1.3.2.Biochemical Test Media

2.1.3.2.1.Czapeck Medium (Shirling and Gottlieb, 1966)

It was prepared by dissolving 48 g in 1000 ml distilled water, sterilized by autoclaving at 121ºC for 15 min and used for bacterial growth.

2.1.3.2.2.lysozymeResistance Medium (Kuster, 1976)

Basal medium

Component	Quantity(g/l)
Peptone	10
Yeast extract	5
D.W	1L

Lysozyme stock solution was made of 10 mg/ml of D.W and sterilized by filtration. Basal medium was sterilized by the autoclave and distributed in 3 ml aliquots in sterile test tubes. Basal medium was supplemented with lysozyme concentrations of (10, 50, 100)µg/ml of D.W as final concentration**.**

2.1.3.2.3.Sugars Utilization Medium (Kuster, 1976)

A) Basal medium

Component	Quantity(g/l)
$(NH_4)_2SO_4$	2.56
KH_2PO_4	2.38
K_2HPO_4	5.56
$MgSO_4.\ 7H_2O$	1

$CuSO_4$. $5H_2O$	6.4
$FeSO_4$. $7H_2O$	1.1
$MnCl_2$. $4H_2O$	7.9
$ZnSO_4$. $7H_2O$	1.5
Agar	20
D.W	1L

B) Carbon sources were prepared using the following sugars

Carbon Sources	
D – glucose	L – inositol
D – mannitol	Mannose
D – xylose	Rhaminose
L –arabinose	Sucrose

Each sugar was sterilized by filtration and added as a final concentration of 1% to basal medium, after adjusted the pH to 7.0 and autoclaved.

2.1.3.2.4.Organic Acids Formation Medium (Kuster, 1976)

Suspension (A)

Component	Quantity(g/l)
Glucose	50
Yeast extract	3
$MgSO_4$. $7H_2O$	0.62
Bromocresol purple	0.3

D.W	1

Suspension (B)

Component	Quantity
Na_2HPO_4. $2H_2O$	1.06
KH_2PO4	0.544
D.W	1

Suspension (C)

Component	Quantity(g/l)
$CaCO_3$	10
D.W	1

Test tubes were labeled with 0.2 ml of suspension C and autoclaved. Solution A and B were autoclave separately, mixed, and 1.8 ml of the mixture was added to each test tube containing suspension C. Inoculated tubes were incubated for 10 days at 30°C.

2.1.3.2.5.Urea Agar Base Medium (Kuster, 1976)

Component	Quantity(g/900ml)
Glucose	1
Na_2HPO_4. $2H_2O$	2
Casein – peptone	1
KH_2PO_4	1.5
$MgSO_4$. $7H_2O$	0.5

NaCl	5
Phenol red	0.012
Urea solution	180
Agar	20
D.W	900ml

After autoclaving of basal medium it was supplemented with the urea solution (20g/ml D.W) sterilized by filtration.

2.1.3.2.6. R2YE Medium (Hopwood *et al*., 1985)

Basal medium

Component	Quantity(g/800 ml)
Sucrose	10.3
K_2SO_4	0.25
$MgCl_2$. $6H_2O$	10.12
Glucose	10
Casamino acids	0.1
D.W	800 ml

It was Prepared by dissolving 2.2 g of agar for each 80 ml basal medium in 250 ml flask. The flasks were closed and autoclaved. At the time of use, the media were melted and 5 ml volume of yeast extract (10%) and R2Y2 solution (2.1.5.4) was added to each flask.

2.1.3.3.Hydrocarbon Utilization Media

2.1.3.3.1.Liquid Mineral Salt Medium (Jimnani *et al.*,2009)

This medium used for screening of bacterial capability to degrade crude oil and diesel as a sole source of carbon and energy, and its consisted of:

Component	Quantity (g/l)
NaCl	1
Na_2HPO_4	1
$(NH_4)_2So_4$	0.5
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.02
$FeCl_3$	0.002
$MnSO_4.2H_2O$	0.002

pH was adjusted to 7.0 and sterilized by autoclaving.

2.1.3.3.2. Solid Mineral Salt Medium

This medium included same ingredients mention in (2.1.3.3.1) with the addition of 2% agar and 10% hydrocarbon contaminated soil. The pH was adjusted to 7.0 and sterilized by autoclaving.

2.1.3.3.3. Modified Yeast Extract Malt Extract Broth

This medium used for cultivation of the bacterium under hydrocarbon condition and consists of the followings:

Component	Quantity(g/l)
Yeast extract	3
peptone	5

Malt extract	3
Hydrocarbon source	10

pH was adjusted to 7.0 and sterilized by autoclaving.

2.1.3.4. *Streptomyces* Cultivation Medium

2.1.3.4.1.YEME Broth (Shirling and Gottlieb, 1966)

This medium used for cultivation of bacteria and consists of the followings:

Component	Quantity(g/l)
Yeast extract	3
peptone	5
Malt extract	3
Glucose	10
Sucrose	30

pH was adjusted to 7.0 and sterilized by autoclaving.

2.1.3.5.Preservation Media

2.1.3.5.1.Gauza Medium (Komagata, 1986)

This medium used for preservation of bacteria and consists of the followings:

Component	Quantity(g/l)
Soluble starch	20
KNO_3	1
NaCl	0.5

$MgSO_4.7H_2O$	0.5
$FeSO_4.H_2O.\ 7H_2O$	0.01
K_2HPO_4	0.5
Agar	20
D.W.	1

pH was adjusted to 7.0 and sterilized by autoclaving.

2.1.3.6.Biosurfactant Production Media

2.1.3.6.1.Luria–Bertanil (LB)Broth

Luria–Bertanil medium was used to study biosurfactant production by the bacteria.

Component	Quantity(g/l)
Casein enzymic hydrolysate	10
Yeast extract	5
Sodium chloride	5

pH was adjusted to 7.0 and sterilized by autoclaving.

2.1.3.6.2.Blood Agar Base Medium (Oxoid)

Blood agar was used for hemolytic blood test and in the screening of biosurfactant producing microorganisms. It was prepared by dissolving 40g blood agar base in1000 ml D.W and sterilized by autoclaving, after cooling (45°C), 5%fresh blood was added and mixed well.

2.1.4.kits

Favor gene biotech corp (Plasmid DNA Extraction Mini Kit).

2.1.5.Buffers and Solutions

2.1.5.1.Gram's stain (Harley and Prescott, 1996)

2.1.5.2.Soil Extract Solution (Shirling and Gottlieb, 1966)

In order to prepare the soil extract, 500 g of soil was mixed with 1 L of D.W and incubated overnight at room temperature. The mixture was filtrated and then centrifuged for 30 min at 6,000 rpm. The supernatant was sterilized through a 0.2µm membrane filter and served as stock.

2.1.5.3.Trace element solution (Hopwood *et al*., 1985)

Component	Quantity(g/l)
$(NH_4)_6Mo_7O_{24}.\ 4H_2O$	0.01
$CuCl_2.\ 2H_2O$	0.01
$FeCl_3.\ 6H_2O$	0.2
$MnCl_2.\ 4H_2O$	0.01
$Na_2B_4O_7.\ 10\ H_2O$	0.01
$ZnCl_2$	0.04
Distilled water	1

2.1.5.4. R2Y2 Medium Solution(Hopwood *et al*., 1985)

Component	Quantity
$CaCl_2.\ 2H_2O$ (3.68 %)	8 ml
KH_2PO_4 (0.5 %)	1 ml
L – proline (20 %)	1.5 ml
NaOH (1 N)	0.5 ml
TES solution (5.73 % pH7)	10 ml
Trace element solution	0.2 ml

2.1.5.5.TLC Solutions

•Alpha-Naphthol Solution

It was prepared by dissolving 1g of Alpha-Naphthol powder in 25 ml methanol and used freshly.

2.1.5.6. Electrophoresis buffers (Sambrook and Russell, 2001)

•Ethidium bromide

It was Prepared at concentration of 10 mg/ml in sterilized D.W. and stored at 4°C until use.

•Gel loading buffer (6X)

Bromophenol blue (0.25% w/v)

Sucrose in H_2O (40% w/v)

•5X Tris – Borate – EDTA (TBE) (pH 8.0)

Component	Quantity (g/l)
Trise-base	54
Boric acid	27.5
EDTA	(0.5M) 20ml
D.w.	1

The working solution was 1 X

2.1.6. Reagents

2.1.6.1. Catalase reagent (Hopwood *et al*., 1985)

This reagent composed of 3% H_2O_2

2.1.6.2. Sugar moiety Reagent (Sadasivam and Manickam, 1996)

It was prepared by dissolving 5% 1- naphthol in alcohol (95%).

2.1.6.3. Biuret test

It was prepared by two solution:

- Dissolving10g of NaOH in 100ml D.W.
- Dissolving 0.1g of CuSO4 in 100ml D.W.

2.2.Methods

2.2.1. Sterilization Methods (Colins and Lyne, 1987)

- **Autoclaving:** Buffers, solutions, and media were sterilized by autoclaving at 121ºC, (15 Ib/in)2 for 15 minutes.
- **Dry heat:** Electric oven was used for glassware sterilization at 180 ºC for 3 hours.
- **Filtration:** Some solutions that cannot withstand heat were sterilized by filtration through Millipore filters (0.2 μm diameter).

2.2.2. Soil Samples Collection (Saadoun *et al*., 2008)

Samples were collected from five different sites contaminated with crude petroleum oil spills in Baghdad. After removing approximately 3 cm of the soil surface materials, 1000 g of soil were collected and placed in plastic bags and stored in the laboratory at 4 ± 1C until use. Collected soil samples were crushed thoroughly, mixed and sieved through a 2 mm pore size mesh to get rid of large debris.

2.2.3.Isolation of Hydrocarbons Degrading Microorganisms

One gram of each sample was suspended in 100 ml of sterile distilled water, agitated on a water-bath shaker (100 rpm at 28ºC for 30 min). Suspension of each soil sample was allowed to settle down and then serial ten-fold dilutions were prepared. Aliquots of 0.1 ml was taken from each dilution and evenly spread with the aid of sterilized L-shaped glass rod over the surface of Soil Extract Glucose Yeast Extract Agar (2.1.3.1.1)in Petri dishes and incubated at 30ºC for 10 days. Bacterial colonies that showed a *Streptomyces*-like appearance were re-streaked on 1SP medium (2.1.3.1.2) to obtain pure colonies used for identification (Shirling and Gottlieb,1966;Nonomura, 1974)

2.2.4. Inoculum Preparation

A single colony of the isolate was inoculated into 30 ml YEME broth and incubated at 30°C for overnight at 150 rpm. After incubating the culture was centrifuged for 10min at 10000 rpm. The cell pellets were washed and re-suspended in phosphate buffer saline (pH 7.0) until the OD at 600 nm was equivalent to 1. One ml of bacterial inoculum (1 OD 600 nm equivalent was used in most experiments).

2.2.5.Identification of Bacterial Isolates

According to the method described by Shirling and Gottlieb (1966) and Saadoun *et al*.,(2007), the following tests were employed to identify the suspected *Streptomyces spp*. isolates.

2.2.5.1.Morphological Characteristics

The morphological characterization of each isolate was first performed by:

2.2.5.1.1.Colony Characteristics (Saadoun *et al*., 2007)

Bacterial isolates grew on ISP medium were characterized morphologically and physiologically according to the International Streptomyces project (ISP)**.**

2.2.5.1.2.Gram's Stain (Harely and Prescott,1996)

A single colony was transferred by a loop to a clean glass slide. The smear was stained with crystal violet, treated with iodine, decolorized by the ethanol (95%), and stained with safranine, then examined by a microscope.

2.2.5.1.3.Number of Spores (Hopwood, 1985)

The hypha *Streptomyces*-like appearance was detected under microscope. Typical Streptomyces hypha carries from 16-32 spores as a specific criterion of this bacteria among it's family.

2.2.6.2.Biochemical Tests

2.2.6.2.1.Catalase Test (Hopwood *et al*., 1985)

Bacterial cells from slant were inoculated on R2YE agar and grown at 30°C for 48 hours . Catalase reaction was made by dropping H_2O_2 of3% (v/v) on bacterial cells and foaming was observed for catalase positive colonies.

2.2.6.2.2.Growth on Czapeck Medium (Shirling and Gottlieb, 1966)

Cultured medium were incubated at 30^0C for 10 days. Positive results were observed after bacteria were able to grow on the medium.

2.2.6.2.3.Lysozyme Resistance Test (Kuster, 1976)

Basal medium supplement with Lysozyme **(2.1.3.2.2)** prepared in tubes were inoculated with bacterial growth and incubated in a shaker incubator in a slanted position. Observation was made after (2 – 4) days. Sensitive strains give clear cut.

2.2.6.2.4.Sugar Utilization Test (Kuster, 1976)

The basal medium with sugar(2.1.3.2.3) was inoculated with bacterial culture, after incubation for 10 days at 30°C.Positive test was recorded by detection bacterial growth in the medium .

2.2.6.2.5.Organic Acid Formation Test (Kuster, 1976)

The organic acids formation medium (2.1.3.2.4),were inoculated with bacterial culture, After incubation at 30°C for 10 days, acid formation was observed by disappearance of $CaCO_3$ and color changing of bromocresol purple to yellow.

2.2.6.2.6.Urea utilization (Kuster, 1976)

The Tubes containing urea agar base medium(2.1.3.2.5) was Inoculated with bacterial culture and incubated at 30°C. Observation for medium color change to pink was made after 2, and 5 days.

2.2.6.2.7.Blood Hemolysis

The blood agar base medium(2.1,3.6.2) was inoculated with bacterial culture and incubated at 30°C for seven days. Blood hemolysis was observed.

2.2.7.Maintenance of Bacterial Isolates (Sambrook and Russell, 2001)

2.2.7.1. Short Term Storage

Bacterial isolates were maintained for periods of few weeks on the surface of YEME agar plates. The plates were tightly wrapped in parafilm and stored at 4ºC.

2.2.7.2. Medium Term Storage

Bacterial isolates were maintained for periods of few months on the surface of Gauza agar plates. The plates were tightly wrapped in parafilm and stored at 4 ºC.

2.2.7.3. Long Term Storage

This was done by adding 1.5 ml of 20%glycerol to the well growth of bacteria in a screw-capped bottle with final volume 10 ml and stored at –20 ºC.

2.2.8. Screening of Utilization Hydrocarbon Compound

2.2.8.1.Utilization Hydrocarbon Compounds in Liquid Medium (Jimnani *et al.*, 2009)

Liquid mineral salt medium (2.1.3.3.1) was used to detect the ability of bacterial isolates to utilize hydrocarbon compounds in which 50 ml of this medium were dispensed in a volumetric flask (250ml), then 1% of each of hydrocarbons, crude oil and diesel, were added separately in each flask. Flasks were inoculated with 1 ml of bacterial inoculum (1 OD600 nm equivalent). Before incubation in a shaking incubator (150 rpm) at 30 ºC for 30 days. The growth was determined by measuring the optical density at wavelength600nm.

2.2.8.2.Utilization Hydrocarbon Compounds on Solid Medium

Solid mineral salt medium (2.1.3.3.2) was used to detect the ability of bacterial isolates to utilize hydrocarbon compounds. The plate were inoculated with bacteria by streaking and incubated in 30 ºC for 7 days.

2.2.9.Utilization of Pure Hydrocarbon Compounds

Mineral salt medium (2.1.3.3.1) was used to investigate the ability of bacterial isolates to utilize different pure hydrocarbon compounds in

which 50 ml of this medium were dispensed in a volumetric flask (250ml), then soluble hydrocarbons(Hexadecane, Cyclohexane, Naphthalene, Toluene and benzene) and solid hydrocarbons (naphthalene, Anthracene, sodium benzoate) were added in concentration 1% (v/v). The DMSO used for dissolving 0.1% of naphthalene, and diethyl ether used for dissolving 0.1% of (Anthracene and sodium benzoate) as stock solutions were discarded by volatilization. Flasks were inoculated with 1 ml of bacterial inoculum (1 OD 600 nm equivalent). Cultures then incubated in a shaking (180 rpm) at 30 ºC for 14 days. The growth was determined by measuring the absorbency at wavelength 600 nm.

2.2.10. Gas Chromatography Analysis for Liquid-Liquid Extraction of Residual Crude oil and Diesel

2.2.10.1.Liquid-Liquid Extraction of Residual Crude Oil (Kannahi and Thara, 2012)

The 250 ml flask contained inoculated 100ml of modified YEME medium in addition of 1% crude oil, incubated for 10 day in shaker incubator at 30 ºC/150 rpm. The extraction of the residual crude oil was done after 10 day by mixing the entire volume of flask with 20ml hexane, then mixed in a separation funnel and allowed to resettled for 1 day. The organic layer was recovered and concentrated then used for gas chromatography.

2.2.10.2. Liquid-Liquid Extraction of Residual Diesel (Marquez-Rocha *et al*., 2001)

The 250 ml flask contained inoculated 100 ml of modified YEME medium in addition of 1% diesel, incubated for 10 day in shaker incubator at 30 ºC/150 rpm. The extraction of the residual diesel was

done after 10 day by mixing the entire volume of flask with equal volume of dichloromethane. This mixture was shacked in a separation funnel and allowed to resettled for 1 day. The organic layer was recovered and concentrated then used for gas chromatography.

