ASSESSMENT OF HYDROCARBON-DEGRADING POTENTIAL OF *PSEUDOMONAS PUTIDA* AND *BACILLUS MEGATERIUM* ISOLATED FROM PETROLEUM REFINERY EFFLUENT

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DEPARTMENT OF MICROBIOLOGY,
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DECLARATION

This dissertation entitled "Assessment of Hydrocarbon-Degrading potential of *Pseudomonas Putida* And *Bacillus megaterium* Isolated From Petroleum Refinery Effluent" has been carried out by me in the Department of Microbiology, Ahmadu Bello University, Zaria under the supervision of Prof. J.B. Ameh and Dr. H.I. Atta and has not been presented in any previous application for a higher degree or published in any book. In the course of writing this dissertation, references were made to various sources of information. These were duly acknowledged.

_____ _____

Khadijah SAIDU (P15SCMC8003) Date

CERTIFICATION

This dissertation entitled "ASSESSMENT OF HYDROCARBON-DEGRADING POTENTIAL OF *PSEUDOMONAS PUTIDA* AND *BACILLUS MEGATERIUM* ISOLATED FROM PETROLEUM REFINERY EFFLUENT" by Khadijah SAIDU (P15SCMC8003) meets the requirement for the award of Master of Science degree in Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This dissertation is dedicated to my entire family and to the blessed memory of my late husband Mohammad Usman, who stood by me during the course of my study.

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ABBREVIATIONS

KRPC	Kaduna Refinery and Petrochemical Company
VOC	Volatile Organic Compounds
SEO	Spent Engine Oil
MSM	Mineral Salts Media
TPH	Total Petroleum Hydrocarbons
HUB	Hydrocarbon-Utilizing Bacteria
PAH	Polycyclic Aromatic Hydrocarbons
PRB	Permeable Reactive Barrier
LNAPLs	Light Non-Aqueous Phase Liquids
USEPA	United State Environmental Protection Agency
BTEX	Benzene, Toluene, Ethylbenzene and Xylene
NNPC	Nigerian National Petroleum Corporation
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
EC	Electrical Conductivity
TDS	Total Dissolved Solids
FMENV	Federal Ministry of Environment
MR-VP	Methyl Red - Voges-Proskauer
ONPG	O-nitro-phenyl-D-galactopyranoside

ABSTRACT

The ability of *Pseudomonas putida* and *Bacillus megaterium* isolated from a petroleum refinery effluent to degrade diesel and spent engine oil was assessed in this study. The effluent sample was collected from the treatment plant in the Kaduna Refinery and Petrochemical Company (KRPC), Nigeria. Diesel was purchased from a Filling Station in Zaria and spent engine oil was collected from a service station during oil change for vehicles in Zaria. The physicochemical properties and heavy metal content of the effluent were determined. Three strains of *Pseudomonas putida* and seven strains of *Bacillus megaterium* were identified among the bacteria isolated using conventional biochemical and phenotypic tests. The strains showing the highest degradation potential after screening were selected for the final biodegradation studies. The ability of the selected strains of *Pseudomonas putida*(C15a) and *Bacillus megaterium* (N9a) and mixed culture to utilize the hydrocarbons in diesel and spent engine oil was assessed over a period of eighteen days, and monitored on a three-day interval using the pH, hydrocarbon-utilizing bacterial count and optical density. It was observed that the organisms were able to utilize diesel and spent engine oil for their metabolic needs at varying degrees as shown by the increase in hydrocarbon-utilizing bacterial (HUB) count and corresponding decrease in oil and grease content as well as pH. *Pseudomonas putida* degraded 98.3% of diesel while *Bacillus megaterium* degraded 81.03%, however, lower degradation of diesel was recorded by the bacterial consortium of 68.97% with their corresponding hydrocarbon utilizing bacterial (HUB) count of 1.85 x 10^7CFU/ml, 1.35 x 10^7CFU/ml and 6.1 x 10^6CFU/ml respectively. *Pseudomonas putida* degraded 75.03% of spent engine oil while *Bacillus megaterium* degraded 66.22% and lower degradation of spent engine oil was recorded by the bacterial consortium (60.86%) with their hydrocarbon utilizing bacterial (HUB) count of 1.35 x 10^7CFU/ml, 1.05 x 10^7CFU/ml and 5.6 x 10^6CFU/ml, respectively at pH range of 6.33 -7.70.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

The contamination of soil and groundwater by hazardous chemicals has become a major concern due to the associated risks to human health and the environment (Ghosh and Tick, 2013).

Oil contamination of soil and water from industrial sources and other activities causes ecological disasters (Che, 2002). Petroleum hydrocarbons are composed of complicated mixtures of non-aqueous and hydrophobic components such as n-alkane, aromatic, resins and asphaltenes. Water/soil contamination with petroleum and petroleum-based hydrocarbons has caused critical environmental and health defects and increasing attention has been paid for developing and implementing innovative technology for cleaning up this contamination (Sathishkumer *et al.,* 2008).

Several techniques including physical, chemical and biological methods have been developed to resolve the problem of petroleum pollution (Darsa *et al.,* 2014). However, the most promising approach being researched so far is microbial degradation since the physico-chemical methods are rather too expensive, not environmentally friendly and do not give a total elimination of the pollutant (Umanu *et al.,* 2013). Microbial degradation (biodegradation) involves the use of microbes to break down potentially-harmful pollutants into harmless products (Hamza *et al*., 2012). This agrees with the report of Onwurah, (2003), who reported that crude oil degrading bacteria such as *Pseudomonas* sp, *Micrococcus* sp. and *Bacillus* sp. could metabolize the toxic components of crude oil, leading to its degradation.

These days, crude oil pollution has become a global problem particularly in industrialized and developing countries, which results in significant decline in soil quality and chronic sub-acute

toxicological effects within ecosystem (Tanee and Albert, 2015). Soil and ground water are often contaminated due to inevitable spillage during oil exploration, transportation, extraction, refining and also from leaking underground storage tanks and pipelines (Xiong *et al*., 2015). There are several clean-up technologies for removing petroleum from the contaminated area. Among them, bioremediation methods are low cost, high efficiency, environmentally friendly and simple technology for long-term restoration of crude oil contaminated sites (Elmahdi *et al*., 2014; Rodríguez *et al*., 2015).

Oil-contaminated wastewater has been identified as one of the most disturbing sources of pollution. This kind of wastewater is widely produced from a variety of sources such as crude oil exploration, automotive garage, oil refinery, petrochemical industry, metal processing, lubricant and car washing. These sources serve as the major contributors to the environmental problems especially in soil and water (Ikhajiagbe and Anoliefo, 2011). Both the waste and unused compounds from the prescribed sources are grouped as oily waste, which is difficult to treat or recycle (Tri, 2002). Petroleum-contaminated soil contains various hazardous materials such as aromatic hydrocarbons and polycyclic aromatic hydrocarbons and they are potentially toxic, mutagenic and carcinogenic (Milic *et al*., 2009). Dramatic increase in production, refining and distribution of crude oil has brought with it an ever-increasing problem of environmental pollution (Ojo, 2006). The persistence of petroleum pollution depends on the quantity and characteristics of hydrocarbon mixture and on the properties of the affected ecosystem. The ability to isolate high numbers of certain oil degrading microorganisms from oil polluted environment is commonly taken as an evidence that these microorganisms are active degraders in that environment (Okerentugba and Ezeronye, 2003). Contamination of water with hydrocarbon waste stimulates indigenous microbial populations, which are capable of utilizing the hydrocarbon substrates as their sole carbon and energy sources thereby degrading the

contaminants. Several bacterial species have been identified as having the ability for oil degradation. In general, *Bacillus* sp. has been identified as petroleum hydrocarbon degrader (Ghazali *et al*., 2004) and is known as naphthalene and pyrene degrader (Ron and Rosenberg, 2014; Zhuang *et al*; 2002).

Wastewater released from petroleum refineries is characterized by the presence of large quantity of petroleum products, polycyclic aromatic hydrocarbons, phenols, metal derivatives, surface active substances, sulfides, naphthylenic acids and other chemicals. Due to ineffective purification systems, the pollutants find their way into nearby water bodies and soil with potentially serious consequences on the ecosystem (Beg *et al*., 2001; Bay *et al*., 2003; Otokunefor and Obiukwu, 2005).

Wastewater may be treated by physico-chemical or biological methods. Biological treatment is preferred over physicochemical as the former is cost effective, efficient and environmentally friendly (Hamza, 2012). Alternative to physico-chemical method is bioaugmentation, where exogenous microbes are introduced to carry out the process (Hamza *et al*., 2012).

Study has shown that most potential bacteria for petroleum hydrocarbon degradation have been isolated from areas contaminated with oil (Chaerun *et al*. 2004). The application of bacterial isolates in degrading hydrocarbons involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate. Microbes are dependent on nutrients for survival and these nutrients will be used to synthesize enzymes for the breakdown of hydrocarbons (Vidali, 2001).

1.2 Statement of Research Problem

In Nigeria, like in other developing countries, the conventional cleaning technologies (physicochemical) are being used for the remediation of refinery wastewater. These methods are

expensive and do not lead to complete degradation of the pollutants. Therefore there is the need for a robust, cost-effective and environmentally friendly cleaning technology (Ojo, 2006; Hamza *et al*., 2009).

The economy of Nigeria is largely hinged on revenue from petroleum and petro-chemicals. Spent engine oil is one of the petro-chemicals reported to be a major and most common soil contaminants in Nigeria. It contains potentially toxic polycyclic aromatic hydrocarbons (Sharifi *et al.,* 2007) and heavy metals (Ahamefule *et al.,* 2014). It is indiscriminately disposed into gutters, water drains, open vacant plots and farms by auto technicians and allied artisans with workshops on the road sides and open places (Aneliefo and Vwioko, 2001). The consequences are the pollution of water bodies, contamination of ground water and toxicity to animals and plants (Ahamefule *et al.,* 2014). Spent engine oil is one of the major environmental pollutants in Nigeria owing to the high demand for lubricant oil (Okonokhua *et al.,* 2007; Ahamefule *et al.,* 2014).

Majority of the soil samples gathered from spent engine oil sites had low water abpsortion rate and retention capacity (Akande *et al.,* 2018). Ahamefule *et al.,* (2014) also made similar observations and they suggested that it may have occurred because of the hydrophobic properties of spent engine oil which impeded the adherence of water molecules to soil particles.

Lands contaminated with spent engine oil are found mostly in developing countries of the world due to the ineffective environmental laws (Adams *et al.,* 2014) which directly affects the rate at which the spent engine oil enters and pollutes the environment (Sulayman *et al.,* 2020). Fatuyi *et al.,* (2011) defined spent engine oil as a pollutant in the environment that causes damage to the ecosystem as well as health hazard to human beings. High percentage of aromatic and aliphatic hydrocarbons, nitrogen, sulphur compounds and metals (Zn, Pb, Cr and Fe) are contained in

spent engine oils than fresh oils. Some of the metals in the spent engine oil could dissolve in water, move through the soil easily and may eventually be found in surface and ground water (Mohd *et al*., 2011; Abdulsalam *et al*., 2012).

Diesel spilled into groundwater and seawater will persist longer and spread immediately to a thin rainbow film and silver sheens. The higher molecular fractions will sink into the deeper part of water (Zhao *et al.*, 2016; Wade *et al.*, 2016; Ji *et al.*, 2018). Diesel-polluted water is said to be more toxic than the originally spilled oil. This is due to the event of physiochemical changes that occure when mixing with water. Even though affected ecosystems have shown multiple signs of pollution, the responses and actions taken by authorities are either too slow or too little (Khalid *et al*., 2021).

The high polycyclic aromatic hydrocarbon content of refinery effluent is an issue of environmental concern owing to the fact that polycyclic aromatic hydrocarbons apart from being persistent in the environment are also toxigenic, carcinogenic and mutagenic (Mrayyana and Battikhi, 2005).

Bioremediation efforts have been hardly, if at all applied in the Nigerian, and possibly West African situation. In view of the fact that Nigeria is now a major player in the oil industry, and with regards to environmental concerns that are increasingly being expressed, it has become more and more important to find solutions to problems of poor environmental management.

1.3 Justification for the Study

Several techniques including physical, chemical and biological techniques have been developed to resolve the problem of petroleum pollution (Darsa *et al.*, 2014). However, the most promising approach being researched so far is microbial degradation since the physico-chemical methods

are rather too expensive, not environmentally friendly and do not give a total elimination of the pollutant (Umanu *et al.,* 2013).

Studies have shown that most potential bacteria for petroleum hydrocarbon degradation have been isolated from areas contaminated with petroleum (Chaerun *et al*., 2004).

It is very necessary to develop and optimize an effective means of degrading hydrocarbons in effluents generated from indigenous refineries and petrochemical plants, because compliance with the stipulated toxicant levels has always been very low in Nigeria (Obot *et al*., 2007).

1.4 Aim of the Study

The aim of this research was to determine the hydrocarbon degrading potential of *Pseudomonas putida* and *Bacillus megaterium* strains isolated from a petroleum refinery effluent.

1.5 Objectives of the Study

The objectives of the study were to:

1. Determine the physicochemical properties and heavy metal content of the petroleum refinery effluent.
2. Isolate and characterize strains of *Pseudomonas putida* and *Bacillus megaterium* from the refinery effluent obtained from Kaduna Refinery and Petrochemical Company (KRPC).
3. Screen the isolates for their capacity to degrade selected petroleum hydrocarbons (diesel and spent engine oil).
4. Assess hydrocarbon degradation by the selected isolates using Hydrocarbon Utilising Bacterial (HUB) counts, optical density and pH.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Petroleum Hydrocarbons

Petroleum or crude oil is a naturally-occurring flammable liquid that is found in geological formations beneath the Earth's surface. A fossil fuel is formed when huge quantities of dead organisms and organic matter get buried underneath sedimentary rock and subjected to intense pressure and heat. The components of petroleum and petroleum products are in thousands. They range in molecular weight from methane to very large uncharacterized components with molecular weight in the thousands. The toxicity of the components varies immensely. The components of these mixtures have only two common properties such as they are derived from petroleum and they contain hydrocarbons, i.e., hydrocarbon functional groups (C-H).

Although there is a considerable variation between the ratios of organic molecules, the elemental composition of petroleum is well-defined – carbon: 83–87%, hydrogen: 10–14%, nitrogen: 0.1–2%, oxygen: 0.05–1.5%, sulfur: 0.05–6.0%, metals - < 0.1% (Helmenstine, 2019).

Petroleum-based products are the major source of energy for industry and daily life. Leaks and accidental spills occur regularly during the exploration, production, refining, transport and storage of petroleum and petroleum products. 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 (Kvenvolden and Cooper, 2003). Release of hydrocarbons into the environment, whether accidentally or due to human activities, is a main cause of water and soil pollution (Holliger *et al.,* 1997).

Natural gas, crude oil, tars and asphalt are types of petroleum hydrocarbons (Frick *et al.,* 1999). The word “petroleum” means “rock oil” or “oil from the earth” (United States Environmental

Protection Agency, 2011). Petroleum used in our daily life plays a vital role in our modern society. It is the main energy source for heating, transportation and manufacturing, but acts as a raw material for plastic and synthetic rubber. Total Petroleum Hydrocarbons (TPHs) are used to describe mixtures of organic compounds found in or derived from crude oil that has the potential to be toxic (Canadian Council of Ministers of the Environment, 2001). It's made up of hydrogen and carbon but may also contain sulfur, nitrogen, heavy metals, and oxygen compounds. Most products that contain total petroleum hydrocarbons (TPHs) can burn easily. Some are clear or light colored liquids that evaporate easily, and others are thick, dark liquids or semi-solids that do not evaporate (Agency For Toxic Substances And Disease Registry, 1999) and many of these products (gasoline, kerosene, etc.) have oily odors. Modern society uses so many petroleum-based products such as gasoline, kerosene, fuel oil, mineral oil and asphalt (ATSDR, 1999).

The composition of petroleum hydrocarbons (PHCs) varies slightly by its source, but the toxic properties are consistent. Chemicals such as benzene and polycyclic aromatic hydrocarbons (PAHs) are extremely toxic components of high concern (Kamath *et al.,* 2007).

Petroleum hydrocarbons are made up of complex of mixtures of hundreds of compounds (Epps, 2006) ranging from light, volatile, short-chained organic compounds to heavy, long-chained, branched compounds. The major hydrocarbon fractions have different environmental fates (ATSDR, 1999) and the degradability of simple hydrocarbons and petroleum fuels decreases as molecular weight and degree of branching increase (Shukla, 2010). The exact composition of petroleum products varies depending upon the source of the crude oil and refining practices used to produce the product.

Once the PHCs are released into the soil, depending on their chemical properties, the complex PHCs mixture separate into individual compounds. Compounds with lower molecular weight,

e.g, BTEX compounds (benzene, toluene, ethylbenzene and the xylenes) which are naturally-occurring constituents of gasoline and diesel, although also present in kerosene and fuel oils, are highly mobile, more volatile and easily leach to the groundwater than PHCs of higher molecular weight (ATSDR, 1999; Kamath *et al.*, 2007). Compared to motor oil, gasoline has lighter compounds which can break down and volatilize more easily.

Generally, hydrocarbons with straight chain and few chains can degrade more readily than those with complex chains (ATSDR, 1999). Compounds with longer chains, such as PAHs and aromatics, tend to bind strongly to soil particles and soil organic matter plays a vital role in the degradation of contaminants (ATSDR, 1999; Kamath *et al.*, 2007).

2.1.1 Types of petroleum hydrocarbons

These can be divided into four major groups: the alkanes, the aromatics, the resins and the asphaltenes. In general, the alkane fraction is the most biodegradable rather than the polar fraction (i.e., the resins and asphaltenes), which is resistant to biological degradation. The aromatic compounds, especially the polycyclic aromatic hydrocarbons (PAHs), are of intermediate biodegradability, but these are of most concern owing to their toxicity and tendency to bioaccumulation (Shivendra and Hardik, 2014).

2.1.2 Factors influencing petroleum hydrocarbon degradation

A number of limiting factors have been recognized to affect the biodegradation of petroleum hydrocarbons. The composition and inherent biodegradability of the petroleum hydrocarbon pollutant are the first and foremost important consideration when the suitability of a remediation approach is to be assessed. Among physical factors, temperature plays an important role in biodegradation of hydrocarbons by directly affecting the chemistry of the pollutants as well as the physiology and diversity of the microbial flora (Nilanjana and Preethy, 2010). Atlas, (1975)

found that at low temperatures, the viscosity of the oil increased while the volatility of the toxic low molecular weight hydrocarbons were reduced, delaying the onset of biodegradation.

Temperature influences petroleum biodegradation by its effect on the physico-chemical properties of the oil, rate of hydrocarbon metabolism by microorganisms and composition of the microbial community (Rahman *et al.,* 2002). Temperature also affects the solubility of hydrocarbons (Nilanjana and Preethy, 2010). Although hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with the decreasing temperature. Highest degradation rates generally occur in the range of 30–40^{0}C in soil environments, 20–30^{0}C in some freshwater environments and 15–20^{0}C in marine environments (Nilanjana and Preethy, 2010). Venosa and Zhu (2003) reported that ambient temperature of the environment affected both the properties of spilled oil and the activity of the microorganisms. Significant biodegradation of hydrocarbons has been reported in psychrophilic environments in temperate regions (Pelletier *et al.,* 2004; Delille *et al.,* 2004).

Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants especially nitrogen, phosphorus, and, in some cases, iron (Cooney, 1984). Some of these nutrients could become a limiting factor, thus, affecting the biodegradation processes. Atlas, (1975) 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. In marine environments, it was found to be more pronounced due to low levels of nitrogen and phosphorous in seawater (Floodgate, 1984). Freshwater wetlands are typically considered to be nutrient deficient due to heavy demands of nutrients by the plants (Mitsch and Gosselink, 1993). Therefore, additions of nutrients were necessary to enhance the biodegradation of oil pollutant (Choi *et al.,* 2002; Kim *et*

al., 2005). On the other hand, excessive nutrient concentrations can also inhibit the biodegradation activity (Chaillan *et al.,* 2006). Chaˆıneau *et al.,* 2(005) reported the negative effects of high NPK levels on the biodegradation of hydrocarbons especially on aromatics. The effectiveness of fertilizers for the crude oil bioremediation in subarctic intertidal sediments was studied by Pelletier *et al.,* 2004. Use of poultry manure as organic fertilizer in contaminated soil was also reported (Okolo *et al.,* 2005) and biodegradation was found to be enhanced in the presence of poultry manure alone. Maki *et al.,* (2005) reported that photo-oxidation increased the biodegradability of petroleum hydrocarbon by increasing its bioavailability and thus enhancing microbial activities.

2.2 Spent Engine Oil

Waste engine oil (WEO) which is also known as used motor oil is produced when fresh engine oil (or motor oil) is subjected to high temperature and high mechanical strain during running of the vehicle for a stipulated time. It is a brown-to-black liquid mixture consisting of low to high molecular weight (C_{16}-C_{36}) aliphatic and aromatic hydrocarbons, poly-chlorinated biphenyls, chlorodibenzofurans, lubricative additives and decomposition products (Koma *et al.,* 2001; Wang *et al.,* 2010) along with heavy metal contaminants such as zinc, lead and chromium, coming from engine parts as they wear away (Munna and Dipa, 2014).

Any lubricating oil that has served its major purpose in engines and is withdrawn from the intended area of application is referred to as the spent engine oil (SEO), being considered unfit for the initial intended purpose (Ameh *et al.,* 2013).

The disposal of spent engine oil (SEO) into gutters, water drains, open vacant plots and farms is a common practice in Nigeria especially by motor mechanics. This oil, also called spent lubricant or waste engine oil, is usually obtained after servicing and subsequently draining from

automobile and generator engines (Anoliefo and Vwioko, 2001) and much of this oil is poured into the soil.

The presence of different types of automobiles and machinery has resulted in an increase in the use of lubricating oil. Spillage of used motor oils such as diesel or jet fuel contaminates the natural environment with hydrocarbons (Husaini *et al.*, 2008). Hydrocarbon contamination of the air, soil and freshwater, especially by PAHs, attracts public attention because many PAHs are toxic, mutagenic and carcinogenic (Clemente *et al.,* 2001; Cerniglia and Sutherland, 2001).

In Nigeria and some developing countries, about 20 million gallons of waste engine oil are generated annually from mechanical workshops and discharged carelessly into the environment (Abioye *et al.,* 2009).

2.2.1 Properties of spent engine oil

There are relatively large amounts of hydrocarbons in spent engine oil which include highly toxic polycyclic aromatic hydrocarbons (PAHs) (Wang *et al.*, 2000). Most heavy metals, such as vanadium, lead, aluminium, nickel, iron, chromium and zinc, which were below detection in fresh lubricating oil, have also been reported by Okonokhua *et al.,* (2007) at higher concentrations (ppm) in spent engine oil (SEO). These heavy metals may be retained in soils in the form of oxides, hydroxides, carbonates or exchangeable cations bound to organic matter in the soil (Obuotor *et al.,* 2016). Nevertheless, this is dependent on the local environmental conditions and on the kind of soil constituents present in the soil-water system.

It was noted by Agbogidi and Ejemete (2005) that SEO has deleterious effects on the biological, chemical and physical properties of the soil, depending on dose, soil type and other factors.

The illegal dumping of used motor oil is an environmental hazard with global ramifications (Blodgett, 2001). Akoachere *et al.* (2008) reported the discharge of used crankcase oil from vehicles as a major cause of oil pollution in Buea, Cameroon. Studies by Thenmozhi *et al.*, (2011) as well as Ugoh and Moneke (2011) have also reported soil pollution due to discharge of used engine oil in Pudukkottai region, India as well as Gwagwalada area, Nigeria, respectively. Various contaminants, such as used engine oil and heavy metals have been found to alter soil biochemistry, including alteration in soil microbial properties, pH, oxygen and nutrient availability (Odjegba and Sadiq, 2002).

Okonokhua *et al.*, (2007) agreed with the submission of Ekundayo *et al.*, (1989) that a marked change in properties occurs in soils polluted with petroleum hydrocarbons, affecting the physical, chemical and microbiological properties of the soil. Oil pollution of soil leads to build up of essential (organic C, P, Ca, Mg) and non-essential (Mg, Pb, Zn, Fe, Co, Cu) elements in soil and the eventual translocation in plant tissues (Vwioko *et al.*, 2006). Although some heavy metals at low concentrations are essential micronutrients for plants, but at high concentrations they may cause metabolic disorders and growth inhibition for most of the plant species (Fernandes and Henriques, 1991). However, plants respond differently to pollutants. Anoliefo and Vwioko (2001) reported that the contamination of soil with spent engine oil caused growth retardation in plants.

Yong (2001) reported that there is a bonding relationship between contaminants and soil surfaces due to sorption forces. The contaminant solutes in solution become attached to the surface of the soil (solids) particles through mechanisms, which seem to satisfy the forces of attraction from the soil solids (surfaces). The heavy metals, being positively charged, are electrostatically attracted to the negative charges on the clay particles (Yong, 2001).

Spent engine oil contains highly toxic polycyclic aromatic hydrocarbons (PAHs) (Wang *et al.*, 2000). The aromatic compounds, especially the polycyclic aromatic hydrocarbons (PAHs), are of intermediate biodegradability, but these are of most concern owing to their toxicity and tendency to bioaccumulation (Shivendra and Hardik, 2014).

Spent engine oil is not easily biodegradable by microorganisms because it contains low amount of saturated hydrocarbons. Crude oil fractions with lower amount of saturated hydrocarbons are more resistant to microbial degradation than the fraction containing higher amount of saturated hydrocarbons (Manal, 2011). Short and medium chain alkanes undergo higher degradation than the longer chain alkanes (Mukred *et al.*, 2008).

Naphthenic acids (NAs), which are one class of compounds in wastewaters from petroleum industries that are known to cause toxic effects, and their removal from oilfield wastewater is an important challenge for remediation of large volumes of petrochemical effluents (Wang *et al.*, 2015).

Lack of essential nutrients, such as nitrogen and phosphorus, is one of the major factors affecting biodegradation of hydrocarbons by microorganisms in soil and water environments. Therefore, the addition of inorganic or organic nitrogen-rich nutrients (biostimulation) is an effective approach to enhance the bioremediation process (Hollender *et al.*, 2003; Semple *et al.*, 2006; Walworth *et al.*, 2007). Positive effects of nitrogen amendment on microbial activity and/or petroleum hydrocarbon degradation have been widely demonstrated by various authors (Jørgensen *et al.*, 2000; Margesin and Schinner *et al.*, 2001; Riffaldi *et al.*, 2006).

2.3 Diesel

Diesel is a complex mixture of chemicals mainly obtained from the distillation of crude oil. Breathing large quantities of diesel vapour or drinking diesel-based fluids may cause non specific

signs and symptoms of poisoning such as dizziness, headache and vomiting. A severe form of lung damage, called pneumonitis, may occur if liquid diesel is inhaled directly into the lungs (United Kingdom Health Protection Agency, 2006).

Among petroleum products, diesel is a complex mixture of alkanes and aromatic compounds that are frequently reported as soil contaminants leaking from storage tanks and pipelines or released in accidental spills (Gallego *et al.,* 2001).

Diesel oil is a common product of crude oil distillation with a very complex composition. It consists mainly of low molecular weight alkanes and polycyclic aromatic hydrocarbons (PAHs) (Bona *et al.,* 2011). The fate of the latter compounds in nature may be of great human health importance, since PAHs have been considered toxic for plants and carcinogenic to humans (Alkio *et al.,* 2005; Reynoso-Cuevas *et al.,* 2008; Bona *et al.,* 2011). In case of an uncontrolled industrial leakage, diesel oil and its constituents might act as a persistent water and soil pollutant.

2.3.1 Properties of diesel

The carbon number of diesel hydrocarbons is between 11 and 25 and the distillation range is between 180 to 380°C. Diesel is composed of four main structural classes of hydrocarbons: n-alkanes or n-paraffins (linear saturated hydrocarbons), isoalkanes or isoparaffins (branched saturated hydrocarbons), cycloalkanes or naphthenes (saturated cyclic alkanes) and aromatics (Marchal *et al*., 2003).

Diesel contains 2000 to 4000 hydrocarbons, which cannot be totally separated by gas chromatography. In fact, only n-alkanes and a few branched hydrocarbons can be identified as separated compounds (Marchal *et al*., 2003).

Petroleum diesel is produced by the fractional distillation of crude oil between 200°C and 350°C at atmospheric pressure, resulting in a mixture of carbon chains that typically contain between 8 and 21 carbon atoms per molecule. Diesel is composed of about 75% saturated hydrocarbons (primarily paraffins including n-, iso- and cycloparaffins) and 25% aromatic hydrocarbons (including naphthalenes and alkylbenzenes). The average chemical formula for common diesel fuel is $C_{12}H_{23}$, ranging approximately from $C_{10}H_{20}$ to $C_{15}H_{28}$. The density of petroleum diesel is about 0.832 kg/l. About 86.1% of the fuel mass is carbon, and when burned, it offers a net heating value of 43.1 MJ/kg (Demirel, 2012).

Diesel consists mainly of low molecular weight alkanes (about 75%) which are easily biodegradable by microbes (Mukred *et al.,* 2008; Demirel, 2012).

Natural attenuation (NA), biostimulation (BS) and bioaugmentation (BA) techniques are used for bioremediation of diesel contaminated soils, since they are cost effective and environmentally friendly methods (Lee *et al.,* 2011). However, NA or BS occasionally show higher remediation efficiencies than BA treatment, according to environmental conditions, such as pollutants, soil types, pH and so on (Devinny and Chang, 2000; Bento *et al.,* 2003).

2.4 Industrial Effluents

As man advances in technology and industry, large amounts of water are used for industrial activities and consequently significant volumes of wastewaters are generated. Based on the type of industry, various levels of pollutants are deliberately released and discharged into the environment directly. Among these industries that discharge their effluents into the aquatic environments are the petroleum oil refineries. As not all refineries have the same processes, the effluents that are produced will have different chemical compositions depending on the type of treatment they received (Wake, 2005).

It is generally recognized that in many developing countries, industrial environmental standards are lacking, and where they do exist, the instruments of control are not efficient. This is largely explained by the absence of reliable and comprehensive system of monitoring of industrial emissions and enforcement of compliance with the industrial standards (Aluyor and Badmus, 2003).

Pollution from industrial disposal and effluent discharges is becoming a serious environmental issue in many developing countries of Africa (Uzoukwu *et al.,* 2004). The ultimate recipient of all forms of pollution is the natural water body (Otaraku and Nkwocha, 2010). Many industries are located near water bodies, presumably, to facilitate easy disposal of effluents and other wastes into them.

Treatment of waste prior to its discharge into the environment is desirable to avoid pollution. In Nigeria, the Federal Environmental Protection Agency (FEPA) has established guidelines and standards for industrial emissions and effluent discharges (FEPA, 1991). Industries are required by law to monitor their effluents to ensure compliance. This involves treatment of the effluents where necessary, before discharge into the environment.

Due to the ineffectiveness of purification systems, wastewaters may become seriously dangerous, leading to the accumulation of toxic products in the receiving water bodies, with potentially serious consequences on the ecosystem (Beg *et al.,* 2003; Otokunefor and Obiukwu, 2005).

The composition of effluent in refinery wastewater depends on the crude quality. It varies with the operating conditions (Benyahia, 2006). In the refinery, non-hydrocarbon substances are removed and the oil is broken down into its various components and blended into useful products. So, petroleum refineries produce large volumes of wastewater including oil-water

brought to the surface during oil drilling, which often contain a recalcitrant compounds and rich in organic pollutants therefore cannot be treated easily and difficult to be treated biologically (Asatekin and Mayes, 2009; Rasheed *et al.,* 2011; Vendramel *et al.,* 2015). Removal of pollutants produced by industrial plants is requirement for reuse of water and obtains to environmental standards (Farajnezhad and Gharbani, 2012). Petroleum wastewaters are a major source of aquatic environmental pollution and are wastewaters originating from industries primarily engaged in refining crude oil, manufacturing fuels and lubricants (Wake, 2005) and petrochemical intermediates (Harry, 1995). Coelho *et al.,* (2006) reported that the volume of petroleum wastewater generated during processing is 0.4–1.6 times the amount of the crude oil processed (Attiogbe *et al.,* 2007).

Liquid wastes generated by refineries consist of chemical components such as oil and grease, phenols, benzene, toluene, ethyl benzene and xylene (BTEX), ammonia, suspended solids, cyanide, sulfide, nitrogen compounds and heavy metals such as iron (Fe), cadmium (Cd), nickel (Ni), chromium (Cr), copper (Cu), molybdenum (Mo), selenium (S), vanadium (V) and zinc (Zn), most of which are known to be toxic at high concentrations (Oaikhena *et al*., 2016).

These effluents are composed of grease and petroleum compounds, which consist of three main hydrocarbon groups: Paraffin [very few carbon atoms (C_1 to C_4) such as methane (CH_4), ethane (C_2H_6) and propane (C_3H_8)], naphthene [such as cyclohexane (C_6H_{12}) and dimethyl cyclopentane (C_7H_{14})] and aromatics [the more carbon atoms and hydrocarbon molecule such as benzene (C_6H_6), toluene (C_7H_8) and xylene (C_8H_{10})] (Wang *et al.,* 2015). In addition, naphthenic acids (NAs), which are one class of compounds in wastewaters from petroleum industries, that are known to cause toxic effects, and their removal from oilfield wastewater, is an important challenge for remediation of large volumes of petrochemical effluents (Wang *et al.,*, 2015).

When a crude oil contains appreciable quantities of sulphur, it is called ‘sour crude’. So, wastewater is a specific stream of petroleum refineries, which contains slowly biodegradable compounds and toxic substances (Coelho *et al.,* 2006). Petroleum wastewater can vary greatly depending on the plant configuration, operation procedures and type of oil being processed (Saien and Nejati, 2007).

2.4.1 Physical properties of industrial effluents

Petroleum processing unavoidably generates considerable volumes of oily sludge. Common sources of these sludges are storage tank bottoms, oil-water separators, flotation and biological wastewater treatment units, cleaning of processing equipment and occasional minor spills on refinery grounds (Dibble and Bartha, 1979; Mihaela, 2010). Oily sludge contains several toxic hydrocarbon constituents, making the sites contaminated by them a major environmental concern, because many of the constituents of oily sludge are carcinogenic and potent immunotoxicants (Mishra *et al.,* 2001). Oily sludge is a complex mixture of total petroleum hydrocarbon (TPH), water and soil particles. The composition of these sludges varies according to their origin, storage and treatment history. The principal physical characteristics of wastewater include solids content, colour, odour and temperature (Alturkmani, 2013).

The total solids in a wastewater consist of the insoluble or suspended solids and the soluble compounds dissolved in water. The suspended solids content is found by drying and weighing the residue removed by the filtering of the sample. When this residue is ignited, the volatile solids are burned off. Volatile solids are presumed to be organic matter, although some organic matter will not burn and some inorganic salts break down at high temperatures. The organic matter consists mainly of proteins, carbohydrates and fats. Between 40 and 65 % of the solids in an average wastewater are suspended. Settleable solids, expressed as millilitre per litre, are those

that can be removed by sedimentation. Usually, about 60% of the suspended solids in a municipal wastewater are settleable (Alturkmani, 2013).

Colour is a qualitative characteristic that can be used to assess the general condition of wastewater. Wastewater that is light brown in colour is less than 6 hour old while a light-to-medium grey colour is characteristic of wastewaters that have undergone some degree of decomposition or that have been in the collection system for some time. If the colour is dark-grey or black, the wastewater is typically septic, having undergone extensive bacterial decomposition under anaerobic conditions. The blackening of wastewater is often due to the formation of various sulphides, particularly, ferrous sulphide. This results when hydrogen sulphide produced under anaerobic conditions combines with divalent metal, such as iron, which may be present. Colour is measured by comparison with standards (Alturkmani, 2013).

The determination of odour has become increasingly important, as the general public has become more concerned with the proper operation of wastewater treatment facilities. The odour of fresh wastewater is usually not offensive, but a variety of odorous compounds are released when wastewater is decomposed biologically under anaerobic conditions (Alturkmani, 2013).

The temperature of wastewater is commonly higher than that of the water supply because warm municipal water has been added. The measurement of temperature is important because most wastewater treatment schemes include biological processes that are temperature dependent. The temperature of wastewater will vary from season to season and also with geographic location (Alturkmani, 2013).

2.4.2 Chemical properties of industrial effluents

Wastewaters released by oil refineries contain large amounts of toxic derivatives such as oil and grease, phenols, sulphides, cyanides, suspended solids, nitrogen compounds as well as heavy

metals such as iron, nickel, copper, selenium, zinc, molybdenum, etc. (Nwanyanwu and Abu, 2010).

The chemical characteristics of wastewater include free ammonia, organic nitrogen, nitrites, nitrates, organic phosphorus and inorganic phosphorus. Chloride, sulphate, pH and alkalinity tests are performed to assess the suitability of reusing treated wastewater and in controlling the various treatment processes (Alturkmani, 2013).

Trace elements, which include some heavy metals, are not determined routinely, but trace elements may be a factor in the biological treatment of wastewater. All living organisms require varying amounts of some trace elements such as iron, copper, zinc and cobalt for proper growth. Heavy metals can also produce toxic effects; therefore, determination of the amounts of heavy metals is especially important where the further use of treated effluent or sludge is to be evaluated. Many of metals are also classified as priority pollutants such as arsenic, cadmium, chromium, mercury, etc (Alturkmani, 2013).

Chromium is the most widely used and discharged into the environment from different sources. Many of the pollutants entering aquatic ecosystems (e.g., mercury, lead, pesticides and herbicides) are very toxic to living organisms. They can lower reproductive success, prevent proper growth and development, and even cause death (Alturkmani, 2013).

However, chromium is not the metal that is most dangerous to living organisms. Much more toxic are cadmium, lead and mercury. These have a tremendous affinity for sulphur and disrupt enzyme function by forming bonds with sulphur groups in enzymes. Protein carboxylic acid (-CO_2H) and amino (-NH_2) groups are also chemically bound by heavy metals. Cadmium, copper, lead and mercury ions bind to cell membranes, thus hindering transport processes through the cell wall (Alturkmani, 2013).

