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Isolation And IdentificationOf Bacteria Capable Of Degrading Crude Oil Under Aerobic Conditions In River Nile State

By

Ebtihag Mohammed Fadllah Sulaiman

B.Se.(Agric)

University of Gezira (1996)

A thesis Submitted to University of Shendi In

Fulfillment Of The Requirement Of The Degree Of

MSC In Environmental Biotechnology

Supervisor:

Dr. Nada Hassan M. Doleib

Jan.2014

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بسم اللة الرحمن الرحيم

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DEDICATION

To my family

And all my friends

Ebtihag

ACKNOLEDGMENT

In great indebtedness and gratitude, I would like to express my appreciation to my supervisor Dr. Nada Hassan M. Doleib for her generous assistance and her useful guidance. And great thanks to teatching staff and my colleagues, in Botany Department-faculty of Science and Technology for their help and all staff in Department of Microbiology in Faculty of Medical Laboratory Sciences in Shendi University. Thanks are also extended to Dr.El Mutaz Nasir Hassan. My love and warmest thanks to all members of my family and I would like to express my great indebtedness to everyone who encouraged me espesal thanks to Ustaze Ahmed Elrasheed and at last and forever my great thanks to my husband.

thank you all

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ABSTRACT

The study was carried out to isolate and identify bacteria that can degrade crude oil in River Nile State. In this study a total of one hundred and twenty five strains of petroleum degrading bacteria were isolated from twenty five soil samples contaminated with hydrocarbons. The samples were collected from different soil polluted areas in Shendi and Atbara cities. In addition , three water samples from Aljayli refinery..Samples were were used to determine their degradation rates of petroleum. Eleven of these isolate showed high degree of degradation for total petroleum hydrocarbons, they were tentatively identified as follows:*Pseudomonas aeruginosa, P. putida, Serratia marcences, Cedeca davicae, Enterobacter cloacae, Yersinia enterocolytica and Citrobacter freundii.*The highest percentage of degradation recorded by *Serratia marcences* which was 72% within ten days under aerobic conditions. The other strains have degradation percent in the range 31%- 62%.The isolate *Citrobacter freundii* which taken as example for a weak degrader (36%).It is realized that it was able to degrade n-alkane more than total petroleum hydrocarbons. This result come through the analyze by Gas Chromatography for the residual oil after degradation for this isolate and the best three isolates. Combination of the best five isolates gave high percentages of degradation than single strains.That is because different isolates can breakdown hydrocarbons chains which is different in their length.

مستخلص البحث

اجريت الدراسه لعزل وتعريف البكتريا المحلله للبترول تحت الظروف الهوائيه في ولاية نهر النيل. فى هذه الدراسة تم عزل مجموع 125 سلالة من البكتريا التى لديها المقدرة علي تحليل البترول من 25 عينة تربة ملوثة بالهيدروكربونات أخذت من مناطق ملوثة مختلفة من مدينة شندى وعطبرة و3 عينات مياة أخذت من مصفاة الجيلي لتحديد معدلات تحليلها للبترول. أحدى عشرمن هذه العزلات أعطت نسبة تحليل عالية للبترول .عرفت هذه العزلات من خلال الاختبارات المورفولوجيه والبيوكيميائيه على انها *Pseudomonas aeruginosa, P.putida, Serratia marcences, Cedeca davicae, Enterobacter cloacae, Yersinia enterocolytica* و *Citrobacter freundii*. *أعلى* نسبة مئوية لتحليل البترول سجلتها سلالة *Seratia marcences* *بنسبة*72% للكربون الكلى فى البترول,خلال عشرة أيام تحت الظروف الهوائية. أما بقية السلالات كانت نسبة تحليلها فى المدى 31%-62%. بالنسبه للعزله*Citrobacter frundiidi* كانت نسبة تحليلها 36%. لكن لاحظنا أن لديها مقدرة عالية على تحليل الالكانات من خلال تحليل كمية البترول المتبقية لها ولافضل ثلاثة عينات بواسطة الغاز كروماتوغرافي. عندما تم دمج أفضل خمس سلالات مع بعضهما البعض لإختبار درجة التحليل وجد أنها أعطت نتيجة أفضل من ما لو كان التحليل بسلالة مفردة. ذلك لأن السلالات المختلفه لديها مقدره على كسر سلاسل كربونيه مختلفة الطول.

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CHAPTER ONE

INTRODUCTION

Crude oil as a complex mixture contains a large number of distinctively different chemicals and is composed of four main fractions: saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes (Hasanuzzaman *et al*., 2007). The contamination of soil caused by accidental leakage or chronic release of crude oil and refined products to the environment occurs yearly with growing industrialization and demands for energy (Udiwal and Patel, 2010).

The amount of natural crude oil seepage was estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year.

If left unaddressed, these spills can adversely affect human health and the environment (CCME, 2008; Sanscartier *et al.*, 2011). Produce water is the water extracted from reservoir along with crude oil, which is the largest waste stream generated in oil field and gas industries. It is a mixture of different organic and inorganic compounds. Due to the increasing volume of waste all over the world in the current decade, the outcome and effect of discharging produced water on the environment has lately become a significant issue of environmental concern.

In Sudan, there is a big problem with the produced water and its separation off before oil transported via pipeline. The single most significant environmental issue for crude oil production facilities in Sudan is the disposal of produce water, especially in Hegleg oil field. Produced water is conventionally treated through different physical, chemical, and biological methods. Plant (Yousaf *et al.*, 2010), earthworm (Natal-da-Luz *et al*., 2012) and microbes were applied for the bioremediation of terrestrial or aquatic ecosystems polluted by crude oil. Microorganisms were given more attention in bioremediation of polluted environments as well as the oil exploitation

considering their adaptation to the extreme environment (Whyte *et al.,* 2002), capabilities to produce biosurfactant (Zheng *et al*., 2012) and metabolic potential to degrade the hydrocarbons. Microorganisms are able to utilize hydrocarbons as the sole carbon and energy sources resulting in the breakdown of crude oil components to low molecular weight compounds (Quek *et al*., 2006).

Bioremediation has been evaluated in several studies as an option to treat the oil pollution resulting from the spillage or leakage of crude oil and fuels in the environment (Ijah and Antai, 2003; Yuste *et al.,* 2000).

The main objectives of this research were to:

1. Determine the feasibility of bioremediation as a treatment option for produced water and refinery sludge.
2. Isolate a range of hydrocarbon degrading bacteria from contaminated soil with hydrocarbon, stored produced water and from refinery sludge.
3. Identify the isolates as far as possible to species level using morphological and biochemical characters.
4. Determine the hydrocarbon biodegradation potential of selected bacterial isolates using pure and mixed culture under controlled laboratory conditions.

CHAPTER TWO

LITERATURE REVIEW

2-1 Petroleum:

Petroleum known as crude oil is naturally occurring flammable liquid which is brown or sometimes black in color. Consisting of a complex mixture of hydrocarbons (about 50-98%) and small amounts of other non-hydrocarbon and mineral impurities, of various molecular weights and other liquid organic compounds. That are found in geologic formations beneath the Earth's surface.(Davis, 1967).

2-1-1 Hydrocarbon Components of Crude Oil:

Crude oils are composed of carbon and hydrogen molecules with typical H/C ratios of 1.5 – 2, containing predominantly aliphatic compounds (linear chains namely ‘paraffin’ or rings namely ‘naphthenic’) and aromatic compounds. The proportions of crude oil vary widely from oil to oil. In average of 527 crude oils, saturates make up to 58.2%, aromatics 28.6%, and polar compounds 14.2% (Tissot and Welte, 1984). Linear alkanes make up an average of 15% - 20% of undegraded crude oil. Branched alkanes, mainly occur in the range of C6 to C8, and pristine (C19H40) and phytane (C20H42). The sulfur content is typically 0.1% in light oils and sometimes more than 5% in heavy oils (Widdel and Amann, 2006).

Crude oils have long been classified as:

1- Paraffins (saturated alkene): They are one of the major constituents of crude oil found in refined petroleum products such as gasoline, kerosene, diesel fuel and heating oil. They categorized according to their chemical structures into alkane and cycloalkanes. There are three major classes of paraffin. These are linear alkenes, branched alkenes and naphthenes.

2- Olefins (also called alkene): defined as any unsaturated hydrocarbon containing one or more pairs of carbon atoms linked by a double bonds.

Olefins are classified in either or both of the following ways:

A-as cyclic or acyclic (aliphatic) olefins, in which the double bond is located between carbon atoms.

B-as monoolefins, diolefins, triolefins, etc., in which the number of double bonds per molecule is, respectively, one, two, three, or some other number.

3-Aromatics: which contain one or more 6-carbon rings with three of carbons containing double bonds. Examples of 1ring aromatics are benzene, tolune ethylbenzene and xylene (BTEX). Multiple ring aromatics (polynuclear) are aromatic compounds with multiple 6-carbon ring molecules. Example of these are naphthalene, anthracene and pyreneand (Wang *et al.*, 1995).

2-1-2 Heteroatom Components: (Non-hydrocarbon components of oil)

Consist of:-

A- Sulfur compounds:

Sulfur compounds might be present in inorganic and organic forms such as dibenzylthiopene (2 benzene rings separated by sulfur atom) in crude oils.

B- Oxygen compounds:

They are responsible for petroleum acidity in particular when they carry carboxylic group ($OH-C_{=}O$) bonded to a benzene ring. They may also from phenolic (OH bonded to benzene ring) groups.

C- Nitrogen compounds:

The nitrogen content of crude oil is generally low. Nitrogen compounds in petroleum may be valued arbitrarily as “basic” or “non-basic”. The relative amounts depend on procedure used. Basic compounds such as pyridine, and non-basic compounds are like indoles, carbazoles and pyrroles. Many of the nitrogen compounds are harmless although they lower the heating value of fuels. Some nitrogen compounds may impart an unpleasant odor or reddish colour to the crude distillation (Viadimira and Kennth,1959).

2-1-3 Oxygenated Compounds:

Oxygenated compounds may be present in crude oil or formed on oxidation as in the course of refining. Oxidation of n-Paraffins at a low pressure of Oxygen and a low temperature in the liquid phase has been carried out. The role of the Catalyst to help the Oxidation process has been investigated. The catalyst used in the process is Cobalt Naphthenate. Products derived from the so called catalytic oxidation of a n-Cetane as reference and n-Hydrocarbons extracted from gas oil as main feed-stock includes: Hydroperoxides converting to Aldehydes, Ketones, Alcohols and Carboxylic Acids. It was found that oxidation of branched chain hydrocarbons at similar conditions results in the production of more hydroperoxides.(Feizabadi,1997).

Crude oil may contain small quantity of fatty acids, which contain one to ten carbon atoms found in cracked distillates. They may produced by thermal decomposition of naphthenic acids.

Naphthenic acids characterized by cyclic structures, are present in crude oils and in straight run distillates –naphthenic acid decomposes as elevated temperatures and seldom appear in cracked products. Most of the naphthenic acids containing 6-carbon structure have been isolated from petroleum. Naphthenic acids are used in manufacture of products like dyes, insecticides, and soap. They may corrode refinery distillation equipment.

Phenols are present in crude oil and its distillates, particularly those obtained from cracking operations. Some of the lower phenols are recovered as by-products. Phenols act as oxidation inhibitors but some might act as accelerators.

2-1-4 Trace Metals:

Ashes of crude oils may contain a long list of metals, metallic and non-metallic elements found in petroleum, they are mostly Nickel and vanadium. Other metal ions reported form crude oils include copper, lead, iron, magnesium, sodium, molybdenum, zinc, cadmium, titanium, manganese,

chromium, cobalt, uranium, aluminum, tin, barium, gallium, silver and arsenic.(Khuhawar *et al.,*2012.; Nafi'u Tijjani *et al.,* 2012.).

2-2-1 Pollution of Environment by Crude Oil:

The use of fossil fuels such as petroleum have a negative impact on Earth's biosphere, releasing pollutants and greenhouse gases into air and damaging ecosystems through events such as oil spills. A vast amount of petroleum hydrocarbons discharge from industries or from accidental oil spills continuously released into the ocean (Prince, 1993).