2.2.11.Production of Biosurfactants by Bacterial Isolates

2.2.11.1.Blood Hemolysis Test (Suthindhiran and Kannabiran, 2009)

Bacterial isolates were plated on blood agar plates and incubation at 30 ºC for 7 days. Hemolytic activity was measured as the presence of definite clear zone around each colony (Carillo *et al.*, 1996; Youssef *et al.*,2004; Suthindhiran, and Kannabiran, 2009).

2.2.11.2.Growing on LB Broth (Gudiña *et al.*, 2012)

A portion of 50 ml LB broth was inoculated with 1ml inoculum (1 OD at 600 nm equivalent) in a 250 ml flask and incubating in a shaking incubator at 30ºCfor 7 days. Bacterial growth was determined by measuring the absorbency at 600 nm. Afterwards, the samples were centrifuged (6000, 20 min, 10 ºC) and cell-free supernatants were used to measure surface tension.

2.2.11.3. Emulsification Index (E24)

The emulsification index (E_{24}) was used to evaluate the emulsifying capacity of different oils and hydrocarbons. The cultures sample was determined by adding 2 ml test oils and 2 ml of cell free broth in test tube, vortexes at high speed for 2 min and allowed to stand for 24h. The emulsification assay was performed with each of castor oil, eucalyptus oil, sesame oil, mustard oil and hydrocarbons such as diesel, crude oil, toluene, and aniline. Emulsions formed was compared with those formed by a 1% (w/v) solution of the synthetic surfactant sodium dodecyl sulphate (SDS) in distill water. The index (E_{24}) was calculated by the

percentage of the height of emulsified layer(cm) divided by the total height of the liquid column multiply by 100 (Kokare *et al.,* 2007**;** Gudiña *et al.,* 2012).

2.2.11.4.Oil Spreading Test (Rodrigues *et al*., 2006)

The selected isolates(S7 and S14) which gave highest degradation ability was subjected to the oil spreading test which compared the result of with other isolates in MSM by measuring the diameter of the clear zones occurred when a drop of a biosurfactant-containing solution is placed on an oil-water surface. The large petri dish (15 cm diameter) contained 50 ml D.W and 20 µl of crude oil add to the water surface ,10 µl of supernatant of culture broth. The diameter of clear zones of triplicate assays from the same sample were determined.

2.2.11.5.Surface Tension Measurement

Surface tension (ST) of the cell free supernatant was measured by the tensiometer. Before the measurement, tensiometer was calibrated with distilled water (72 mN/m), chloroform (27.1 mN/m) and glycerol (64 mN/m),then the obtained reading was recoded at the moment when surface-immersed ring breaks away from the liquid surface. Surface tension readings were then recorded. All the measurements were performed at room temperature 20 ºC.

2.2.12.Optimization of Biodegradation Process (Kokare *et al*., 2007)

The optimization experiments were carried out by dispensing 100ml of modified YEME broth (2.1.3.3.3) in a 250 ml volumetric flasks, inoculated with 1% of well growth culture of bacterium and also added 1% (v/v) of hydrocarbon sources (crude oil and diesel) were added to the flasks separately, before incubation in a shaker incubator (150 rpm) at 30

ºC for 10 day. After incubation, surface tension was measured for each samples as mentioned above.

2.2.12.1.Effect of pH

The production medium was adjusted to different pH values (5,6,7,8 and 9) to determine the optimum value for biodegradation.

2.2.12.2.Effect of Temperature

To observe the effect of different temperature on biodegradation, cultures were incubated at different temperatures (15, 30, 37 and 45°C).

2.2.12.3.Effect of Agitation

Effect of agitation on biodegradation was detected by incubating inoculated fermentation medium with the isolates at different agitation speeds (50, 100, 150 and 200 rpm).

2.2.12.4.Effect of Salt Concentration

Experiments on the effect of salt concentration were carried out by using varied concentrations of NaCl (1, 2, 3, 4 and 5 w/v).

2.2.13.Partial Purification and Characterization of Biosurfactant Produced by *Streptomyces*

2.2.13.1.Isolation and Purification of Biosurfactant

Biosurfactant production was carried out in flasks containing500 ml of LB medium. Each flask was inoculated with 5 ml of a pre-culture and incubated in 30°C in shaking incubator for 150 rpm for 10 days. The cells were harvested by centrifugation (6000 rpm, 20 min, 10 °C) and biosurfactant production was determined by tensiometer. To recover the biosurfactant, the cell-free supernatant was subjected to acid

precipitation. the pH was adjusted to 2.0 by HCl (6M) and left overnight at 4ºC. Afterwards, the precipitate was collected by centrifugation (6000 rpm, 20 min, 4 °C) and washed twice with acidified water (pH 2.0). The crude biosurfactant was dissolved in a minimal amount of demineralized water and the pH was adjusted to 7.0 using NaOH 1 M. Finally, the crude biosurfactant solution was freeze-dried and stored at 20 °C for further use (Gudiña *et al*., 2012).

2.2.13.2.Biochemical detection tests

- **Sugar moiety (Sadasivam and Manickam, 1996)**

Two drops of sugar moiety reagent (2.1.6.2) were added to 2ml of purified biosurfactant solution and mixed well, then the tube was inclined and about 1 mlof concentrated sulphric acid was added along the side of the tube . appearance of red-violet ring at the junction of the two liquids indicates it contain sugar moiety.

- **Biuret test (Sadasivam and Manickam, 1996)**

To 2 ml of purified biosurfactants solution 2 ml of NaOH was added and mixed well followed by adding two drops of $CuSO_4$ solution were added. Observation of violet or pink color indicates presence of peptide bond**.**

2.2.13.3.Characterization of Biosurfactants

2.2.13.3.1.Thin layer chromatography Method (Nishanthi *et al*., 2010)

Screening for detect biosurfactant production was done by thin layer chromatography method. Biosurfactant was characterized by thin layer chromatography using silica-gel (20cm x 20cm, Merck). The solvent systems used was differed based on the components tested. The TLC plates were spotted with biosurfactant extracts and developed with the

following: solvent 1, petroleum ether - diethyl ether-acetic acid (80:20:1 v/v/v) for neutral lipids, solvent 2, chloroform-methanol- water (65:25:4v/v/v) for polar lipids, and solvent 3, ethyl acetate-acetic acid-methanol water (12:3:3:2v/v/v) for carbohydrate compounds. After developing, the spots were visualized with standard reagents. The lipid components were detected as yellow spots after placing the plates in a closed jar saturated with iodine vapors. Carbohydrate components were detected as red spots on the plates after spraying with an alpha- naphthol solution followed by concentrated sulphuric acid. Also used UV-light to confirm our results.

2.2.13.3.2.Fourier Transformed Infrared Spectroscopy Analysis

Nature and chemical structure of the biosurfactant were examined using the Fourier transformed infrared spectroscopy (FTIR) in order to characterize the chemical nature of a compound. FTIR spectrometry, an advanced type of infrared spectrometry, gives usually the functional groups found in the compound in order to propose a possible chemical structure of the test compound.

2.2.14.Plasmid Extraction (Favor gene Biotech Corp.)

Plasmid DNA was extracted from selected *Streptomyces* isolates(S7 and S14) by using plasmid DNA Extraction mini kit (appendix-1).

2.2.15.Gel Electrophoreses (Sambrook and Russell, 2001)

Agarose powder (0.8%) was dissolved in TBE buffer and the slurry was heated in boiling water bath until dissolved. The solution was cooled to 50 °C and ethidium bromide was added to a final concentration 0.5µg/ml.

The warm agarose solution was poured into mold and immediately the comb was clamped, the teeth of which will form the samples wells,

into position near one end of the gel. After the gel was completely set (30-45 min at room temperature), carefully the comb was removed and just enough electrophoreses buffer was added to cover the gel to a depth about 1 mm.

Each sample of DNA was mixed with 15 µ1 volume of loading buffer and were loaded into the wells of the submerged gel.

DNA bands were visualized by using U.V. Transilluminater (illumination at 302 nm).

2.2.16.Plasmid curing (Padan*et al*. 1981; Rean, 2010)

Curing experiment was performed on *Streptomyces* isolates by using low pH as curing agent. Bacterial cells were grown in 10 ml YEME broth, then 0.1 ml of each culture was inoculated in each of the series of 30 ml fresh YEME broth flasks adjusted the pH to (3, 3.5, 4, 4.5 and 5). All flasks were incubated in a shaking incubator (120 rpm) at 30°C for 10 days.

Growth density in the flask was observed by naked eye and compared with the control to determine the effect of low pH that inhibits the growth of the bacterial cells to be considered as the minimum inhibitory concentration (MIC).

Samples were taken from flasks that still grow under low pH stress, and diluted appropriately. Then 0.1 ml from the proper dilution was spreaded on YEME agar plates and incubated in 30 °C for 10 days to score the survived colonies. The survived colonies were replica plated (using tooth pick) on YEME agar plates (master plate) and test for its ability to grow on the mineral salts agar plate supplemented with crude oil and diesel contaminated soil.

Chapter Three

Results and Discussion

3. Results and Discussion

3.1.Isolation of Hydrocarbons Degrading Microorganism

Five soil samples contaminated with diesel and oil spills were collected from different regions in Baghdad city to isolate the hydrocarbon degrading bacteria. They were grown on soil extract glucose yeast extract agar at 30 ºC for 10 days.

A total of 14 suspected isolates were obtained which subjected to the cultural and microscopic characterization for primary genus identification.

When these isolates were recultured in mineral salt medium supplemented with 1% crude oil and diesel separately, their ability to degrade the hydrocarbon was varied, with two isolates (S7 and S14) as the most efficient.

3.2. Ability of Isolates to Utilize Hydrocarbon Compounds

All isolates (14) were recultured in liquid and on solid medium. Results in the Table (3.1) indicate that all isolates were able to grow and utilize crude oil and diesel in variable ranges as a sole of carbon and energy. Isolates S7 and S14 showed significant growth density compared with other isolates throughout the incubation period.

The isolates thrived in the crude oil and diesel substrate when supplied as the sole source of carbon and energy. Utilization of the diesel resulted in an increase in the number of colony forming units per ml with a concomitant visual gradual reduction in the diesel layer and substantial disappearance of the diesel with prolonged incubation.

Table (3.1). Growth density of bacterial isolates grown in mineral salt medium (pH 7) supplemented with 1% of hydrocarbon for 30 days of shaking incubation (150 rpm) at 30^{o}C

Symbol of isolate	Growth in crude oil	Growth in diesel
S 1	+	+
S 2	+	+
S 3	+	+
S 4	+	+
S 5	+	+
S 6	+	+
S 7	+++	+++
S 8	+	++
S 9	+	+
S 10	+	+
S 11	+++	++
S 12	+	+++
S 13	+	+
S 14	+++	+++

(+) Slight growth ($O.D_{600}$ = 0.1-0.3).

(++) Moderate growth ($O.D_{600}$ = 0.4-0.6).

(+++) Good growth ($O.D_{600}$ = 0.8-1).

(Control) mineral salt medium supplemented with 1% of hydrocarbon without bacterial inoculum ($O.D_{600}$ = 0.03)

Das and Mukherjee (2007) mention that the biosurfactant micelles can increase mass transfer due to increasing pollutant pseudo solubility in aqueous environments. It is possible that biosurfactant micelles may coalesce with microbial membranes and deliver a contaminant molecule directly to the outer membrane of a cell (Miller and Bartha1989). At the appropriate critical micellization concentration, mass transfer can be improved due to interfacial tension reductions between aqueous and organic phases, and the associated increases in solubility. Here it is believed that hydrophobic cells adhered to hydrocarbon droplets release an emulsan coat to mark a used oil droplet with a hydrophilic repulsive layer (Brown, 2007).

Gold (1985) from another point of view, thought that the petroleum contain a class of molecules known as hopanoids, which are commonly found in bacterial cell walls. He indicated that these fuels at some point originated at least in part from microorganisms and that biodegradation of these fuels has always been occurring to some extent. The 'biological evidence' within these hydrocarbons could be the reason for the adaptation of microorganisms (Surridge, 2007).

3.3. Identificationof Bacterial Isolates

The two isolates of highest degradation ability were selected for depending on morphology (colony characteristics, Gram's stain and number of spores) and biochemical tests. Results show that colonies appearance differed depending on the selective or differential medium used (Shirling and Gottlieb 1966; Saadoun *et al*., 2007). colonies of S7 isolate appeared on medium in diameters of (5-7) mm, discrete and butyrous, initially relatively smooth surfaced, lately grow to velvety aerial mycelia, color of aerial mycelia was dark brown and white on most of the media, colonies of S14 isolate were smaller in diameters (3 –5)

mm, discrete and butyrous, initially relatively leathery smooth surfaced, lately grow to velvety aerial in white color on most of the media. Biochemical characterizations of bacterial isolates are listed in the table (3.2) indicated these bacterial isolates were *Streptomyces sp.*

Table (3.2). Biochemical characterizations of bacterial isolates growing on International Streptomyces project medium**.**

Test	Bacterial isolates	
	S7	S14
Growth on Czapeck Medium	+red	+ gray
Lysozyme Resistance	+	+
Sugar Utilization		
glucose, arabinose, xylose, sucrose, mannose	+	+
Rhamnose, inositol	_	+
Organic Acid Formation	_	+
Urea utilization	+	+
Blood Hemolysis	α	β
catalase production	+	+

(+) mean positive results,

(-) mean negative results.

Barab´as *et al*. (2001) recorded that *Streptomyces* were the most abundant and the most efficient bacterial species in utilizing crude oil and different hydrocarbon compounds.

3.4. Utilization of Different Pure Hydrocarbon Compounds

Isolates S7 and S14 showed the ability to utilize hydrocarbon compounds as a sole source of carbon and energy. Results in Table (3.3) indicate that the two isolates were able to utilize hexadecane (small chain n-alkane),benzene and sodium benzoate (single ring aromatic compounds) and low degradation ability in phenol.

Isolate S7 gave highest ability to utilize all of hydrocarbon compounds, while S14 isolate showed a divergence ability. In addition ,

S7 was characterized by its good ability to grow on aromatic hydrocarbons When the growth was noticed after one week of incubation on these compounds.

Moreover, both isolates showed a good utilization of aliphatic hydrocarbons, which are broken by their simple compositions, low toxicity and easy to be utilized by the hydrocarbon utilizer (Barabás *et al.*, 1995). It was observed from the results that the utilization rate of aromatic hydrocarbons decreased whenever the chemical structure of these aromatic hydrocarbons became more complicated. It was also noticed that S14 was able to utilize hexadecane, benzoate, phenol and benzene, but failed to grow on others. This may be explained that the ability of microorganisms to utilize hydrocarbon compounds is decreased with increasing the complexity of these hydrocarbons. The environmental persistence of aromatic hydrocarbons become longer with increasing the number of benzene rings of these compounds (Mueller *et al*., 1996; Haritash and Kaushik, 2009; Chaudhary *et al*., 2011).

Table (3.3). Growth of bacterial isolates on different pure hydrocarbon compounds at 30 C for 14 days.

Hydrocarbon compound	Bacterial growth measured by $O.D_{600}$	
	S 7	S 14
Hexadecane	++++	++
Sodium benzoate	++	++
Naphthalene	+++	_
Anthracene	+++	_
Cyclohexane	++	_
Phenol	+	+
Tetrahydrofuran	++++	+
Benzene	+++	++
Toluene	++	_

(-) No growth.

(+) Slight growth ($O.D_{600}$ = 0.1-0.3).

(++) Moderate growth ($O.D_{600}$ = 0.4-0.6).

(+++) Good growth ($O.D_{600}$ = 0.7-0.9).

(++++) Heavy growth (O.D600 = 1-2).

(Control) mineral salt medium supplemented with 1% of hydrocarbon without bacterial inoculum ($O.D_{600}$ = 0.01)

Bacterial degradation of alkanes usually occurs through the sequential oxidation of one or both terminal methyl groups of the molecule, first to an alcohol, then to an aldehyde, and finally to a fatty acid (Mercedes *et al*.,2001).

Radwan, (1998) showed that the *Streptomyces* was one of the most common bacteria consuming aromatic hydrocarbons. It was reported that *Streptomyces* spp. were able to degrade anthrecene, through oxidizing it

to 3-hydroxyl-2-naphthoate (one of the naphthalene metabolites),which was further oxidized to salicylate and catechol that enter the tri-carboxylic acid cycle.

According to the results obtained , only isolate S7 was able to utilize cyclohexane(alicyclic compound). Alicyclic compounds are characterized by their high toxicity toward microbial cells and their complex structure, therefor; biodegradation of alicyclic compounds as a sole source of carbon and energy (Cerniglia, 1992). On the other hand, isolate S14 failed to utilize tetrahydrofuran as a sole source of carbon and energy.

Furthermore, the increase in the compound complexity might increase the hydrophobicity and association with water molecules, which decreases the enzyme – substrate interaction, leading to less enzymatic reactions.

There is no doubt that the chemical structures of hydrocarbons compounds not only have an extensive effect on other utilization by different bacterial isolate, but the genetic diversity of these isolates also affect the biodegradability of hydrocarbon compounds. As well as, some of bacteria may contain different genetic elements like transposone, harboring biodegradative genes that might participate in diverging the utilization of hydrocarbons (Chakrabarty,1996).