2.4.3 Microbiological properties of industrial effluents

Microorganisms are vital for the efficient functioning of any ecosystem; hence, factors that affect their metabolism, composition and abundance are of great concern. Monitoring microbial responses has been recommended as an early warning indicator of ecosystem stress as microbes respond promptly to environmental perturbations (Nweke *et al.,* 2007). Measurement of microbial enzyme activity is used in the assessment of ecotoxicological impacts of environmental substrates. In this regard, dehydrogenase activity has been widely used. The dehydrogenase assay is an effective primary test for assessing the potential toxicity of chemicals to microbial activities (Ghaly and Mahmoud, 2006). In this assessment, dehydrogenase activity (DHA) is measured using the reduction of 2, 3, 5-triphenyltetrazolium chloride (TTC) to triphenylformazan (TPF). Determination of their ability to reduce TTC to the formazan product after exposure to test compounds, compared to the control situation, enables the relative toxicity of the chemicals to be assessed (Nwanyanwu and Abu, 2010).

Ammonia is the initial product of the decay of nitrogenous organic wastes, and its presence frequently indicates the presence of such wastes. It is a normal constituent of some sources of groundwater and is sometimes added to drinking water to remove the taste and odour of free chlorine (Alturkmani, 2013).

Hydrogen sulphide (H_2S) is a product of the anaerobic decay of organic matter containing sulphur. It is also produced in the anaerobic reduction of sulphate by microorganisms and is developed as a gaseous pollutant from geothermal waters. Wastes from chemical plants, paper mills, textile mills and tanneries may also contain H_2S. Nitrite ion (NO^{-2}) occurs in water as an intermediate oxidation state of nitrogen. Nitrite is added to some industrial processes to inhibit corrosion; it is rarely found in drinking water at levels over 0.1 mg/l. Sulphite ion (SO_3^{-2}) is

found in some industrial wastewaters. Sodium sulphite is commonly added to boiler feed-waters as an oxygen scavenger (Alturkmani, 2013).

If water of high organic matter content or biochemical oxygen demand (BOD) value flows into a river, the bacteria in the river will oxidize the organic matter, thereby consuming oxygen from the water faster than it dissolves back in from the air. If this happens, fish will die from lack of oxygen; a consequence known as 'fish kill'. A stream must have a minimum of about 2 mg/l of dissolved oxygen to maintain higher life forms (Attiogbe *et al.,* 2009). In addition to this life-sustaining aspect, oxygen is important because the end products of chemical and biochemical reactions in anaerobic systems often produce aesthetically displeasing colours, tastes and odours in water (Attiogbe *et al.,* 2009) .

The most widely used parameter of organic pollution measurement applied to both wastewater and surface water is the 5-day biochemical oxygen demand (BOD_5) (Metcalf and Eddy, 1995). The biochemical oxygen demand is the amount of oxygen, expressed in mg/l or part per million (ppm), that microorganisms take from water when they oxidize organic matter. The test is performed at a defined temperature (normally 20°C) and for a standard period, which is usually 5 days (hence, BOD_5) but can be longer for specific purposes. During the 5day period of the BOD test, the microorganisms oxidize mainly the soluble organic matter present in the water. Very little oxidation of solid (insoluble) matter occurs in that short time (Hach *et al.,* 1997; Attiogbe *et al.,* 2009).

2.5 Bioremediation

Bioremediation is the use of living organisms to degrade or detoxify hazardous wastes into harmless substances such as carbon dioxide, water and cell biomass. Bioremediation is one of the most promising technological approaches to the problem of hazardous waste, which relies on

microorganisms such as bacteria and/or fungi, to transform hazardous chemicals into less toxic or nontoxic substances. Such biological transformation is more attractive than direct chemical or physical treatment. Microorganisms directly degrade contaminants rather than merely transferring them from one medium to another, employ metabolic degradation pathways and can be used in-situ to minimize disturbance of the cleanup site. Hence, microorganisms can be effective, economical and non-disruptive tools for eliminating hazardous chemicals (Rončević *et al.,* 2005; Maletić *et al.,* 2009).

Bioremediation is suggested for treating contaminated soil sites because of its low cost and ability to convert contaminants to harmless end products. Bioremediation can be described as the conversion of chemical compounds by living organisms, especially micro organisms, into energy, cell mass and biological waste products. The rates of uptake and mineralization of many organic compounds by a microbial population depends on the concentration of the compound (Rahman *et al.,* 2002). High concentrations of hydrocarbons can be associated with heavy, undispersed oil slicks in water, causing inhibition of biodegradation by nutrient or oxygen limitation or through toxic effects exerted by volatile hydrocarbons. It is a technology for removing pollutants from the environment, thus restoring the original natural surroundings and preventing further pollution (Sasikumar and Papinazath, 2003).

Most of the physical and chemical methods employed, which in spite of cost, do not always ensure that the contaminants are completely removed (Ajao *et al.,* 2013). Bioremediation is the most desirable approach for cleaning up many environmental pollutants. It is a pollution control technology that uses biological systems to catalyze the degradation or transformation of various toxic chemicals to less harmful forms (Ashoka *et al.,* 2002). Most studies on the metabolism of organic contaminants have been performed with bacteria especially in the context of bioremediation (Ajao *et al.,* 2013).

The streams are difficult to treat due to large concentrations of oil (Aljuboury *et al.,* 2017). The biological treatment methods are attractive due to their cost effectiveness, diverse metabolic pathways and versatility of microorganisms (Ajao *et al.,* 2013).

The continuous development and improvement of microbial remediation technology has also provided a new method for the remediation of petroleum hydrocarbon pollution, which has attracted much attention (Dombrowski *et al.,* 2016; Dvoˇrák *et al.,* 2017).

2.5.1 Types of bioremediation

The wastewater from petroleum industries and refineries mainly contains oil, organic matter and other compounds. The treatment of this wastewater can be carried out by physical, chemical and biological treatment processes. Treatment of petroleum wastewater has two stages: firstly, pre-treatment stage to reduce grease, oil and suspended materials. Secondly, an advanced treatment stage to degrade and decrease the pollutants to acceptable discharge values. Most studies (Aljuboury *et al.,* 2017) are focused on degradation of some pollutants found in the petroleum wastewater such as organic materials, phenols, sulphides and ammonia.

There are two major bioremediation techniques with regards to site of application: *Ex-situ* and *in-stu* bioremediation techniques.

2.5.1.1 Ex-situ bioremediation techniques

These techniques involve excavating pollutants from polluted sites and subsequently transporting them to another site for treatment. Ex-situ bioremediation techniques are usually considered based on the cost of treatment, type of pollutant, degree of pollution, geographical location and geology of the polluted site. Performance criteria, which also determine the choice of ex-situ bioremediation techniques, have been described (Philip and Atlas, 2005).

Biopile:

Biopile-mediated bioremediation involves above-ground piling of excavated polluted soil, followed by nutrient amendment and sometimes aeration, to enhance bioremediation by basically increasing microbial activities. The components of this technique are aeration, irrigation, nutrient and leachate collection systems, as well as a treatment bed. The use of this particular ex-situ technique is increasingly being considered due to its constructive features including cost effectiveness, which enables effective biodegradation on the condition that nutrient, temperature and aeration are adequately controlled (Whelan *et al.,* 2015). The application of biopile to polluted sites can help limit volatilization of low molecular weight (LMW) pollutants; it can also be used effectively to remediate polluted extreme environments such as the very cold regions (Gomez and Sartaj 2014; Dias *et al.,* 2015; Whelan *et al.,* 2015). The flexibility of biopile allows remediation time to be shortened as heating system can be incorporated into biopile design to increase microbial activities and contaminant availability, thus, increasing the rate of biodegradation (Aislabie *et al*., 2006). Furthermore, heated air can be injected into biopile design to deliver air and heat in tandem, in order to facilitate enhanced bioremediation. In another study, Sanscartier *et al.,* (2009) reported that humidified biopile had a very low final TPH concentration compared to heated and passive biopiles as a result of optimal moisture content, reduced leaching and minimal volatilization of less degradable contaminants. In addition, it was reported (Chemlal *et al.,* 2013) that biopile could be used to treat large volume of polluted soil in a limited space. Biopile setup can easily be scaled up to a pilot system to achieve similar performance obtained during laboratory studies. Important to the efficiency of biopile is sieving and aeration of contaminated soil prior to processing (Delille *et al.,* 2008). Bulking agents such as straw, saw dust, bark or wood chips and other organic materials have been added to enhance remediation process in a biopile construct (Rodrı ´guez-Rodrı ´guez *et al.,* 2010).

Although biopile systems conserve space compared to other field ex-situ bioremediation techniques, including land farming, robust engineering, cost of maintenance and operation, lack of power supply especially at remote sites, which would enable uniform distribution of air in contaminated piled soil via air pump are some of the limitations of biopiles. More so, excessive heating of air can lead to drying of soil undergoing bioremediation, which will result in inhibition of microbial activities, and promote volatilization rather than biodegradation (Sanscartier *et al.,* 2009).

Windrows:

As one of *ex-situ* bioremediation techniques, windrows rely on periodic turning of piled polluted soil to enhance bioremediation by increasing degradation activities of indigenous and/or transient hydrocarbonoclastic bacteria present in polluted soil. The periodic turning of polluted soil, together with addition of water bring about increase in aeration, uniform distribution of pollutants, nutrients and microbial degradative activities, thus, speeding up the rate of bioremediation, which can be accomplished through assimilation, biotransformation and mineralization (Barr, 2002). Windrow treatment when compared to biopile treatment, showed higher rate of hydrocarbon removal; however, the higher efficiency of the windrow towards hydrocarbon removal was as a result of the soil type, which was reported to be more friable (Coulon *et al.,* 2010). Nevertheless, due to periodic turning associated with windrow treatment, it may not be the best option to adopt in remediating soil polluted with toxic volatiles.

Bioreactor:

Bioreactor, as the name implies, is a vessel in which raw materials are converted to specific product(s) following series of biological reactions. There are different operating modes of bioreactor, which include batch, fed-batch, sequencing batch, continuous and multistage. The

choice of operating mode depends mostly on market economy and capital expenditure. Conditions in a bioreactor support natural process of cells by mimicking and maintaining their natural environment to provide optimum growth conditions. Polluted samples can be fed into a bioreactor either as dry matter or slurry; in either case, the use of bioreactor in treating polluted soil has several advantages compared to other *ex-situ* bioremediation techniques. Excellent control of bioprocess parameters (temperature, pH, agitation and aeration rates, substrate and inoculum concentrations) is one of the major advantages of bioreactor-based bioremediation. The ability to control and manipulate process parameters in a bioreactor implies that biological reactions within can be enhanced to effectively reduce bioremediation time. Importantly, controlled bioaugmentation, nutrient addition, increased pollutant bioavailability and mass transfer (contact between pollutant and microbes), which are among the limiting factors of bioremediation process, can effectively be established in a bioreactor, thus, making bioreactor-based bioremediation more efficient. Further, it can be used to treat soil or water polluted with volatile organic compounds (VOCs) including benzene, toluene, ethylbenzene and xylenes (BTEX). The flexible nature of bioreactor designs allows maximum biological degradation while minimizing abiotic losses (Mohan *et al.*, 2004). Short or long-term operation of a bioreactor containing crude oil-polluted soil slurry allows tracking of changes in microbial population dynamics, thus, enabling easy characterization of core bacterial communities involved in bioremediation processes (Chikere *et al.*, 2012; Zangi-Kotler *et al.*, 2015). Furthermore, it allows the use of different substances as biostimulant or bioaugmenting agent including sewage sludge. In addition, bioreactor being an enclosed system, genetically-modified microorganisms (GEM_s) can be used for bioaugmentation after which the organisms (GEM_s) can be destroyed before treated soils are returned to field for landfilling. This containment of GEM_s in a bioreactor, followed by destruction, will help ensure that no foreign gene escapes into an environment after

bioremediation. With bioreactor, the role of biosurfactant was found to be insignificant due to efficient mixing associated with bioreactor operations (Mustafa *et al.,* 2015). Moreover, bioreactor-based bioremediation is not a popular full-scale practice due to some reasons. Firstly, due to bioreactor being *ex-situ* technique, the volume of polluted soil or other substances to be treated may be too large, requiring more manpower, capital and safety measures for transporting pollutant to treatment site, therefore, making this particular technique cost ineffective (Philp and Atlas, 2005). Secondly, due to several bioprocess parameters or variables of a bioreactor, any parameter that is not properly controlled and/or maintained at optimum, may become a limiting factor; this in turn, will reduce microbial activities and make bioreactor-based bioremediation process less effective. Lastly, pollutants are likely to respond differently to different bioreactors; the availability of the most suitable design is of paramount importance. Above all, cost of a bioreactor suitable for a laboratory or pilot-scale bioremediation makes this technique to be capital intensive.

Land farming:

Land farming is amongst the simplest bioremediation techniques owing to its low cost and less equipment requirement for operation. In most cases, it is regarded as *ex-situ* bioremediation, while in some cases, it is regarded as *in-situ* bioremediation technique. This debate is due to the site of treatment. Pollutant depth plays an important role as to whether land farming can be carried out *ex-situ* or *in-situ*. In land farming, one thing is common, polluted soils are usually excavated and/or tilled, but the site of treatment apparently determines the type of bioremediation. When excavated polluted soil is treated on-site, it can be regarded as *in-situ*; otherwise, it is *ex-situ* as it has more in common with other *ex-situ* bioremediation techniques (Christopher *et al.,* 2016). It has been reported that when a pollutant lies <1 m below ground surface, bioremediation might proceed without excavation while pollutant lying >1.7 m needs to

be transported to ground surface for bioremediation to be effectively enhanced (Nikolopoulou *et al.,* 2013). Generally, excavated polluted soils are carefully applied on a fixed layer support above the ground surface to allow aerobic biodegradation of pollutant by autochthonous microorganisms (Philp and Atlas 2005; Paudyn *et al.,* 2008; Volpe *et al.,* 2012; Silva-Castro *et al.*, 2015). Tillage, which brings about aeration, addition of nutrients (nitrogen, phosphorus and potassium) and irrigation are the major operations, which stimulate activities of autochthonous microorganisms to enhance bioremediation during land farming (Christopher *et al.,* 2016). Nevertheless, it was reported that tillage and irrigation without nutrient addition in a soil with appropriate biological activity increased heterotrophic and diesel-degrading bacterial counts, thus, enhancing the rate of bioremediation; dehydrogenase activity was also observed to be a good indicator of biostimulation treatment and could be used as a biological parameter in land farming technology (Silva-Castro *et al.,* 2015). Similarly, in a field trial, Paudyn *et al.,* (2008) reported 80 % contaminant (diesel) removal by aeration using rototilling approach at remote Canadian Arctic location over a 3-year study period; this further demonstrates that in land farming technique, aeration plays crucial role in pollutant removal especially at cold regions. Land farming is usually used for remediation of hydrocarbon-polluted sites including polyaromatic hydrocarbons (Silva-Castro *et al.,* 2012; Cerqueira *et al.,* 2014); as a result, biodegradation and volatilization (weathering) are the two remediation mechanisms involved in pollutant removal. Land farming bioremediation technique is very simple to design and implement, requires low capital input and can be used to treat large volume of polluted soil with minimal environmental impact and energy requirement (Maila and Colete, 2004). Although the simplest bioremediation technique, land farming like other *ex-situ* bioremediation techniques has some limitations, which include: large operating space, reduction in microbial activities due to unfavourable environmental conditions, additional cost due to excavation and reduced efficacy in

inorganic pollutant removal (Khan *et al.*, 2004; Maila and Colete, 2004). Moreover, it is not suitable for treating soil polluted with toxic volatiles due to its design and mechanism of pollutant removal (volatilization), especially in hot (tropical) climate regions. These limitations and several others make land farming-based bioremediation time consuming and less efficient compared to other *ex-situ* bioremediation techniques.

One of the major advantages of *ex-situ* bioremediation techniques is that they do not require extensive preliminary assessment of polluted site prior to remediation; this makes the preliminary stage short, less laborious and less expensive. The excavation features of *ex-situ* bioremediation tend to disrupt soil structure; as a result, polluted and surrounding sites alike experience more disturbances (Christopher *et al.*, 2016). Generally, *ex-situ* bioremediation techniques tend to be faster, easier to control and can be used to treat wide range of pollutants (Prokop *et al.*, 2000).

2.5.1.2 In-situ bioremediation techniques

These techniques involve treating polluted substances at the site of pollution. It does not require any excavation; therefore, it is accompanied by little or no disturbance to soil structure. Ideally, these techniques ought to be less expensive compared to *ex-situ* bioremediation techniques, due to no extra cost required for excavation processes; nonetheless, cost of design and on-site installation of some sophisticated equipment to improve microbial activities during bioremediation is of major concern. Some *in-situ* bioremediation techniques might be enhanced (bioventing, biostimulation, bioaugmentation, biosparging and phytoremediation) while others might proceed without any form of enhancement (intrinsic bioremediation or natural attenuation). *In-situ* bioremediation techniques have been successfully used to treat chlorinated solvents, dyes, heavy metals, and hydrocarbons-polluted sites (Folch *et al.*, 2013; Kim *et al.*,

2014; Frascari *et al.*, 2015; Roy *et al.*, 2015). Notably, the status of electron acceptor, moisture content, nutrient availability, pH and temperature are amongst the important environmental conditions that need to be suitable for a successful *in-situ* bioremediation to be achieved (Philp and Atlas, 2005). Unlike *ex-situ* bioremediation techniques, soil porosity strongly influences the application of in-situ bioremediation to any polluted site.

Bioventing:

This technique involves controlled stimulation of airflow by delivering oxygen to unsaturated (vadose) zone in order to increase bioremediation, by increasing activities of indigenous microbes. In bioventing, amendments are made by adding nutrients and moisture to enhance bioremediation with the ultimate goal being to achieve microbial transformation of pollutants to a harmless state (Philp and Atlas, 2005). This technique has gained popularity among other *in-situ* bioremediation techniques especially in restoring sites polluted with light spilled petroleum products (Hohener and Ponsin, 2014).

Biostimulation:

This technique utilizes indigenous microbial populations to remediate contaminated soils with and without addition of nutrients. It involves stimulating naturally occurring microbial communities, providing them with nutrients and other needs, to break down a contaminant. In many studies (Christopher *et al.*, 2016) on PHC bioremediation, natural attenuation by microorganisms was improved significantly by biostimulation and optimizing conditions such as temperature, moisture, pH, aeration and addition of nutrients such as agroindustry sludge, fertilizers, manure and crop residue. For instance, Agarry *et al.*, (2010) used a combination of poultry manure, piggery manure, goat manure and NPK fertilizer to remediate soil containing mixture of 10% (w/w) kerosene, diesel and gasoline. The authors observed 73%, 63%, 50% and

39% TPH degradation in poultry manure, piggery manure, goat manure and NPK fertilizer added soils after 4 weeks, respectively. Similarly, Coulon *et al.* (2005) studied the effects of nutrients and temperature on degradation of PHC in artificially-contaminated sub-Antarctic soil with crude oil. The authors reported that increasing the temperature from 4°C to 20°C and addition of oleophilic fertilizer containing N and P increased the hydrocarbon degrading microbial abundance and TPH degradation. After 180 days, the total alkane degradation was 77- 95% and total PAHs was 80%.