Soil contaminated with petroleum has a serious hazard to human health, causes organic pollution of ground water (which limits its use), economic loss, environmental problems, and decreases the agricultural productivity of the soil.The extensive use of petroleum products leads to the contamination of almost all compartments of the environment, (Jyothi *et al.,* 2012). The problem faced by oil refineries is the safe disposal of oily sludge generated during the processing of crude oil. Improper disposal of oily sludge leads also to environmental pollution, particularly soil contamination and poses a serious threat to ground water. Many of constituents of oily sludge are carcinogenic and potent immune -toxicants (Propst *et al.*, 1999). The concern stems primarily from health risks, direct contact with contaminated soil, vapors from the contaminants, and from secondary contamination of water supplies within and underlying the soil (Thapa *et al.,* 2012). Environmental impact of petroleum is often negative because it is toxic to almost all forms of life. It capable of causing serious damages to humans and the ecosystem (Latha and Kalaivani., 2012).

In Sudan, the most significant environmental issue for crude oil production is the disposal of produced water (UNEP, 2007). Produced water defined as the water that exists in subsurface formations brought to the surface during oil and gas production. It contains a large volume of sodic water, contaminated

with hydrocarbons and treatment chemicals, disposing into a sensitive and vulnerable environment (Savanna zone).

The volume of water became very large particularly in the later years of production. Heglig facility alone currently generates over ten million cubic meters of produce water annually; in addition to sea transport oil spill risks.

2-2-2 Oil-Spills:

Oil spills is one of the most serious problems could occur in marine environment. In the last years, a large number of ecosystems have been changed by the growing influence of human activity. Contamination with oil spills is the major global concern today. The quantity of oil spilled during accidents has ranged from a few hundred tons to several hundred thousand tons. Approximately five million tons of crude oil and refined oil enter the environment each year because of anthropogenic sources such as oil spills (Malatova, 2005).

Examples of that, Exxon Valdez oil spill in Prince William Sound, Alaska, was an anthropogenic perturbation occurring between two major natural perturbation, the Nahodka oil spill, the Erica spill (1999) and the Prestige spill (2002), The super tanker Torrey Canyon sank in the English Channel (1967) have recently increased the attention of environmentalists, chemists, biotechnologists and engineers (Malatova,2005). Shipping accidents have a serious impact on the surrounding environment. A great part of the oil pollution problem results from the fact that the major oil-producing countries are not the major oil consumers. It follows that massive movements of petroleum have to be made from areas of high production to those of high consumption (Thapa *et al.,* 2012).

Oil spills at sea are generally much more damaging than those on land, since they can spread for hundreds of nautical miles in a thin oil slick which can cover beaches with a thin coating of oil. This can kill sea birds, mammals, shellfish and other organisms it coats. Oil spills on land are more readily

containable if a makeshift earth dam can be rapidly bulldozed around the spill site before most of the oil escapes, and land animals can avoid the oil more easily.

2-2-3 Treatment of the Crude Oil-Polluted Sites:

The traditional treatment of oily wastewater, such as containment and collection using floating booms, adsorption by natural or synthetic materials, etc., cannot degrade the crude oil thoroughly (Liang Zhang, *et al.*, 2005).

Conventional remediation methods include physical removal of contaminated material. These methods also use chemicals, especially shoreline cleaners, which are often organic solvents with or without surfactants (Riser-Roberts 1992).

Mechanical and chemical methods generally used to remove hydrocarbons from contaminated sites have limited effectiveness and it could be very expensive. 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 (Malatova, 2005; Salleh *et al.,* 2003).

Methods of Remediation contaminated soils:

A- Mechanical technologies: commonly used for soil remediation include burying, evaporation, dispersion, and washing.

B - Chemical methods: contain acid and base hydrolysis, oxidation and reduction fixation, solvent extraction and adsorption.

C- Physical methods: these are involved incineration, irradiation and deep-well injection.

D- Biological methods: these include landfills, activated sludge treatment, microbial degradation and soil injection procedures.

However, these technologies can lead to incomplete decomposition of contaminants. Moreover, the addition of chemicals, which makes the process expensive and environmentally damaging, may involve physical and or

chemical processes. Moreover, the methods require further disposal steps to remove the toxic compounds generated (Oboh *et al.,* 2006; Aldous, 1991).

2-3-1 Bioremediation:

Bioremediation is synonymous with Bioreclamation (Sanning, 1985) and Biotreatment (Lapinskas, 1989). Natural bioremediation (Biodegradation) is the breaks down of organic compounds to minerals (Woodward, 1988). Biodegradation of crude oil by microbial appears to be the natural process by which the bulk of the polluting oil is used as an organic carbon source, causing the breakdown of petroleum components to lower molecular compounds or transformed into the other organic compounds such as biosurfactants (Chhatre *et al*., 1996). Bioremediation is a manage process in which microbiological processes are used to degrade or transform contaminants to less toxic or nontoxic forms (Cauwenberghe and Root, 1998). The process of bioremediation, defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities, it is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry (Das and Chandran,2010; Jyothi *et al.,* 2012).

In the last few years, as landfills have become more and more scarce and concomitantly more and more cost prohibitive, interest in biological methods to treat organic wastes has increased (Molnaa and Grubbs,2001; Meintanis *etal.,* 2005). Bioremediation is becoming an attractive alternative for cleaning up soil systems contaminated with petroleum and other hydrocarbons (Marquez-Rocha *et al*., 2000, Molnaa and Grubbs, 2001).

Most organic chemicals and many inorganic ones are subject to enzymatic attack through the activities of living organisms. Most of modern society's environmental pollutants are included among these chemicals, and the actions of enzymes on them are usually lumped under the term biodegradation. (Thapa *et al*., 2012).

2-3-2 Microorganisms Capable of Degrading Hydrocarbons (Historical view):

The ability of microorganisms to transform and degrade many types of pollutants in different matrixes (soil, water, sediments and air) has been widely recognized during the last decades (Ilyina *et al.,* 2003). Microorganisms with the ability to degrade crude oil are ubiquitously distributed in soil and marine environments (Marquez-Rocha *et al.*, 2000; Jyothi *et al.,* 2012,). Some members of these populations degrade alkanes, some aromatics, while others decompose both paraffinic and aromatics hydrocarbons transforming them into products such as carbon dioxide, water and biomass or other less harmful end-products (Salleh *et al.,* 2003).

Many research workers have isolated and identified bacteria, yeasts and molds that could breakdown petroleum products and xenobiotic such as pesticides. Diverse groups of bacteria and fungi have the ability to degrade petroleum hydrocarbons. ZoBell (1946) in his review noted that more than 100 species representing 30 microbial genera had been shown to be capable of utilizing hydrocarbons. In polluted fresh water ecosystems bacteria, yeasts and filamentous fungi all appear to be important hydrocarbon degraders. In northwest Atlantic coastal waters and sediment, Mulkins-phillips and Stewart (1974) found hydrocarbon-utilizing bacteria of genera *Nocardia*, *Pseudomonas*, *Flavobacterium*, *Vibrio* and *Achromobacte*r. Bartha and Atals (1977) listed 22 genera of bacteria, one alga genus and 41 genera of fungi that had been demonstrated to utilize petroleum hydrocarbons. The most important genera of hydrocarbon utilizers in aquatic environments were *Pseudomonas, Achromobacter, Arthrobacter, Micrococcus, Nocardia, Vibrio, Actinetobacter, Brivibacterium, Corynebacterium, Flavobacterium, Candida, Rhodotorula and Sporobolomeces*.

Iqbal *et al.*, (1991) isolated *Bacillus, Pseudomonas, Enterobacter and Escherichia* in Pakistan**.** Bacterial genera belonging to the species *Arthorobacter, Bacillus, Micrococcus, Pseudomonas and Staphylococcus*

were also isolated (Hashem, 1996). In Egypt Kheiralla and Roshdy (1998) found that petroleum fraction gasoil inhibit the growth of a great number of bacteria and fungi, particularly gram-positive bacteria. The microbes were originally isolated from soils at Sues. In Canada many microorganisms have been isolated, which were capable of aerobic or anaerobic Pentachlorophenol breakdown. These include *Flaobacerium sp., Rhodococcus sp, Arthrobacter sp, Pseudomonas sp, Sphingomonas and Mycobacterium sp,. Mycobacterium* and *Pseudomonas* strains were isolated in Canada from a creosote-contaminated soil (Stelmack *et al.*, 1999). In France, *Pseudomonas putida* was isolated from petroleum-contaminated soil by enrichment in a medium containing meta-xylen as the sole carbon source (Hallier *et al.,* 1999). Microorganisms, namely, *Arthrobacter, Burkholderia,Mycobacterium, Pseudomonas, Sphingomonas, and Rhodococcus* were found to be involved for alkylaromatic degradation in a polluted tropical stream in Lagos, Nigeria (Adebusoye *et al.,* 2007).

Walker *et al*., (1975) isolated an alga, *Prototheca zopfi* capable of utilizing crude oil and mixed hydrocarbon substrate it exhibited extensive degradation of n-alkanes and isoalkanes as well as aromatic hydrocarbons. Cerniglia *et a*l., (1980) observed that nine cyanobacteria, five green algae, one red alga, one brown alga, and two diatoms could oxidize naphthalene. Protozoa, by contrast, could not utilize hydrocarbons.

Cerniglia and Perry (1973) found that several fungi (*Penicillium and Cunningghamella spp.*) exhibited greater hydrocarbon biodegradation than bacteria (*Flavobacterium, Brevibacterium and Arthrobacter spp*.). McAllister *et al., (1996)* isolated two fungi those were *Phanerochaete sp and Trametes sp. Asperigllus terreus* has been isolated as dominant fungal species in oil polluted desert soil in Kuwait and was able to degrade petroleum oil hydrocarbon (Al Gounaim and Diab, 1998). In Saudi Arabia, twenty fungal species were isolated from both petroleum- contaminated and uncontaminated

sandy soils, *Aspergillus sp.* was the most dominant species followed by *Penicillium and Cladosporium.* (Hashem, 1996).

Recent studies continue to expand the list of microbial species capable of degrading petroleum hydrocarbons. Fungal genera, namely, *Amorphoteca*, *Neosartorya*, *Talaromyces,*and *Graphium* and yeast genera, namely, *Candida, Yarrowia,* and *Pichia* were isolated from petroleum contaminated soil and proved to be the potential organisms for hydrocarbon degradation (Das and Chandran 2010).

Walker *et al., (*1973*)* compared the abilities of bacteria and fungi to degrade hydrocarbons. The following genera were included in their study: *Candida, Sorobolomyces, Hansenula, Aureobasidium, Rhodotorula Cladosporium, Penicillium, Aspergillus, Pseudomonas, Vibro, Acinetobacter, Leucothrix, Nocardia, and Rhizobium* .Bacteria and yeasts showed decreasing abilities to degrade alkanes with increasing chain length. Filamentous fungi did not exhibit preferential degradation of particular chain lengths.

Both Gram-negative and Gram-positive thermophilic bacteria have been demonstrated to be capable of hydrocarbon utilization. Some isolated thermophiles were obligate hydrocarbon utilizers and could not grow on other carbon sources.

The genetics information for hydrocarbon degradation in these organisms was generally found to occur on plasmids. *Pseudomonas* species used for genetic engineering and the first successful test case was in the United States (Chakrabarty *et al.,* 1973).

2-3-3 Bacterial Bioremediation:

Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in environment (Rahman *et al.,* 2003; Brooijmans *et al.,* 2009; Das and Chandran 2010). Several bacteria known to feed exclusively on hydrocarbons (Das and Chandran, 2010). Bacterial genera, namely, *Gordonia*, *Brevibacterium*, *Aeromicrobium, Dietzia*,

Burkholderia, and *Mycobacterium* isolated from petroleum contaminated soil proved to be the potential organisms for hydrocarbon degradation (Chaillan *et al.,* 2004). The degradation of polyaromatic hydrocarbons by *Sphingomonas* reported by Das and Chandran, (2010). *Alcaligenes*, *Corynebacterium, Flavobacterium*, *Achromabacter, Pseudomonas, Arthrobacter Micrococcus, Nocardia, and Mycobacterium* appear to be the most consistently isolated hydrocarbon degrading bacteria from soil (Malik and Ahmed, 2011).