Barab´as *et al*. (2001) have isolated three *Streptomyces* strains (*S. griseoflavus, S. parvus*, and *S.plicatus*) from the Kuwait Burgan oil field with the ability to utilize n-hexadecane, n-octadecane, kerosene, and crude oil as sole carbon and energy sources.

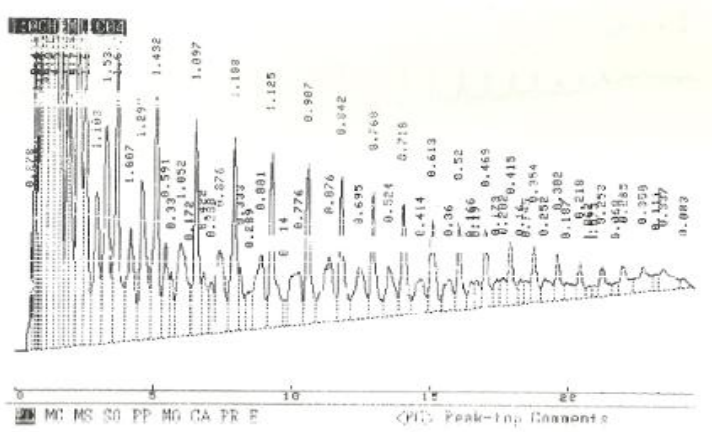
Jimnani *et al*. (2009) reported *Streptomyces* utilization of different hydrocarbons namely -diesel, engine oil, kerosene, benzene, toluene and n-hexane vary widely among the bacteria isolates. This might be due to

the fact that the bacteria isolated were able to use the hydrocarbons as substrate for growth by probably releasing extra cellular enzymes and acids which are capable of breaking down the recalcitrant hydrocarbon molecules, by dismantling the long chains of hydrogen and carbon, there by converting petroleum into simpler forms or products that can be absorbed for growth and nutrition of the bacteria (Alexander, 1999).

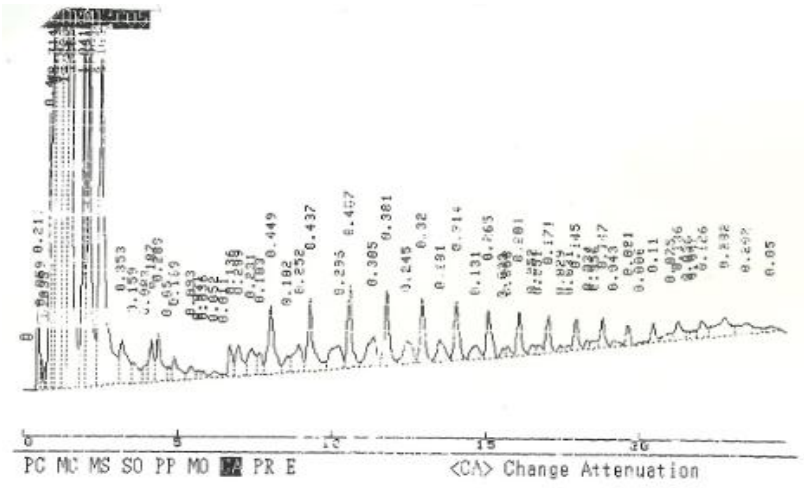
Kannahi and Thara (2012) showed that the *Streptomyces* species were capable of degrading organic solvents is the development of tolerance against them by alteration in the composition of their cytoplasmic and outer membranes/cell surface protein.

3.5. Liquid-Liquid Extraction of Residual Crude oil and Diesel by GC

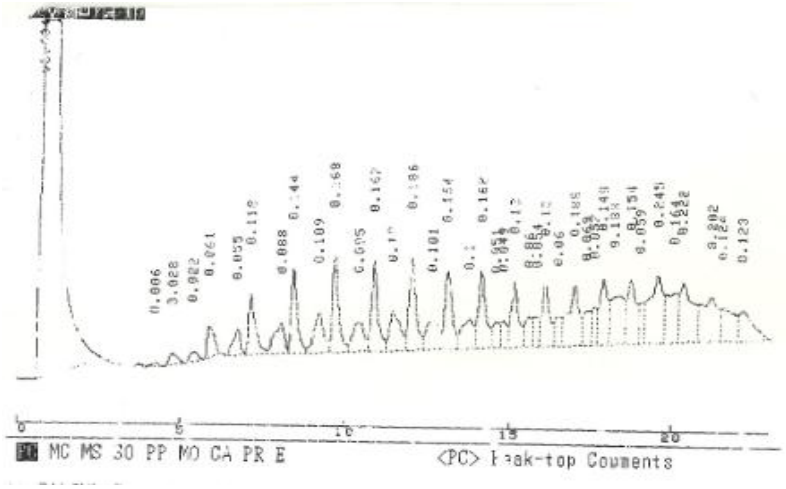
Gas chromatography was done to identify the ability of the two isolates to degrade crude oil and diesel. Figures (3.1) (3.2) show significant changes upon both compound structures. Most of long and complex carbon chains were broken into simpler type, in addition to the disappearance of some compounds during the process.



A.

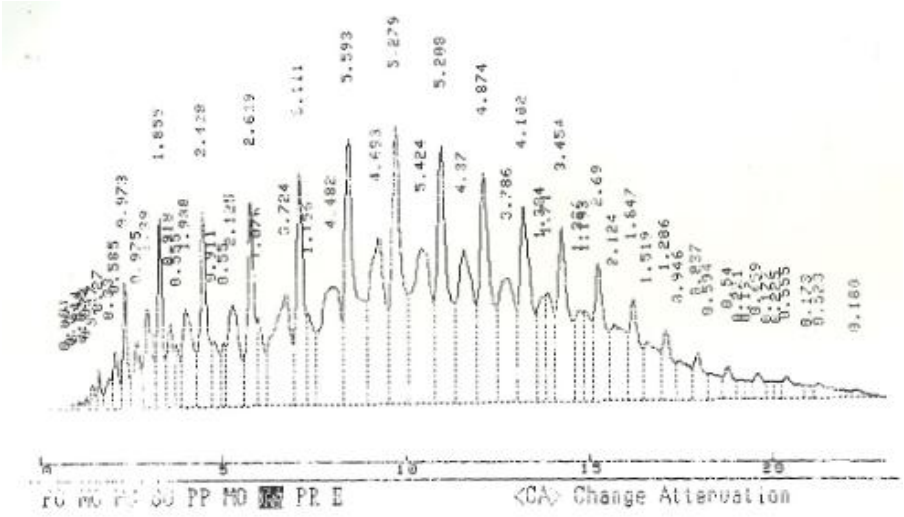


B.

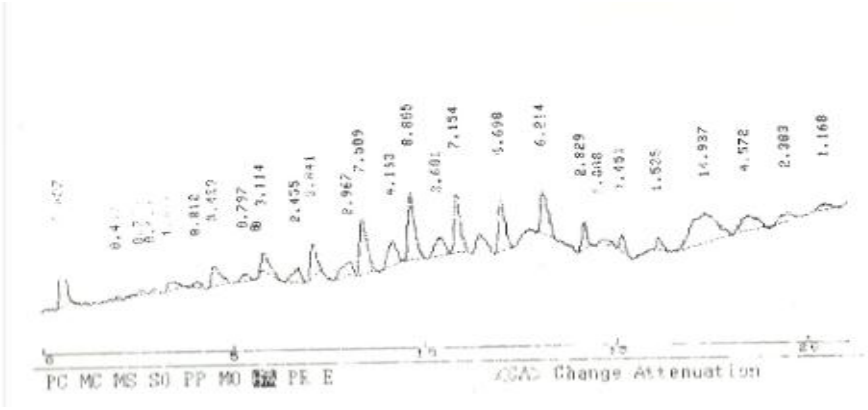


C.

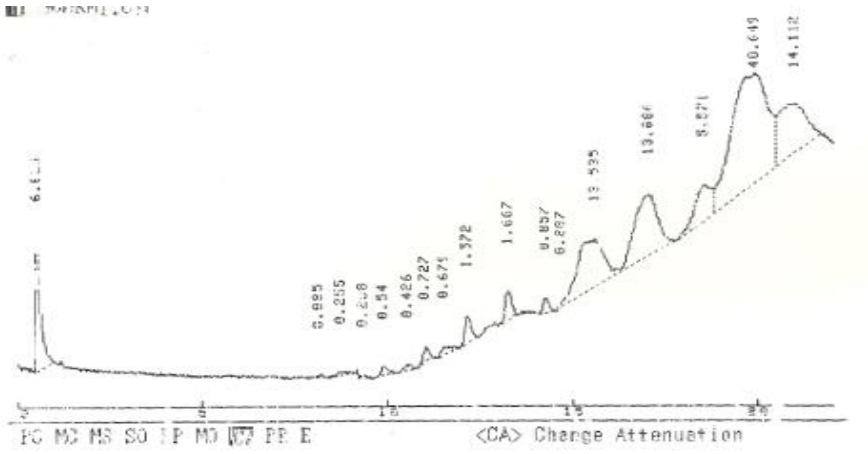
Figure (3.1). GC chromatograms of crude oil inoculated with two local *Streptomyces* isolates S7 and S14.(A. control, B. S7 and C. S14).



A.



B.



C.

Figure (3.2). GC chromatograms of diesel inoculated with two local *Streptomyces* isolates S7 and S14.(A. control, B. S7 and C. S14).

Malatova, (2005) mentioned that the analysis of crude oil by gas chromatographs is a powerful measurement to evaluate the biodegradation process and reveals the differences in efficiency of degradation for each bacterial isolates (*Pseudomanos sp*., *Acinetobacter baumannii* and *Serratia marcescens*). Gas chromatographs showed significant differences in the composition of hydrocarbons at the end of the experiment.

3.6. Ability of Bacterial Isolates to Produce Biosurfactants

Streptomyces isolates (S7 and S14) were examined for their ability in production of biosurfactants which was detected by blood hemolysis test, oil spreading test, Emulsification index (E24)and surface tension measurement.

3.6.1. Blood Hemolysis

Results of blood hemolysis test showed that the two isolates(S7 and S14) have complete and partial hemolysis of blood on blood base agar plates(β and α) respectively indicating production of lipase enzyme.

3.6.2. Oil Spreading

Results of oil spreading test showed that the isolates S7 and S14 lowered the surface activities by forming clear zones of 8.5 cm and 6 cm respectively, biosurfactant on an oil-water surface. This test it is considered to be very sensitive for detection and it has some other advantages like requiring small volume of samples, rapid and easy to be carried out, and do not require specialized equipment (Youssef *at el*., 2004; Plaza *at el*., 2006).

3.6.3. Emulsification Index (E24)

Another approach for screening potential biosurfactant-producing microorganisms is the estimation of emulsification index (E24). After 24 h, emulsification activity was increased. Results in table (3.4) show that the highest emulsification activities (83 and 66.6) mN/m were recorded when crude oil was used , while the lowest (37 and 40) mN/m when castor oil was used by the two isolates S7 and S14, respectively.

Table (3.4). Emulsification index % (E_{24}) for different oils and hydrocarbons**.**

Emulsification Index % (E_{24})								
Isolate	Castor oil	Eucalyptus oil	Sesame oil	Mustard oil	diesel	Toluene	Aniline	Crude oil
S 7	37	66	50	43	50	50	-	83
S 14	40	43	50	40	50	50	-	66.6

Lipase activity represents a criterion for hydrocarbon utilization. By using different types of oils from different sources indicate the wide spectrum of lipase enzyme(s) produced by those two isolates, when the E_{24} range was (40-66.6).

Pines and Gutnick (1986) indicated that the production of bioemulsifier was induced by the addition of hydrocarbons or oils. Production of bioemulsifier occurs by degradation of hydrocarbons. These are utilized as a nutrient by bacteria in limited nutrient condition.
Kokare *et al* (2007) observed that toluene as a substrate showed maximum activity against all test oils and hydrocarbons. Toluene in 1% (v/v) concentration as a substrate gave maximum activity against all test oils and hydrocarbons.

3.6.4. Surface Tensionm

Results in table (3.5), show reduction in surface tension from 72 in the control to 29 mN/m and 47.50 mN/m by S7 and S14, respectively. Desai and Banat (1997) mention that the microbial candidates for biosurfactant production are expected to decrease surface tension to around 40mN/m.

Table (3.5). Surface tension (mN/m) Measurements of the two local *Streptomyces* isolates

sample	Surface tension (mN/m)	
	Isolate S7	Isolate S14
Control	72	72
Cell free broth	29.71	47.50

Due to the efficiency of surface-active compounds produced by *Streptomyces* spp. a number of studies were conducted towards this field (Banat *et al*., 2001;Deepika *et al*., 2009).

Rahaman *et al* (2002) and Lakshmipathy *at el* (2010) observed that the microbial molecules which exhibit high surface activity and emulsifying activity are classified as biosurfactants. The amphipathic nature of these agents helps in reducing the surface tension of media.These agents not only helpful in the uptake and utilization of hydrocarbons by the organisms but also facilitate the biodegradation of toxic hydrocarbons.

3.7. Optimization of Biodegradation Process

3.7.1. Effect of pH

Modified YEME medium was prepared at different pH values (5,6,7,8 and 9) in an attempt to determine the optimum pH required for growth of S7 and S14 isolates on 1% crude oil and diesel . The obtained results as shown in Figure (3.3) elucidated that the optimum reduction in the surface tension occurred at pH 7. The minimum reduction (28.91mN/m) for S7 isolate was reached after 10 days,as well asS14 isolate.

Figure (3.4) shows the best reduction in surface tension in degradation of diesel when S7 isolate grown for 10 days. While the degradation rate of S14 at pH (8 and 9) shows the highest reduction in surface tension comparing with other readings.

In other studies, it was shown that the pH 7 was optimum for the activity and biosurfactants production after a time period of 14 day. Production of the biosurfactants also was enhanced at alkaline pH 8 (Navon-Venezia *et al.*, 1995; Kokare *et al.*, 2007).

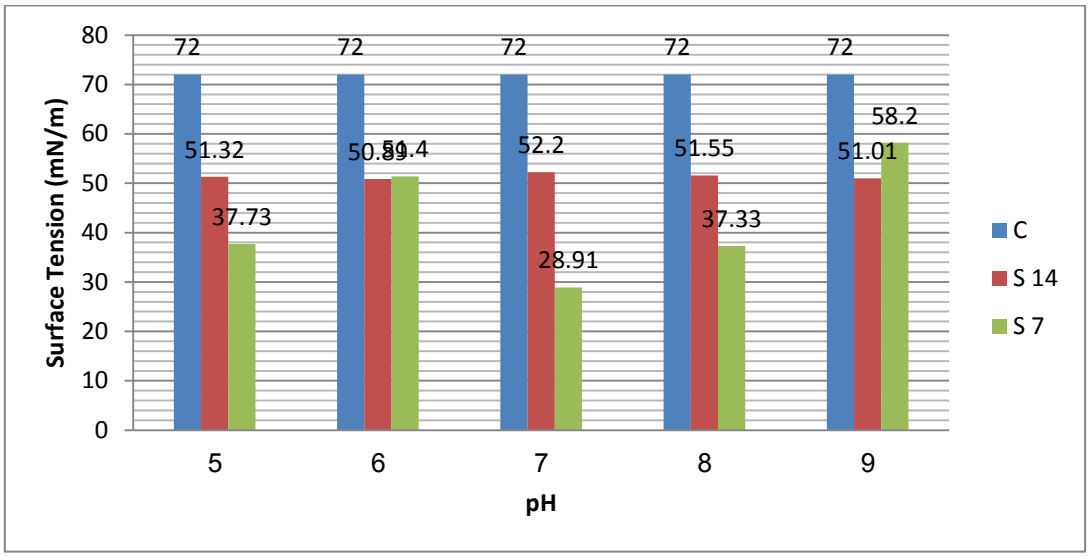


Figure (3.3). Effect of pH on degradation by S7 and S14 grown in modifying medium containing 1% of crude oil in shaker incubator (150rpm), at 30°C for 10 days.