Bioaugmentation:

Bioaugmentation involves addition of specific microorganisms, native or exogenous, to the contaminated sites for effective bioremediation. Bioaugmentation can be carried out by inoculating whole cells or encapsulating the cells in a carrier material. For instance, Catia *et al.* (2010) compared biodegradation of phenol by free and encapsulated cells of *Aspergillus* sp. strain isolated from a crude oil-contaminated soil. The authors observed no significant difference in degradation potential between the two types but encapsulated cells adapted faster in batch cultures as compared to free cells. Gene bioaugmentation is also practiced wherein the inoculated culture transfers remediation genes to indigenous microorganisms. The observation that introduced organisms have low survival rates following bioaugmentation led to investigations of use of naturally-occurring horizontal gene transfer processes for introduction of remediation genes into a contaminated site. Horizontal gene transfer may occur via transformation, transduction, conjugation or exchange of genetic material such as plasmids (Gentry *et al.*, 2004). Mishra *et al.* (2001) reported the use of indigenous bacterial consortium grown in a laboratory as an inoculum to stimulate in-situ bioremediation of oily-sludge-contaminated soil. Bento *et al.* (2005) used a consortium of *Bacillus* species such as *B. cereus, B. sphaericus, B. fusiformis, B. pumilus, Acinetobacter junii* and *Pseudomonas* species for the degradation of hydrocarbons in

soil. The microbial flora degraded light fractions (C_{12}- C_{23}) more efficiently than heavy (C_{23}–C_{40}) fractions of TPH. Furthermore, they compared the effectiveness of other methods namely, natural attenuation, biostimulation with bioaugmentation and found the latter treatment most effective.

Bioslurping:

This technique combines vacuum-enhanced pumping, soil vapour extraction and bioventing to achieve soil and groundwater remediation by indirect provision of oxygen and stimulation of contaminant biodegradation (Gidarakos and Aivalioti, 2007). The technique is designed for free products recovery such as light non-aqueous phase liquids (LNAPLs), thus, remediating capillary, unsaturated and saturated zones. It can also be used to remediate soils contaminated with volatile and semi-volatile organic compounds. The system uses a ‘‘slurp’’ that extends into the free product layer, which draws up liquids (free products and soil gas) from this layer in a manner similar to that of how a straw draws liquid from any vessel. The pumping mechanism brings about upward movement of LNAPLs to the surface, where it becomes separated from water and air. Following complete free products removal, the system can easily be made to operate as a conventional bioventing system to complete remediation process (Kim *et al.*, 2014). In this technique, excessive soil moisture limits air permeability and decreases oxygen transfer rate, thereby in turn reducing microbial activities. Although the technique is not suitable for remediating soil with low permeability, it saves cost due to less amount of groundwater resulting from the operation, thus, minimizes storage, treatment and disposal costs (Philp and Atlas, 2005).

Biosparging:

This technique is very similar to bioventing in that air is injected into soil subsurface to stimulate microbial activities in order to promote pollutant removal from polluted sites. However, unlike

bioventing, air is injected at the saturated zone, which can cause upward movement of volatile organic compounds to the unsaturated zone to promote biodegradation. The effectiveness of biosparging depends on two major factors namely: soil permeability, which determines pollutant bioavailability to microorganisms and pollutant biodegradability (Philp and Atlas, 2005).

Biosparging is similar to bioventing, which involves supply of air under pressure in the saturated zone so as to vaporize the volatile contaminants. The vaporized contaminants get carried to the unsaturated zone where they are degraded by microorganisms. Gasoline components such as benzene, toluene, ethylbenzene and xylenes (BTEX) have been successfully degraded using biosparging with natural micro flora mainly *Candidauts magnetobacterium*, Flavobacteriales bacterium and Bacteroidetes bacterium (Kao *et al.,* 2008).

Phytoremediation:

This technique relies on the use of plant interactions (physical, biochemical, biological, chemical and microbiological) in polluted sites to mitigate the toxic effects of pollutants. Depending on pollutant type (elemental or organic), there are several mechanisms (accumulation or extraction, degradation, filtration, stabilization and volatilization) involved in phytoremediation. Elemental pollutants (toxic heavy metals and radionuclides) are mostly removed by extraction, transformation and sequesteration. On the other hand, organic pollutants (hydrocarbons and chlorinated compounds) are predominantly removed by degradation, rhizoremediation, stabilization and volatilization, with mineralization being possible when some plants such as willow and alfalfa are used (Meagher, 2000; Kuiper *et al.,* 2004).

Phytoremediation is the use of plants for *in-situ* remediation of contaminated soil, sludge, sediment and groundwater through any of the mechanism: extraction, filtration, stabilization, degradation and evapotranspiration. A number of plants, which have extensive fibrous roots such

as common grasses, corn, wheat, soyabean, peas and beans, were studied for their rhizoremediation potential (Glick, 2003). Several trees of family Salicaceae (poplar and willow), which can grow fast and possess a deep rooting ability, were planted to bioremediate soil polluted with 20,000 mg/kg gasoline and diesel compounds to a depth of 3 m (Trapp *et al.,* 2001). However, the authors observed that at high concentrations, e.g., >5000 mg/kg hydrocarbons, willows could not be used for phytoremediation due to toxic effects. Thus, use of willows for bioremediation was restricted and localized for low hydrocarbon contamination.

Pradhan *et al.,* (1998) used phytoremediation technology for the treatment of soil contaminated with PAHs. Three plant species, alfalfa (*Medicago sativa*), switch grass (*Panicum virgatum*) and little bluestem grass (*Schizachyrium scoparium*) were found to remediate 72% of total PAH within 6 months. White *et al.* (2006) studied phytoremediation of two to four ring alkylated PAHs in crude oil-contaminated soil using treatment systems involving combination of fescue (*Lolium arundinaceum*), rye grass (*Lolium multiflorum* L.) and Bermuda grass (*Cynodon dactylon* L.). The authors reported a degradation pattern of 2-ring > 3-ring > 4-ring, which decreased with increased alkylation of larger ringed structures. They also observed an increase in the degradation with addition of plants and fertilizer. Tanee and Akonye (2009) observed that *Vigna unguiculata* could remediate 5% (v/w) crude oil-polluted soil by reducing the total hydrocarbon contents by 54% within two months along with an improved growth and yield of the bean crop in the phytoremediated soil.

After phytoremediation, plants can then be subsequently harvested, processed and disposed of (Lee *et al.,* 2008). The plants usually influence rhizosphere microbial community which can play a role in rhizoremediation. However, members of plants are also reported to produce enzymes such as cytochrome P450 and peroxidase involved in the metabolism of n-alkanes. Vega-Jarquin

et al. (2001) showed that the cell cultures of *Cinchona robusta* and *Dioscorea composita* could take up and metabolize n-hexadecane with the formation of hexadecanol and hexadecanoic acid. They further reported that the levels and activities of cytochrome P450 and peroxidase were increased, suggesting their role in biotransformation of n-hexadecane.

Phytoremediation is limited to the surface area and depth occupied by the roots. Secondly, the time required is also more as the plants involved grow slowly with low biomass. The leaching of contaminants into the groundwater cannot be avoided in plant-based systems of remediation (Kuiper *et al.,* 2004).

Permeable reactive barrier (PRB):

This technique is mostly perceived as a physical method for remediating contaminated groundwater due to its design and mechanism of pollutant removal. Nevertheless, researchers (Thiruvenkatachari *et al.,* 2008; Obiri-Nyarko *et al.,* 2014) reported that biological reaction is one of the several mechanisms

In general, PRB is an *in-situ* technique used for remediating groundwater polluted with different types of pollutants including heavy metals and chlorinated compounds (Christopher *et al.,* 2016). In this technique, a permanent or semi-permanent reactive barrier (medium), mostly made up of a zero-valent iron (Garcı ´a *et al.,* 2014; Zhou *et al.,* 2014), is submerged in the trajectory of polluted groundwater. As polluted water flows through the barrier under its natural gradient, the pollutants become trapped and undergo series of reactions resulting in clean water in the flow through (Thiruvenkatachari *et al.,* 2008; Obiri-Nyarko *et al.,* 2014). Ideally, the barriers are usually reactive enough to trap pollutants, permeable to allow the flow of water but not pollutants, passive with little energy input, inexpensive, readily available and accessible (De Pourcq *et al.,* 2015).

2.5.2 Merits of bioremediation

1. It is a publicly accepted treatment of polluted soil because it is based on natural process. Microbes that metabolize contaminants increase in population when the contaminant is present, and decline in population when the contaminant is degraded. The products from treatment are usually harmless products; such as water, carbon dioxide and cellular biomass.
2. It requires very less effort as bioremediation is theoretically meant to completely degrade a wide range of pollutants on-site without causing major disruption of normal activities. This eliminates the risks involved with transportation of pollutants off -site and the potential threats to human health and the environment that can arise during transportation.
3. It is a cost effective process compared to other technologies used for clean-up of hazardous wastes.
4. Bioremediation is meant to completely eliminate specific pollutants without the risks of transferring contaminants from one environmental medium to another (water, land and air).
5. Eco-friendly and sustainable.
6. Nonintrusive, potentially allowing for continued site use.
7. Relative ease of implementation.
8. Effective way of remediating natural ecosystem from a number of contaminants and act as environmental friendly option.

2.5.3 Demerits of bioremediation

1. It is limited to those compounds that are capable of undergoing bioremediation. Not every compound is susceptible to rapid and complete degradation.

2. The products of biodegradation may potentially be even more persistent or toxic than the original contaminants.
3. Biological processes are usually extremely specific and require the presence of microbes that are capable of metabolizing the contaminants. In order for the microbes to be present, the appropriate environmental conditions, appropriate level of nutrients and contaminants need to be met.
4. It is difficult to extrapolate from bench and pilot-scale studies to full-scale field operations.
5. More research needs to be completed to create technologies that can adapt, because the real environment contains contaminants that are mixed, unevenly distributed and in different phases (solids, liquids and gases).
6. It is time consuming, usually takes longer time than other treatment options such as excavati on or incineration.
7. Requires very skilled human power.

2.6 Hydrocarbon-Degrading Bacteria

Although oil pollution is difficult to treat, petroleum hydrocarbon-degrading bacteria have evolved as a result of existing in close proximity to naturally-occurring petroleum hydrocarbons in the environment. Such organisms are candidates for the treatment of oil pollutants (Margesin *et al.,* 2003; Ron and Rosenberg, 2014; Lea-Smith *et al.,* 2015). Therefore, bacteria have been screened and utilized to degrade waste products produced by the food, agricultural, chemical and pharmaceutical industries. In recent years, the use of bacteria to deal with environmental pollutants has become a promising technology because of its low cost and eco-friendly nature (Guerra *et al.,* 2018).

Most petroleum hydrocarbons encountered in the environment are ultimately degraded or metabolized by indigenous bacteria because of their energetic and carbon needs for growth and reproduction, as well as the requirement to relieve physiological stress caused by the presence of petroleum hydrocarbons in the microbial bulk environment (Hazen *et al.,* 2010; Kleindienst *et al.,* 2015a). Indeed, many studies have revealed that there is a large number of hydrocarbon-degrading bacteria in oil-rich environments, such as oil spill areas and oil reservoirs (Hazen *et al.,* 2010; Yang *et al.,* 2015), and that their abundance and quantity are closely related to the types of petroleum hydrocarbons and the surrounding environmental factors (Fuentes *et al.,* 2015; Varjani and Gnansounou, 2017).

Recent studies have identified bacteria from more than 79 genera that are capable of degrading petroleum hydrocarbons (Tremblay *et al.,* 2017); several of these bacteria such as *Achromobacter, Acinetobacter, Alkanindiges, Alteromonas, Arthrobacter, Burkholderia, Dietzia, Enterobacter, Kocuria, Marinobacter, Mycobacterium, Pandoraea, Pseudomonas, Staphylococcus, Streptobacillus, Streptococcus* and *Rhodococcus* have been found to play vital roles in petroleum hydrocarbon degradation (Margesin *et al.,* 2003; Chaerun *et al.,* 2004; Jin *et al.,* 2012; Nie *et al.,* 2014; Varjani and Upasani, 2016; Sarkar *et al.,* 2017; Varjani, 2017; Xu *et al.,* 2017). Alkanindiges sp., have been reported to exhibit rare-to-dominant bacterial shifts that are strongly affected by environmental constrains such as diesel pollution (Fuentes *et al.,* 2015). Similarly, some obligate hydrocarbonoclastic bacteria (OHCB), including *Alcanivorax, Marinobacter, +Thallassolituus, Cycloclasticus, Oleispira* and a few others (the OHCB), showed a low abundance or undetectable status before pollution, but were found to be dominant after petroleum oil contamination (Yakimov *et al.,* 2007). These phenomena suggest that these microorganisms are crucial to the degradation of petroleum hydrocarbons, and that they significantly influence the transformation and fate of petroleum hydrocarbons in the environment. Although some bacteria

have been reported to have a broad spectrum of petroleum hydrocarbon degradation ability, *Dietzia* sp. utilizes n-alkanes (C_6–C_{40}) and other compounds as the sole carbon sources (Wang *et al.,* 2011) and *Achromobacter xylosoxidans* works well on a variety of monoaromatic and polyaromatic hydrocarbons (Ma *et al.,* 2015), almost no bacteria can degrade the entire petroleum hydrocarbon fraction. Indeed, most bacteria can only effectively degrade or utilize certain petroleum hydrocarbon components, while others are completely unavailable (Chaerun *et al*., 2004; Varjani, 2017). This can be attributed to the fact that different indigenous bacteria have different catalytic enzymes; thus, their roles in oil-contaminated sites also vary widely. This also implies that the remediation of petroleum hydrocarbon contamination requires the joint action of multiple functional bacteria to achieve the best environmental purification effect (Dombrowski *et al.,* 2016).

Numerous genera of bacteria are known as good hydrocarbon degraders. Most of them belong to *Aeromonas, Alcaligenes, Acinetobacter, Arthobacter, Bacillus, Brevibacterium, Flavobacterium, Geobacillus, Micrococcus, Mycobacterium, Ochrobactrum, Pseudomonas, Rhodococcus, Sphingomonas, Thermus* and *Xanthomonas* species (Plaza *et al.*, 2008).

Rahman *et al.*, (2002) used *Micrococcus* sp, *Corynebacterium* sp, *Flavobacterium* sp and *Pseudomaonas* sp individually as well as their consortium for degradation studies. They observed that the bacterial consortium degraded 78% of 1% crude oil, which was higher than the percentage degradation by individual cultures. Schippers *et al.* (2005) reported two novel crude oil degrading bacteria (*Microbacterium oleivorans* sp. and *Microbacterium hydrocarbonoxydans* sp). Verma *et al.* (2006) tested the ability of three bacterial strains (*Bacillus* sp, *Acinetobacter* sp and *Pseudomonas* sp) from contaminated soil in Ankleshwar (21°60' N 73°00' E), India for their ability to degrade the complex mixture of petroleum hydrocarbons (such as alkanes, aromatics,

resins and asphaltenes), sediments, heavy metals and oily sludge. Wook *et al.* (2006) isolated a psychrotrophic *Rhodococcus* sp. from an oil-contaminated ground water, which could degrade various petroleum hydrocarbons such as crude oil, diesel oil and gasoline by almost 90% within 20days and was able to grow in 7% NaCl at 4°C.

Auffret *et al.* (2009) reported two strains (*R. wratislaviensis* and *R. Aetherivorans*) that could degrade more than 15 petroleum compounds from a mixture of benzene, toluene, ethylbenzene, m-xylene, p-xylene, o-xylene, octane, hexadecane, 2,2,4-trimethylpentane (isooctane), cyclohexane, cyclohexanol, naphthalene, methyl tert-butyl ether (MTBE), ethyl tert-butyl ether (ETBE), tert-butyl alcohol (TBA), and 2-ethylhexyl nitrate (2-EHN). The co-culture degraded 13 compounds completely. Interestingly, these strains had broad degradation capacities towards the more recalcitrant compounds like MTBE, ETBE, isooctane, cyclohexane and 2-EHN. Olajide and Ogbeifun (2010) studied the hydrocarbon-degrading potential of *Proteus vulgaris* strain isolated from fish samples. John and Okpokwasili (2012) studied the utilization of kerosene, diesel, jet fuel and engine oil as carbon sources by autotrophic nitrifying bacteria (*Nitrosomonas* and *Nitrobacter s*pecies) and reported that mixed culture of the isolates degraded 52% of crude oil followed by 40% by *Nitrosomonas* sp. and 20% by *Nitrobacter* sp.

The mechanisms of toxicity to microbial membranes caused by hydrocarbons have well been studied and comprehensively reviewed (Ramos *et al.*, 2002; Van Hamme *et al.*, 2003). The accumulation of toxic hydrocarbons in the membrane increases membrane fluidity (allowing the leakage of macromolecules such as RNA, phospholipids, proteins), increases membrane swelling and reduces the normal functioning of membrane-associated proteins (Norman *et al.*, 2004; Zahir *et al.*, 2006). The accumulation of hydrocarbons results in the disruption of bilayer stability and membrane structure, causing a loss of membrane function and ultimately cell death. Despite this extreme toxicity, hydrocarbon-tolerant Gram-negative and Gram-positive bacteria that are

capable of growing in a two-phase water-hydrocarbon system have been isolated (Segura *et al.*, 2008). Many of these tolerant bacterial species, including the first strain isolated, were Gram-negative bacteria such as *Pseudomonas putida* (Kim *et al.*, 1998) or closely related *Pseudomonas* sp. (Segura *et al.*, 2008). Gram-positive bacteria such as *Bacillus* (Zahir *et al.*, 2006; Segura *et al.*, 2008), *Rhodococcus* (Plaza *et al.*, 2008), *Staphylococcus* (Zahir *et al.*, 2006) and *Exiguobacterium* (Segura *et al.*, 2008) have also been found to be hydrocarbon-tolerant, although limited investigation has occurred towards understanding the mechanisms of their hydrocarbon tolerance. Because of the highly impermeable outer membrane of Gram-negative bacteria, it was generally accepted that this type of bacteria are more tolerant to hydrocarbons than Gram-positive bacteria (Isken *et al.*, 1998). However, several Gram-positive bacteria have seemed to be more resistant (Matsumoto *et al.*, 2002; Zahir *et al.*, 2006; Segura *et al.*, 2008). Because of the different experimental set-ups used in the published literature, it has been difficult to compare the hydrocarbons tolerance of different strains, and extremely difficult to compare hydrocarbons tolerance between Gram-positive and Gram-negative strains (Segura *et al.*, 2008).

2.6.1 *Pseudomonas putida*

Pseudomonas putida is a Gram-negative, obligately aerobic, rod-shaped, non-spore forming and motile by one or several polar flagella bacterium, with a cell size of 0.5-1.0 × 1.5-5.0 μm (Palleroni, 2005).