The predominant degraders of organ pollutants in the toxic zone of contaminated areas are chemo-organotrophic species able to use a huge number of natural and xenobiotic compounds as carbon sources and electron donors for the generation of energy. Mixed cultures carry out more extensive biodegradation of petroleum than pure cultures (Sun *et al.*, 2004; Trindade *et al.*, 2004; Ghazali *et al.*, 2004; Malatova, 2005; Oteyza *et al.*, 2005; Gerdes *et al.*, 2005).

Although many bacteria are able to metabolize organic pollutants, a single bacterium does not possess the enzymatic capability to degrade all or even most of the organic compounds in polluted soil. Mixed microbial communities have the most powerful biodegradative potential because genetic information of more than one organism is necessary to degrade the complex mixtures of organic compounds present in contaminated areas (Malik and Ahmed, 2011; Das and Chandran, 2010).

2-4 Metabolisms of Hydrocarbon:

Bacteria can live at substantial depth within a sediment column (Parkes *et al.*, 1994). Oil biodegradation by bacteria can occur under both toxic and anoxic conditions (Zengler *et al.*, 1999). Microorganisms can break down petroleum hydrocarbon, in presence of oxygen or in its absence (Grishchenkov *et al.,* 1999).

The first process, called aerobic, Bacteria utilize oxygen as the primary electron acceptor. It gives carbon dioxide and water as the final products

(Jyothi, *et al.,* 2012). Aerobic degradation usually proceeds more rapidly and considered more effective than anaerobic degradation. One reason is that aerobic reactions require less free energy for initiation and yield more energy per reaction (Thapa *et al.*, 2012; Malatova, 2005). The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions (Das and Chandran, 2010).

Hydrocarbons broken down by a series of enzyme-mediated reactions (Thapa *et al.,* 2012). Oxygen serves as an external electron acceptor, while an organic component of the contaminating substance functions as the electron donor or energy source. The general degradation pathway for an alkane involves sequential formation of an alcohol, an aldehyde and a fatty acid. Fatty acid cleaved, releasing carbon dioxide and forming a new fatty acid that is two carbon units shorter than the parent molecule in a process known as beta-oxidation. The initial enzymatic attack involves a group of monooxygenases.

The general pathway for aromatic hydrocarbons involves cis-hydroxylation of the ring structure forming a dihydric alcohol (diol) (e.g. catechol) using dioxygenase. The ring oxidatively cleaved by dioxygenases, forming a dicarboxylic acid (e.g. muconic acid). Oxidation of substituted aromatics generally proceeds by initial beta-oxidation of the side chain, followed by cleavage of the ring structure. The degradative pathway for a highly branched compound, such as pristane or phytane, may proceed by omega oxidation forming a dicarboxylic acid, instead of only monocarboxylic acid. (Malatova, 2005).

In the second processes, they utilize an alternative electron acceptor such as nitrate or sulfate. It gives some partial breakdown products. The nature of which will depend on the microbe concerned. The anaerobic process, called fermentation, can gives end products such as methane, alcohols, organic acids and perhaps carbon dioxide (Dirar, 2000).

During bioremediation, microbes utilize chemical contaminants in soil as an energy source through oxidation-reduction reactions, metabolize the target contaminant into useable energy for microbes. By-products (metabolites) released back into the environment are typically in a less toxic form than the parent contaminants. Hydrocarbon loses electrons and oxidized while oxygen gains electrons then reduced. The result is formation of carbon dioxide and water (Nester *et al*., 2001).

When oxygen is limited in supply or absent, as in saturated or anaerobic soils or lake sediment, anaerobic (without oxygen) respiration prevails. Generally, inorganic compounds such as nitrate, sulfate, ferric iron, manganese, or carbon dioxide serve as terminal electron acceptors to facilitate biodegradation (State of Mississippi, Department of Environmental Quality, 1998).

2-5- The Advantages and Disadvantages of Bioremediation on Site and in Situ:

In many ecosystems, there is already an adequate indigenous microbial community capable of extensive oil biodegradation, if environmental conditions are favorable for oil-degrading metabolic activity (Malatova, 2005). When few or no indigenous degradative microorganisms exist in a contaminated area and practically does not allow time for the natural enrichment of suitable population, inoculation may be a convenient option (Ilyina *et al.,* 2003). Bio-augmentation is the addition of a group of indigenous microbial strains or genetically engineered microbes to treat the contaminated soil. It is effective where native microorganisms not identified in the soil or do not have the metabolic capability to perform the remediation process (Thapa *et al.,* 2012).

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. Ojo (2006) reported that the application of a consortium of native bacterial species in bioremediation processes has long been desired in Nigeria because they would be cost effective and efficient in terms of acclimation time.

Secondly, the ability to utilize hydrocarbons distributed among a diverse microbial population. This population occurs in natural ecosystems and either independently or in combination metabolizes various hydrocarbons.

Nutrient availability, especially nitrogen and phosphorus, seems to be the most limiting factor. It was confirmed that these nutrients enhance growth of microorganisms, which leads to more rapid decomposition of contaminants (Chaineau *et al*., 2005; Malatova, 2005; Coulon *et al*., 2005.) Soil type is an important consideration when determining bioremediation suited approach to a particular situation.

On Site bioremediation refers to treatment of soil in place. Although microorganisms presented in contaminated soil, they cannot necessarily be there in the numbers required for bioremediation of the site. Their growth and activity must be stimulated (Thapa, *et al.,* 2012). In situ biostimulation treatments usually involve bioventing, in which oxygen and/or nutrients pumped through injection wells into the soil. It is imperative that oxygen and nutrients distributed evenly throughout the contaminated soil (Salleh *et al.,* 2003).

Soil texture directly affects the utility of bioventing, as much as permeability of soil to air and water is a function of soil texture. Fine-textured soils like clays have low permeability, which prevents biovented oxygen and nutrients from dispersing throughout the soil. It is also difficult to control moisture content in fine textured soils because their smaller pores and high surface area allow it to retain water. Fine textured soils are slow to drain from

water-saturated soil conditions, thus preventing oxygen from reaching soil microbes throughout the contaminated area (US EPA, 2006).

In Situ bioremediation causes minimal disturbance to the environment at the contamination site. In addition, it incurs less cost than conventional soil remediation or removal and replacement treatments because there is no transport of contaminated materials for off-site treatment.If the challenges of bioremediation, particularly of in situ techniques, could be overcome, bioremediation has potential to provide a low cost, non-intrusive, natural method to render toxic substances in soil less harmful or harmless over time. Currently, research is being conducted to improve and overcome limitations that hinder bioremediation of petroleum hydrocarbons.

2-6 Factors Affecting Bioremediation:

Petroleum hydrocarbons may be completely biodegraded within a few hours or days depending upon many factors (Malik and Ahmed, 2011). Most of the petroleum compounds are biodegradable but the process is varying according to the prevailing conditions (Thapa, *et al.,* 2012; Malik and Ahmed, 2011). The indigenous microorganisms in the soil or water can clean the contamination with petroleum very slowly and may take years. Inoculations with bacteria with hydrocarbon biodegradation capabilities can precede the process. (Marquez-Rocha *et al*., 2000).

The extent of hydrocarbon biodegradation in contaminated soils is critically dependent upon many factors such as: creation of optimal environmental conditions to stimulate biodegradative activity, predominant petroleum hydrocarbon types in contaminated matrix and bioavailability of contaminants to microorganisms and petroleum hydrocarbon compounds bind to soil components, which it's difficult to removed or degraded. Hydrocarbons differ in their susceptibility to microbial attack. However, the constituents of oil differ distinctly in volatility, volubility, and susceptibility

to biodegradation. Some compounds are easily degraded, some resist degradation and some are non-biodegradable (Thapa *et al.,* 2012).

The chemical structure of hydrocarbons affects its biodegradation into two ways. Firstly, the molecule may contain groups or substituents that cannot react with available or inducible enzymes. Secondly, the structure may determine the compound to be in a physical state where microbial degradation does not easily occur. Usually, the larger and more complex structure of a hydrocarbon, the more slowly oxidized. In addition, the degree of substitution affects the degradation. Compounds that contain amine, methoxy and sulfonate groups, ether linkages, halogens and branched carbon chains are generally persistent. Adding of aliphatic side-chains increases the susceptibility of cyclic hydrocarbons to microbial attack (Riser-Roberts, 1992).

The susceptibility of hydrocarbons to microbial degradation generally ranked as follows:

Linear alkanes > branched alkanes > small aromatics > cyclic alkanes.

Some compounds, such as hydrocarbon with high molecular weight and polycyclic aromatic hydrocarbons (PAHs), might not be degraded at all (Das and Chandran, 2010).

Microorganisms have limits of tolerance to particular environmental conditions, as well as optimal conditions for pinnacle performance. Factors that affect success and rate of microbial biodegradation include nutrient availability, moisture content, pH, and temperature of the soil matrix. (Walworth et *al*., 2005).

2-6-1 Temperature:

Temperature plays an important role in biodegradation of hydrocarbons by directly affecting chemistry of pollutants as well as the physiology and diversity of microbial flora (Meintanis, *et al.,* 2005).Temperature influences the rate of biodegradation by affecting rate of enzymatic activity of

microorganisms. Generally, “speed of enzymatic reactions in the cell approximately doubles for each 10^{oc} rise in temperature” (Nester *et al*., 2001).

Most bacteria found in soil, including many bacteria that degrade petroleum hydrocarbons, are mesophiles, which have an optimum temperature ranging from 25 ^{oc} to 45 ^{oc} (Nester *et al*., 2001).

Temperature influences petroleum biodegradation by its effect on physical and chemical composition of oil, rate of hydrocarbon metabolism by microorganisms, and composition of the microbial community (Atlas, 1981). At low temperatures, viscosity of oil increases which determines the spreading and dispersion of hydrocarbon mixture and also surface area available for microbial attack. The variability in the physicochemical character of hydrocarbons causes changes in behavior of individual hydrocarbons as well as its mixtures (Malatova, 2005).

2-6-2 Oxygen:

Degradation of hydrocarbons by bacteria and fungi involve oxidation of the substrate by oxygenases, for which molecular oxygen is required. Aerobic conditions are therefore necessary of microbial oxidation of hydrocarbons in environment. Conditions of oxygen limitation normally do not exist in the upper levels of water column in marine and freshwater environments. Aquatic sediments, however, are generally anoxic except for a thin layer at the surface of the sediment. The availability of oxygen in soils is dependent on rates of microbial oxygen consumption, the type of soil, soil waterlogged, and the presence of utilizable substrates, which lead to oxygen depletion.

Anaerobic degradation of petroleum hydrocarbons by microorganisms has been shown in some studies to occur only at negligible rates, and its ecological significance has been generally considered to be minor (Joseph and Rita, 1990). However, microbial degradation of oxidized aromatic compounds such as benzoate, and of halogenated aromatic compounds such as the

halobenzoates, chlorophenols, and polychlorinated biphenyls, has been shown to occur under anaerobic conditions. Recent evidence also indicates that microbial consortia from soil and sludge are capable of metabolizing unsubstituted and alkyl-substituted aromatics, including benzene, toluene, xylene, 1,3-dimethylbenzene, cenaphthene, and naphthalene, in the absence of molecular oxygen.

2-6-3 Nutrient Availability:

Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants especially nitrogen, phosphorus, and in some cases iron (Joseph and Rita, 1990). Atlas, 1985, reported that when a major oil spill occurred in marine and freshwater environments, the supply of carbon was significantly increased and the availability of nitrogen and phosphorus generally became the limiting factor for oil degradation.

Important inorganic nutrients include nitrogen and phosphorus that are necessary for microbial activity and cell growth. Treating of petroleum-contaminated soil with nitrogen increase cell growth rate, decrease microbial lag phase, help to maintain microbial populations at high activity levels, and increase rate of hydrocarbon degradation (Walworth *et al.*, 2005). However, excessive nutrient concentrations can also inhibit the biodegradation activity (Chaillan *et al.*, 2004).

2-6-4 Moisture Content:

All soil microorganisms require moisture for cell growth and function. Availability of water affects diffusion of water and soluble nutrients into and out of microorganism cells. However, excess moisture, in saturated soil, is undesirable because it reduces the amount of available oxygen for aerobic respiration. Anaerobic respiration, which produces less energy for microorganisms (than aerobic respiration) and slows the rate of biodegradation, becomes the predominant process. Soil moisture content "between 45 and 85 percent of water-holding capacity (field capacity) of soil

or about 12 percent to 30 percent by weight" is optimal for petroleum hydrocarbon degradation (USA, EPA, 2006, "Landfarming").