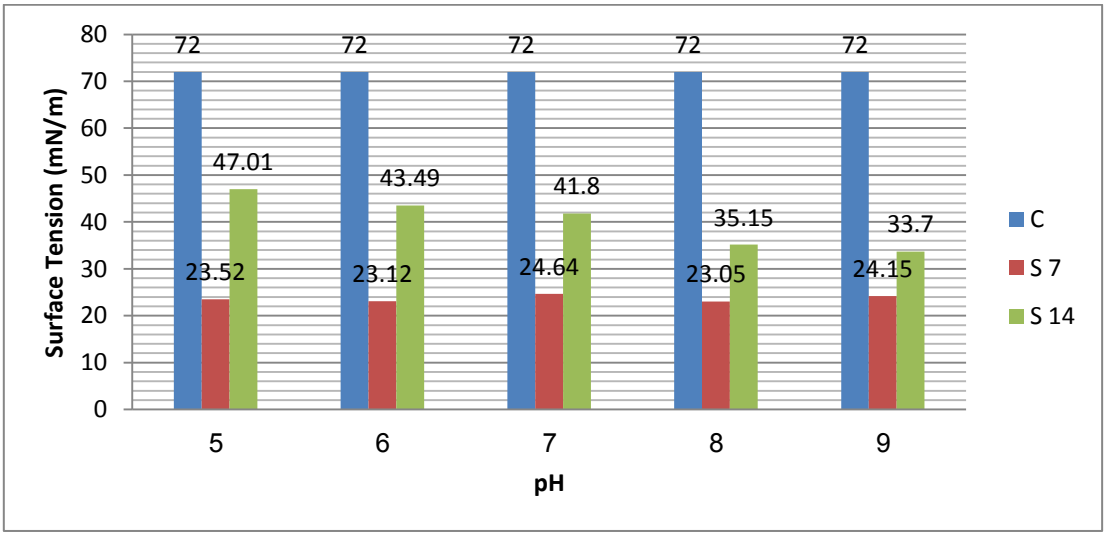


Figure (3.4). Effect of pH on degradation by S7 and S14 grown in modifying medium containing 1% of diesel in shaker incubator (150 rpm), at 30°C for 10 days.

3.7.2. Effect of Temperature

After *Streptomyces* isolates were grown and incubated at different temperatures (15, 30, 37 and 45 °C), results in figure (3.5) illustrate that the reduction in the surface tension of S7 isolate occur when the medium was supplemented 1% crude oil at temperatures (30, 37 and 45)°C was the same, whereas, at 45°C, the S14 showed gave best surface tension reduction and consumption of hydrocarbon.

Results shown in Figure (3.6)indicated that the best temperatures for S7 were (30, 37 and 45 °C) but the S14 show the best reduction at (45 °C) when grown in 1% diesel for 10 days.

At 15 °C, S7 and S14 growth in both crude oil and diesel, was lower than at other incubation temperatures.

3.7.3. Effect of Agitation

According to the results in the figures (3.7) and (3.8), S7 isolate have highest reduction in surface tension and biosurfactant production in the agitation rates of (50,100, 150 and 200) rpm. On the other hand, S14 isolate had similar agitation rates in crude oil agitation, but in the diesel degradation, 150 rpm was the optimum.

As showed in the results mentioned above, the degradation increased when the agitation speed increased and up to a limit. This may be attributed to several reason: oxygen availability is important for oxidation process which provided by agitation,

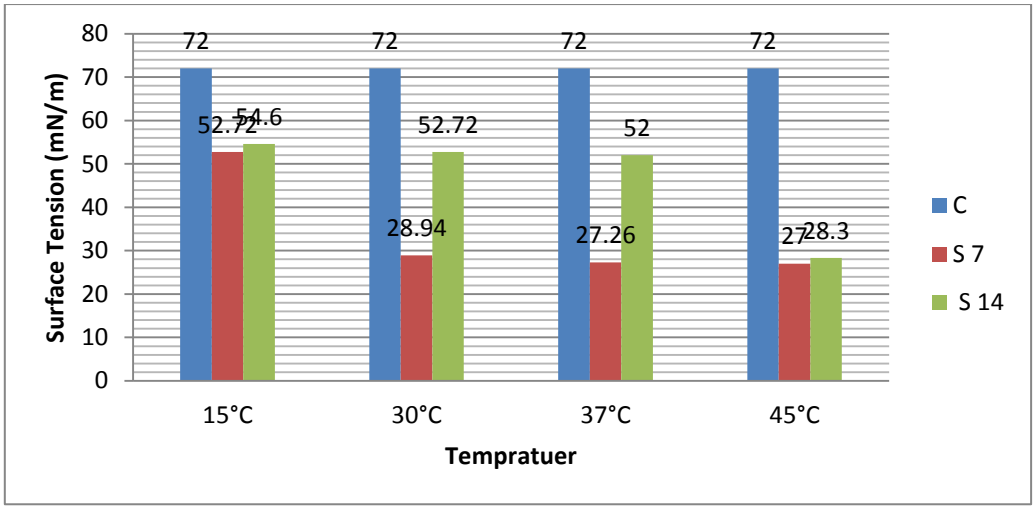


Figure (3.5). Effect of temperature on degradation by S7 and S14 grown in modifying medium containing 1% of crude oil in shaker incubator (150 rpm) at 30°C for 10 days.

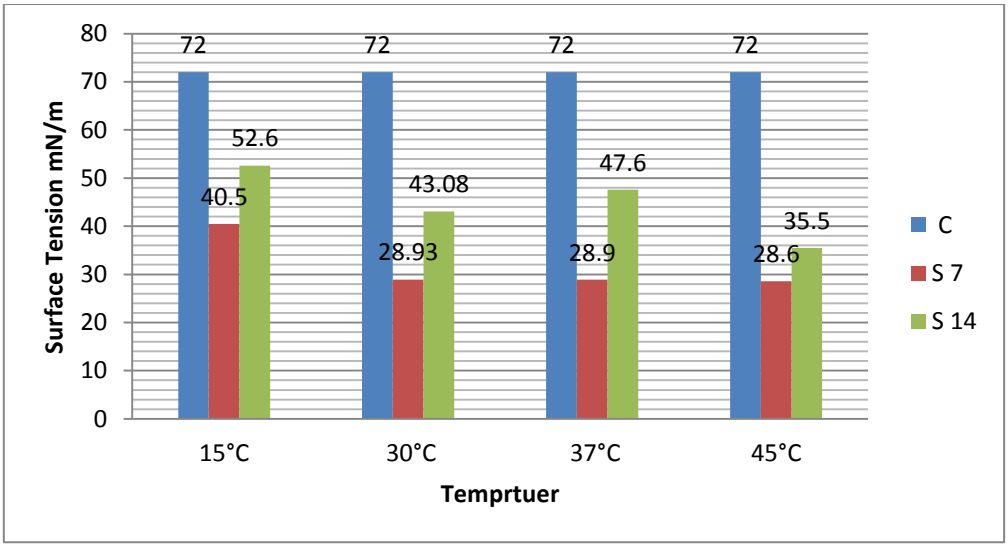


Figure (3.6). Effect of Temperatures on degradation by S7 and S14 grown in modifying medium containing 1% of Diesel in shaker incubator (150 rpm) at 30°C for 10 days.

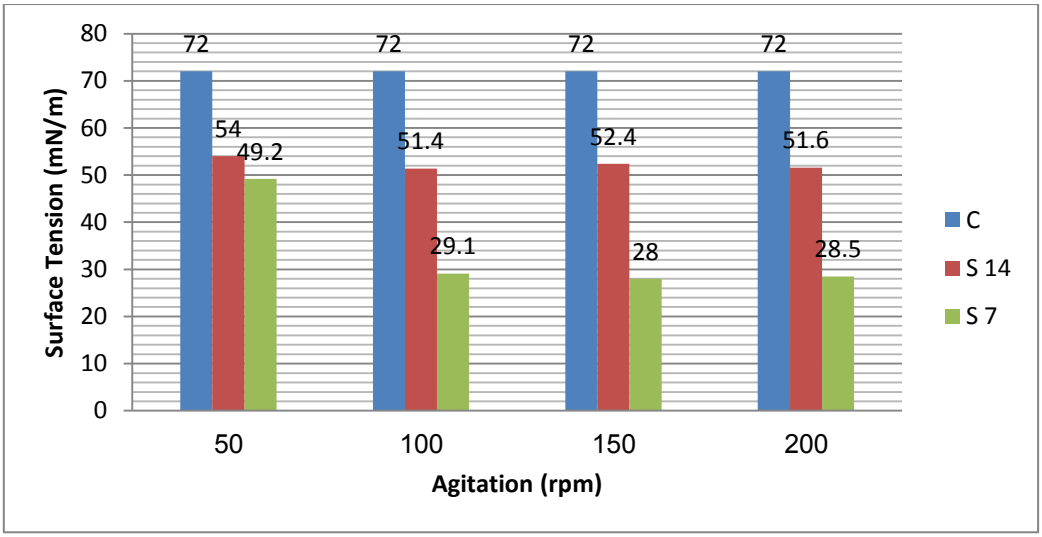


Figure (3.7). Effect of Agitation on degradation by S7 and S14 grown in modifying medium containing 1% of Crude oil in shaker incubator at 30°C for 10 days**.**

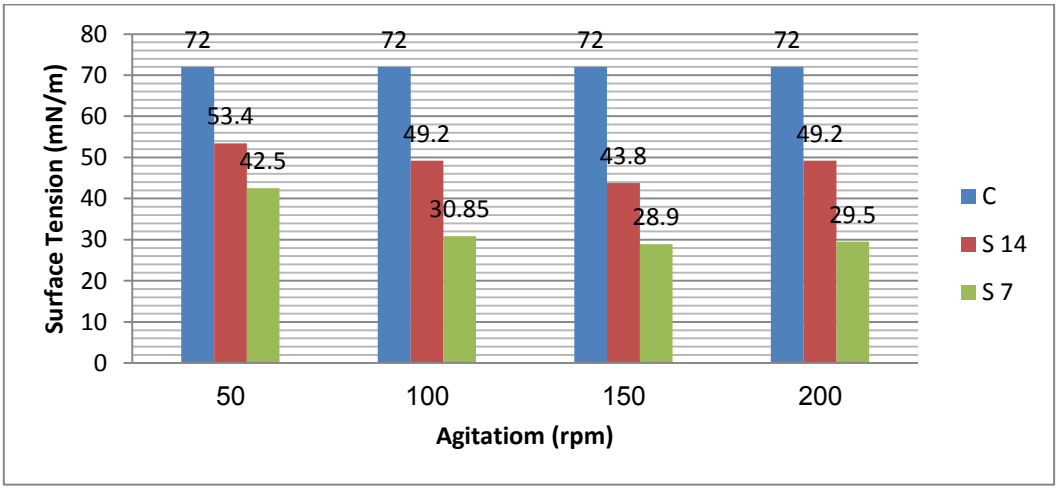


Figure (3.8). Effect of Agitation on degradation by S7 and S14 grown in modifying medium containing 1% of Diesel in shaker incubator at 30°C for 10 days.

the nutrients distribution in the liquid medium was improved by agitation for mixing the nutrients with bacterial culture, increase emulsification capability due to distribution of biosurfactants, and increase in the chance of hydrocarbon attachment with cell wall of bacteria.

3.7.4. Effect of Salt Concentration

Different concentrations (1,2,3,4 and 5)% of salt were used to determine the optimum salt concentration effecting hydrocarbons degradation by S7 and S14 isolates . Results in figure (3.9) indicate that the S7 isolate reached optimum surface tension reduction (28.91mN/m) by growing with 1 %NaCl concentration for 10 days. while it is not effect by S14 isolate with all concentrations. Figure (3.10) showed that the two isolates not effected by different salt concentrations in the degradation of diesel.

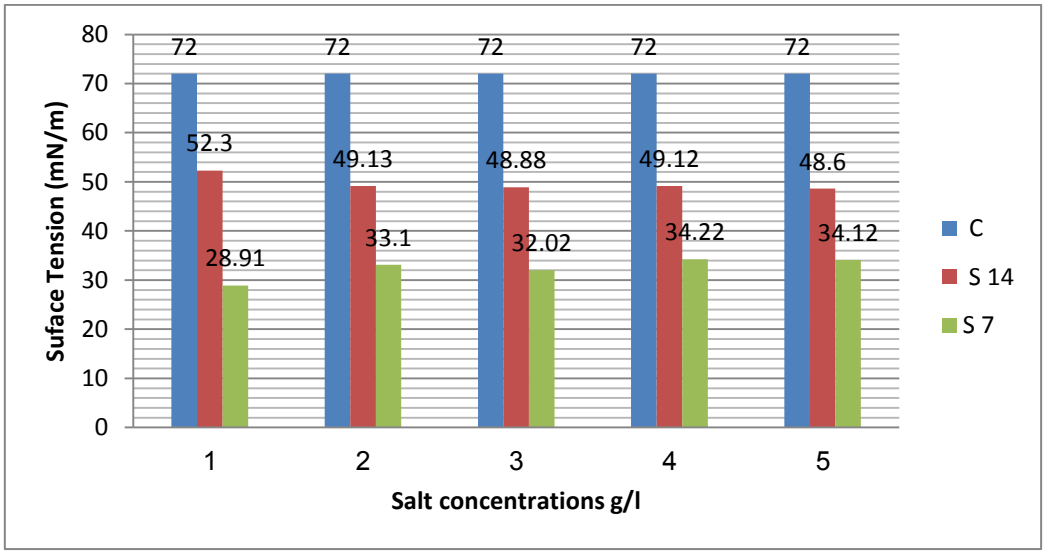


Figure (3.9). Effect of salt concentrations on degradation by S7 and S14 grown in modifying medium containing 1% of crude oil in shaker incubator (150 rpm) at 30°C for 10 days.

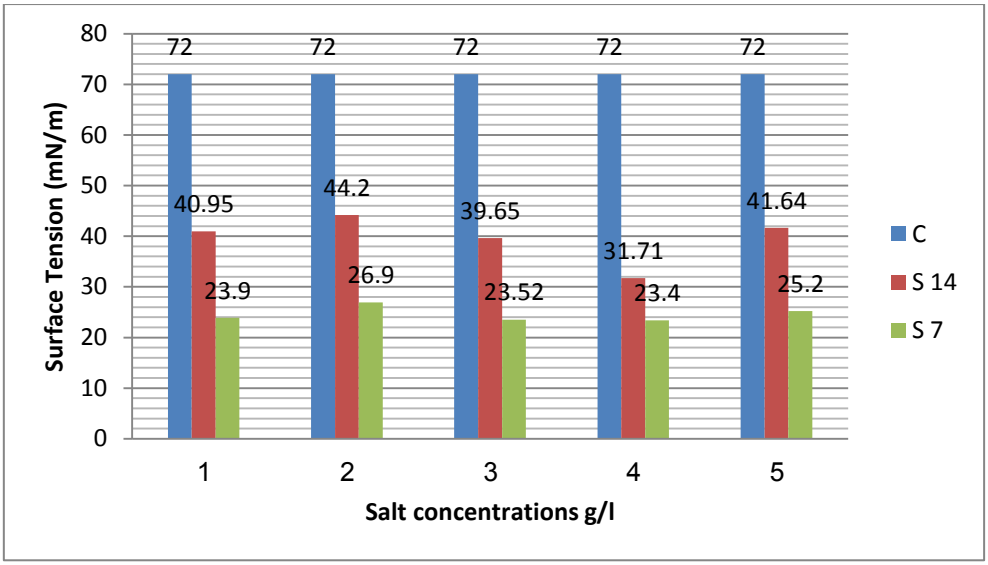


Figure (3.10). Effect of salt concentrations on degradation by S7 and S14 grown in modifying medium containing 1% of diesel in shaker incubator (150 rpm) at 30°C for 10 days**.**

3.8. Partial Purification and Characterization of Biosurfactant

3.8.1. Isolation and Purification of Biosurfactant

The surface-active compound was obtained from the cell-free culture supernatant of *Streptomyces* by acid precipitation method followed by the purification step. Results indicate that the biosurfactant was finally obtained in a white precipitate form, and yields (0.5, 1)g/L of culture medium by S7 and S 14, respectively.

3.8.2. Biochemical detection tests

3.8.2.1. Sugar moiety

This test was followed in an attempt to determine whether the sugar moiety is present in the tested molecules or not. Negative result was obtained from this test indicating that this product is possibly a lipopeptide. This result was potentially agreed with the other researches that demonstrated that most of the bioemulisfiers belonged to *Streptomyces tendae* produced alasan, streptofactin, particulate surfactant, and biosurfactant PM (Richter *et al.*, 1998). In another study concerning

biosurfactants produced *Streptomyces* sp. referred that it was mainly a glycolipid compound (Gamian *et al*., 1996).

3.8.2.2. Biuret test

The presence of peptide bonds in the structure of biosurfactant was determined throughout this sensitive test to evaluate if it was a lipopeptide compound . A pink color was observed indicating a positive result and the examined compound was a lipopeptide molecules.

3.8.3. Characterization of Biosurfactants

3.8.3.1. Thin layer chromatography (TLC) Analysis

Hydrolysates from crude oil and diesel were extracted by diethyl ether and subjected to TLC to analyze the types of component might be produced by such process. Figures (3.11, 3.12, and 3.13) show TLC of hydrolysates. Lysates were found to be polar and non-polarlipids, proteins, and carbohydrates. They were produced after 10 days of propagation in the modified YMD medium.

The Figures showed the lipids and carbohydrates spots of two isolates S7 and S14 which revealing convert the complex hydrocarbon to more simple compounds.