P. putida is found in most soil and water habitats where there is availability of oxygen (Danhorn and Fuqua, 2007; Olapade *et al.* 2005; Palleroni 2005), but it may be best suited to live in terrestrial environments (Olapade *et al.* 2005). It has been isolated from various sources including but not limited to:

- **Soils and rhizosphere**: Polluted soils (Timmis, 2002) and rhizospheres of rice, sugarcane and peas (De Castro *et al.,* 2010; Silva *et al.,* 2009; Sutra *et al.,* 2000; Hassan *et al.,* 2011; Berggren *et al.* 2005)
- **Animals**: Frog skin (Flechas *et al.* 2012) and kidney samples collected from ornamental fish of various species, sampled for the presence of bacteria (Rose *et al.* 2013),
- **Aquatic environments:** Marine environments with oil contamination (Jalal *et al.* 2012), Freshwater and wastewater mixed liquor samples of the Kat River in Fort Beaufort and the Tyume River in Alice, South Africa (Igbinosa *et al.* 2012).
- **Humans:** Homemakers' hands were sampled in Manhattan, USA and *P. fluorescens*/*putida* was the most prevalent bacterium (Aiello *et al.,* 2003).
- **Built environments:** Security swipe cards in hospitals in United Kingdom (Sultan *et al.,* 2009); household surfaces, water, drains and garbage (Remold *et al.* 2011).
- **Food:** Refrigerated raw cow and goat milk samples (Callon *et al.* 2007), spoiled refrigerated meat and fish (Speranza *et al.* 2010).
- **Others:** Isolated in air sample from dusts from polluted harbor air in Rouen, France (Duclairoir Poc *et al.* 2014).

Pseudomonas putida has the ability to proliferate and to be competitive in a variety of environments (reviewed in Cray *et al.* 2013), and can also persist in environments with low nutrient availability (Palleroni, 2005). Factors known to affect the persistence of *Pseudomonas putida* in terrestrial environments include both the soil type and the water content of the substrate (Mirleau *et al.,* 2005). Additionally, the characteristic flagellar motility of the species is known to contribute to its dispersal in hydrated soil and aquatic environments (reviewed in Dechesne *et al.,* 2010).

The ability of *Pseudomonas putida* to metabolize hydrocarbon compounds may be transferable to other bacteria in the environment, as conjugation can occur with *Pseudomonas putida* in the environment and in contaminated industrial sites. *Pseudomonas putida* intra-species transfer of the TOL plasmid pWWO coding for alkylbenzoate metabolism and toluene and xylene degradation has been reported to occur in soils (Greated *et al.*, 2002; OECD, 1997).

Furthermore, transfer of the pHCL catabolic plasmid coding for hydrocarbon degradation from the terrestrial *Pseudomonas putida* strain to marine bacteria (*Micrococcus luteus* and *M. varians*) was seen successfully in *in-situ* and *in-vitro* conjugation testing, allowing for possible routes of bioremediation by natural marine species (Latha and Lalithakumari, 2001).

P. putida can persist and grow in heavy metal-contaminated water and sequester heavy metals via biosorption and bioaccumulation (Kamika and Momba, 2013). Resistances/tolerances to the following heavy metals have been observed: mercury (Zhang *et al.,* 2012) as well as methylmercury, copper, lead, nickel, chromate, zinc, cobalt, manganese and barium over a wide range of pH and temperature (Cabral *et al.,* 2013).

Pseudomonas putida is metabolically diverse, which could make it of commercial interest in a variety of industries, particularly in the degradation of xenobiotic compounds. A search of the public domain and the Canadian Intellectual Property Office Patent Database (CIPO, 2014) yielded the following potential uses of other naturally-occurring strains of *Pseudomonas putida*:

- Activated sludge in wastewater treatment processes (Pramanik *et al.,* 2011).
- Wastewater treatment of pharmaceutical drugs containing a mix of antibiotic drugs including amoxicillin and cefadroxil (Krifa *et al.,* 2013).

The fuel-eating bacteria, known as *Pseudomonas* sp., have evolved a taste for hydrocarbons, which are the major component of fossil fuels. Degradation of oils by *Pseudomonas* sp. is the best carrier based inoculums. These bacteria are found in different environments such as soil, water as well as plant and animal tissues. Various different species of this bacterium are opportunistic pathogens that affect humans, other animals and plants (Shivendra and Hardik, 2014).

Pseudomonas sp. are ubiquitous in soil and water. They comprise a taxon of metabolically versatile organisms, which are capable of utilizing a wide range of simple and complex organic compounds. They are known to be involved in biodegradation of natural or man-made toxic chemical compounds. *Pseudomonas* sp. is a prolific producer of a number of extra cellular enzymes (like lipase) (Shivendra and Hardik, 2014).

2.6.3 *Bacillus megaterium*

Bacillus megaterium is a rod-like, Gram-positive, mainly aerobic, spore-forming bacterium, found in widely diverse habitats from soil to seawater, sediment, rice paddies, honey, fish, and dried food. With a cell length of up to 4 µm and a diameter of 1.5 µm, *B. megaterium* is amongst the biggest known bacteria.

In 1884, De Bary named *B. megaterium* "big beast" because of its large size with a volume approximately 100 times that of *Escherichia coli*. It can grow in simple media on over 62 carbon sources out of 95 tested including all tricarboxylic acid cycle intermediates, formate and acetate (Patricia *et al.*, 2007).

Bacillus megaterium (BM) is a well known soil microbe It is a Gram-positive strain, which has the potential to to act in the phytoremediation mechanisms of the sites polluted with heavy metals *Bacillus megaterium* is able to reduce toxic Cr(VI) to non-toxic Cr(III) and to accumulate

Cu(II) and Pb(II) . However, the selective accumulation capacity of several heavy metals, such as Mn, Co, Cd, Ni, Cu, Zn, Hg, Pb, U, Ra and Po by *Bacillus megaterium* from uranium waste pile, has since been reported in the literature (Ioana *et al.*, 2011).

Bacillus megaterium has been industrially employed for over 50 years since it possesses some very useful and unusual enzymes, and a high capacity for the production of exoenzymes. It is also a desirable cloning host for the production of intact proteins since it does not possess external alkaline proteases and can stably maintain a variety of plasmid vectors. Genetic tools for this species include transducing phages and several hundred mutants covering the processes of biosynthesis, catabolism, division, sporulation, germination, antibiotic resistance and recombination. The seven plasmids of *B. megaterium* strain QM B1551 contain several unusual metabolic genes that may be useful in bioremediation. (Patricia *et al.*, 2007)

Altogether, a "toolbox" of hundreds of genetically characterized strains, genetic methods, vectors, hosts and genomic sequences make *B. megaterium* an ideal organism for industrial, environmental and experimental applications. (Patricia *et al.*, 2007)

2.7 Studies on Biodegradation of Petroleum Hydrocarbons

Biodegradation of petroleum hydrocarbons is a complex process that depends on the nature and amount of the hydrocarbons present. Petroleum hydrocarbons can be divided into four classes: the saturates, the aromatics, the asphaltenes (phenols, fatty acids, ketones, esters and porphyrins) and the resins (pyridines, quinolines, carbazoles, sulfoxides and amides) (Shivendra and Hardik, 2014). Different factors influencing hydrocarbon degradation have been reported by Cooney *et al.* (1984). One of the important factors that limit biodegradation of oil pollutants in the environment is their limited availability to microorganisms. Petroleum hydrocarbon compounds bind to soil components, and they are difficult to be removed or degraded (Barathi and

Vasudevan, 2001). Hydrocarbons differ in their susceptibility to microbial attack. The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes (Ulrici, 2000). Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all (Atlas and Bragg, 2009).

Microbial degradation is the major and ultimate natural mechanism by which one can clean up the petroleum hydrocarbon pollutants from the environment (Lal and Khanna, 1996). The recognition of biodegraded petroleum-derived aromatic hydrocarbons in marine sediments was reported by Jones *et al.* (1983). They studied the extensive biodegradation of alkyl aromatics in marine sediments, which occurred prior to detectable biodegradation of n-alkane profile of the crude oil and the microorganisms namely *Arthrobacter, Burkholderia, Mycobacterium, Pseudomonas, Sphingomonas* and *Rhodococcus* were found to be involved in alkyl aromatic degradation. Microbial degradation of petroleum hydrocarbons in a polluted tropical stream in Lagos, Nigeria was reported by Adebusoye *et al.* (2007). Nine bacterial strains, namely, *Pseudomonas fluorescens*, *P. aeruginosa*, *Bacillus subtilis*, *Bacillus* sp., *Alcaligenes* sp., *Acinetobacter lwoffi*, *Flavobacterium* sp., *Micrococcus roseus* and *Corynebacterium* sp. were isolated from the polluted stream, which could degrade crude oil.

Hydrocarbons in the environment are biodegraded primarily by bacteria, yeasts and fungi. The reported efficiency of biodegradation ranged from 6% to 82% for soil fungi, 0.13% to 50% for soil bacteria and 0.003% to 100% for marine bacteria (Nilanjana and Preethy, 2010). Many scientists (Nilanjana and Preethy, 2010) reported that mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil in soil, fresh water, and marine environments.

Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in an environment (Rahman *et al.,* 2003). Several bacteria are even known to feed exclusively on hydrocarbons (Yakimov *et al.,* 2007). Floodgate (1984) listed 25 genera of hydrocarbon-degrading bacteria and 25 genera of hydrocarbon-degrading fungi, which were isolated from marine environment. A similar compilation by Bartha and Bossert (1984) included 22 genera of bacteria and 31 genera of fungi. In earlier days, the extent to which bacteria, yeasts and filamentous fungi participate in the biodegradation of petroleum hydrocarbons was the subject of limited study, but appeared to be a function of the ecosystem and local environmental conditions (Leahy and Colwell, 1990). Crude petroleum oil from petroleum-contaminated soil from North East India was reported by Das and Mukherjee (2007). *Acinetobacter* sp. was found to be capable of utilizing n-alkanes of chain length C_{10}–C_{40} as a sole source of carbon (Throne-Holst *et al.,* 2007). 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* was reported by Daugulis and McCracken (2003).

Fungal genera, namely *Amorphoteca, Neosartorya, Talaromyce,* 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 (Chaillan *et al.,* 2004). Singh (2006) also reported a group of terrestrial fungi, namely, *Aspergillus, Cephalosporium*, and *Pencillium*, which were also found to be the potential degraders of crude oil hydrocarbons. The yeast species, namely *Candida lipolytica, Rhodotorula mucilaginosa, Geotrichum* sp, and *Trichosporon mucoides*, isolated from contaminated water, were noted to degrade petroleum compounds (Bogusławska-Was and Da˛browski, 2001).

Though algae and protozoa are the important members of the microbial community in both aquatic and terrestrial ecosystems, reports are scanty regarding their involvement in hydrocarbon biodegradation. Walker *et al.,* (1975) isolated an alga, *Prototheca zopfi* which was capable of utilizing crude oil and a mixed hydrocarbon substrate and exhibited extensive degradation of n-alkanes and isoalkanes as well as aromatic hydrocarbons. Cerniglia *et al.*, (1980) reported that nine cyanobacteria, five green algae, one red alga, one brown alga and two diatoms could oxidize naphthalene. Protozoa, by contrast, had not been shown to utilize hydrocarbons.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of Samples

The effluent sample was collected from Kaduna refinery and petrochemical company (KRPC), in a sterile amber-coloured glass bottle (500 ml). Five hundred millilitres (500mL) of diesel was purchased from a Filling Station, in Zaria in clean one litre plastic gallon. Spent engine oil (500mL) was collected from a service station during oil change for vehicles in Zaria in clean one litre plastic container. The samples were transported to the Department of Microbiology, Ahmadu Bello University Zaria for analysis.

3.2 Determination of Physicochemical Properties of the Petroleum Refinery Effluent

The physicochemical parameters that were analyzed included: pH, temperature, turbidity, electrical conductivity (EC), total dissolved solids (TDS), dissolved oxygen (DO), biological oxygen demand (BOD), chemical oxygen demand (COD), nitrate, phosphate and sulphate, organic carbon and total nitrogen using Standard methods as described by APHA (1995).The following heavy metals were also analyzed: chromium, manganese, lead, cadmium and zinc.

3.2.1 pH, temperature, electrical conductivity and total dissolved solids

The pH, temperature, electrical conductivity and total dissolved solids were determined by using the HANNA combo tester (H198130, Denver, USA). Five millilitres of the effluent sample was dispensed into a beaker, following the manufacturer's instructions and the electrodes were connected to each meter were submerged in the beaker containing the culture. The value for each of the parameters tested were recorded and expressed as $^{\circ}$C (temperature), µS/cm (electrical conductivity) and mg/L (total dissolved solids) (APHA, 1995).

3.2.2 Dissolved oxygen and biological oxygen demand

Dissolved oxygen and biological oxygen demand were determined using HANNA instrument (HANNA 3200, Denver, USA). Dissolved oxygen was determined by inserting the instrument into the two hundred millilitres of the effluent sample at the point of collection (DO_1) while the BOD was carried out by transferring the sample into a BOD bottle and incubated for five days at 20^oC in the dark. After five days, the instrument was inserted into the incubated sample and the level of residual oxygen was recorded (DO_2). The value of residual DO was then subtracted from the initial DO value to obtain the BOD of the samples (Radojavic and Bashkin, 1999).

The BOD was calculated using the formula below:

$$BOD\ (mg/L) = DO_1 - DO_2$$

3.2.3 Chemical Oxygen Demand

This was determined using dichromate reflux method. Here, $HgSO_4$ (mercuric sulphate) (0.4g) was weighed out into a reflux flask and twenty millilitres of the effluent sample was added with distilled water. Ten millilitres of standard potassium dichromate was added and three glass beads were dropped into the reflux flax. Thirty millilitres of conc.H_2SO_4 containing silver sulphate ($AgSO_4$) was carefully added and mixed properly. The whole content was refluxed for one hour, cooled and diluted to one hundred millilitres with distilled water. Three drops of ferroin indicator were added and excess dichromate was titrated against standard ferrous ammonium sulphate (the colour change was sharp from blue-green to brown-green and then to reddish-brown). A blank containing twenty millilitres of distilled water was treated and refluxed in the same manner (APHA, 1995).

The COD was calculated using the formula below:

$$COD\ (mg/l) = \frac{(A-B)C \times 8000}{\text{Vol. of sample (ml)}}$$

Where A= Titration for blank (7.2mL)
B= Titration for sample (6.2 mL)
C= Normality of titrant (ferrous ammonium sulphate). It is a constant (0.25N).

3.2.4 Nitrates

The effluent sample was adjusted to pH 7.0 and 100ml of the vigorously shaken effluent sample was measured using a measuring cylinder into a clean glass Petri dish, placed on a water bath at 100 $^{\circ}$C and evaporated to dryness. The resulting residue was dissolved using a bent glass rod with 2ml of phenol disulfonic acid measured with a pipette and transferred into Nessler's tube. Six millilitres of ammonium hydroxide (NH_4OH) was measured by using a pipette into the preparation and the solution was diluted with ninety two millilitres of distilled water. The preparation was filtered with a filter paper placed in a funnel into a conical flask. A blank was prepared in the same manner using distilled water instead of the sample. The *752 UV model* spectrophotometer reading was adjusted to zero mark and set at 410nm. The cuvette were rinsed with distilled water and filled with the blank and the test sample and properly fixed into the wells respective of the spectrophotometer. The spectrophotometer was standardised with the blank and the transmittance of the test sample was measured and compared with the standard curve. The nitrate concentration in the samples was calculated using the formula below (APHA, 1995):

$$\text{Nitrate } (NO_3)^- \text{ (mg/l)} = \frac{\text{Nitrate } (NO_3)^- \text{ (mg)} \times 1000}{\text{Vol. of sample (ml)}}$$

3.2.5 Sulphate

The effluent sample was thoroughly mixed by shaking vigorously and one hundred millilitres was dispensed into 250ml Erlenmeyer flask. Five millilitres of conditioning reagent was added. A bar magnet was dropped inside the sample and mixed on the magnetic stirrer. As the solution was being stirred, a spoonful of barium chloride crystals was added and timing began immediately. It was left on the magnetic stirrer for one minute at a constant speed after which it

was removed. A blank was prepared using 100ml of distilled water without adding barium chloride. The same procedure was repeated for the blank. The blank was poured inside a cuvette and placed inside the *752 UV model* spectrophotometer. The spectrophotometer was standardised using a wavelength of 420nm and both the transmittance and absorbance were adjusted to zero. After standardizing the spectrophotometer, the sample was placed inside and the reading was taken and compared with the standard.

The sulphate concentration was calculated using the formula below and estimated by comparing the reading with a standard curve (APHA, 1995).

$$\text{Sulphate } (SO_4)\ (mg/l) = \frac{SO_4\ (mg) \times 1000}{\text{Vol. of sample (ml)}}$$

3.2.6 Phosphate

3.2.6.1 Preliminary sample treatment

One drop of phenolphthalein indicator (0.05ml) was added to one hundred millilitres of the effluent sample and concentrated hydrochloric acid solution was added drop wise until the colour turned pink.

3.2.6.2 Colour development

One hundred millilitres (100ml) of treated effluent sample (in section 3.2.6.1) was dispensed into a 250ml Erlenmeyer flask and 4.0ml of molybdate reagent was added and mixed thoroughly. One millilitre (1ml) of stannous chloride reagent was added drop-wise. Distilled water was used to prepare a blank in the same way. After 10 minutes, the absorbance of the colour developed was measured spectophotometrically at 690nm using spectrophotometer. The phosphate content of the samples was read off the standard curve and the concentration was obtained using the formula (APHA, 1995):

$$\text{Phosphate } (PO_4)\ (mg/l) = \frac{PO_4(mg) \times 1000}{\text{Vol. of sample (ml)}}$$

3.2.7 Heavy metal analyses

The determination of heavy metals (chromium, manganese, lead, cadmium and zinc) was performed with a bulk scientific 205 atomic absorption spectrophotometer. The instrument's setting and operational conditions were done in accordance with manufacturer's specifications. The instrument was calibrated with analytical-grade metal standard stock solutions (1 mg/l) in replicate. One hundred and fifty millilitres (150 ml) of the effluent sample was transferred to a beaker, 5 ml concentrated HNO_3 was added and the mixture was evaporated to dryness on a hot plate. Two millilitres of concentrated HNO_3 was added to dissolve the residues on the walls of the beaker. The distilled digested sample was filtered and made up to fifty millilitres and analyzed using Atomic Absorption Spectroscopy (AAS). A blank was prepared in the same manner using distilled water instead of the sample (Radojavic and Bashkin, 1999).

3.3 Isolation and Characterization of *Pseudomonas putida* and *Bacillus megaterium*

3.3.1 Isolation of *Pseudomonas putida* and *Bacillus megaterium* from petroleum refinery effluent

The mineral salts medium (MSM), which was used throughout this study, consisted of the following composition per litre: $Na_2PO_4.7H_2O$ (64g), KH_2PO_4 (15g), NaCl (2.5g), NH_4Cl (5.0g), $MgSO_4$ (0.12g), $CaCl_2$ (5.5g). The salts were dissolved in a litre of distilled water and adjusted to a pH of 7.0. The preparation was sterilized by autoclaving at 121^{o}C for 15 minutes (Musa *et al.*, 2015).