2-6-5 Soil pH:

Soil pH is among the important because most microbial species can survive only within a certain pH range. Furthermore, soil pH can affect availability of nutrients. Biodegradation of petroleum hydrocarbons is optimal at a pH 7 (neutral); the acceptable range is pH 6 – 8 (USA EPA, 2006).

2-6-6 Soil Type:

Contaminants can absorb to soil particles, rendering some contaminants unavailable to microorganisms for biodegradation. Thus, in some circumstances, bioavailability of contaminants depends not only on the nature of the contaminant but also on soil type. Hydrophobic contaminants, like petroleum hydrocarbons, have low solubility in water and tend to absorb strongly in soil with high organic matter content. In such cases, surfactants utilized as part of the bioremediation process to increase solubility and mobility of these contaminants (State of Mississippi, Department of Environmental Quality, 1998. Additional research findings of the existence of thermophilic bacteria in cool soil also suggest that high temperatures enhance the rate of biodegradation by increasing the bioavailability of contaminants. It is suggested that contaminants absorbed to soil particles are mobilized and their solubility increased by high temperatures (Perfumo *et al.*, 2007).

2-7 Successful Bioremediation Programs:

Several innovative and successful bioremediation programs have been conducted by Solmarcorporation. (Molnaa, and Grubbs) *http://books.google.com* in conjunction with various environmental engineering firms and remediation contractors, i.e.

A- Bioremediation selected as the method of choice to clean up an abandoned refinery site in southern California. The thirty tow acre site located in a prime industrial area and the goal was to clean the site to a low enough

level that commercial building could be built. The site was sectioned off into several treatment zones, and a bioremediation program was begun using a consortia of microorganisms supplied. While areas were treated, other areas taken out of service until the entire tank farm dismantled within a period of one year.

B- Bioremediation was the method of treatment opted for to treat 1500 cubic yards of diesel contaminated soil at the former King's Truck Stop in Sacramento, C A. The project reduced the diesel contaminant levels from 3000 PPM to less than 30 PPM in approximately 62 treatment days.

C- In 2002: The Indonesian Ministry of Environment granted approval for CPI (Chevron Pacific Indonesia) to begin full-scale bioremediation treatment operations in Sumatra. In 2003-present: Using bioremediation technology, CPI has successfully treated more than half a million cubic meters of soil, the equivalent in size to 200 Olympic-sized swimming pools. The treated soil used for re-greening of 60 hectares of land in Riau Province, Sumatra the equivalent of approximately 75 football fields.

CHAPTER THREE

MATERIALS AND METHODS

3-1-1 Collection of Samples:

Soil samples were collected from petroleum hydrocarbons contaminated soils from Shendi and Atbara, River Nile State, Sudan. A scoop was used to remove debris of organic particles from the surface of the soil. One Kg soil sample at a depth of 0-10 cm. was collected from each site at random with sterile spatulas.

Twenty three soil samples were collected from different sites in Shendi. Eighteen samples from the Industrial Station and coded, (D), 4 samples from Nile company old location petrol storage near the center of Shendi University and coded (S) and one sample from Aljayli workshop coded (W). Two samples were collected from White Nile Company, Atbra and coded (N and G).

Water samples collected from Aljayli refinery particularly from water treatment sinks of produced water (code A, B, and C) in sterile container. Crude oil was obtained from Aljayli refinery.

3-1-2 Sterilization of Glassware:

Before sterilization, glassware were washed thoroughly and left to dry; then they were sterilized in a hot dry oven at 160°C for 3 hours (Harrigan, 1998).Instruments and tools such as loops, needles and forceps were sterilized by flaming directly or after dipping in ethanol.

3-1-3 Sterilization of Media:

Nutrient agar and basal salt media were sterilized by autoclaving according to Harrigan, 1998.

3-1-4 Growth of Bacteria in Crude Oil and Isolation of Microorganisms:

One gram (1g) of soil sample was suspended in 250 ml Erlenmeyer flask containing 100 ml of basal salt medium (APP 1) with 1% of crude oil as sole carbon and energy source after sterilization by autoclaving at 121^{oc} for 15

minutes. The mixture was shaken for 10 hours daily on a rotary shaker at room temperature 25^{oc} for 10 days. After this period a volume of 0.1 ml of each flask was spread on nutrient agar medium (APP 2) in Petri dishes then was incubated at 37^{oc} for 48 hours. Colonies were then picked out and purified for further studies. Growth of bacteria indicates the capability of degradation (Elkhir, 2007).

The degree of degradation estimated visually. The visual observation was based on:

A- The change in the color of crude oil and the clearance of the medium.

B- Microbial growth.

C- The disappearance of the oily surface.

The results were recorded in the form of pluses (1-4 pluses) (Elkhir, 2007).

3-2-Identification of Bacteria:

Identification of pure culture of the best isolate was carried out according to Harrigan (1998), Cheesbrough (2000) and Bergey's Manual of Systematic Bacteriology (1984). The purified colonies were subjected to the following tests:

3-2-1 Gram's Staining Method:

An overnight colony was picked carefully with sterile wire loop. The colony was emulsified in a drop of physiological saline (0.85% NaCl) placed on a clean slide and spread evenly to make a thin film. The slide was allowed to dry. The smear was fixed by using flame. Then the smear was stained with crystal violet solution for 2 minutes, then rinsed rapidly with water and iodine solution was added and the smear was blotted dry. Slide was washed with 95% ethanol for 15 seconds, then rinsed with tap water and at last stained with 1% safranine for 20 seconds then rinsed with tap water and examined under microscope (Harrigan 1998).

3-2-2 Catalase Test:

This test was done to demonstrate the presence of catalase enzyme that catalyzes the release of oxygen from hydrogen peroxide. One ml of 3% hydrogen peroxide solution was placed in a small clean test tube. A loopful of 24 hr culture was added. The release of bubbles (of oxygen) indicated the presence of catalase in the culture under test (Harrigan, 1998).

3-2-3 Oxidase Test:

A piece of filter paper was impregnated with oxidase test solution (Tetra methyle-p- phenylene diamine hydrochloride). Then a loopful of 24 hours culture was streaked onto the filter paper. A positive reaction was indicated by purple color after 10-15 seconds. Any later reaction was recorded as negative (Harrigan, 1998).

3-2-4-Motility Test:

A tube of motility medium (APP3) was inoculated with 24 hours culture. This was done aseptically using a straight wire loop to half depth of the tube and incubated at 37^{oc}. After incubation and during growth, motile bacteria will migrate from the line of inoculation to form turbidity in the surrounding medium; non-motile bacteria will grow only along the line of inoculation.

3-2-5-Fermentation of Carbohydrate:

According to Harrigan (1998), the medium used to detect fermentation of sugar is 1.5g peptone water, 1.0g test sugar, 1.0 ml Andrade's indicator and 100 distilled water. Durham tubes were inverted and tubes incubated at 37°C for 7 days and examined every 2 days for acid or acid plus gas.

3-2-6-Voges - Proskauer Test:

Five ml of glucose phosphate broth (containing: 5.0 g peptone, 5.0 g glucose, 5.0 g dipotassium hydrogen phosphate, and 1000 ml distilled water) were inoculated with the tested cultures, and then incubated at $37^{o}C$ for two days. Half ml of culture was added to 0.5 ml 5 % naphthol solution and 0.5 ml

16% KOH. Development of a red coloration at the surface was recorded as positive reaction.

3-2-7-Hydrolysis of Starch:

Nutrient agar containing 0.2% soluble starch was sterilized by autoclaving at 121^{oc} for 20 minutes. The poured solidified and dried plate of the medium was inoculated with spot of the culture in the center of dish and incubated at 37^{oc} for 2-4 days. The plate was flooded with 5 ml of iodine solution which consists of 5g iodine, 10g potassium iodide and 100ml distilled water (Barnett, 2000). A clear zone around the growth indicated starch utilization.

3-2-8-Reduction of Nitrate to Nitrite:

Nitrate peptone water is the medium consisting of peptone water and 0.2g potassium nitrate. It was distributed in test tubes in 5 ml quantities. To each a Durham tube was added in an inverted position and was sterilized by autoclaving and then inoculated by adding a small amount of culture and incubated at 37^{oc} for 2-7 days. One ml each of two reagents (Griess-llosvay's reagent) (Harrigan, 1998) was added to the culture. The development of a red color within a few minutes indicated the presence of nitrite. Gas in Durham tubes showed positive results. The negative results were confirmed by the addition of a small quantity of zinc dust to the tube. This is to reduce any nitrate still present to nitrite. Thus, the development of a red color indicates that nitrate remains.

3-2-9-Utilization of Citrate:

A slope of Simmon's citrate agar (Oxoid) with bromthymol blue as a pH indicator was incubated by streaking over the surface with a loopful of culture and incubated at 37°C for up to 7 days. The change of the medium from green to bright blue was indication of positive test.

3-2-10-Growth Temperature Tests:

Test tubes containing nutrient broth were sterilized by autoclaving then inculcated with a small amount culture of different isolates. Tubes were incubated at 42 ^{oc} and 5 ^{oc}. Growth was indicated by turbidity of culture media.

3-3- Analysis of the Oil:

Oil was extracted from cultures by liquid – liquid extraction as follows: Sixty ml of normal hexane was added to fermentation flask and shaken well until the oil dissolved. The mixture was pure in separating funnel. Bacterial cells were removed by centrifugation at 30.000 for 15 min. The normal hexane was evaporated with heat at 70^{oc}. (Elkhir, 2007).

The samples to be analyzed were injected into a gas chromatograph in Central Petroleum Laboratories, Khartoum. Di- Chloromethylen was added to the samples to be lighter.

Method:	Iana crude oil std Gailli (12\8\2012).
Instrument:	Varian 3800 GC.
Module Address:	44, 8410 Auto sampler.
That is equipped with:	25m long. PONA column
Coating with Sil Pona:	0.21mm.
Injection Mode:	Split.

3-3-1Condition of the Test:

Temperature:	250^{oc}.
Carrier Gas:	N2.
Flow Control Mode:	Pressure.
Pressure:	80.4 kpa.
Total Flow :	24.0ml\min.
Column Flow:	1.00ml\min.
Linear Velocity:	25.0cm\sec.
Purge flow:	3.0ml\min.

Column temperature program was setup as.

initial temperature ;	40.^{0C} .
Equilibration Time:	3.0 min.
Total program Time:	76.50 min.
And detecter chanal temperature:	300. ^{OC}.
Sampling Rate:	40 msec.
Makeup Gas:	N2.
Makeup flow:	30.0 ml/min.

3-3-2 Calculations:

% of oil degradation =

[(n-Alkane control – n-Alkane treatment) / n-Alkane control] x 100.

CHAPTER FOUR

RESULTS AND DISCUTION

4-1 The Relative Observed Performance of Bacterial Isolates on Crude oil Degradation:

The screening was done as described in 3-1-4.All of bacterial isolates shown in tables1,2,and 3 were grown on crude oil as a sole source of carbon and energy. Many researchers isolates bacteria from contaminated soil capable of degrading light crude oil, Palittapongarnpim *et al.,* (1998) isolated bacteria from contaminated soil in Bangkok in Thailnd, Nor Eldin and Elsanousi (2005) isolated bacteria associated with soil water contaminated by oil from Khartoum state. Microorganisms such as bacteria, fungi, yeast and microalgae can degrade petroleum hydrocarbons. Bacteria play the central role in hydrocarbon degradation (Malatova, 2005).Biodegradation of oil pollutants is not a new concept as it has been intensively studied in controlled condition (Sugiura *et al*., 1997; Chaillan *et al*., 2004) and in open field experiments (Chaŷneau *et al*., 2003; Gogoi *et al*., 2003).

4-2 Visual Estimation of Biodegradation of Crude Oil:

The degree of degradation estimated visually as in 3-1-4. The results were recorded in the form of pluses, to compare the performance of isolates. The percentage of 4 pluses (++++), three pluses (+++) and two pluses (++) in each group were computed. in the case of isolates from Industrial Station in Shendi (Table 1) there are 4 isolates recorded 4 pluses (++++) which made 8% giving very good performance. In the same table those isolates scoring three pluses (+++) made 26% of total isolates which giving good performance, 38% recorded for isolates with two pluses (++) giving fair performance and 28% for weak isolates.