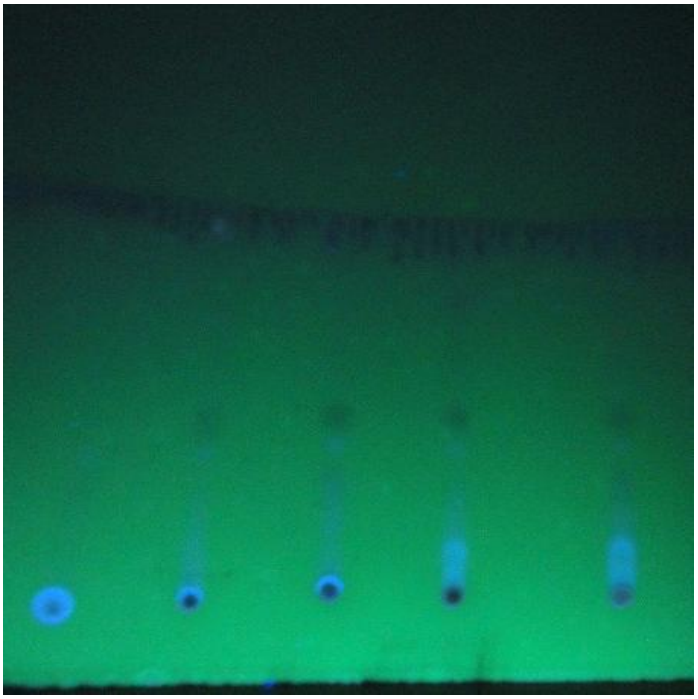


Figure (3.11). Polar lipids chromatography for hydrolysates produce in culture media by the two *Streptomyces* isolates 7 and 14. The first spot is control, the second two spotes are hydrolysates of isolate No. 7, while the others are from isolate No. 14.

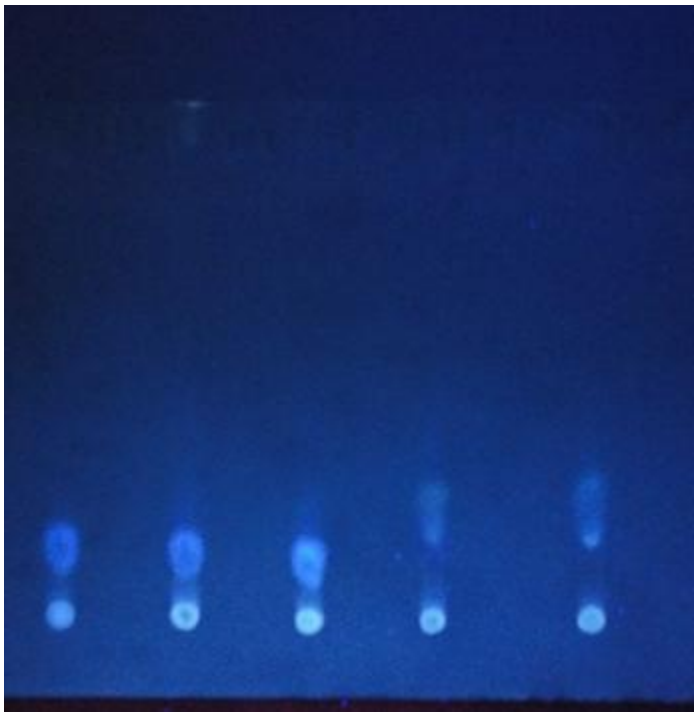


Figure (3.12). Non-Polar lipids chromatography for hydrolysates produce in culture media by the two *Streptomyces* isolates 7 and 14. The first spot is control, the second two spots are hydrolysates of isolate No. 7, while the others are from isolate No.14

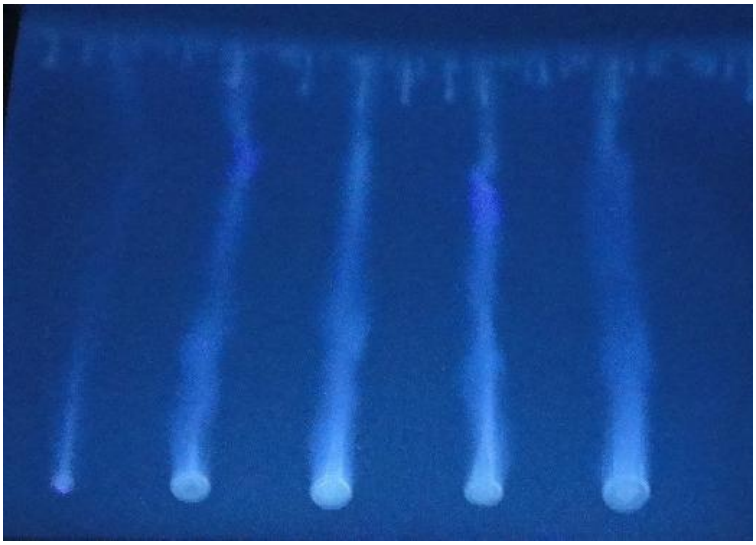
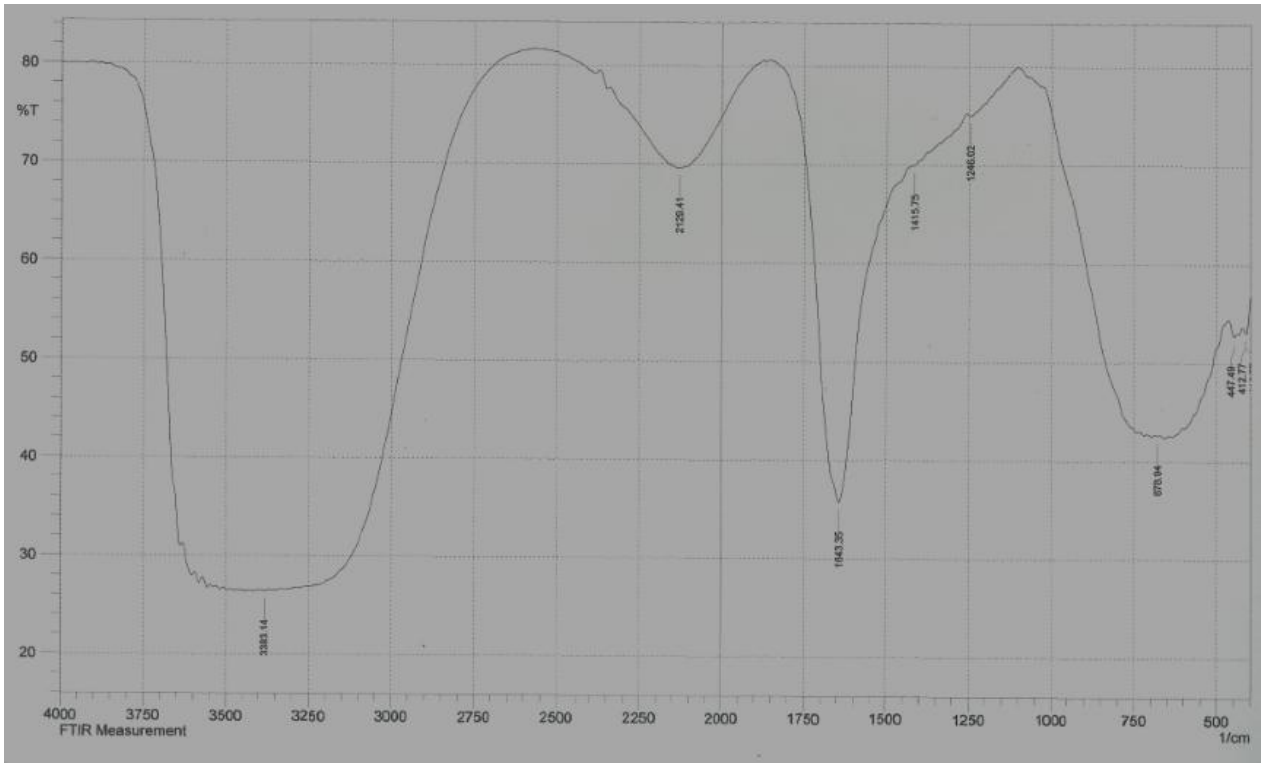


Figure (3.13). Carbohydrate chromatography for hydrolysates produce in culture media by the two *Streptomyces* isolates 7 and 14. The first spot is control, the second two spots are hydrolysates of isolate No. 7, while the others are from isolate No.14

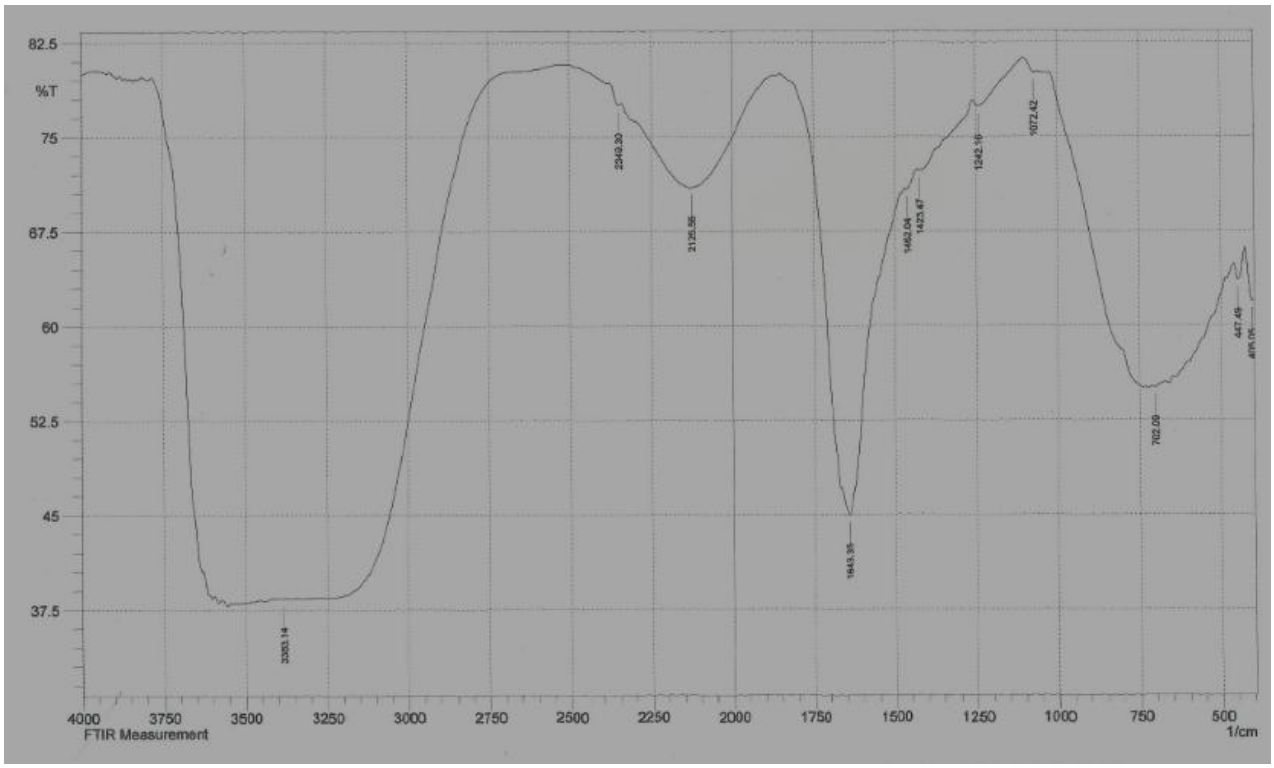
3.8.3.2. Fourier transformed infrared spectroscopy (FTIR) Analysis

The range of FTIR spectrum from 500-700 nm was used to determine the rate of change upon both crude oil and diesel. Figure (3.14) shows an obvious changes on specific regions referring to the complex chains and aromatic rings in both compounds. A widening areas were observed at these regions indicating the increase of these compounds concentrations after bacterial hydrolysis for crude oil and diesel. FTIR Spectrum in the figures of the isolation fraction in CH_2Cl_2 illustrate the following characterized bands:

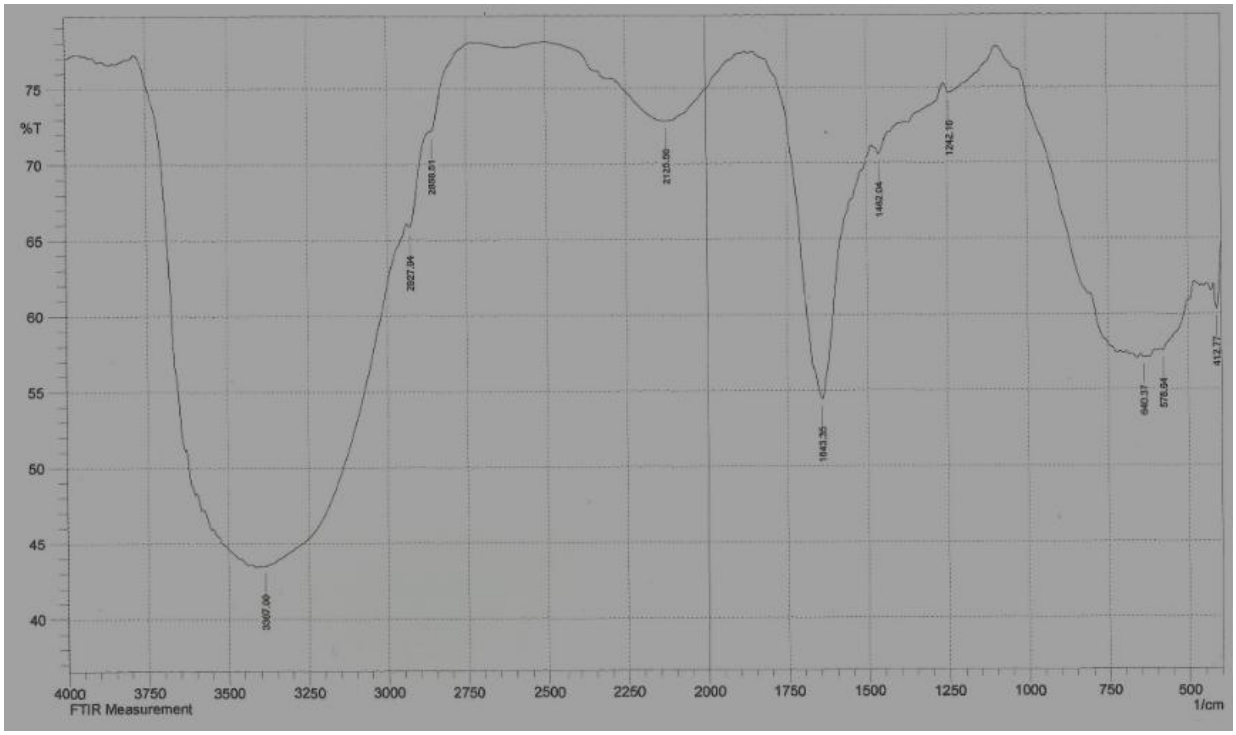
- Broad band at (3383, 3387, and 3414) cm-1 resulting from the C-H bands stretching mode suggest the presence of an aliphatic chain (CH3CH2)n.
- Bands at (1643) cm-1 which may be due to the stretching mode of the N-C bond present in amide group.


80
%T
70
60
50
40
30
20
3383.14
2129.41
1643.35
1415.75
1246.02
670.94
447.49
412.77
4000
3750
3500
3250
3000
2750
2500
2250
2000
1750
1500
1250
1000
750
500
FTIR Measurement
1/cm

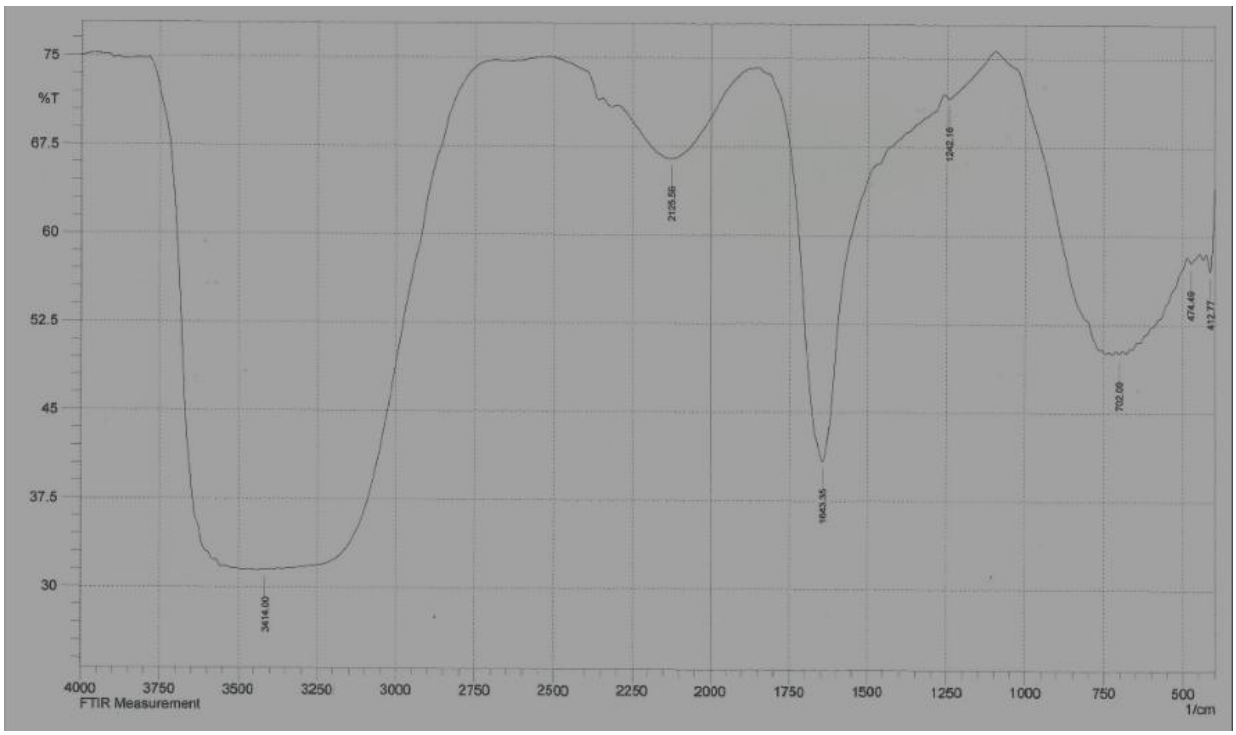
A.