Two hundred milliliters (200ml) of the prepared MSM medium contained in a 500ml Erlenmeyer flask was inoculated with 1% (v/v) of the effluent sample. The flask was incubated for 7 days at room temperature on a shaker. Ten-fold serial dilutions were carried out for all suspensions of

enrichment samples. Each dilution (0.1ml) was inoculated unto the surface of freshly-prepared cetrimide agar plates and nutrient agar plates (prepared according to manufacturer's instructions) using spread plate method. The plates were incubated at 37ºC for 24 hours. The resulting bacterial colonies were examined for size, shape, margin, consistency and pigmentation. Distinctive colonies were then sub-cultured unto nutrient agar plates by streaking for purification. The resulting pure isolates were sub-cultured unto a nutrient agar slant medium and kept in a refrigerator for further studies (Manal, 2011; Musa *et al.*, 2015).

3.3.2 Gram's staining and identification of *Pseudomonas putida* and *Bacillus megaterium*

Slides with heat fixed smear were placed on tray, gently flooded with crystal violet and allowed to stand for one minute. The slides were tilted and gently rinsed with water. Smears were then flooded with Lugol's iodine and allowed to stand for one minute, and then rinsed. The smears were decolorized using acetone for 10 seconds until the alcohol ran almost clear and then rinsed. The slides were gently flood with safranin to counter-stain and allowed to stand for 45 seconds, rinsed and allowed to air dry. The slides were examined microscopically by using 100x oil immersion to determine the Gram's reaction, behaviour and colonial morphology of the cells. Spore staining test was carried out for all the isolates that are Gram positive rods (Cowan and Steel, 2003).

3.3.2.1 Biochemical Characterization

The isolates were biochemically identified following the scheme described by Cowan and Steel (2013). Motility, citrate, urease, nitrate reduction, starch hydrolysis, carbohydrate fermentation, endospore staining, indole and MR-VP tests were carried out for *Bacillus megaterium* isolates while motility, citrate, urease, nitrate reduction, starch hydrolysis, carbohydrate fermentation and oxidase tests were carried out for *Pseudomonas putida* isolates.

Endospore staining:

Smear of organisms to be tested for presence of endospores was prepared on clean glass slides, air dried and heat fixed. Small pieces of blotting papers (absorbent paper) were placed over the smear and the slide was placed on a wire gauge on a ring stand. The blotting papers were saturated with malachite green stain solution and steamed for five minutes. As the papers began to dry, two drops of malachite green were added to keep them moist. After five minutes, the slides were carefully removed. The blotting papers were removed and the slides were allowed to cool to room temperature. The slides were thoroughly rinsed with tap water. The smears were stained with safranin for two minutes. Both sides of the slides were rinsed to remove secondary stain and slides were air-dried. The slides were viewed under microscope using 100x oil immersion to determine the presence of spores in the isolates (Cowan and Steel, 2003).

Motility test:

A stab was aseptically made with inoculating needle to a depth of about one third the total volume of the culture medium. The culture was then incubated at 35^{o}C for 24hrs. Where the culture turned cloudy (turbid) after incubation, it means that the organism was motile but where growth was restricted to the line of inoculation and the rest of the culture remained clear, then, the organism was non-motile (Cowan and Steel, 2003).

Citrate utilization test:

This test was carried out by inoculating the colonies of the isolates on Simmons' citrate agar slant and the inoculated slants were incubated at 35^{o}C for 72 hours and examined for development of a deep blue colour, which indicated a positive reaction (Cowan and Steel, 2003).

Urease test:

Urease test was carried out by inoculating urea agar slant with the colonies of the isolates and the slant was incubated at 35^{o}C for 48 hours and examined for the development of red colour indicated a positive reaction (Cowan and Steel, 2003).

Nitrate reduction test:

This was carried out by inoculating nitrate broth with loopful colonies of the isolates and incubated at 37^{o}C for 48hrs. One (1) millilitre of reagent A (sulphanilic acid) was added, followed by one millilitre of reagent B (α-napthol amine) and examined. A red colour showed the presence of nitrite and thus, showed that nitrate has been reduced and indicating a positive reaction.

To tubes that didn't show red colour within five minutes, powdered zinc (5 mg/ml) was added and allowed to stand and then examined for red colour, which indicated that nitrate was present in the medium (i.e., not reduced by the organism) and absence of red colour indicated nitrate was absent in the medium (i.e. reduced by the organism to nitrite, which in turn was itself reduced (Cowan and Steel, 2003).

Starch hydrolysis test:

Colonies of the isolates were inoculated on nutrient agar containing 0.2% soluble starch and the plates were incubated at 30^{o}C for 24hrs. After incubation, the plates were then flooded with Lugol's iodine and examined for zone of hydrolysis which indicates a positive test (Cowan and Steel, 2003).

Oxidase test:

This was carried out for Gram negative rod isolates by placing a piece of filter paper in a clean Petri dish and three drops of freshly-prepared oxidase reagent was added. Using a piece of glass

rod, a colony of the test organism was smeared on the filter paper and examined for the development of a blue-purple colour within ten seconds which indicated a positive test and absence of blue-purple colour within ten seconds indicated a negative test (Cowan and Steel, 2003).

Indole test:

Indole test was carried out for Gram positive rods by inoculating colonies of the isolates into 1% peptone water and then the inoculated peptone was incubated at 37^{o}C for 24 hours. After 24 hours of incubation, three drops of Kovac's reagent was added and shaken and examined. A positive reaction was indicated by the development of a red colour in the reagent layer above the broth while a negative reaction was indicated by a yellow colour (Cowan and Steel, 2003).

3.3.3 Phenotypic authentication of bacterial isolates using Microgen kits

Isolates tentatively identified as *Bacillus megaterium* and *Pseudomonas putida* on the basis of biochemical characteristics were authenticated using Microgen kits; (Bioproducts Co; U.K) Bacillus-ID kit for *Bacillus megaterium* and GNA and GNB enterobacteriacae-ID kit for *Pseudomonas putida*. Isolates thus authenticated were stored in the refrigerator for further studies.

3.4 Screening the Selected Isolates for Their Capacity to Degrade Various Hydrocarbons

Standardization of inoculum

Suspension of *Pseudomonas putida* and *Bacillus megaterium* corresponding to $1.8x10^{9}$ CFU/ml were prepared separately in normal saline and compared with a McFarland standard (6.0). Colonies were added to the normal saline until it was as turbid as the McFarland standard to obtain a bacterial population density of $1.8x10^{9}$ CFU/ml (Atta, 2009).

Fifty millilitres (50ml) each of MSM prepared as previously described (3.3.1) was dispensed into twelve (12) 100ml Erlenmeyer flasks and autoclaved at 121^{o}C at 15atm for 15 minutes. The inoculum for each isolate (standardized using McFarland standard (6) corresponding to 1.8x10^{9} CFU/ml) was inoculated into the sterile medium at 10% (v/v) and diesel was added at 0.5% (v/v). The eleventh and the twelveth flasks served as control (containing only MSM and MSM +diesel, respectively). The flasks were incubated at room temperature on an orbital shaker for seven days. Absorbance readings (at 540nm) were obtained on the first and the seventh day of incubation. Increase in absorbance was used as a measure of increase in bacterial growth and their corresponding ability to degrade diesel. The set-up was repeated for spent engine oil (Atta, 2009). (Table 3.1).

3.5 Biodegradation Studies

Pseudomonas putida C15a and *Bacillus megaterium* N9a were observed to be the most efficient strains and as such, were selected for further studies. Four hundred milliliters (400ml) of MSM was prepared as previously described and one hundred milliliters (100ml) each of the MSM was dispensed into four (4) 250ml Erlenmeyer flasks and isolates of *Pseudomonas putida, Bacillus megaterium* and consortium (McFarland standard 6 was used to standardise the inoculums size) were added to the sterile medium at 10% (v/v) and enriched with 0.5% (v/v) diesel as source of hydrocarbon. The fourth flask, which served as the control, contained only MSM and diesel. The set-up was repeated for spent engine oil (Table 3.2).

The set-up were incubated on a rotary shaker at 150rpm at ambient temperature for a period of eighteen days and, at three days intervals, the ability of the isolates to degrade hydrocarbons was studied by determining the hydrocarbon-utilizing bacterial (HUB) count, optical density and changes in pH of the culture (Okerentugba and Ezeronye, 2003).

Table 3.1: Set-up for screening the authenticated isolates

Erlenmeyer flask	Contents
C2b	*Pseudomonas putida* (10% v/v) + Diesel (0.5% v/v) + Mineral salts medium
C11b	*Pseudomonas putida* (10% v/v) + Diesel (0.5% v/v) + Mineral salts medium
C15a	*Pseudomonas putida* (10% v/v) + Diesel (0.5% v/v) + Mineral salts medium
N8a	*Bacillus megaterium* (10% v/v) + Diesel (0.5% v/v) + Mineral salts medium
N8b	*Bacillus megaterium* (10% v/v) + Diesel (0.5% v/v) + Mineral salts medium
N17b	*Bacillus megaterium* (10% v/v) + Diesel (0.5% v/v) + Mineral salts medium
N16a	*Bacillus megaterium* (10% v/v) + Diesel (0.5% v/v) + Mineral salts medium
N9b	*Bacillus megaterium* (10% v/v) + Diesel (0.5% v/v) + Mineral salts medium
N10b	*Bacillus megaterium* (10% v/v) + Diesel (0.5% v/v) + Mineral salts medium
N9a	*Bacillus megaterium* (10% v/v) + Diesel (0.5% v/v) + Mineral salts medium
MSM + diesel	Diesel (0.5% v/v) + Mineral salts medium
MSM only	Mineral salts medium only

The set-up was repeated for spent engine oil

Table 3.2: Set-up for biodegradation studies

Erlenmeyer flask	Contents
DB	*Bacillus megaterium* (10% v/v) + Diesel (0.5% v/v) + Mineral salts medium
DP	*Pseudomonas putida* (10% v/v) + Diesel (0.5% v/v) + Mineral salts medium
DC	*Bacillus megaterium* (10% v/v) + *Pseudomonas putida* (10% v/v *)* + Diesel (0.5% v/v) + Mineral salts media
DCN	Diesel (0.5% v/v) + Mineral salts medium
SB	*Bacillus megaterium* (10% v/v) + Spent engine oil (0.5% v/v) + Mineral salts medium
SP	*Pseudomonas putida* (10% v/v) + Spent engine oil (0.5% v/v) + Mineral salts medium
SC	*Bacillus megaterium* (10% v/v) + *Pseudomonas putida* (10% v/v) + Spent engine oil (0.5% v/v) + Mineral salts medium
SCN	Spent engine oil (0.5% v/v) + Mineral salts medium

3.5.1 Hydrocarbon-utilising bacterial (HUB) count

An aliquot (1ml) of the broth culture from each flask was inoculated into test tubes containing 9 ml of sterile distilled water. Then, a ten-fold dilution was carried out up to dilution 10^5, and then 0.1ml of the dilution 10^5 was aseptically inoculated on nutrient agar medium using the spread plate method (Atta, 2009) and incubated at 35^0C for 24 hours, the colonies obtained were counted, recorded and expressed as CFU/mL

3.5.2 Optical density

Five milliliters (5ml) of the broth culture was aseptically transferred from each flask into test tubes, and the residual hydrocarbons extracted by adding 5ml dichloromethane (DCM) and centrifuged at 5000 rpm for five minutes. The resulting supernatant was read at a wavelength of 250nm using a *Cary 300 UV-Vis model* spectrophotometer (Nwankwegu *et al.,* 2016). The residual hydrocarbon was calculated after determining the amount of hydrocarbon from a prepared standard using known amounts of hydrocarbon; using the formula below:

$$\text{Percentage degradation} = \left[\frac{\text{RESIDUAL HYDROCARBON (control)} - \text{RESIDUAL HYDROCARBON (treatment)}}{\text{RESIDUAL HYDROCARBON (control)}}\right] \text{x } 100$$

(Manal, 2011).

3.6.3 pH

The pH was determined using the HANNA combo tester (H198130, Denver, USA). Five millilitres of the culture was dispensed into beaker and briefly, following the manufacturer's instructions, the electrodes were connected to each meter submerged in the beaker containing the culture. The value read for the sample by the tester were observed and recorded (APHA, 1995).

3.7 Data Analysis

The data obtained were subjected to analysis of variance (ANOVA) with regards to HUB count, pH and optical density using the Microsoft Excel (2016 version) to determine if there was significant difference in the treatments. Mean variables were compared using the Newman Keuls test at probability level, p = 5%.

CHAPTER FOUR

4.0 RESULTS

Table 3.1 shows set-up for screening the authenticated isolates. Isolates that showed to be *Pseudomonas putida* and *Bacillus megaterium* were screened for their ability to degrade hydrocarbons by using optical density. *Pseudomonas putida* C15a and *Bacillus megaterium* N9a showed higher optical density than others, hence were selected for further studies

Table 3.2 shows the set-up for biodegradation studies. DCN (containing only MSM and diesel) and SCN (containing only MSM and spent engine oil) flasks served as control.

Table 4.1 shows the physicochemical properties of the refinery effluent sample. The pH value of the refinery effluent was 6.8 with temperature of 26.2^{o}C. Total dissolved solids, turbidity, chemical oxygen demand, biological oxygen demand and dissolved oxygen were found to be above FMENV standard while nitrate, sulphate and total carbon were observed to be below the standard.

Table 4.2 shows the concentration of each heavy metal (ppm) in the refinery effluent. The concentrations in the effluent samples measured in ppm ranged from 0.0ppm to 1.034ppm. The order of the metals in the effluent are Zn >Pb> Cr > Cd >Mn with zinc having the highest concentration (1.034ppm) and manganese not detectable in the effluent sample (0.0 ppm).

The cultural characteristics, Gram's reaction and biochemical reactions of the bacterial isolates are presented in Table 4.3. Three strains of Gram negative and seven strains of Gram-positive were obtained. *Pseudomonas putida* produced transluscent greenish colonies on centrimide agar. *Pseudomonas putida* were not able to hydrolyse starch and oxidase-positive while *Bacillus megaterium* were able to hydrolyse starch and negative to indole and oxidase. All the isolates were motile and tested positive to citrate and catalase.

Table 4.1: Physicochemical properties of petroleum refinery effluent samples

Physicochemical Parameters	Value attained	FMENV (1999) Standard
Dissolved Oxygen (mg/L)	200	≥ 10
Biological Oxygen Demand (mg/L)	100	≤ 10
Chemical Oxygen Demand (mg/L)	1200	≤ 10
Electrical Conductivity (µS/cm)	620	≤ 400
Total Dissolved Solids (mg/L)	950	≤ 500
Turbidity (NTU)	179	≤ 10
pH	6.8	6.0-9.0
Temperature ($^{\circ}$C)	26.2	≤ 40
Nitrate (mg/L)	6.81	≤ 10
Phosphate (mg/L)	27.12	≤ 5
Sulphate (mg/L)	4.82	≤ 50
Organic carbon (mg/L)	10.77	≤ 50
Total carbon (mg/kg)	1.54	≤ 10

Table 4.2: Heavy metal concentrations of petroleum refinery effluent samples

Heavy metals	Value attained (mg/L)	FMENV (1999) Standard (mg/L)
Manganese	0.000	< 1
Lead	0.456	< 1
Cadmium	0.007	< 1
Zinc	1.034	< 1
Chromium	0.009	< 1

Table 4.3: Cultural, Gram's reaction and biochemical characteristics of the bacterial isolates

Isolate number	Cultural Characteristics	Gram Reaction	MOT	STA	CIT	OXI	CAT	IND	Tentative identity
C2b	Small round greenish colonies	Gram negative rods	+	-	+	+	+		*Pseudomonas* sp.
C11b	Small transluscent green colonies	Gram negative rods	+	-	+	+	+		*Pseudomonas* sp.
C15a	Flat translucent greenish colonies with pigmentation	Gram negative rods	+	-	+	+	+		*Pseudomonas* sp.
N8a	Cream flat dry colonies	Gram positive rods	+	+	+	-	+	-	*Bacillus* sp.
N8b	Cream flat dry colonies	Gram positive rods	+	+	+	-	+	-	*Bacillus* sp.
N9a	Cream flat dry colonies	Gram positive rods	+	+	+	-	+	-	*Bacillus* sp.
N9b	Cream flat dry colonies	Gram positive rods	+	+	+	-	+	-	*Bacillus* sp.
N10b	Yellow flat dry spreading colonies.	Gram positive rods	+	+	+	-	+	-	*Bacillus* sp.
N16a	Yellow, flat dry spreading colonies.	Gram positive rods	+	+	+	-	+	-	*Bacillus* sp.
N17b	Spreading cream coloured colonies	Gram positive rods	+	+	+	-	+	-	*Bacillus* sp.

KEY: MOT: motility; STA: starch; CIT: citrate; OXI: oxidase; CAT: catalase; IND: indole; +: positive; -: negative

Three strains of *Pseudomonas putida* and seven strains of *Bacillus negaterium* were authenticated using Enterobacteriaceae (GNA+GNB) and Microgen® *Bacillus* Kits, respectively. The percentage probabilty for the isolates ranged from 71.38% to 98.39% as shown in Table 4.4.

The ability of *Pseudomonas putida* and *Bacillus megaterium* isolates to degrade various hydrocarbons is presented in Table 4.5. All the bacterial isolates produced turbidity indicating ability of each to utilize the hydrocarbons, but to varying degrees. *Pseudomonas putida* C15a showed the highest percentage increase in optical density in media containing diesel and spent engine oil while *Bacillus megaterium* N9a showed highest percentage increase in optical density in media containing diesel and spent engine oil. As such, *Pseudomonas putida* C15a and *Bacillus megaterium* N9a were the isolates used in further studies.

Table 4.4: Phenotypic characteristics of isolates of *Pseudomonas putida* and *Bacillus megaterim* based on microgen identification kit

Isolate code	Octal code	Probability (%)	Identity
C2b	640522001	90.5	*Pseudomonas putida*
C11b	640522001	90.5	*Pseudomonas putida*
C15a	640722001	90.5	*Pseudomonas putida*
N8a	61270234	91.09	*Bacillus megaterium*
N8b	61270234	91.09	*Bacillus megaterium*
N17b	64270224	94.94	*Bacillus megaterium*
N16a	61270221	92.45	*Bacillus megaterium*
N9b	63070225	71.38	*Bacillus megaterium*
N10b	74270224	98.39	*Bacillus megaterium*
N9a	60070223	82.16	*Bacillus megaterium*

Table 4.5: Absorbance values of *Pseudomonas putida* and *Bacillus megaterium* during hydrocarbon (spent engine oil and diesel) utilization

Isolate code	Identity	Percentage degradation (%)	
		Spent engine oil	Diesel
C2b	*Pseudomonas putida*	56.64	57.74
C11b	*Pseudomonas putida*	-37.85	-27.45
C15a	*Pseudomonas putida*	70.86	69.08
N8a	*Bacillus megaterium*	1.50	-18.8
N8b	*Bacillus megaterium*	44.88	3.1
N17b	*Bacillus megaterium*	9.64	12.5
N16a	*Bacillus megaterium*	-35.55	- 4.1
N9b	*Bacillus megaterium*	48.45	-22.8
N10b	*Bacillus megaterium*	22.73	-19.4
N9a	*Bacillus megaterium*	50.54	48.4

Absorbance values taken at 540nm for all the treatments.