Table 2 shows estimation performance of oil degradation by bacteria isolated from old Nile Company old location in Shendi, White Nile Petroleum Company in Atbar a and from Aljayli work shop in Shendi.

Five of the isolates showed very good performance, which gave 23.9% of the isolates with very good performance, 4.8% recorded for good performance, 28.6% for fair performance and 42.9% for weak performance. Table 3 shows estimation performance of oil degradation by bacteria isolated from Aljayli refinery.23.1% of the isolate showed fair performance and 76.9% for weak isolate. These isolates were taken from treated produced water. The isolates were weak in utilizing hydrocarbon individually there was no very good performance, the original isolation flask of these isolates give high degree of degradation in short time. Individual microorganisms are capable of degrading only a limited number of crude oil components. Thus, more extensive degradation of oil resulted where the presence of metabolically diverse microbial communities in the soil. The result is similar to those reported by Atlas (1981), Frontera-suau *et al.,* (2002).

In table 1 there is 17 isolates gave a high degree of degradation individually and in table 2, 6 isolates gave a high degree of degradation individually. This finding agrees with the result found by Elkhir (2007) who isolated bacteria from different location including Aljayli refinery to test their degradation ability.

Table 1 and 2 show high growth of bacteria on crude oil, compared to isolates from table 3. The isolates in table 1 and 2 have been obtained from very old petrol-contaminated sites. The isolates had high ability to degrade crude oil may be due to the fact that the changes in microbial community structure and diversity as a response to environmental stress. (MacNaughton *et al.,*1999 Lawton,1994.

For the 125 isolates we can say 7.2% were very good, 11.2% were good, 22.4% were fair and 59.2% were weak, these results shows in (Fig1).

Table1.Visual estimation of oil degradation degree by bacteria isolated from soil samples of Industrial station in Shendi city (D).

Code of source	Code of Isolate	The degree of Degradation
D.1	D.1.2	+++
	D.1.6	++
	D.1.7	+
D.2	D.2.9	+
	D.2.10	++++
D.3	D.3.15	++
D.4	D.4.20	+
	D.4.21	+++
D.5	D.5.26	++
	D.5.29	+++
	D.5.30	++
	D.5.31	++
D.6	D.6.34	+++
D.7	D.7.41	++
D.8	D.8.42	+++
	D.8.44	+
	D.8.45	++
D.9	D.9.46	+
	D.9.47	++
	D.9.48	+
	D.9.49	++++
D.10	D.10.50	++
	D.10.51	++
D.11	D.11.54	++++
	D.11.55	++++
	D.11.56	++
	D.11.57	++
	D.11.58	++
	D.11.60	+++
D.12	D.12.61	+
	D.12.64	+++

Table 1. Continued:

Code of source	Code of Isolate	The degree of degradation
D.13	D.13.65	+
	D.13.66	++
	D.13.67	+++
D.15	D.15.74	+
	D.15.75	+
	D.15.76	+++
D.16	D.16.77	+
	D.16.78	++
	D.16.81	++
	D.16.82	+
	D.16.83	++
D.17	D.17.84	+
	D.17.85	+++
	D.17.86	++
	D.17.87	+
D.18	D.18.88	++
	D.18.89	+++
	D.18.90	+++
	D.18.91	+++

+ Weak

++ Fair

+++ Good

++++ v.good

+++++ Excellent

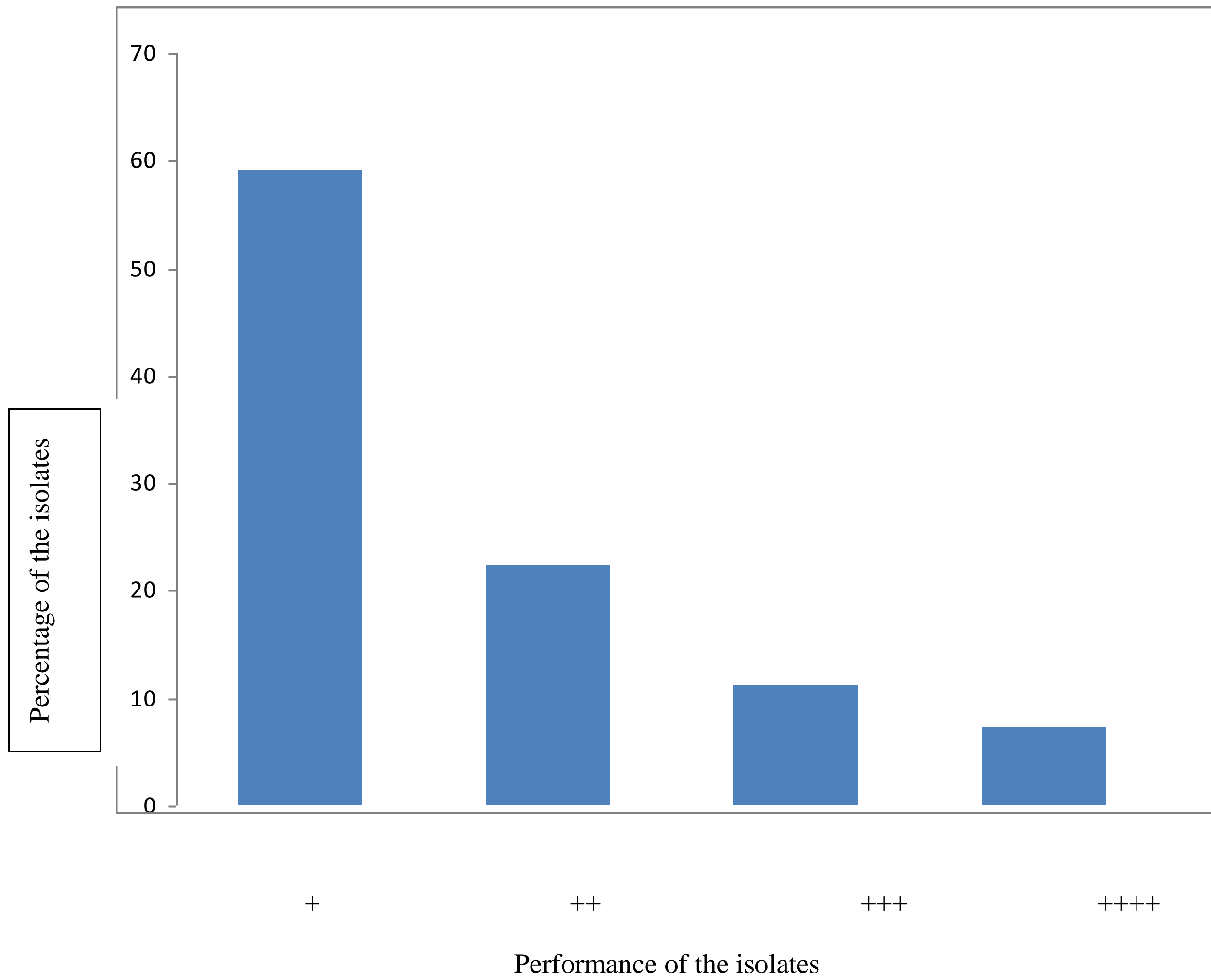
Table 2. Visual estimation of the degree of oil degradation by bacteria isolates from samples of soil from Nile company old location in Shendi (S) and White Nile Petroleum Company in Atbara (N,G). and from Aljayli work shop in Shendi (W).

Code of source	Code of isolate	Degree of degradation
S.19	S.19.92	++
	S.19.93	++
S.20	S.20.94	++++
	S.20.95	++++
	S.20.96	++++
	S.20.97	+
S.21	S.21.98	+
	S.21.99	+
	S.21.100	+
S.22	S.22.101	++
	S.22.102	
W.23	W.23.103	++
	W.23.104	++
	W.23.105	++
	W.23.106	+
N.27	N.27.120	++++
	N.27.121	+
	N.27.122	++++
	N.27.123	+
G.28	G.28.124	+
	G.28.125	+

Table 3. Visual estimation of the degree of oil degradation by bacteria isolates from samples of water from Aljayli refinery(A,B,C)

Code of source	Code of isolate	Degree of degradation
Samples isolated from produced water		
A.24	A.24.107	+
	A.24.108	++
	A.24.109	++
	A.24.110	+
	A.24.111	+
B.25	B.25.112	+
	B.25.113	+
	B.25.114	+
	B.25.115	+
C.26	C.26.116	+
	C.26.117	++
	C.26.118	+
	C.26.119	+

Fig (1)



Visual Estimation of Oil Degradation by Isolated Bacteria

4-3 Degradation percentage by weight:

The best eleven isolates were selected for further studies namely D.1.2, D.2.10, D.9.49, D.11.54, D.11.55. (From industrial station in Shendi), S.20.94, S.20.95, S.20.96. (from Nile Company old location in Shendi), W.23.103.(from Aljayli work shop in Shendi) N.27.120, and N.27.122.(from White Nile Petroleum Company in Atbra). These then used in experiments to see the degree of degradation they attained in an oil-mineral medium, the results were presented in table (4). The percentage degradation calculated after 10 days of growth. The degree of degradation was measured according to the methods described in 3-3. There was a variation in degradation depending on the isolate, isolate N27.122 showed the greatest percentage of degradation it gave 72%, while isolate N27.120 recorded 62%. Five more isolates had percentage of degradation range from 51% to 58%, one isolate had 40% and tow isolates had 33% and 31%. Other combinations between isolates gave degradation ratio ranged from 61% to 36%.In many ecosystems there is already an adequate indigenous microbial community capable of extensive oil biodegradation, provided that environmental conditions are favorable for oil-degrading metabolic activity, 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 (Malatova, 2005).

4-4-1Tentative Identification of bacteria:

The studying of the diversity of indigenous microorganisms capable of degrading pollutant (such as crude oil polycyclic aromatic hydrocarbon and polychlorinated biphenyl) in different environment is taking a great interest nowadays. Thus in this study we tried to tentatively identified the bacterial isolates.

Table 4. Estimation of degradation percentage by weight.

Code of isolate	Weight after degradation	Loss of weight after degradation	Percentage of degradation
D.1.2	0.67	0.33	33%
D.2.10	0.49	0.51	51%
D.9.49	0.60	0.40	40%
D.11.54	0.49	0.51	51%
D.11.55	0.69	0.31	31%
S.20.94	0.42	0.58	58%
S.20.95	0.44	0.56	56%
S.20.96	0.48	0.52	52%
W.23.103	0.64	0.36	36%
N.27.120	0.38	0.62	62%
N.27.122	0.28	0.72	72%

Weight of crude oil before degradation was 1 gm.

4.4.2. Morphological characteristics of the isolates:

All the isolated strains were gram negative and single short rod. Colonies on nutrient agar were moderate in size, smooth with rounded edges. Some of the colonies produced green pigment in basal medium.

4.4.3 Some biochemical tests of the isolates:

Table 5 and table 6 showed some biochemical tests of the isolates. They were catalase Positive, some oxidase positive and some aren't, metabolized glucose fermentatively in Hugh and Leifson medium.

Isolate S20.94 and S20.95:

Those two isolates identified as *Pseudomonas aeruginosa* their biochemical tests were shown in table 6. They produced green pigment, which diffused in the medium, and could grow at 42°C but not at 5°C, and those didn't produced green pigment and couldn't grow at 42°C nor at 5°C they tentatively identified as *Pseudomonas putida*.

Similar results found by Elkhir (2007) and Nour Eldin and Elsanousi (2005), they isolated this species from Khartoum state and they found that it was effective in degrading crude oil. Latha and Kalaivani (2012) isolated these species from an Indian contaminated hydrocarbon soil and tested it their ability for degradation. The same organisms isolated by Mittal and Singh (2009) from contaminated sites they found that out of twenty isolates, only five strains were potent hydrocarbon degraders and capable of degrading crude oil, particularly most of representative fractions of petroleum. Singer and Finnerty (1984) reported that among the hydrocarbon utilizing bacteria *Pseudomonas aeruginosa* was one of the most frequently isolated from hydrocarbon-impacted environment.