82.5
%T
75
67.5
60
52.5
45
37.5
3383.14
2129.58
2049.30
1643.35
1452.04
1423.47
1242.16
1072.42
702.09
447.49
4000
3750
3500
3250
3000
2750
2500
2250
2000
1750
1500
1250
1000
750
500
FTIR Measurement
1/cm

B.



C.



D.

Figure (3.14).FTIR analysis of hydrolysate produce by isolate

A. S7 grown on crude oil. B. S14 grown on crude oil.

C. S7 grown on diesel. D. S14 grown on diesel**.**

From the above FTIR spectral data and results of the biochemical tests it can be concluded that the structure of biosurfactant produced by the local isolated *Streptomyces* is potentially a lipopeptide. *Streptomyces tendae* produces an extracellular hydrophobic peptide, referred to as streptofactin, which is a mixture of structurally related peptides (Richter *et al*., 1998; Kiran *et al*., 2010). Depending on the IR spectra with a variety of analytical techniques, detailed information about the already known biosurfactants were studied by a number of investigators which have demonstrated the lipopeptide nature of these compounds (Javaheri *et al.*, 1985; Nitschke and Pastore, 2006; Ghojavand *et al.*, 2008).

Lipopeptide biosurfactants produced by *Bacillus* strains include, among others, surfactin and lichenysin, which reduce surface tension to values around 26 and 36 mN/m, with emulsification indexes of about 60% (Yakimov *et al*., 1995; Abdel-Mawgoud *et al*., 2008).

On other study *Pseudomonas* strains produced viscosin, a peptidolipid biosurfactant that lowers surface tension to 27 mN/m (Neu and Poralla, 1990).

3.9.Plasmid Isolation

In order to determine the plasmid profile of *Streptomyces* isolates, the Favor Prep (Plasmid DNA Extraction Mini Kit) was used. Results in lane 2 and 5as showed in the Figure (3.15 A) indicated that S7 and S14, , isolates containing a small plasmid DNA bands.

Saadoun *et al* (2008) indicated that a different *Streptomyces* isolates containing plasmids with different size have different set of genes or the plasmids are the same plasmids but there were transferred between the soil population of bacteria by conjugation or transformation.

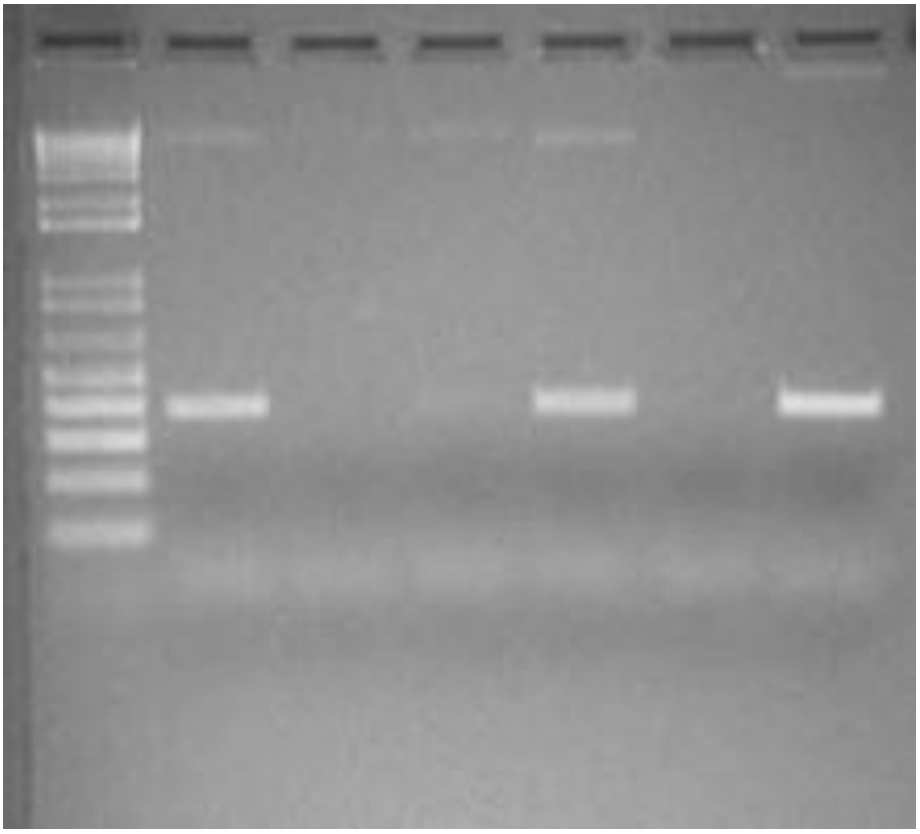
3.10. Role of *Streptomyces* Plasmid(s) in Biodegradation

3.10.1.Plasmid Curing

In order to study the role of plasmid(s) of *Streptomyces* sp. in degradation of hydrocarbons, curing experiment was performed. These bacteria was investigated to find the role of their plasmid(s) in hydrocarbons degradation. Isolates S7 and S14 were the most efficient isolates, by giving growth density and growth yield more than other isolates.

From this treatment of curing agent, appropriate dilutions were made and spread on YEME agar plates, then 30 colonies were tested on the selective medium (mineral salts medium containing hydrocarbons) in order to determine the cured colonies, which cannot degrade hydrocarbons. After repeatedly plated of the selected colonies, results indicated the colony survived on master plate and grown on mineral salts agar plate supplemented with crude oil and diesel contaminated soil. So that the ability of degradation may be not be depended on plasmid existence a sole source of carbon and energy.

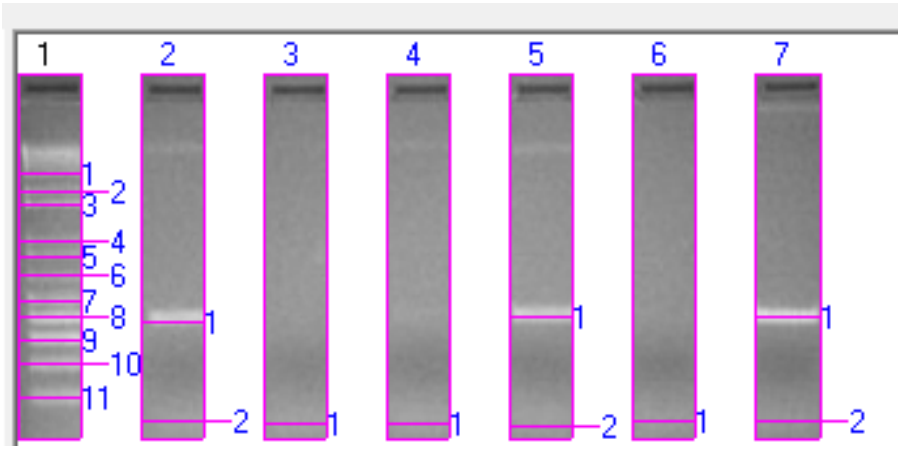
The results indicated that there was a change in the plasmid patterns between the cured and the original isolate. Cured isolates (S7 and S14) were lost their plasmid DNA bands as shown in the figure (3.15 B), and this may be due to the fact that plasmid DNA bands could not be responsible for degrading hydrocarbons in the two isolates (S7 and S14). In other word, gene(s) responsible for degradation are located on chromosomal genome or controlled by elements located on the plasmid (s) in these bacteria.



← **Plasmid**

1 2 3 4 5 6 7

A



	M.W.						
	L1	L2	L3	L4	L5	L6	L7
1	10.000	2.737			3.000		3.000
2	9.000						
3	8.000						
4	7.000						
5	6.000						
6	5.000						
7	4.000						
8	3.000						
9	2.000						
10	1.000						
11							

B

Figure (3.15):

A. Gel electrophoresis of isolated plasmid from bacterial isolates S7 and S14 Migrated on agarose gel (0.8%) in TBE bufferat (5V/cm). Lane(1)ladder marker1000bp, Lane(2) S7, Lane(3 and 4) cured S7, Lane (5 and 7) S14, and Lane(6) curedS14**.**

B. In this figure used captured photoshop for isolated plasmids**.**

According to this results, plasmid DNA may be a burden on microorganisms and may lie toward expulsion of these plasmids unless it interferes with viable process. Using this fact and avoiding alteration of DNA if chemical curing is used our attempt to cure plasmid(s) of our *Streptomyces* sp. by physical stress for showed no effect on this isolate to utilize hydrocarbons. This may be explained in two ways: (i) the plasmid DNA may play no rule in hydrocarbon utilization and genes responsible for such trait may be located on chromosomal DNA; (ii) giant plasmids which are known to be found in *Streptomyces* may play a part in this process was not cured because of its size that reach 700 KB and / or is could be integrated with chromosomal DNA (Kinashi *et al.*, 1987; Chater and Hopwood, 1993;Floriano and Bibb, 1996; Hopwood, 1999; Saadoun, 2002).

Saadoun and his colleagues (2008) mention that the molecular assessment of the ability of *Streptomyces* to degrade hydrocarbons is usually done by specific genes located on the bacterial genome. One of the most studied genes is the *alkB* gene which codes for a membrane-bound monooxygenase or alkane hydroxylase that plays the first step in alkane degradation process. (Maeng *et al.*1996; Murrell *et al*. 2000; Hamamura *et al*. 2001; Maier *et al*. 2001; Smits *et al*. 2002).

Kontro and his colleges (2005)showed that after cultivation of *Streptomyces* isolates in gradient pH media (4-11.5), that pH 7 to slightly above was optimum for complete growth. so heindicated that,in pH adaptation, cytoplasmic pH is maintained close tothe neutrality over the whole growth pH range by cellmembrane proteins, which function as primary protonpumps, and are also involved in respiration and energytransduction. In the absence of active pH regulation protonsequilibrate across the membrane, and the intracellular pHequates with that outside the cell. (Padan *et al*. 1981).

Rean (2010) indicated that the presence of proteins in cell membrane is important as control mechanism on substance entering or leaving the cell. Any factor affecting these proteins may pose a stress on the living cell. In acidic environment, the prevalence of H+ may change NH2 to NH3 at the end of peptide chain which makes the compound positively charge. This may affect the active sites of enzymes the may lead to inhibition of enzyme activity (Hewiston, 2009).

Chapter Four

Conclusions and Recommendations

4.1. Conclusions:

1. Soil contaminated with oil spills was rich with bacteria capable to degrade hydrocarbons.
2. Ability of two local *Streptomyces* isolates to degrade hydrocarbons was enhanced by growing in modified YEME medium of 1% NaCl and of pH (7-8), containing 1% crude oil or diesel at 45 °C, incubated in shaking incubator at (150-200)rpm for 10 days.
3. Biochemical detection and FTIR analysis indicated that the biosurfactants, nature was lipopeptide.
4. Gas chromatography analysis for liquid-liquid extraction of residual crude oil and diesel showed significant differences in the composition of hydrocarbons compounds.
5. The trait of degradation of hydrocarbons was not controlled by plasmid in the two local isolates.

4.2. Recommendations:

1. Further studies are required to identifying the lipase enzyme sequencing capable to degrade hydrocarbon.
2. Studying the biochemical pathways of hydrocarbons degradation by microorganisms and characterize the intermediate compounds.
3. Further studies by using bioreactor for hydrocarbon degradation.
4. Another attempt by using mixed culture of bacteria and notice their synergisms ability on hydrocarbons degradation.
5. Applying the purified *Streptomyces* biosurfactants in different application such as antimicrobial antifungal , and anticancer activity.
6. Identification of Streptomyces isolates S7 and S14 by using PCR and DNA sequence.

References

Abdel-Mawgoud; A.M.; Aboulwafa; M.M.; and Hassouna; N.A.H. (2008). Characterization of surfactin produced by *Bacillus subtilis* isolate BS5. Applied Biochemistry and Biotechnology 150. 289-303.

Alexander; M. (1991). Research needs in bioremediation. Environmental science and technology. 26(12): 1972- 1973.

Alexander; M. (1999). Biodegradation and bioremediation; 2nd ed. Academic Press; London; England.

Al-Gafari; R.N. (2004). Characterization; classification; genomic analysis and establishment of phylogenetic tree among local isolates of antibiotic producing streptomycetes. Ph.D. Thesis. Dep. College of science , Al-Nahrain University.

Al-Gafari; R.N.; Al-Jibori; G. H.; Abdel-Malik; W.(2010). Detection the influence of plasmid involvement in hydrocarbon utilization by a *Streptomyces* sp. Isolated from contaminated soil surrounding local generators. Tikrit Journal of Pure Science Vol. 15 No.(1)

Al- Gafari; R. N.(2004). Characterization, classification, genomic analysis and establishment of phylogenetic tree among local isolates of antibiotic producing Streptomycetes. Ph.D. Thesis. College of science, Al-Nahrain University.

Al-Hadhrami; M.N.; Lappin-Scott; H.M. and Fisher; P.J. (1995). Bacterial survival and n-alkanes degradation withen Omani crude oil and a mousse. Mar. Poll. Bull. 30: 403-408.

Al-Hadrami; M. N.; Lappin-Scott; H.M.; and Fisher; P. J. (1995). Bacterial survival and n-alkanes degradation within Omani crude oil and a mousse. Mar. Poll. Bull. 30: 403-408.

Al-Maghrabi; I.M.A.; Ibn; Aqil; A.O.; Isla; M.R.; and Chaalal; O. (1994). Use of thermophilic bacteria for bioremediation of petroleum contaminants. Energy Sources. 21: 17-29.

Anton; J. A.; Adam; P. and Cannon; J. (2000). Molecular evidence for biodegradation of macromolecules. Geochim. Cosmochim. Acta. 64(20): 3525-3537.

Atlas; R.M. (1981): Microbial degradation of petroleum hydrocarbons an environmental perspective. 45: 180-209.

Atlas; R.M. (1991). Microbial hydrocarbon degradation – bioremediation of oil spills. Chem.Tech. Biotechnol., 52: 149-156.

Atlas; R.M. and Bartha; R. (1973). Fate and effect of polluting petroleum on the marine environment. Residue Rev. 49-59.

Banat; I.M.; Makkar; R.S.; Cameotra; S.S. (2000). Potential commercial applications of microbial surfactants. Appl. Microbiol. Biotechnol. 53; 495–508.

Barab´as; G.; Vargha; G.; Istv´an; M.S.; Penyige; A.; Damjanovich; S.; Szöllösi; J.; Matk´o; J.; Hirano; T.; M´atyus; A.; and Szab´o; I. (2001). n-Alkane uptake and utilization by *Streptomyces* strains. Kluwer Academic Publishers. Printed in the Netherlands. Antonie van Leeuwenhoek 79: 269–276

Barabás; G.y.; Sorkhoh; N.A.; Fardia; f.; and Radwan; S.S. (1995). *n*-Alkane utilization by oligocarbophilic actinomycete strains from oil-polluted Kuwaiti Desert soil. Actinomycetologia 9: 13–18

Barkay; T.; Venezia; S. N.; Ron; E. Z.; and Rosenberg; E. (1999). Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier alasan . Appl. Environ. Microbiol. 65:2697-2702.

Bartha; R. (1986). Biotechnology of petroleum pollutant biodegradation; Microbial Ecology. 12: 155-172.

Battershill; C.; Jaspars; M. and Long; P. (2005). Marine biodiversity: new drugs from the ocean depths. Biologist 52:107–114

Be´rdy; J. (2005). Bioactive microbial metabolites: a personal view. J Antibiot 58:1–26.

Blunt; J.W.; Copp; B.R.; Munro; M.H.G.; Northcote; P.T. and Prinsep; M.R. (2007) Marine natural products. Nat Prod Rep 24:31–86.

Brenner; D.J.; Krieg; N.R.; Staley; J.T. (2001). Bergeys's manual of systematic bacteriology; 2nd edition; Vol. 2: The Proteobacteria; Part A: Introductory Essays.

Britton; L.N. (1984). Microbial degradation of aliphatic hydrocarbons. In: Microbial degradation of organic compounds. (Ed. Gibson; D.T.) pp.: 89-130. Marcel Dekker, INC. New York.

Brown; D.G. (2007). Relationship between micellar and hemi-micellar processes and the bioavailability of surfactant-solubilized hydrophobic organic compounds. Environ Sci Technol 41:1194–1199

Bull; A.T.; and Stach; J. E. M. (2007). Marine actinobacteria: new opportunities for natural product search and discovery. Trends in Microbiol., 15: 491-499.

Cameotra; S.S.; Makkar; R.S. (2004) Recent application of biosurfactant as biological and immunological molecules. Curr Opin Microbiol 7:262–266

Capelli; S. M.; Busalmen; P.J.; and Sánchez; R.S.(2001). Hydrocarbon bioremediation of a mineral-base contaminated waste from crude oil extraction by indigenous bacteria. International Biodeterioration and Biodegradation,47:233-238.