Figures 4.1 and 4.2 show hydrocarbon-utilizing bacterial (HUB) count of the samples (diesel and spent engine oil) during the biodegradation. The isolates of *Pseudomonas putida, Bacillus megaterium* and consortium exhibited different growth patterns in diesel and spent engine oil. The controls had no growth all through the 18 day period.

Different HUB counts were observed in the treatments during the degradation of hydrocarbons in the diesel as shown in Figure 4.1. Highest population density was observed at day 15 for all the treatments. Highest population density was observed in the treatment containing *Pseudomonas putida* (1.85 x 10^7CFU/ml) followed by *Bacillus megaterium* (1.35 x 10^7CFU/ml) while consortium had the lowest population density (6.1 x 10^6 CFU/ml). A decline in population was observed in all the treatments at day 18.

Figure 4.2 shows hydrocarbon-utilizing bacterial counts of spent engine oil. The highest population density of 1.35 x 10^7CFU/ml was observed in the treatment inoculated with *Pseudomonas putida*, followed by *Bacillus megaterium* (1.05 x 10^7CFU/ml) and 5.6 x 10^6CFU/ml for the consortium at day 15. A decline in population density was observed in all the treatments at day 18.

The treatments for the diesel showed higher hydrocarbon-utilizing bacterial counts than the treatments for the spent engine oil. There was significant difference in all the treatment sets containing diesel and spent engine oil ($P<0.05$).

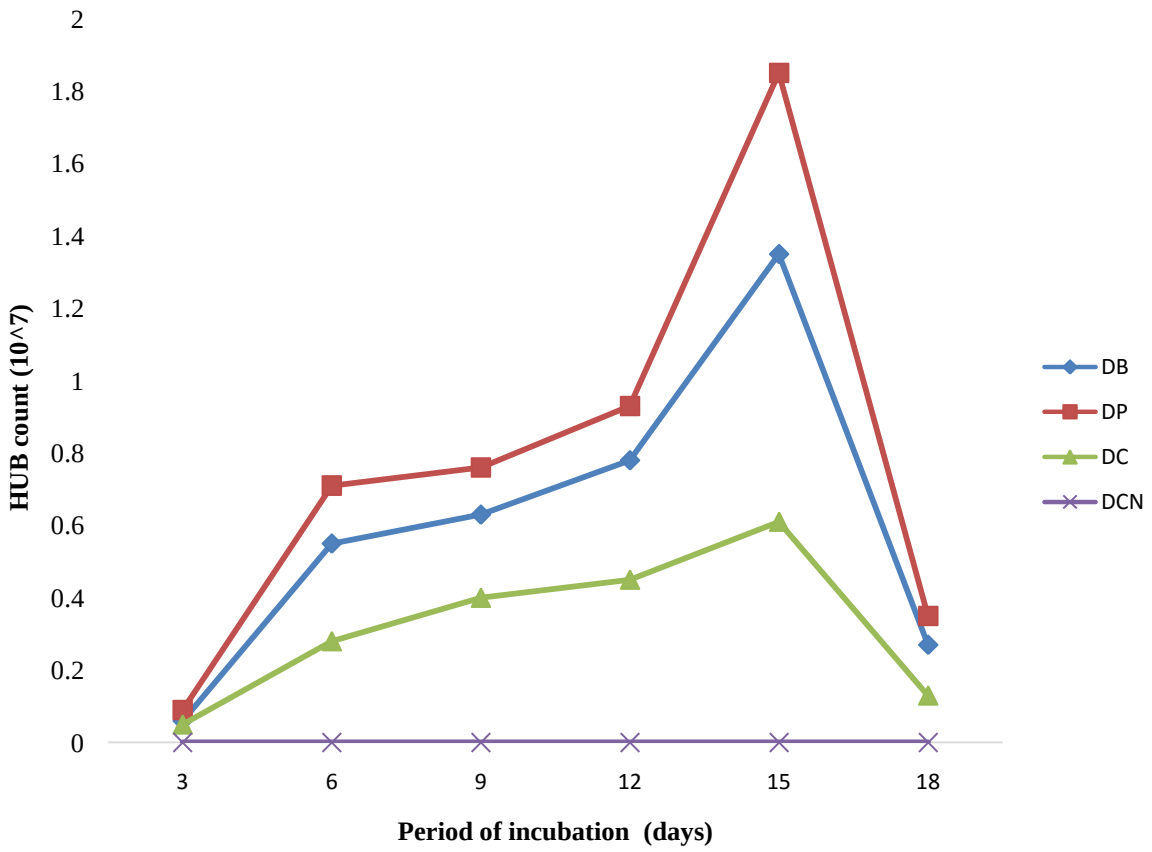

Figure 4.1: Hydrocarbon-utilizing bacterial counts of the isolates during degradation of diesel

Key:

DB: Treatment containing only *Bacillus megatarium* and diesel

DP: Treatment containing only *P*seudomonas *putida* and diesel

DC: Treatment containing consortium of *Bacillus megatarium*, *Pseudomonas putida* and diesel

DCN: Treatment containing only MSM and diesel (control)

There is significant difference for all the treatments ($P<0.05$).

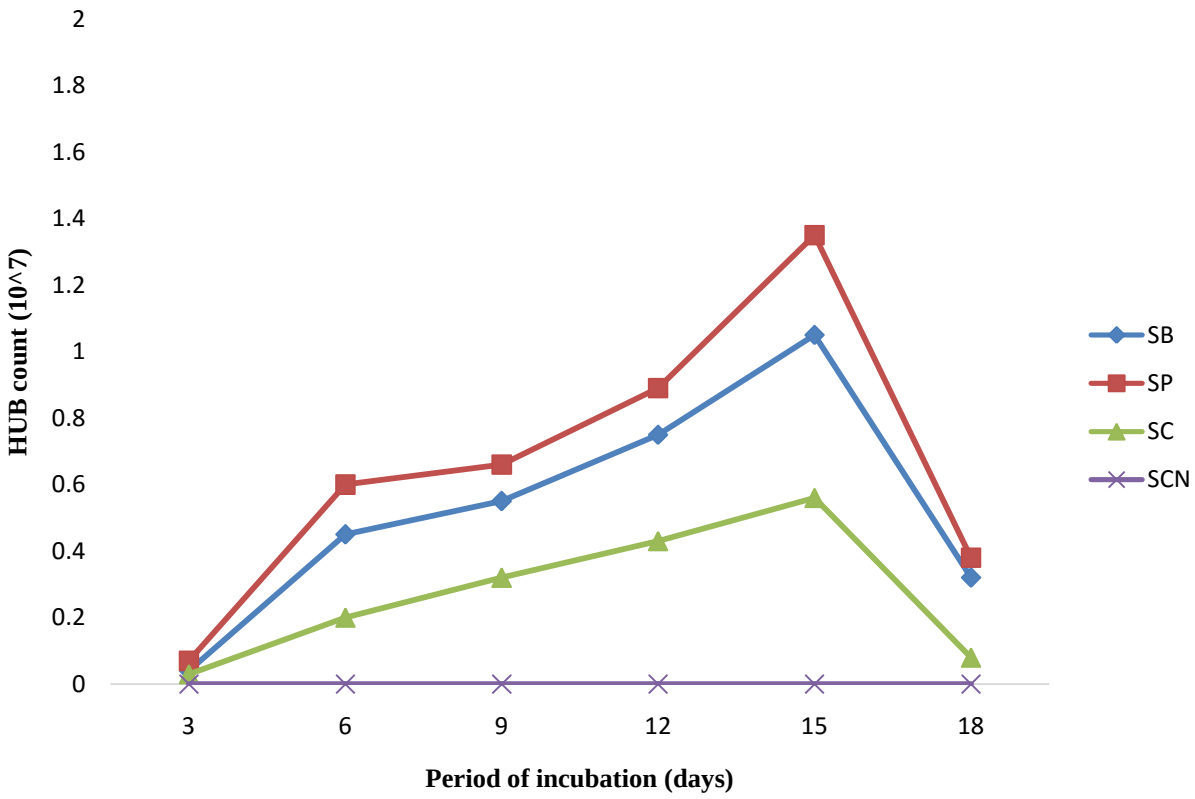

Figure 4.2: Hydrocarbon-utilizing bacterial counts of the isolates during degradation of spent engine oil

Key:

SP: Treatment containing only *Pseudomonas putida* and spent engine oil

SB: Treatment containing only *Bacillus megatarium* and spent engine oil

SC: Treatment containing consortium of *Bacillus megatarium*, *Pseudomonas putida* and spent engine oil

SCN: Treatment containing only MSM and spent engine oil (control)

There is significant difference for all the treatments ($P<0.05$).

Figure 4.3 shows the percentage amount of diesel degraded by *Bacillus megaterium, Pseudomonas putida* and the consortium. The percentage of hydrocarbon removal increased with the experimental period. *Pseudomonas putida* showed highest percentage amount of hydrocarbon degraded (98.3%) followed by *Bacillus megaterium* (81.03%), while the consortium showed the least degradation capacity (68.97%). However, there was significant difference in all the treatments ($P<0.05$).

Figure 4.4 shows the percentage amount of spent engine oil degraded by the isolates. The rate of spent engine oil degradation by *Bacillus megaterium, Pseudomonas putida* and the consortium was relatively low compared to degradation of diesel all through the period of incubation. *Pseudomonas putida* showed highest percentage amount of hydrocarbon degraded (75.03%), while the consortium showed the least degradation capacity (60.86%). There was significant difference in the percentage degradation ($P<0.05$).

Pseudomonas putida showed highest degradation capacity in both diesel and spent engine oil, while the consortium exhibited lowest degradation capacity. However, the treatment sets containing diesel as sole source of carbon recorded better degradation than the treatment sets containing spent engine oil at the end of the biodegradation period.

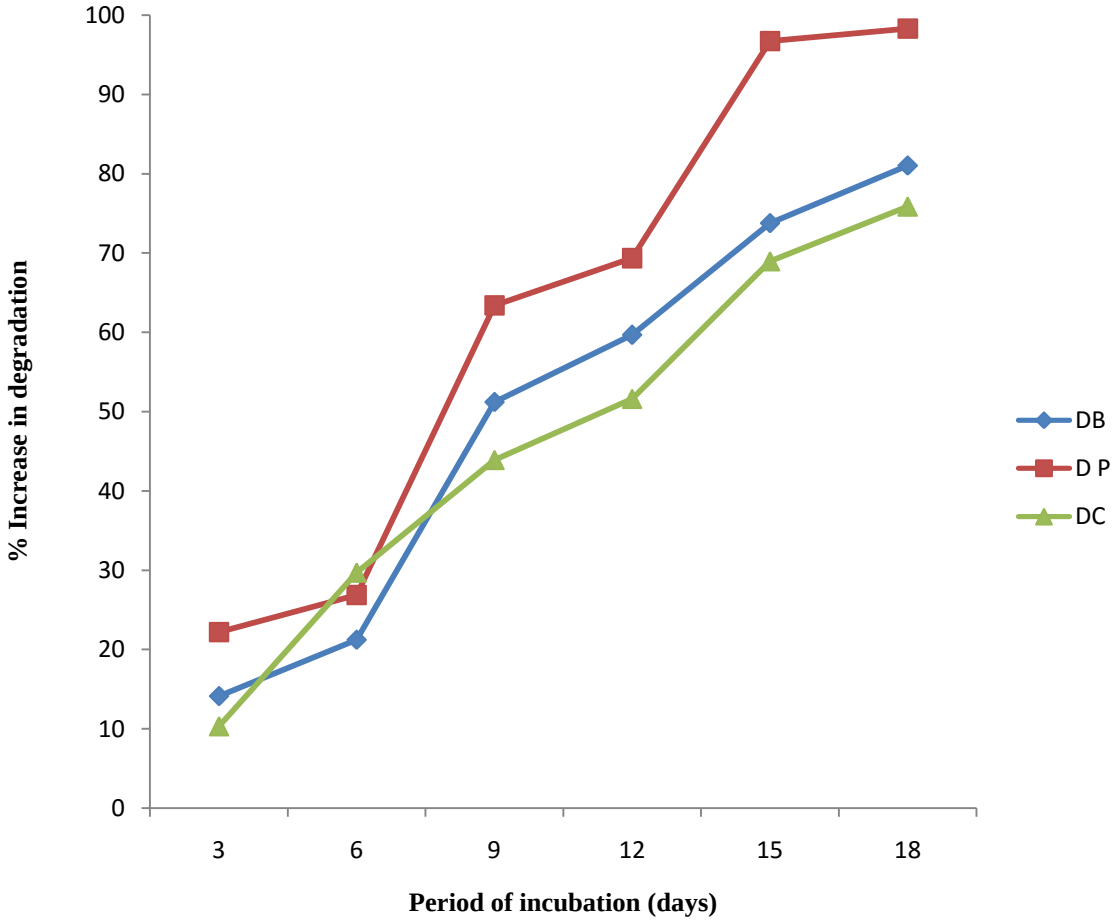

Figure 4.3: Percentage degradation of diesel by *Pseudomonas putida* and *Bacillus megaterium*

Key:

DB: Treatment containing *Bacillus megatarium* and diesel,

DP: Treatment containing *Pseudomonas putida* and diesel,

DC: Treatment containing *Bacillus megatarium*, *Pseudomonas putida* and diesel

There was significant difference for all the treatments. ($P<0.05$)

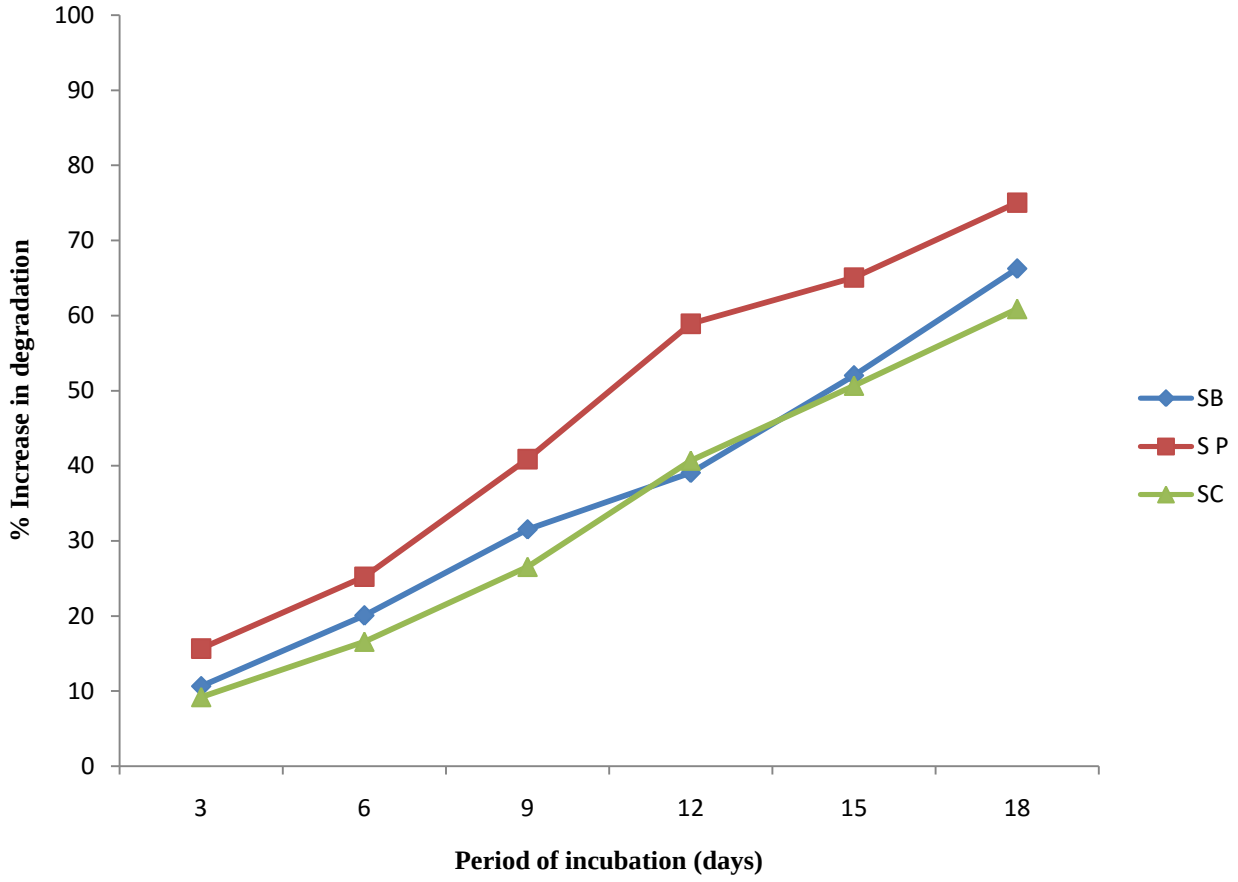

Figure 4.4: Percentage degradation of spent engine oil by *Pseudomonas putida* and *Bacillus megaterium*

Key:

SP: Treatment containing only *Pseudomonas putida* and spent engine oil

SB: Treatment containing only *Bacillus megatarium* and spent engine oil

SC: Treatment containing consortium of *Bacillus megatarium*, *Pseudomonas putida* and spent engine oil

There is significant difference for all the treatments ($P<0.05$).

Figures 4.5 and 4.6 show different pH values exhibited by the samples during the biodegradation. It was observed that pH reduces as biodegradation progresses, during the period of incubation up to day 18. There was no significant difference ($P>0.05$) in the pH values of the treatments containing diesel, but there was significant difference ($P<0.05$) in the treatment sets containing spent engine oil.