S20.96 and N27.120:

This isolates tentatively identified like *Pseudomonas putida*. The results agreed with a number of studies, Alan *et al.* (1975) studied the growth of *Pseudomonas putida* PPG6 on purified n-alkanes. Chakrabarty *et al.* (1973)

and Nieder and Shapiro (1975) reported that *Pseudomonas putida* strains grown on n-alkanes of 6 to 10 carbon atoms by virtue of the transmissible OCT plasmid. *Pseudomonas putida* could degraded n-alkanes Margesin and Shinner, (2001). Also Faizal *et al.* (2005) isolated *P. putida* from an activated sludge.

Pseudomonas appears to be the ubiquitous bacteria found in oil contaminated soils and soil in general. These bacteria could adapt to many different hydrocarbons. They are responsible for degrading most of the aromatics in gasoline, although the efficiency in degrading aromatics hydrocarbons can vary among strains (Malatova, 2005).

Table 6 showed Some biochemical tests for the isolates N27.122, D1.2, D2.10, D9.49, D11.54, D11.55 and W23.103. Referenced to Bergey's Manual of Systematic Bacteriology (1984) and Harrigan, (1998). Those isolates are catalase-positive, oxidase-negative, rod-shaped bacteria and they motile. Facultatively anaerobic, they ferment glucose rapidly with or without gas production with exception of strain D11.55 they reduced nitrate to nitrite. Strains were tentatively identified as follows: N27.122 as *Serratia marcences*, strain D1.2 and D2.10 as *Cedeca davicae,* 11.54 and D9.49 as *Enterobacter cloacae*, D11.55 as *Yersinia enterocolytica* and W23.103 as *Citrobacter freundii*.

Isolate N27.122:

Isolate N27.122 identified as *Serratia marcences* which exhibited the highest emulsification ability and though the strongest adherence to the oil from all investigated microorganisms in this study. Same result found by Malatova (2005) and Ijah (1998). In spite of the fact that *Serratia* is not considered to be a main representative of hydrocarbon degraders, this bacteria has been isolated from oil polluted soil and groundwater contaminated with gasoline.

Table 5. Some biochemical tests for the isolates *Pseudomonas spp* S20.94, S20.95, S2096, and N27.120.

Tests	S20.94	S2095	S20.96	N27.120
Shape	Rod	Rod	Rod	Rod
Gram reaction	-ve	-ve	-ve	-ve
Motility	+	+	+	+
Growth on air	+	+	+	+
Catalase	+	+	+	+
Oxidase	+	+	+	+
Carbohydrate	O	O	O	O
Acid from:				
Glucose	+	+	+	+
Lactose	-	-	-	-
Maltose	-	-	-	-
Mannitol	+	+	-	-
Fructose	+	+	+	+
Indole	-	-	-	-
Lysine	-	-	-	-
Xylose	+	+	+	+
Citrate	-	-	-	-
Urease	-	-	-	-
Starch hydrolysis	-	-	-	-
Nitrate to nitrite	+	+	-	-
Voges Proskauer	-	-	-	-
Pigment	+	+	-	-
Growth at 42°C	+	+	-	-
Growth at 5°C	-	-	-	-
Utilization of citrate	+	+	+	+
Tentative genus	*p. aeruginosa*	*P. aeruginosa*	*P. putida*	*P. putida*

Table:6 Biochemical tests for the isolates N27.122, D1.2, D2.10, D9.49, D11.54, D11.55 and W23.103.

Tests	N27.122	D1.2	D2.10	D11.54	D9.49	D11.55	W23.103
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram reaction	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Motility	+	+	+	+	+	+	+
Growth on air	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-
Acid from:							
Lactose	-	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Sucrose	-	+	+	+	+	+	-
Malonate utilization	-		+	+	+	+	-
Indole	-	-	-	-	-	-	-
Lysine	+	-	-	-	-	-	-
Utilization of citrate	+	+	+	-	-	-	+
Voges Proskauer	+	-	-	+	+	+	+
Nitrate to nitrite	+	+	+	+	+	-	+
Pigment	+			+	+		
Tentative genus	*Serratia marcences*	*Cedecea davicae*		*Enterobacter cloacae*		*Yersinia enterocolytica*	*Citrobacter freundii*

It has showed very good crude oil and gasoline degradability. Ibrahim *et al*., (2013) isolated *Serratia marcescens* as crude oil utilizing bacteria from Nigeria soil by enrichment method. A novel bacterial strain, *Serratia sp*. BF40, was isolated from crude oil contaminated saline soils Binzhou, China, Its salt-tolerance, surface activity and has the ability to degrade crude oil in saline soils (Wu *et al*., 2012). The microbial desulphurization process by *Serratia* can be suggest significant reducing sulphur content in dibenzothiophene(DBT) and showed promising potential for reduction of the sulfur content in diesel oil.(Casullo de Araújo *et al.,* 2012).

Isolates D2.10 and D1.2

These isolates were identified as *Cedecea Davicae* . The enteric bacteria in the family Enterobacteriaceae are mainly regarded as inhabitants of animal guts .The ability of this group to degrade high-molecular-weight PAH compounds appears to be an unusual feature, as this phenomenon has been associated with typical soil bacteria. However, very few reports have indicated utilization of aromatic compounds by enterobacteria, particularly those of the genera Klebsiella, Enterobacter, Escherichia, and Hafnia Although there are several reports of bioremediation of high-molecular-weight PAHs, research pertaining to biodegradation of these substances by enteric bacteria has been relatively rare (Sarma *et al*., 2004).

Isolates D11.54and D9.49

These isolates were identified as *Enterobacter cloacae*. Toledo (2006) isolated fifteen bacterial strains from solid waste oil samples, which was selected due to their capacity of growing in the presence of hydrocarbons. The isolates were identified by PCR of the 16S rDNA gene using fD1 and rD1 primers these included Enterobacter sp. NaCl-tolerant Enterobacter cloacae grown on crude oil as the sole carbon source, the total petroleum hydrocarbon (TPH) degradation percentage was 7.94% with mutant MU-1 in the presence of 7.5% NaCl (Xiufu *et al*., 2010). Two bacterial strains *Enterobacter cloacae*

and *Pseudomonas* spp. were chosen for mineralization and respirometry test, performed to evaluate biodegradability potential of Complex mixture of hydrocarbons, they were capable of degrading petroleum components and utilize the oil as source of carbon and energy (Ahmed, 2013). Also Mashreghi *et al., (*2012) mentioned that the bacterium *Enterobacter cloacae* was capable of biodegrading mazut (as a source content of various hydrocarbons) efficiently and was of first bacterial strain cracking mazut.

Isolate D11.55

This isolate identified as *Yersinia enterocolytica*. Lawson *et al.,* (2013) isolated, identify and assess the degradation potential of hydrocarbon utilizing bacteria in four Ghanaian soils Yersinia was among those isolates. *Yersinia spp* were also identified as bacteria involved in petroleum hydrocarbon degradation. In the work of Van-Hamme and Odumeru (2002), *Yersinia spp* was found to be among the dominant isolates growing on petroleum hydrocarbons.

Isolate W23.103

This isolate identified as *Citrobacter freundii,* the same organism isolated by Olukunle and Boboye, (2012) in Nigeria. And isolated by Ahmed *et al*.,(2010) in Bangladesh, found that all the isolates showed 50% or more degradation of kerosene except F.S19a (*C. freundii*) which showed only 14.13% degradation. Also isolated from contaminated soil in south Africa by Singh and Lin, (2007).

4-5 Analysis of residual hydrocarbons in liquid culture by gas chromatography:

For more precise results the best performing three isolates from the 10 listed in table 11,and one isolate which had weak performance, Gas chromatography was used to detect residual oil in fermentation flasks. The biodegradation of petroleum oil was confirmed by gas chromatography profile. Gas chromatography as mentioned in material and methods (3-3) was

confined to n-parraffins (n-c8-nc44).The ability of biodegradation for crude oil was examined for four isolates of bacteria with regard to their degradation ability for n-alkanes. The analysis profile of crude oil before fermentation was taken as the standard to compare fermented oil with.

4-5-1 *Serratia marcences* (Isolate N27.122):

Table 7 shows the control crude oil by Gas Chromatography and the measured area for each alkane component. And figure 2 shows size on the curve. The results of degrading crude oil GC compared with the control.The calculations were done as in material and methods (3-3-2). Table. 8 shows degree of degradation of crude oil by Gas Chromatography and the percentage of n-alkane removed by the isolate *Serratia marcences* Average percentages of this isolate was 57.89%. The G C shows the degradation of n-alkanes C8-C44. The result shows that nC8 to nC16 was nearly completely degraded as these are small molecules.

It recognized also that degradation percentage decreased gradually from nC17-nC38, until 5%. However, nC40 recorded 27%. When we compare Fig 3 with the control (Fig 2) we notice decrease in the peaks of a wide range of compounds from nC8-nC28, then it decrease. Generally, it is believed that microbes preferably degrade or metabolize C8–C15 n-alkanes followed by C16–C36 n-alkanes due to the simplicity of these hydrocarbons. Alkanes are generally easily biodegraded particularly those of relatively shorter chains due to their lower hydrophobicity. Straight chain alkanes (C10-C24) are most rabidly degraded (Salleh *et al.,* 2003, Binupriya *et al.,* 2007). Malatova (2005) analyzed Leepershank crude oil and mentioned that isolates were able to degrade the medium chain length alkanes hydrocarbons with 10-14 carbons. *Serratia marcescens* and *Acnietobacter baumannii* gave the best degradation percentage among tested isolates proximately 80 %.

Table 7.Control of crude oil by Gas Chromatography:

Alkane component	Measured area of the component in crude oil	Area%
nC6 n-hexane	66713	0.1782
nC7 n-heptane	335485	0.8961
nC8 n-octane	822499	2.197
nC9 n-Nonane	1192901	3.186
nC10 n-Decane	887360	2.370
nC11 n-Undecane	1584860	4.233
nC12 n-Dodecane	1698068	4.535
nC13 n-Tridecane	1820730	4.863
nC14 n-Tetradecane	2072584	5.536
nC15 n-Pentadecane	2006026	5.358
nC16 n-Hexadecane	2132861	5.697
nC17 n-Heptadecane	2326175	6.213
Pyristane	394382	1.053
nC18 n-Octadecane	2093045	5.590
Phytane	256803	0.686
nC19 n-Nnonadecane	2179590	5.822
nC20 n-Eicosane	2036219	5.439
nC21 n-Uncosane	1881026	5.024
nC22 n-Docosane	162873	4.349
nC23 n-Tricosane	1534511	4.099
nC24 n-Tetracosane	1291346	3.449
nC25 n-Pentacosan	1365840	3.648
nC26 n-Hexcaosane	1057487	2.825
nC27 n-Heptacosane	837190	2.236
nC28 n-Octacosane	765854	2.046
nC29 n-Nonacosane	640559	1.711
nC30 n-Triacontane	446707	1.193
nC31 n-Untriacontane	428830	1.145
nC32 n-Dotriacontane	314117	0.8390
nC 33 n-Tritriacontane	234129	0.6254
nC34 n-Tetratriacontane	197864	0.5285
nC35 n-Pentatriacontane	203648	0.5439
nC36 n-Hexatriacontane	118495	0.3165
nC37 n-Heptatriacontane	9584	0.2564
nC38 n-Octatriacontane	140561	0.3754
nC39 n-Nonatriacontane	104073	0.2780
nC40 n-Tetracontane	132825	0.3548
nC41 n-Ùntetracontane	83320	0.2225
nC42 n-Dotetracontane	24157	0.0645
nC43 n-Tritetracontane	2531	0.0068
nC44 n-Tetratetracontane	4088	0.0109