Carillo; P.; Mardarz; C. and Pitta-Alvarez; S. (1996). Isolation and selection of biosurfactant producing bacteria. World Journal of Microbiology and Biotechnol., 12: 82- 84.

Cerniglia; C. E. (1992**).** Biodegradation, Kluwer Academic publisher, Netherland **3**:351-368.

Cerniglia; C. E.; (1992). Biodegradation; Kluwer Academic publisher, Netherland 3:351-368.

Chater; K. F.; and Hopwood; D. A. (1994). Streptomyces. In: Bacillus subtilis and other gram positive bacteria. Biochemistry, Physiology, and Molecular genetics.

Chater; K. F.; and Howard; D. A. (1993). *Streptomyces*: In *Bacillus* and other gram positive bacteria; biochemistry; physiology; and molecular genetics. Sonenshein; A.; Hoch; J. A.; and Losick; R. (eds). Am. Soc. Microbiol

Chater, K.F. and Losik, R. (1997). Mycelial life style of *Streptomyces coelicolor* A3 (2) and its relatives. In: Shapiro JA and Dworkin M (Eds) Bacteria as multicellular organisms. Oxford University Press, Oxford.

Chaudhary; P.; Sharma; R.; Singh; S.B.; and Nain; L. (2011). Bioremediation of PAH by Streptomyces sp. Bull Environ Contam Toxicol ., 86:268–271.

Clarke; P.H. (1984). The evolution of degradative pathways. In microbial degradation of organic compounds, Gibson, D.T.(Ed.), pp:11-27, Marcel Dekker, Inc.

Colins;C.H. and Lyne; P.M.(1987).Microbial Methods (5 th ed). Butter Worth and Co. Publisher Ltd. London.

Coon; M.J. (2005). Omega oxygenases: nonheme-iron enzymes and P450 cytochromes. Biochem Biophys Res Commun 338:378–385.

Creniglia; C.E. (1984). Microbial metabolism of polycyclic aromatic hydrocarbons. Ada. Appl. Microbiol. 30: 31-71.

Das; K.; Mukherjee; A. K. (2007) .Differential utilization of pyrene as the sole source of carbon by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains: role of biosurfactants in enhancing bioavailability. J Appl Microbiol 102:195–203

Deepika; T.L. and Kannabiran; K. (2009). A report on anti dermatophytic activity of actinomycetes isolated from Ennore coast of Chennai, Tamil Nadu, India. International Journal of Integrative Biology, 6: 132-136.

Desai; J.D.; and Banat IM (1997). Microbal production of surfactants and their commercial potential. Microbiol. Mol. Biol. Rev. 61: 47-64.

Devereux; R.; and Sizemore; R.K. (1982). Plasmid incidence in marine bacterial isolate from petroleum polluted sites on different petroleum hydrocarbon. Mar. Poll. Bull. 13: 198-202.

Euzéby; J.P. (2008). "Genus *Streptomyces*". List of Prokaryotic names with Standing in Nomenclature. Retrieved 2008-09-28.

Fehling; R.H.; Buchanan; G.O.; Mincer; T.J.; Kauffman; C.A.; Jensen; P.R. and Fenical; W. (2003). Salinosporamide A: a highly cytotoxic proteasome inhibitor from a novel microbial source; a marine bacterium of the new genus Salinospora. Angew Chem Int Ed 42:355–357.

Fiechter; A. (1992). Biosurfactants: moving towards industrial application. Trends. Biotechnol. 10: 208-217.

Floriano; B.; and Bibb; M. J. (1996). afs R is a pleiotropic but conditionally required regulatory gene for antibiotic production in Streptomyces coelicolor A3(2). Mol. Microbiol. 7: 837.

Fritsche; W.; and Hofrichter; M. (2000). Aerobic degradation by microorganisms. In: Rehm; H.J.; Reed; G. (eds) Biotechnology 11b, Environmental Processes II. Wiley-VCH, Weinheim

Gamian ; A.; Mordarskaa; H.; Ekielb; L.; Ulrichc; J.; Szponara; B.; and Defayed; J. (1996). Structural studies of the major glycolipid from

Saccharopolyspora genus. Elsevier J. ;Carbohydrate Research; Volume 296; Issues 1–4; ; Pages 55–67

Gandhimathi; R.; K. Seghal; T.A. Hema; S. Joseph; R.T. Rajeetha and P.S. Shanmughapriya; (2009). Production and characterization of lipopeptide biosurfactant by a sponge- associated marine actinomycetes *Nocardiopsis alba* MSA10. Bioprocess Biosystems Engineering; DOI 10.1007/s00449-009-0309-x.

Ghojavand; H.; Vahabzadeh; F.; Roayaei; E.; and Shahraki; A.K. (2008). Production and properties of a biosurfactant obtained from a member of the *Bacillus subtilis* group (PTCC 1696). Journal of Colloid and Interface Science 324; 172e176.

Gibson; D.T. (1984). Microbial degradation of organic compounds. The University of Texas at Austin; Texas.

Gold; T. (1985). The origin of natural gas and petroleum, and the prognosis for future supplies. Annu. Rev. Energy, 10: 53-77.

Gudiña; E.J.; Pereira; J.F.B.; Rodrigues; L.R.; Coutinho; J.A.P.; and Teixeira; J.A. (2012) . Isolation and study of microorganisms from oil samples for application in Microbial Enhanced Oil Recovery. International Biodeterioration and Biodegradation; 68; 56-64

Hamamura; N.; Yeager; C. M.; and Arp; D. J. (2001). Two distinct monooxygenase for alkane oxidation in Nocardioides sp. Strain CF8. Appl Environ Microbiol 67:4992–4998

Hambrick; G.H.; Delaune; R.D. and Patrick; W.H. (1980). Effect of estuarine sediment pH and oxidation – reduction potential on microbial hydrocarbon degradation. Appl. Environ. Microbiol. 40(2): 365-369.

Harely; P.J.; and Prescott; M. L. (1996). Laboratory exercises in microbiology; ASM press; Washington.

Haritash; A.K.; and Kaushik; C.P. (2009). Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. J Hazard Mater 169:1–15

Harowitz; A.; Gutnick; D. and Rosenberg; E. (1975). Sequential growth of bacteria on crude oil. Appl. Microbiol. 30(1): 10-19.

Harpal; S.A.; Richard; R.C.; and Nemeth; J. C. (1982). Environment International[J];1:285-291

Hewiston; J. (2009). Effect of pH of the solution on cell membrane. Letters in Appl. Microbiol., **53**(2),122-128.

Hinchee; E.R.; and Kitte; A.J. (1995). Applied Bioremediation of Petroleum Hydrocarbons. Columbus (OH): Battelle Press.E

Hohnstock; A. M.; Stuart-Keil; K. G.; Kull; E. E.; and Madsen; E. L. (2000). Naphthalene and donor cell density influence field conjucation of naphthalene catabolism plasmids. Appl. Environ. Microbial. 66:3088-3092.

Hommel; R.K. (1990). Formation and physiological role of biosurfactants produced by hydrocarbon-utilizing microorganisms. Biodegradation; 1: 107-119.

Hommel; R.K. (1990). Formation and physiological role of biosurfactants produced by hydrocarbon-utilizing microorganisms. Biodegradation; 1: 107-119.

Hopwood; D. A. (1999). Forty years of genetic with *Streptomyces*: from in vivo through in vitro to in silico. Microbio;. 154: 2183.

Hopwood; D.A.; Bibb; M. J.; Chater; K. F.; Kieser; T.; Bruton; C. J.; Kieser; H. M.; Lydiate; D. J.; Smith; C. P.; and Ward; J. M. (1985). Genetic manipulation of *Streptomyces*. A Laboratory Manual. John Innes Inc; Norwich; England.

Hughes; C.C.; Prieto-Davo; A.; Jensen; P.R. and Fenical; W. (2008). The marynopyrroles; antibiotics of an unprecedented structure class from a marine *Streptomyces sp.* Org Lett 10:629–631.

Jain; R. K.; Kapur; M.; Labana; S.; Lal; B.; Sarma; P. M.; Bhattacharya; D.; and Thakur; I.S. (2005). Microbial diversity: application of microorganisms for the biodegradation of xenobiotics. Current Science 89(1): 101-112.

Javaheri; M.; Jenneman; G.E.; McInerney; M.J.; and Knapp; R.M.; (1985). Anaerobicproduction of a biosurfactant by Bacillus licheniformis JF-2. Applied and Environmental Microbiology 50; 698e700.

Jensen; P.R.; Mincer; T.J.; Williams; P.G.; and Fenical; W. (2005). Marine actinomycete diversity and natural product discovery. Antonie Van Leeuwenhoek 87:43–48.

Jimnani; K.; meshram; N.; and shrivastava; R. (2009). Hydrocarbon utilization by bacteria as a sole source of carbon. International Research Journal ISSN-0975-3486 RNI : RAJBIL 01488 V. I * ISSUE—1

Johnsen; A. R.; Wick; L. Y.; and Harms; H. (2005). Principles of microbial PAH-degradation in soil. Env. Pollution. 133: 71-84.

Kannahi; M.; and Thara; V.(2012). Effect of petroleum on seed germination in *Vigno radiata* and its degradation by *Pseudomonas fluorescens* and *Streptomyces* isolates. Journal of Chemical and Pharmaceutical Research; 4(7):3387-3390

Kieser; T.; Bibb; M.J.; Buttner; M.J.; Chater; K.F.; and Hopwood; D.A. (2000). *Practical Streptomyces Genetics* (2nd ed.). Norwich; England: John Innes Foundation. ISBN 0-7084-0623-8.

Kim; S. J.; Choi; D. H.; Sim; D.S.; and Oh; Y. S. (2005). Evaluation of bioremediation effectiveness on crude oil-contaminated sand**.** Chemosphere; 59:845-852.

Kinashi; H.; Shimaji; M.; and sakai; A. (1987). Giant linear plasmids in *Streptomyces*; which code for antibiotic biosynthesis genes. *Nature;* 328: 454

Kiran; S. G.; Anto Thomas; T.; Selvin; J.; Sabarathnam; B.; Anto Thomas; A.; and Lipton; P. (2010). Optimization and characterization of a new lipopeptide biosurfactant produced by marine *Brevibacterium aureum* MSA13 in solid state culture. Bioresource Technology 101; 2389–2396

Kokare; C. R.; Kadam; S. S.; Mahadik; K. R.; and Chopade; B. A.(2007). Studies on bioemulsifier production from marine *Streptomyces* sp. S1. Indian Journal of Biotechnology; Vol 6; pp 78-84.

Komagata; K. (1986). JMC catalogue of strains. (3rd) edition; Japan.

Kontro; M.; Lignell; U.; Hirvonen; M. R.; and Nevalainen; A.(2005). pH effects on 10 Streptomyces spp. growth and sporulation depend on nutrients. Letters in Applied Microbiology; 41; 32–38 doi:10.1111/j.1472-765X.2005.01727.x

Kotani; T.; Yurimoto; H.; Kato; N.; and Sakai; Y. (2007). Novel acetone metabolism in a propane-utilizing bacterium. J Bacteriol 189:886–893.

Kuster; B. (1976). Chromogenicity of actinomycetes. In: Actinomycetes the Boundary Microorganisms. Arai; T. (ed). Toppan.

Lakshmipathy; D.; Prasad; A. A. S.; and Kannabiran; K. (2010). Production of Biosurfactant and Heavy Metal Resistance Activity of Streptomyces Sp. VITDDK3-a Novel Halo Tolerant Actinomycetes Isolated from Saltpan Soil. Advances in Biological Research 4 (2): 108-115; ISSN 1992-0067

Leahy; J.G.; and Colwell; R.R. (1990). Microbial degradation of hydrocarbons in the environment. Microbiol. Rev. 54(3): 305-315.

Lindow; S.E.; Panopoulos; N. J.; and Mcfarland; B. L. (1989). Genetic engineering of bacteria from managed and natural habitates.Science 244:1300-1306.

Madigan; M. and Martinko; J. (eds.) (2005). Brock Biology of Microorganisms (11th ed.). Prentice Hall. ISBN 0-13-144329-1.

Maeng; J. H.; Sakai; Y.; Tani; Y.; and Kato; N. (1996). Isolation and characterization of a novel oxygenase that catalyzes the first step of n-alkaneoxidation in Acinetobacter sp. strain M-1. J Bacteriol 178:3695–3700

Maier; T.; Foerster; H. H.; Asperger; O.; and Hahn; U. (2001). Molecular characterization of the 56-kDa CYP153 from Acinetobacter sp. EB104. Biochem Biophys Res Commun 286:652–658

Malatova; K. (2005). Isolation and characterization of hydrocarbon degrading bacteria from environmental habitats in western new york state. A thesis submitted in partial fulfillment of the requirement for the Degree of Master of Science in Chemistry.

Margesin; R.; and Schinner; F. (2001). Biodegradation and bioremediation of hydrocarbons in extreme environments. Appl. Microbiol. Biotech. 56: 650-663.

Martin; J.P. and Focht; D.D. (1977). Biological properties of soils. In Elliott LF and Stevenson FJ (Eds) Soils for management of organic wastes and waste waters. American Society of Agronomy Madison; WI; USA

Marquez-Rocha; F.J.; Hernadez-Rodriguez; V.; and Teresalamela; M.A. (2001). Biodegradation of diesel oil in soil by a microbial consortium. Kluwer Academic Publishers. Printed in the Netherlands. Water, Air, and Soil Pollution 128: 313–320

Martinkova; L.; Uhnakova; B.; Patek; M.; Nesvera; J.; and Kren; V.(2009). Biodegradation potential of the genus Rhodococcus. Environ. Int. 35; 162–177.

Meintanis; C.; Chalkou; K.I.; Kormas; K.A.; and Karagouni; A.D. (2006). Biodegradation of crude oil by thermophilic bacteria isolated from a volcano island. Biodegradation 17:3–9.

Mercedes M.; Mari; N.; Theo; H.M.; Smits; Jan; B.; and Rojo; F. (2001). The alkane hydroxylase gene of Burkholderia cepacia rr10 is under catabolite repression control. J Bacteriol 183:4202–4209

Merino; J.; and Bucala; V. (2007). Effect of temperature on the release of hexadecane from soil by thermal treatment. J Hazarad Mat 143:455–461.

Miller; R.M.; and Bartha; R. (1989). Evidence from liposome encapsulation for transport-limited microbial-metabolism of solid alkanes. Appl Environ Microbiol 55:269–274

Mills; A.M.; Bonner; S.J.; McDonald; J.T.; Page; A.C.; and Autenrieth; L.R.(2003). Intrinsic bioremediation of a petroleum-impacted wetland. Marine Pollution Bulletin;46:887-899.

Mitra; S. (1996). Genetic engineering: principles and practice. Pp: 314-316. First published. Rajir Beri for McMillan; india Limited. New Delhi.

Mueller; J.G.; Cerniglia; C.E.; and and Pritchard; P.H. (1996). Bioremediation of environments contaminated by polycyclic aromatic hydrocarbons. In: Crawford RL; Crawford LD *et al* (eds) Bioremediation: principles and applications. Cambridge University Press; Cambridge; MA; pp 125–194

Murrell; C. J.; Gilbert; B.; and Mcdonald; I. R. (2000). Molecular biology and regulation of methane monooxygenase. Arch Microbiol 173:325–332

Naas; E. M. H.; Al-Muhtaseb; S. A. and Makhlouf; S. (2009). Biodegradation of phenol by Pseudomonas putida immobilized in polyvinyl alcohol (PVA) gel. J. Hazard. Mater. 164; 720–725.

Navon-Venezia; S.; Zosim; Z.; Gottlibe; A.; Leggman; R.; Carmeli; S.; Ron; E. Z.; and Rosenberg; E. (1995). Alsan; a new bioemulsifier from *Acinetobacter redioresistens*; *Appl Environ Microbiol*; 61; 3240-3244.

Neu; T.R. and Poralla; K. (1990). Emulsifying agent from bacteria isolated during screening for cells with hydrophobic surfaces. Appl Microbiol Biotechnol32: 521–525

Nishanthi; R.; Kumaran; S.; Palani;P.; Chellaram;C.; Prem Anand; T.; and Kannan; V. (2010). Screening of biosurfactants from hydrocarbon degrading bacteria. Journal of Ecobiotechnology 2/5 : 47-35, ISSN 2077-0164

Nitschke; M.; and Pastore; G.M.; (2006). Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater. Bioresource Technology 97; 336e341

Nonomura; H. (1974). Key for classification and identification of 485 species of the streptomycetes included in the ISP. *J. Ferm. Technol.*; 52: 78-92.

Okerentugba; P.O. and Ezeronye; O.V. (2003). Petroleum degrading potentials of single and mixed microbial cultures isolated from rivers and refinery effluent in Nigeria. African. J. Biotech. 2(9): 288-292.

Omura; S.; H. Ikeda; J.; Ishikawa; A.; Hanamoto; C.; Takahashi; M.; Shinose; Y.; Takahashi; H.; Horikawa; H.; Nakazawa; T.; Osonoe; H.; Kikuchi; T.; Shiba; Y. ;Sakaki; and M. Hattori. (2001). Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. Proc. Natl. Acad. Sci. USA 98:12215–12220.