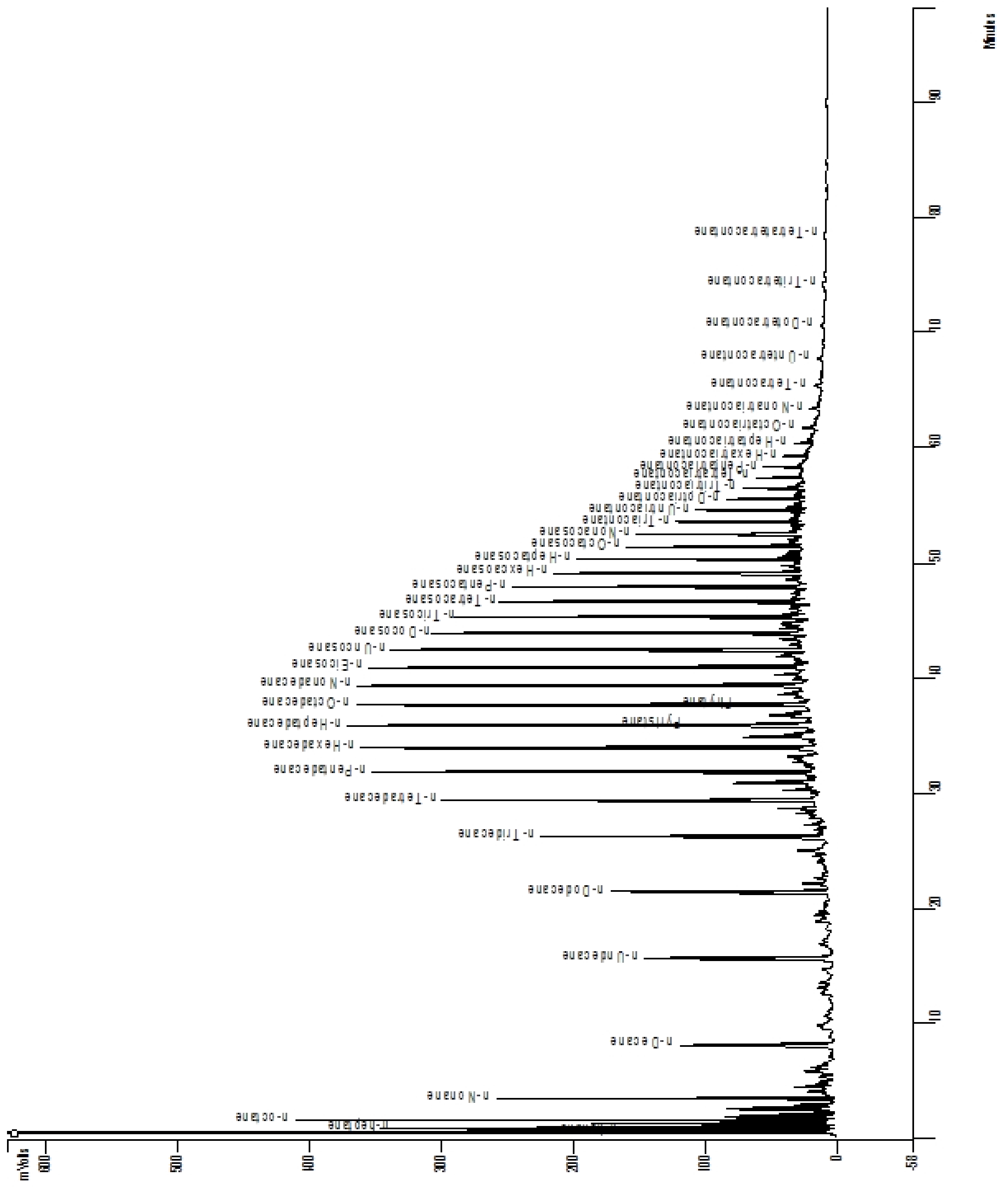


Fig 2:Chromatograms of petroleum control

Table 8-.Degree of degradation of crude oil by Gas Chromatography for isolate *Serrated marcences* N27.122.

Alkane component	Control area	Area of N27.122	Degradation area by N27.122	Percentage of degradation
nC6 n-hexane	66713	-	-	-
nC7 n-heptane	335485	-	-	-
nC8 n-octane	822499	13034	809465	98
nC9 n-Nonane	1192901	84137	1108764	92
nC10 n-Decane	887360	42520	844840	95
nC11 n-Undecane	1584860	14264	1570596	99
nC12 n-Dodecane	1698068	3370	1694698	99
nC13 n-Tridecane	1820730	3955	1816775	99
nC14 n-Tetradecane	2072584	6839	2065745	99
nC15 n-Pentadecane	2006026	30578	1975448	98
nC16 n-Hexadecane	2132861	151732	1981129	92
nC17 n-Heptadecane	2326175	347699	1978476	85
Pyristane	394382	111007	283375	71
nC18 n-Octadecane	2093045	421882	1671163	79
Phytane	256803	84557	172246	67
nC19 n-Nnonadecane	2179590	460502	1719088	78
nC20 n-Eicosane	2036219	451733	1584486	77
nC21 n-Uncosane	1881026	412622	1468404	78
nC22 n-Docosane	162873	364574	-	-
nC23 n-Tricosane	1534511	361881	1172630	76
nC24 n-Tetracosane	1291346	346993	944353	73
nC25 n-Pentacosan	1365840	437744	928096	67
nC26 n-Hexcaosane	1057487	390611	666876	63
nC27 n-Heptacosane	837190	345978	491212	58
nC28 n-Octacosane	765854	416781	349073	45
nC29 n-Nonacosane	640559	426330	214229	33
nC30 n-Triacontane	446707	343531	103176	23
nC31 n-Untriacontane	428830	375690	53140	12
nC32 n-Dotriacontane	314117	298870	15247	4.8
nC 33 n-Tritriacontane	234129	248684	-	-
nC34 n-Tetratriacontane	197864	276942	13204	6
nC35 n-Pentatriacontane	203648	292830	-	-
nC36 n-Hexatriacontane	118495	148056	-	-
nC37 n-Heptatriacontane	95984	127898	-	-
nC38 n-Octatriacontane	140561	133471	7090	5
nC39 n-Nonatriacontane	104073	109954		
nC40 n-Tetracontane	132825	96933	35892	27
nC41 n-Ùntetracontane	83320	89197	-	-
nC42 n-Dotetracontane	24157	90784	-	-
nC43 n-Tritetracontane	2531	35169	-	-
nC44 n-Tetratetracontane	4088	35843	-	-
Average				57.89%

Fig 3: isolate *Serrated marcences* N27.122

Leepershank crude oil consists of medium chain length hydrocarbons and a very low percentage of branched alkanes. No aromatic compounds were detected during analysis of this oil. As the results indicated, all isolates were able to degrad the medium chain length alkanes. The best degradation approximately 80 % for TC1(*Serratia marcescens*) and CL7(*Acnietobacter baumannii*)) was observed on hydrocarbons with 10-14 carbons. The degradation percentage slightly decreased with increasing numbers of carbons. Branching and the addition of side chains significantly influenced the utilization of these hydrocarbons by the various isolates. The highest utilization of these compounds was seen with strain TC1.

4-5-2 Isolate *Pseudomonas putida* N27.120

The result of degradation is shown in table 9 Fig 4.The average percentage for this isolate is 62.38%.The degradation percentage of nC8-nC15 by this isolate was 99%-96%. It decreased from nC16-nC-nC34, however, degradation increased again until 86% for nC42. In addition nC35-nC37 completely disappears. Comparing the results obtained showed a decrease in the peaks of a wide range compounds from nC8-nC42. In addition, this isolate is more active than N27.122 in the range nC8-nC15and the range nC41-nC44. In contrast, Elkhir (2007) *Pseudomonas putida* gave average percentage of 94.61; in addition, he reported that n-alkane nC7-nC13 and nC38-nC42 were completely degraded. this result in agreement with that of Mittal and Singh (2009), they reported that most of the lighter alkane fraction were metabolized by *Pseudomonas* strains, also lighter alkane degraded percentage was 54%and 49% for branched alkane from 40% and 44% in ten days.

4-5-3 Isolate *Pseudomonas aeruginosa* S20.94.

The result of this isolate is shown in table10 Fig 5. The average percentage 70.47%. It showed high degradation percentage of the rang form nC8-nC17 (99%-91%) except nC9 which recorded (84%).Percentage

decreased for nC18-nC38, then increased till(91%) for nC40. The result in contrast with that found by Elkhir (2007) who reported that nC7- to nC10 completely degraded using *Pseudomonas aeruginosa* and overall average percentage was 95.74 for nC7- to nC42. Norman *et al*., (2004) showed the decrease of peaks from nC10- nC34 after 42 days. That indicates that the isolates in this study were more effective in degradation.

Bacteria in the genera Pseudomonas have an ability to utilize a diverse range of substrates including those found in petroleum. A large amount of work has been performed on the alkane oxidation genes in Pseudomonas, which allow bacteria with these genes to grow on alkanes as a sole carbon source (Kaplan, 2002).

4-5-4 Isolate *Citrobacter freundii* W23.103.

The average percentage is 59.89%.(Table11) This isolate show lower degradation of nC8 and nC9, but high degradation of nC10-nC13 it recognized in table 12 and Fig 6, The degradation decreased gradually from nC23-nC34, but nC35 gave 16%. We noticed high percentage for nC41 and nC42.

The degradation percentage slightly decreased with increasing numbers of carbons. Branching and the addition of side chains significantly influenced the utilization of these hydrocarbons by the various isolates.

The results of crude oil degrading by the four above isolates identify them as efficient oil degraders and they could reduce n-alkanes more than the total petroleum as show in table 4 when compared with the result of gas chromatography. In table 4, the degradation measured by weight for the total petroleum but in gas chromatography only the n-alkanes fraction was measured. For example isolate W23.103, its percentage of degradation by weight was 36% but its average percentage of degradation by G C was 59.89%.

Table 9 -Degree of degradation of crude oil by Gas Chromatography for isolate *Pseudomonas putida* N27.120.

Alkane component	Control area	Area of N27.120	Degradation area by N27.120	Percentage of degradation
nC6 n-hexane	66713			
nC7 n-heptane	335485			
nC8 n-octane	822499	10729	811770	98
nC9 n-Nonane	1192901	4686	1188215	99
nC10 n-Decane	887360	12839	874521	98
nC11 n-Undecane	1584860	7241	1577619	99
nC12 n-Dodecane	1698068	2378	1695690	99
nC13 n-Tridecane	1820730	2814	1817916	99
nC14 n-Tetradecane	2072584	6019	2066565	99
nC15 n-Pentadecane	2006026	78166	1927860	96
nC16 n-Hexadecane	2132861	273053	1859808	87
nC17 n-Heptadecane	2326175	495349	1830826	78
Pyristane	394382	177544	216838	54
nC18 n-Octadecane	2093045	544241	1548804	73
Phytane	256803	129490	127313	49
nC19 n-Nnonadecane	2179590	616653	1562937	71
nC20 n-Eicosane	2036219	542429	1493790	73
nC21 n-Uncosane	1881026	482875	1398151	74
nC22 n-Docosane	162873	419629		
nC23 n-Tricosane	1534511	393620	1140891	74
nC24 n-Tetracosane	1291346	370011	921335	71
nC25 n-Pentacosan	1365840	482853	882987	64
nC26 n-Hexcaosane	1057487	408876	648611	61
nC27 n-Heptacosane	837190	367031	470159	56
nC28 n-Octacosane	765854	398080	367774	48
nC29 n-Nonacosane	640559	388025	252534	39
nC30 n-Triacontane	446707	323964	122743	27
nC31 n-Untriacontane	428830	347031	81799	19
nC32 n-Dotriacontane	314117	256010	58107	18
nC 33 n-Tritriacontane	234129	211861	22268	9
nC34 n-Tetratriacontane	197864	184660	13204	6
nC35 n-Pentatriacontane	203648	244347		
nC36 n-Hexatriacontane	118495	125936		
nC37 n-Heptatriacontane	95984	119403		
nC38 n-Octatriacontane	140561	126047	14514	10
nC39 n-Nonatriacontane	104073	57282	46791	44
nC40 n-Tetracontane	132825	47104	81799	19
nC41 n-Ùntetracontane	83320	18369	64951	77
nC42 n-Dotetracontane	24157	3305	20852	86
nC43 n-Tritetracontane	2531			
nC44n-Tetratetracontane	4088	2142	1946	47
Average				62.38%