Padan; E.; Zilberstein; D. and Schuldinger; S. (1981). pH homeostasis in bacteria. Biochim Biophys Acta 650; 151–166.

Pagnout; C.; Frache; G.; Poupin; P.; Maunit; B.; Muller; J.F.; and Ferard; J.F.; (2007). Isolation and characterization of a gene cluster involved in PAH degradation in Mycobacterium sp. strain SNP11: expression in Mycobacterium smegmatis mc2155. Res. Microbiol. 158; 175–186.

Pines; O.; and Gutnick; D. L. (1986). Role of emulsan in growth of *Acinetobacter calcoaceticus* RAG-1 on crude oil; *Appl Environ Microbiol;* 51; 661-663.

Plaza; G. A.; Zjawiony; I.; and Banat; I. M. (2006). Use the Different Methods for Detection of Thermophilic Biosurfactant-Producing Bacteria from Hydrocarbon-Contaminated and Bioremediated Soils; Journal of Petroleum Science and Engineering; 50; 71-77.

Plaza; G.A.; Ulfig; K.; and Brigmon; R.L. (2005). Surface active properties of bacterial strains isolated from petroleum hydrocarbon-bioremediation soil. Polish J. Microbiol. 54(2): 161-167.

Radwan; S.S.; Barabás; G.y.; Sorkhoh; N.A.; Damjanovich; S.; Szabó; I.; Szöllösi; J.; Matkó; J.; Penyige; A.; Hirano; T.; and Szabó; I.M. (1998). Hydrocarbon uptake by *Streptomyces*. FEMS Microbiol. Lett. 169: 87–94

Rahaman; K.S.M.; Banat; I.M.; Thahira; J.; Thayumanavan; T. and Lakshmanaperumalsamy; P. (2002). Bioremediation of gasoline contaminated soil by bacterial consortium with poultry litter; coir pith and rhamnolipid biosurfactant. Bioresource Technology; 81: 25-32.

Rehm; H.J.; and Reiff; I. (1981). Mechanisms and occurrence of microbial oxidation of long chain alkanes. 19: 175-215.

Richard; J.Y.; and Vogel; M.T.(1999). Characterization of a soil bacterial consortium capable of degrading diesel fuel. International Biodeterioration and Biodegradation ;44:93-100.

Richter; M.; Willey; M.; Suessmuth; R.; Jung; G.; and Fiedler; H.P. (1998) Streptofactin; a novel biosurfactant with aerial mycelium inducing activity from *Streptomyces tendae* Tue 901/8c. FEMS Microbiol Lett163: 165–171.DOI: 10.1016/s0378-1097(98)00167-0

Riedlinger; J.; Reike; A.; Za¨hner; H.; Krismer; B.; Bull; A.T.; Maldonado; L.A.; Ward; A.C.; Goodfellow; M.; Bister; B.; Bischoff; D.; and Fiedler; H. P. (2004). Abyssomicins; inhibitors of the para-aminobenzoic acid. J Antibiot 57:271–279.

Rodrigues; L. R.;Teieira; J. A.; Mei; H. C.; and Oliveira; R. (2006). Physiochemical and Functional Characterization of a Biosurfactant produced by *Lactococcus lactis* 53; Colloids and Surfaces B: Biosurfactants; 49;79-86.

Rodrigues; L.; Banat; I.M.; Teixeria; J.; and Oliveira; R. (2006) Biosurfactants potential application in medicine. J Antimicrob Chemother 57:609–618

Rosenberg; E.; Ron; E.Z. (2001). Natural roles of biosurfactants. Environ Microbiol. 3:229–236

Saadoun; I .(2002). Isolation and characterization of bacteria from crude petroleum oil contaminated soil and their potential to degrade diesel fuel. J Basic Microbiol 6:420–428

Saadoun; I.; Alawawdeh; M.; Jaradat; Z.; and Ababneh; Q. (2008). Growth of *Streptomyces* spp. from hydrocarbon-polluted soil on diesel and their analysis for the presence of alkane hydroxylase gene (*alkB*) by PCR. World J Microbiol Biotechnol; 24:2191–2198. DOI 10.1007/s11274-008-9729-z

Saadoun; I.; Wahiby; L.; Ababneh; Q.; Jaradat; Z.; Massadeh; M.; and AL-Momani; F. (2007). Recovery of soil streptomycetes from arid habitats in Jordan and their potential to inhibit multi-drug resistant *Pseudomonas aeruginosa* pathogens. World J Microbiol Biotechnol doi: 10.1007/s11274-007-9451-2

Sadasivam; S.; and Manickam; A. (1996). Biochemical methods. New Age International Publeshers; New Delhi.

Sambrook; J.; and Russell; D. W. (2001). Molecular cloning. A Laboratory Manual; Gold Spring Harbor Laboratory; New York.

Sanchez; O.; Ferrera; I.; Vigues; N.; Tirs; G.; Grimalt; J.; and Mas; J. (2006). Presence of opportunistic oil-degrading microorganisms operating at the initial steps of oil extraction and handling. Inter. Microbiol. 9: 119-124.

Seghal; G.K.; Hema; T.A.; Gandhimathi; R.; Joseph; S.; Anto; T.T.; Rajeetha; R.T.; and Natarajaseenivasana; K. (2009). Optimization and production of a biosurfactantfrom the sponge-associated marine fungus*Aspergillus ustus* MSF3. Colloids and SurfacesB: Biointerfaces; 73: 250-256.

Singh; S. N.; Kumari; B.; and Mishra; S. (2012). Microbial Degradation of Xenobiotics, Chapter 17:Microbial Degradation of Alkanes. Environmental Science and Engineering, Springer-Verlag Berlin Heidelberg. DOI: 10.1007/978-3-642-23789-8_17

Shirling; E.B. and Gottlieb; D. (1966). Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol*. 16; 313-340.

Smits; T.H.M.; Balada; S.B.; Witholt; B.; and van Beilen; J. B. (2002) Functional analysis of alkane hydroxylases from Gram negative and Gram positive bacteria. J Bacteriol 184:1733–1742

Stanley; T.W.; Elizabeth; M.; Sharpe; J.; and Holt; G. (2001). Bergeys's manual of systematic bacteriology; 2nd edition. Williams and Wilkins. ISBN: 978-0-387-98771-2.

Strohl; W.R. (2005). Antimicrobials. In: Bull AT (ed) Microbial diversity and bioprospecting. ASM; Washington DC; pp 336–355.

Stuart-keial; K. G.; Honustock; A. M.; Drees; P. K.; Herric; J. B.; and Madsen; E. L. (1998). Plasmids responsible for horizontal transfer of naphthalene catabolism genes between bacteria at a coal tar-contaminated site are homologous to pDTG1 from *pseudomonas putida* NCIB 9816-4. Appl. Enviro. Microbial. 64: 3633-3640.

Surridge; A. K. J. (2007). Denaturing gradient gel electrophoresis characterisation of microbial communities in polycyclic aromatic hydrocarbon and polychlorinated biphenyl contaminated soil. PhD Thesis. University of Pretoria; South Africa. pp. 1-133.

Suthindhiran; K. and Kannabiran; K. (2009). Hemolytic activity of *Streptomyces VITSDK1* spp. isolated from marine sediments in Southern India. Journal de Mycologie Médicale; 19: 77-86.

Synder; L.; and Champness; W. (1997). Molecular genetics of bacteria. Pp: 105-147. American society for microbiology; Washington; DC.

Szöllösi; J.; Matkó; J.; Penyige; A.; Hirano; T. and Szabó; I.M. (1998). Hydrocarbon uptake by *Streptomyces*. FEMS Microbiol. Lett. 169: 87–94

Taylor; L.H.; Latham; S.M.; Woolhouse; M.E.J. (2001). Risk factors for human disease emergence. Philos Trans R SocLond BBiol Sci 356:983–989.

Towell; M.G.; Bellarby; J.; Paton G.I.; Simon; C.F.; Pollard; J.T.; and Semple; K.T. (2011) Mineralisation of target hydrocarbons in three contaminated soils from former refinery facilities. Environ Pollut 159:515–523.

Van Hamme; J. D.; Singh; A.; and Ward; O.P. (2003). Recent advances in petroleum microbiology. Microbiol Mol Biol Rev 67: 503–549

Wallnofer; P.R.; and Engelhardt; G. (1984). Aromatic and heterolytic hydrocarbon. In: Biotechnology. 9Eds. Rhm; H.J. and Reed; G. Vol. 69. pp: 277-329.

Ward; D.M. and Brock; T.D. (1978). Hydrocarbon degradation in hypersaline environment. Appl. Environ. Microbiol. 35: 353-359.

Watanabe; K. (2001). Microorganisms relevant to bioremediation. Curr. Opin. Biotechnol. 15: 2-9

Waksman; S. A.; and Hinrici; A. T. (1943). The nomenclature and classification of the actinomycetes. J. Bacteriol. 46, 337

Weissenfels; W. D.; Beyer; M.; and Klein; J. (1990). Degradation of fluoranthene by pure bacterial cultures. Appl. Microbiol. Biotechnol. 32; 479–484.

Yakimow; M.M.; Timmis; K.N.; Wray; V.; and Fredrickson; H.L.; (1995). Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface Bacillus licheniformis BAS50. Applied and Environmental Microbiology 61; 1706e1713.

Youssef; N. H.; Ducan; K. E.; Nagle; D. P.; Savage; K. N.; Knapp; R. M.; and Mclnernery; M. J. (2004). Compraision of Methods to Detect Biosurfactant Production by Diverse Microorganisms; Journal of Microbiological Methods; 56(3); 339-346.

Zhang; Y.; and Miller; R.M. (1994). Effect of a *Pseudomonas* rhaminolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. Environ. Microbiol. 60: 2101- 2106.

المصادر العربيه

ريان مازن فيصل (2010). الأس الهيدروجيني المنخفض محيدا لمحتوى ال.DNA لجرثومه *Streptomyces* مقارنه بمحيدات أخرى. مجلة علوم الرافدين، المجلد 21 ، العدد 1، ص 41-54

الخفاجي، زهرة محمود ناصر .(2008) التقنيات الحيوية المايكروبية (توجهات جزيئية). وزارة التعليم العالي والبحث العلمي، جامعة بغداد، العراق.

Appendix

- Transfer 1 ml of well-grown bacteria culture to a microcentrifuge tube (not provided).
- Descend the bacteria by centrifuging for 1-2 min and discard the supernatant completely.
- Add 250 µl of FAPD1 Buffer containing RNase A and lysozyme (Provided by user) (for final concentration of 1 mg/ml) to the pellet and re-suspend the cells completely by pipetting. And incubate the sample mixture at 37 ° C for 15 min.

  • Make sure that RNase A has been added into FAPD1 Buffer when first open.

  • Make sure that lysozyme has been added into FAPD1 for final concentration of 1 mg/ml.

  • No cell pellet should be visible after resuspension of the cells.
- Add 250 µl of FAPD2 Buffer and gently invert the tube 5 times to lyse the cells and incubate at room temperature for 2 min.

  • Do not vortex, vortex may shear genomic DNA. If necessary, continue inverting the tube until the lysate become clear.

  • Do not proceed this step over 5 min.
- Add 350 µl of FAPD3 Buffer and invert the tube 5 times immediately but gently.

  • Invert immediately after adding FAPD3 Buffer will avoid asymmetric precipitation.
- Centrifuge for 10 min. During centrifuging, place a FAPD Column in a Collection Tube.
- Transfer the suspernatant carefully to FAPD Column. Centrifuge for 1 min then discard the flow-through.

  • Do not transfer any white pellet into the column

- Add 400 μl of W1 Buffer to FAPD Column. Centrifuge for 1 min then discard the flow-through.
  • Make sure that ethanol (96-100 %) has been added into W1 Buffer when first open.
- Add 750 μl of Wash Buffer to FAPD Column. Centrifuge for 1 min then discard the flow-through.
  • Make sure that ethanol (96-100 %) has been added into Wash Buffer when first open.
- Centrifuge for an additional 5 min to dry the column.
  • Important step ! This step will remove the residual liquid completely that will inhibit subsequent enzymatic reaction.
- Place FAPD Column to a new 1.5 ml microcentrifuge tube (not provided).
- Add 50 μl ~ 100 μl of 65 °C of Elution Buffer or ddH2O to the membrane center of FAPD Column. Stand the column for 1 min.
  • Important step ! For effective elution, make sure that the elution solution is dispensed on the membrane center and is absorbed completely.
  • Important : Do not Elute the DNA using less than suggested volume (50ul). It will lower the final yield.
- Centrifuge for 1 min to elute plasmid DNA.
- Store plasmid DNA at 4 °C or -20 °C.

الخلاصه

تم جمع خمس عينات من الترب الملوثة بتسرب النفط لسنوات عديدة من أماكن مختلفة في بغداد .تم الحصول من هذه العينات على أربعة عشر عزلة باعتبارها المحللات الأولية للنفط وزيت الغاز .تم اختبار هذه العزلات مرارا في وسط تكون من النفط الخام والديزل كمصدركربوني لضمان قدرتها على تحليل هذه المركبات .كانت العزلات الأكثر كفاءةS7 ، S8، S11، S12، S14لكن العزلتان S7, S14 اعطت أعلى كفاءة،لذا فقدتم اختبار هاتين العزلتين لدراسه كثلفه نموها حيث وجد انهما كانتا متغايرتين في ذالك.

وفقا لكثافة النمو، فقد شخصت هاتين العزلتين اعتمادا على الخصائص المظهرية وشكل المستعمرة، والكيموحيوية. أظهرت النتائج انهما ينتميان الى جنس الستربتومايسيس ولهما القدره استخدام مصادر نقية مختلفة من الهيدروكربونات، وأظهرت النتائج ان العزله S7 كانت أفضل من S14 العزله عند نموها على (Hexadecane, Tetrahydrofuran, Anthracene, and Naphthalene).

لدى التحقق من الظروف المثلى لتحلل النفط الخام والديزل من قبل العزلتين .S14 S7 تحسن قابليتها عندما زرعتا في وسط YEME لدرجات مختلفة من الاس الهيدروجيني، ودرجة الحرارة، والتحريك، وتراكيز ملح كلوريد الصوديوم على 1٪ من النفط الخام والديزل كل على حدة لمدة عشرة أيام.

اظهر عزل وتنقية biosurfactant التي انتجتها عزلتي S14 , S7 الستربتومايسيس بطريقة الترسيب الحامضي وجود (0.5، 1) غم / لتر biosurfactant ، على التوالي .وقد أشارت الاختبارات البيوكيميائية وكشف تحليل FTIR طبيعةكونbiosurfactant بانه عباره عنlipopeptide.

وأظهر تحليل كروماتوغرافيا الغاز للسائل المتبقي من النفط الخام والديزل فروق ذات دلالة واضحة في تركيب سلسلة الهيدروكربون حيثتم كسر معظم سلاسل الكربون الطويلة والمعقدة في لأبسط نوع، ولوحظ اختفاء بعض المركبات خلال هذه العملية.

لدى دراسة محتويات البلازميد للعزلات,S7, S14 أظهرت النتائج أن هذه العزلات احتوت على بلازميد صغير ,من أجل دراسة دور البلازميد في تحلل الهيدروكربونات، تم تحييد

البلازميد باستخدام طريقة الحموضة العالية وظهر أن الحامض النووي البلازميدي ليس له دور في التحليل لأن قدرة كلا العزلتين على تحلل المواد الهيدروكربونية لم تتغير.

الاهداء

اهدي ثمره جهدي المتواضع هذا

الي سيد الخلق ونور الهدى ورسول المحبه

محمد(صلى الله عليه وعلى اله وصحبه وسلم)

الى من كلت انامله ليقدم لي لحظه سعاده

الي من علمني ان ارتقي سلم الحياه بالحكمه والصبر

عائلتي

نور

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

﴿يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ﴾

صدق الله العظيم

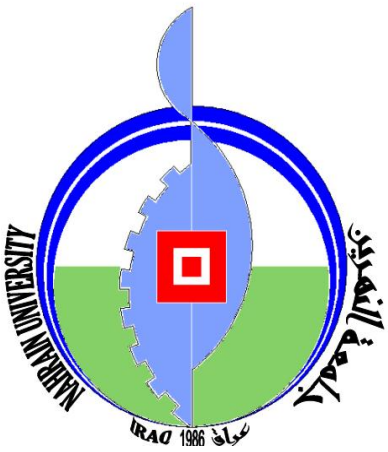
سوره المجادلة / جزء من الاية (١١)

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كلية العلوم



التحليل الحيوي للترب الملوثة بالبترول والوقود بواسطة عزلات بكتريا الستربتومايسيس

رسالة

مقدمة الى كلية العلوم - جامعة النهرين وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الأحيائية

مِن قبِل

نور عبد اللطيف عبدالله

بكالوريوس تقانة احيائية – كليه العلوم – جامعه النهرين (٢٠١٠)

بإشراف

د. رحاب صبحي رمضان (مدرس)

د. رباح نجاح جبار (استاذ مساعد)

شعبان , ١٤٣٤ حزيران , ٢٠١٣