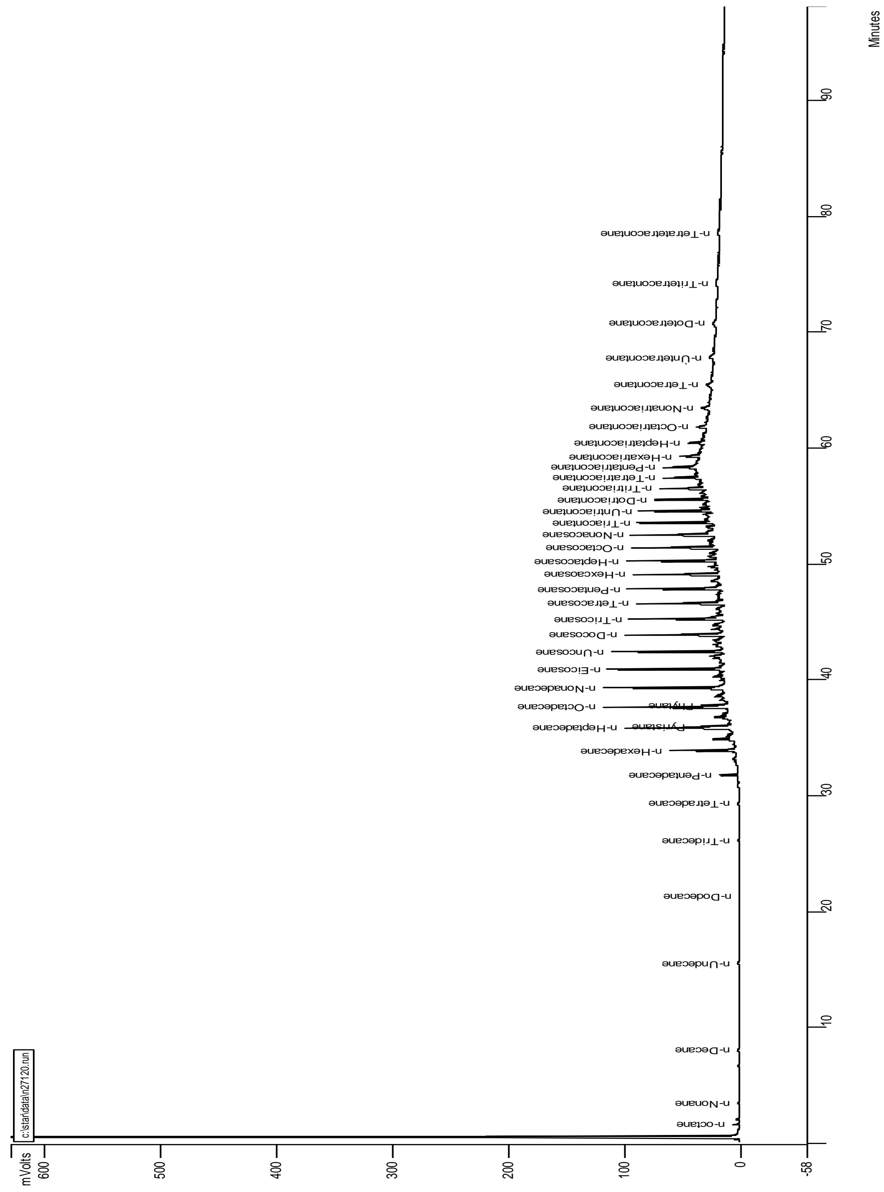


Fig 4: isolate N27.120 ***Pseudomonas putida***

Table 10 Degree of degradation of crude oil by Gas Chromatography for isolate S20.94 *Pseudomonas aeruginosa*

Alkane component	Control area	Area of S20.94	Degradation area by S20.94	Percentage of degradation
nC6 n-hexane	66713	-	-	
nC7 n-heptane	335485	-	-	-
nC8 n-octane	822499	16531	805968	97
nC9 n-Nonane	1192901	181998	1010903	84
nC10 n-Decane	887360	60277	827083	93
nC11 n-Undecane	1584860	14593	1570267	99
nC12 n-Dodecane	1698068	3603	1694465	99
nC13 n-Tridecane	1820730	4323	1816407	99
nC14 n-Tetradecane	2072584	12169	2060415	99
nC15 n-Pentadecane	2006026	49088	1956938	97
nC16 n-Hexadecane	2132861	91189	2041672	95
nC17 n-Heptadecane	2326175	203139	2123036	91
Pyristane	394382	233029	161353	40
nC18 n-Octadecane	2093045	252056	1840989	87
Phytane	256803	143029	113774	44
nC19 n-Nnonadecane	2179590	316867	1862723	85
nC20 n-Eicosane	2036219	366151	1670068	82
nC21 n-Uncosane	1881026	313168	1567858	83
nC22 n-Docosane	162873	267804	-	-
nC23 n-Tricosane	1534511	250326	1284185	83
nC24 n-Tetracosane	1291346	233716	1057630	81
nC25 n-Pentacosan	1365840	321617	1044223	76
nC26 n-Hexcaosane	1057487	241186	816301	77
nC27 n-Heptacosane	837190	178420	658770	78
nC28 n-Octacosane	765854	229552	536302	70
nC29 n-Nonacosane	640559	212199	428360	66
nC30 n-Triacontane	446707	178424	268283	60
nC31 n-Untriacontane	428830	204072	224758	52
nC32 n-Dotriacontane	314117	154909	159208	50
nC 33 n-Tritriacontane	234129	126644	107485	45
nC34 n-Tetratriacontane	197864	123942	73922	37
nC35 n-Pentatriacontane	203648	170772	32876	16
nC36 n-Hexatriacontane	118495	97833	20662	17
nC37 n-Heptatriacontane	95984	-	-	-
nC38 n-Octatriacontane	140561	133836	6725	4
nC39 n-Nonatriacontane	104073	43906	60167	57
nC40 n-Tetracontane	132825	11635	121190	91
nC41 n-Ùntetracontane	83320	14322	68998	82
nC42 n-Dotetracontane	24157	4723	19434	80
nC43 n-Tritetracontane	2531	2699	-	-
nC44 n-Tetratetracontane	4088	2401	1687	41
Average				70.47%

Fig5: isolate S20.94 ***Pseudomonas aeruginosa***

Table11-Degree of degradation of crude oil by Gas Chromatography for isolate *Citrobacter freundii*) W23.103

Alkane component	Control area	Area of W23.103	Degradation area by W23.103	Percentage of degradation
nC6 n-hexane	66713			
nC7 n-heptane	335485			
nC8 n-octane	822499	109874	712625	86
nC9 n-Nonane	1192901	273066	919835	77
nC10 n-Decane	887360	42032	845328	95
nC11 n-Undecane	1584860	10025	1574835	99
nC12 n-Dodecane	1698068	13843	1684225	99
nC13 n-Tridecane	1820730	81598	1739132	95
nC14 n-Tetradecane	2072584	298176	1774408	85
nC15 n-Pentadecane	2006026	446219	1559807	77
nC16 n-Hexadecane	2132861	508031	1624830	76
nC17 n-Heptadecane	2326175	673501	1652674	71
Pyristane	394382	261766	132616	33
nC18 n-Octadecane	2093045	639694	1453351	69
Phytane	256803	159118	97685	38
nC19n-Nnonadecane	2179590	695734	1483856	68
nC20 n-Eicosane	2036219	589417	1446802	71
nC21 n-Uncosane	1881026	511932	1369094	72
nC22 n-Docosane	162873	440149		
nC23 n-Tricosane	1534511	411030	1123481	73
nC24 n-Tetracosane	1291346	384304	907042	70
nC25 n-Pentacosan	1365840	476396	889444	65
nC26 n-Hexcaosane	1057487	383081	674406	63
nC27 n-Heptacosane	837190	299056	538134	64
nC28 n-Octacosane	765854	348134	417720	54
nC29 n-Nonacosane	640559	314253	326306	50
nC30 n-Triacontane	446707	252018	194689	43
nC31 n-Untriacontane	428830	274187	154643	36
nC32 n-Dotriacontane	314117	205104	109013	34
nC 33 n-Tritriacontane	234129	166126	68003	29
nC34 n-Tetratriacontane	197864	141413	56451	28
nC35 n-Pentatriacontane	203648	170949	32699	16
nC36 n-Hexatriacontane	118495	93765	24730	20
nC37 n-Heptatriacontane	9584	80818	15166	15
nC38 n-Octatriacontane	140561	45826	94735	67
nC39 n-Nonatriacontane	104073	64366	39707	28
nC40 n-Tetracontane	132825	38111	94714	71
nC41 n-Ùntetracontane	83320	6172	77148	92
nC42 n-Dotetracontane	24157	2203	21954	90
nC43 n-Tritetracontane	2531	2256	275	10
nC44 n-Tetratetracontane	4088	2150	1938	47
Average				59.89%

Fig 6: isolate W23.103 ***Citrobacter freundii***

4-6 Efficiency of Mixed Bacterial Strains on Degradation

Mixed cultures of the best four isolates that represented higher degradation performance by weight and tested by GC for residual oil were used in this experiment. The result indicates that individual bacterial cultures showed less growth and degradation than mixed cultures. The results were shown in table 12. This in agreement with that found by Elkhir (2007).

Binupria *et al.,* (2007) reported that mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil or diesel fuel. Various organisms have the capability of degrading various forms of hydrocarbons. Westlake (1982) noted that no single microbial species has the enzymatic ability to metabolize more than two or three classes of compounds typically found in crude oil.

Salleh *et al.,* (2003) examined Three microbial consortia consisting of 3,5,8 bacterial strains obtained from hydrocarbon-polluted soils, it was seen that the consortium consisting the highest number of bacterial strains was most effective at removing weathered diesel from soil, this in agreement with our result.

Biological treatment methods for petroleum hydrocarbon remediation, generally relies upon the cooperation of more than a single bacterial species. Mixed cultures carry out more extensive biodegradation of petroleum than pure cultures. Microbial degradation depends on their distributions in crude oil. High concentrations of hydrocarbons associated with heavy oil can even inhibit biodegradation. It has been expected that only one microbial could not biodegrade crude oil thoroughly, corresponding well with the results shown.

Table 12: Degree of growth of combination of strains on crude oil:

Isolate	Estimation of Degradation	Strains Combinations	Estimation of Degradation
1-N27.120	++++	N27.122+N27.120	+++++
2-S20.94	++++	N27.122+S20.94	+++++
3-S20.95	++++	N27.122+S20.95	+++++
4- S20.96	++++	N27.122+S20.96	+++++
		N27.122+The four isolates	+++++

CONCLUSION

In conclusion, biodegradation of crude oil contaminants is the conversion of chemical compounds by microorganisms into energy, cell mass and biological products. In the current study, bacteria that able to utilized hydrocarbon were isolated from contaminated petroleum aged soil as to be efficient crude oil degrader. Those bacterial isolates identified tentivly as *Pseudomonas aeruginosa, P. putida, Serratia marcences, Cedeca davicae, Enterobacter cloacae, Yersinia enterocolytica and Citrobacter freundii.*The best three isolates that showed high degradation performance by weight, were chosen for gas chromatography analysis to detect residual oil in fermentation. Average degradation percentage were as follows: *Serratia marcences* 57.89%, *P. putida*, 62.38%, and *P. aeruginosa* 70.47%.

Mixed cultures of the best four isolates showed high degradation percentage by weight. The result indicates that individual bacterial cultures showed less growth and degradation than mixed cultures. The three effective bacterial isolates could be studied under molecular level and packaged for future remediation.

RECOMMENDATION

The following recommendations can be suggested:

1- Further studies of the isolates are needed.

2- Conduct and promote further research in the area of bioremediation in Sudan to solve the problem of produced water contamination.

3-Establish culture bank collection of oil-degrading microorganisms.

4 -Research could be carried on combination of oil degrading microorganisms and phytoremediation in produce water aeries.

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APPENDISES

Appendix (1)

1- Basal salts mineral medium (BSM) g/l:

K_2HPO_4	1.37g
KH_2PO_4	0.68g
NH_4NO_3	1.0g
$MgSO_4$ $7H_2O$	0.1g
$FeSO_4 7H_2O$	0.03g

Appendix (2)

Nutrient Agar:

Peptone	5.0g
Lablemco meat	1.0g
Yeast extracts	2.0g
Sodium chloride	5.0g
Agar	15.0g
Distilled water	1 liter
Ph	7.2-+0.2

Appendix (3)

Motility Medium:

Gelatin	80g
Peptone	10g
Beef extract	3g
NaCl	5g
Agar	4gm
Distilled water	1liter