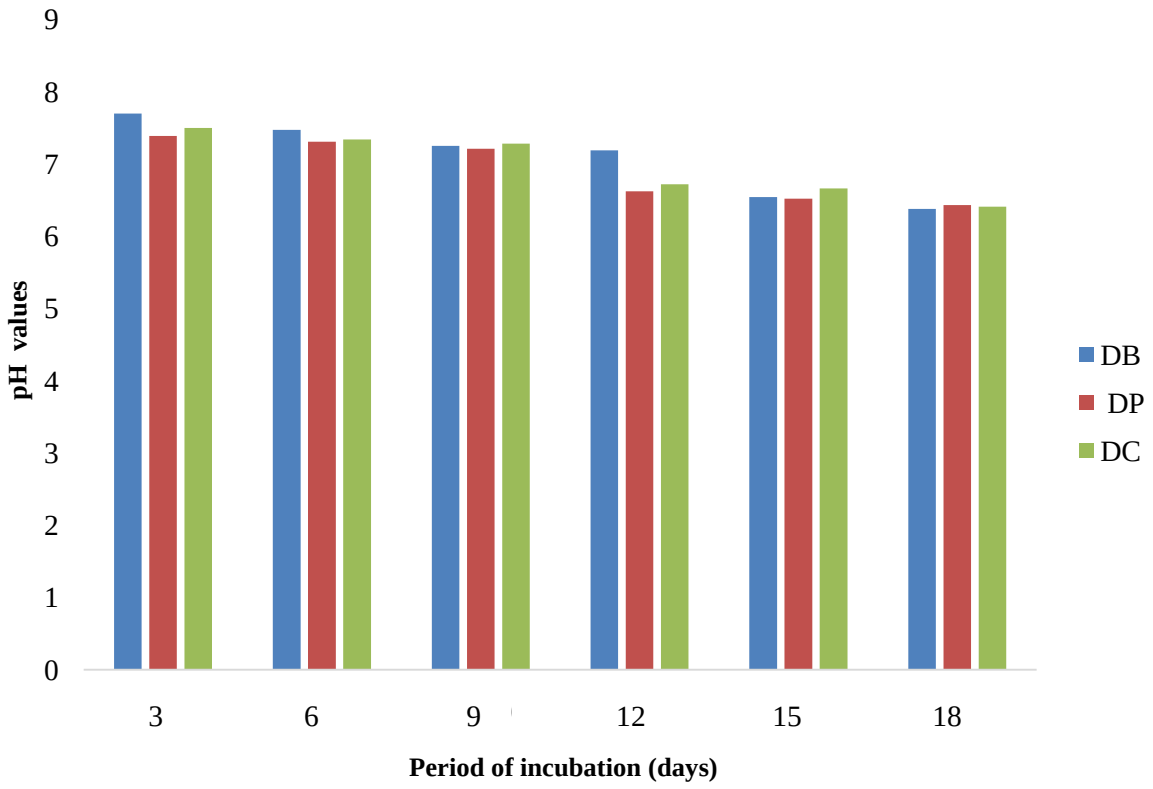

Figure 4.5: Change in pH during degradation of diesel by *Pseudomonas putida* and *Bacillus megaterium*

Key:

DB: Treatment containing only *Bacillus megatarium* and diesel

DP: Treatment containing only *Pseudomonas putida* and diesel

DC: Treatment containing consortium of *Bacillus megatarium*, *Pseudomonas putida* and diesel

There is no significant difference for all the treatments. (P>0.05)

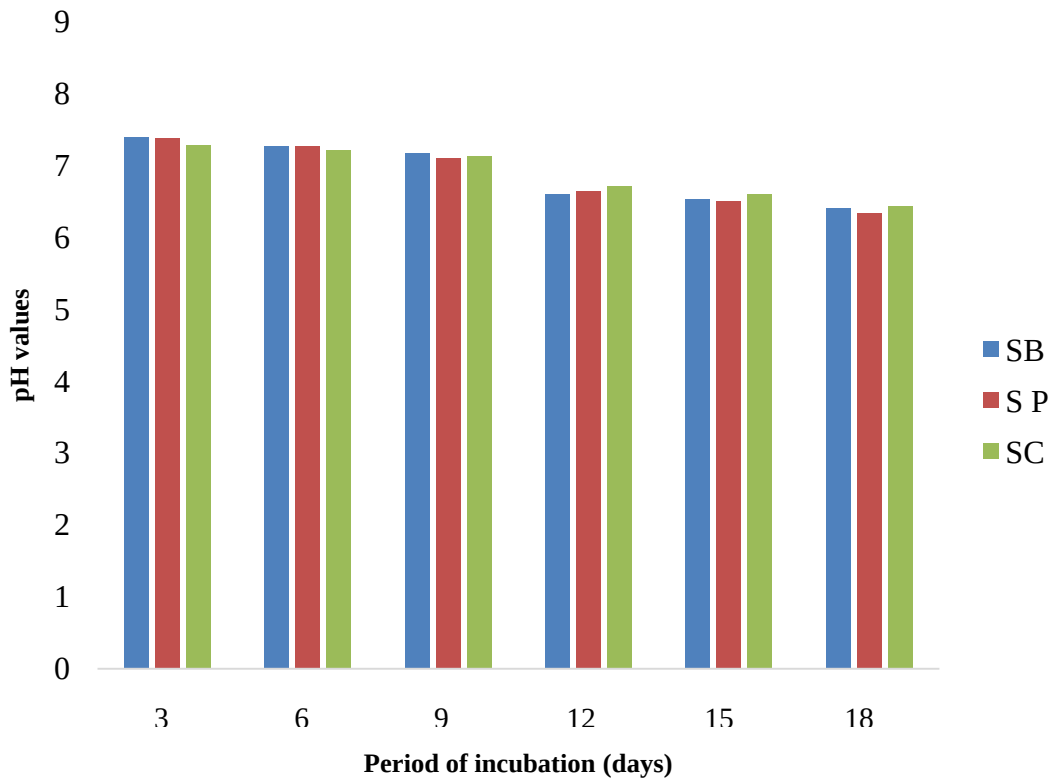

Figure 4.6: Change in pH during degradation of spent engine oil by *Pseudomonas putida, Bacillus megaterium*

Key:

SP: Treatment containing only *Pseudomonas putida* and spent engine oil

SB: Treatment containing *Bacillus megatarium* and spent engine oil

SC: Treatment containing consortium of *Bacillus megatarium*, *Pseudomonas putida* and spent engine oil

There is significant difference for all the treatment. ($P<0.05$)

CHAPTER FIVE

5.0 DISCUSSION

5.1 Physicochemical Properties of Petroleum Refinery Effluent

This finding revealed that the dissolved oxygen (DO) value obtained was very high and above the stipulated limit of (DO ≥ 10mg/L) set by Federal Ministry of Environment, Nigeria (FMEnv) for refinery effluents in Nigeria. The fact that the effluents were coming fresh from the refining tower could suggest that active microbial degradation of the organics that would have drastically reduced the level of the dissolved oxygen had not set in. The bacteria are still in the lag phase and adjusting to a new environment (Attioghe *et al*., 2007).

Temperature, nitrate, sulphate and phosphate are at levels considered not hazardous to the environment based on FMEnv standards (temperature ≤ 40^0C, nitrate ≤ 10 mg/L, sulphate ≤ 50mg/L and phosphate ≤ 50mg/L). This could be attributed to several factors, which include season of the year and time of sampling as well as other chemicals used in the refining process (Otokunefor and Obiukwu, 2005).

Total dissolved solids (TDS) appeared to be high and above FMEnv standard (≤500mg/L). The time of sampling and season of the year could be the reason for the high amounts of dissolved solids and low temperature recorded. This is because rainfall plays an important role in increasing the suspended and dissolved solids of wastewater due to constant washing of top soil materials into the effluent receiving pond while temperature of wastewater is usually a function of the prevailing environmental temperature (Uzoekwe and Oghosanine, 2011).

The turbidity, BOD and COD values obtained were higher than FMEnv standards (turbidity ≤ 10 NTU, BOD ≤ 10 mg/L and COD ≤ 10 mg/L). The high turbidity could be attributed to the cumulative effects of the dissolved solids contained in the effluent. The high BOD value could

be as a result of the presence of pollutants of organic origin especially hydrocarbons. The COD value obtained, in the same vain, is a measure of oxygen demand exerted by both the organic component and reduced inorganic component of the effluent (Uzoekwe and Oghosanine, 2011). Excessive turbidity in water can cause problem for water purification processes such as flocculation and filtration, which may increase treatment cost (Uzoekwe and Oghosanine, 2011). Again, turbidity causes decrease in photosynthetic process since turbidity precludes deep penetration of light in water (Muoghalu and Omocho, 2000).

BOD test is useful in determining the relative organic waste loading of water. High value therefore indicates the presence of large amount of organic pollutants and relatively higher level of microbial activities with consequent depletion of oxygen content (Uzoekwe and Oghosanine, 2011). The high values of BOD observed in this study are causes for worry because the continuous discharge of wastewater containing organic matter into receiving water bodies could result in depletion of oxygen as microorganisms engage in biochemical decomposition of the organic pollutants (Attioghe *et al*., 2007). This problem could lead to the inadequate maintenance of higher life forms. In addition, oxygen availability is important because the end-products of chemical and biochemical reactions in anaerobic systems often produce aesthetically-displeasing colours, tastes and odours in water (Attiogbe *et al*., 2007). The findings in this work are in agreement with that of Uzokwe and Oghosanine (2011), who also reported high values of BOD in raw petroleum refinery effluents. The effluent is a potential source of environmental hazard based on the results obtained in this study and as such, must be treated thoroughly before discharge to avoid drastic alteration of the physicochemical properties of the receiving sites. High or increased turbidity of the effluent receiving water body could reduce the rate of aeration from the atmosphere and penetration of sunlight, which can contribute to a continuous build-up of oxygen deficit with consequent devastating effect on the aquatic organisms. Proliferation of

aquatic organisms that require sunlight to thrive could also be reduced drastically as penetration of sunlight become very minimal (Attioghe *et al*., 2007).

The petroleum refinery effluent was highly polluted as most of the physicochemical parameters analysed were found in concentrations considered to be deleterious. Therefore, it will be unsafe to discharge such effluent into receiving sites without adequate treatment to reduce the pollutants levels. However, some of the parameters were found to be within the permissible limits set by the Federal Ministry of Environment (FMEnv, 1991) for refinery effluent in Nigeria.

Mean values of heavy metals determined were found to range from 1.034ppm for Zinc(Zn) to non-detectable level in manganese (Mn) (Zn >Pb> Cr > Cd >Mn). The metals except zinc were within the stipulated limits set by Federal Ministry of Environment (FMEnv, 1991) for refinery effluent in Nigeria. Several researchers have demonstrated that heavy metal contamination can cause shifts in microbial populations. Heavy metals indirectly affect soil enzymatic activities by shifting the microbial community, which synthesizes enzymes (Shun-hong *et al.*, 2009). Heavy metals exhibit toxic effects towards soil biota by affecting key microbial processes and decrease the number and activity of soil microorganisms (Mora *et al.,* 2005). Heavy metals can affect the microbial reproduction and cause morphological and physiological changes. The exposure of microorganisms to metals always inhibits microbial growth and activity (Huang *et al.*, 2010). Ashraf and Ali (2007) also reported that the heavy metals exert toxic effects on soil microorganisms hence, results in the change in the diversity, population size and overall activity of the soil microbial communities. They also observed that the heavy metals (Cr, Zn and Cd) pollution influenced the metabolism of soil microbes in all cases. In general, an increase in metal concentration adversely affects soil microbial properties, e.g., respiration rate and enzyme activity, which appear to be very useful indicators of soil pollutions. Heavy metals of specific concern to surface water systems are cadmium, chromium, mercury, lead and arsenic. These

heavy metals can bind to the surface of microorganisms and may even breach inside the cells (Gupta *et al.*, 2016). Heavy metals are the potent inhibitors of biodegradation (Kratochvil and Volesky, 1998). These metals cannot be degraded, and eventually persists in the environment. The toxic properties of heavy metals result mainly from the interaction of metals with proteins (enzymes) and inhibition of metabolic processes. Heavy metals such as copper, cadmium, lead, zinc, nickel, mercury and chromium when collected in soils and water bodies above their threshold value becomes toxic to plants, animals, humans and aquatic life (Kumar *et al.*, 2014). Each heavy metal has exclusive bio functions or bio toxicities. Heavy metal-toxicity mechanisms to microbes include protein denaturation (Hg, Pb and Cd), inhibition of cell division (Hg, Pb, Cd and Ni), cell membrane disruption (Hg, Pb, Zn, Ni, Cu and Cd), inhibition of enzyme activity (Hg, Pb, Zn, Ni, Cu and Cd), transcription inhibition (Hg, Pb and Cd) and DNA damage (Hg, Pb, Cd and As) (Gupta *et al.*, 2016). Extreme levels of essential metals though can be harmful to the living organism. Metal such as cadmium is tremendously toxic and was shown to induce the DNA breakage (Baker, 1999).

5.2 Isolation of *Pseudomonas putida* and *Bacillus megaterium*

The ability to isolate *Pseudomonas putida* and *Bacillus megaterium* from refinery effluent shows that the organisms were able to adapt and thrive in environments polluted with petroleum hydrocarbons. Also, their ability to survive in mineral medium enriched with 1% (v/v) of refinery effluent as the sole source of carbon and energy during seven days of enrichment technique is important and suggests the synthesis of enzymes by these organisms involved in crude oil metabolism (Manal, 2011). This finding showed that *Pseudomonas putida* and *Bacillus megaterium* were able to adapt to hydrocarbon polluted sites and that enrichment technique is useful in isolation of various culturable oil degrading bacteria from contaminated sites. Species of either *Bacillus* or *Pseudomonas* or both have been consistently isolated from oil polluted soil

and implicated in crude oil biodegradation (Sathishkumer *et al.,* 2008; Idise *et al.,* 2010; Agarry and Ogunleye, 2012; Vinothini *et al.,* 2015; Riskuwa-Shehu and Udeme, 2016). Most of the studies indicated that the effective ability of *Bacillus* and *Pseudomonas* species in crude oil biodegradation is caused by the active enzyme system for hydrocarbon metabolism. Besides, *Bacillus* species produce spores, which help the bacteria to withstand harsh conditions and germinate when conditions become favourable (Riskuwa-Shehu and Udeme, 2016). The mechanisms employed by the autochthonous microorganisms to achieve this feat includes synthesis of inducible enzymes, mutations such as single nucleotide change or DNA re-arrangement that results in degradation of the compound and acquisition of genetic information from closely related or phylogenetically distinct population within the hydrocarbon-challenged community (Salam *et al*., 2011).

5.3 Screening of the Isolates for Their Capacity to Degrade Various Hydrocarbons

During the period of authentication of the biodegradation of diesel and spent engine oil by the isolates, *Pseudomonas putida* C15a (70.86% and 69.08%) and *Bacillus megaterium* N9a (50.54% and 48.4%) were observed to exhibit the highest potential for biodegradation because they produced higher optical densities than others. The growth of the isolates indicate the occurrence of enzyme induction which enabled growth in all the media due to prior exposure from the polluted sites. This is in agreement with previous reports that previous exposure of microorganisms enhanced their ability to degrade hydrocarbons (Okerentugba and Ezeronye, 2003; VanHamme *et al.,* 2003; Idise *et al.,* 2010). Increase in optical density was related with the bacterial growth, which also shows the extent of degradation of the hydrocarbons. The difference in the ability of the various strains of *Bacillus megaterium* and *Pseudomonas putida* to grow on mineral medium containing diesel and spent engine oil may be attributed to better autochthonous adaptation with corresponding better developed enzyme system for hydrocarbon metabolism of

certain strains than others and the presence of different catabolic genes involved in hydrocarbon degradation in the bacterial species (Darsa, 2014). These isolates have also been demonstrated by other researchers who isolated either *Bacillus* or *Pseudomonas* species or both from petroleum polluted sites to be hydrocarbon degraders (Okerentugba and Ezeronye, 2003; Idise *et al.,* 2010; Sarma and Sarma, 2010; Nwanyanwu and Abu, 2012; Ebrahimi *et al.,* 2012; Riskuwa-Shehu and Udeme, 2016). Due to their diversity, metabolic versatility and tolerance to many xenobiotics, *Pseudomonas* are key players in bioremediation. Important criteria that make *Pseudomonas* so versatile in metabolism and suitable for bioremediation include Chemotaxis towards pollutants by way of transport system protein, broad specificity of enzymes involved in primary attack on xenobiotics make them capable of degrading structurally analogous compounds, dioxygenases attack the thermodynamically highly stable compounds and incorporate two atoms of oxygen forming catechol and protocatechuate from a number of compounds, simultaneous functioning of several enzymes involved in ring cleavage which is the rate limitating step in degradation of xenobiotics, genes coding for a number of of catabolic peripheral pathways are borne on plasmids referred to as degradative (D) plamids and *Pseudomonas* are capable of degrading organic solvents develop tolerance to solvents by altering the composition of their inner and outer membrane proteins (Kahlon, 2016).

5.4 Biodegradation

Different growth patterns were observed in all the treatments during degradation of the diesel and spent engine oil. During day 3 of the incubation period, there was little growth recorded for all the treatment sets. This may be attributed to the fact that, the bacteria was in the lag phase of growth. However, an increase in population density was observed in all the treatments during the subsequent period of incubation before eventually a decline occured. The decline in growth could be attributed to a depletion of carbon source for energy and growth requirements. The

highest growth rate was observed at day 15 for all the treatment sets containing diesel (DB: 1.35 x 10^7CFU/ml; DP: 1.85 x 10^7CFU/ml; DC: 6.1 x 10^6 CFU/ml) and spent engine oil (SB: 1.05 x 10^7CFU/ml; SP: 1.35 x 10^7 CFU/ml; SC: 5.6 x 10^6 CFU/ml). The control treatment sets for both samples (DCN and SCN) showed no bacterial growth all through the period of biodegradation, these treatments were devoid of the isolates as they only contained diesel and spent engine oil. Higher hydrocarbon-utilizing bacterial counts was observed in treatment sets containing diesel than spent engine oil. This could be attributed to the constituents of the samples used. Diesel consists mainly of low molecular weight alkanes (about 75%), which are easily biodegradable by microbes, whereas spent engine oil consist of polycyclic aromatic hydrocarbons (PAHs) (Wang *et al.,* 2000), which are highly toxic and have tendency to bioaccumulation (Shivendra and Hardik, 2014). Hence, the lower HUB count exhibited by the treatments containing spent engine oil probably reflects its lower content of saturated hydrocarbons and high aromatic content (Okerentugba and Ezeronye, 2003). The utilization of the oil resulted in increase in the population densities of the various bacterial strains. The growth rate of the organisms correlated with the rate of oil degradation, suggesting that the breakdown of the hydrocarbons resulted in the provision of utilizable compounds required for their growth. Ajao *et al.* (2013) also reported an increase in log number of cells with increase in degradation efficiency. A decrease in population density occurred at day 18 for all the isolates and this could be attributed to decrease in the amount of utilizable hydrocarbons consequently resulting in competition, build up of toxic substances such as organic acids and other metabolic products that may be unfavourable to the growth of the organism. The bacterial growth reached the stationary phase and moved into the death phase in almost all the cases. This shows that the bacterial isolates utilized and degraded the hydrocarbons (Okerentugba and Ezeronye, 2003). It was observed that the treatments increased in population with increase in the rate of degradation but decreased progressively as

the incubation continued. Using one-way analysis of variance to compare hydrocarbon utilizing bacteria count, it was observed that all the treatment set containing diesel and spent engine had significant difference.

From the findings, it was observed that the strains of *Pseudomonas putida* and *Bacillus megaterium* had varying hydrocarbon degradation capacity for diesel and spent engine oil. However, *Pseudomonas putida* showed higher ability to degrade both diesel and spent engine oil than *Bacillus megaterium* and the consortium. The difference in the rate of hydrocarbon degradation may be due to the presence of different catabolic genes and better developed enzyme systems in the bacterial species involved in hydrocarbon degradation and thus utilizing the hydrocarbons as carbon and nitrogen source (Marchal *et al.,* 2006). Moreover, owing to the presence of these catabolic enzymes, bacterial strains are extremely well equipped to make adaptive changes for their survival in adverse environments (Heinonsalo *et al.,* 2000). This is in agreement with the findings of Sathishkumer *et al.* (2008); Nwanyawu and Abu (2010) and Vinothini *et al.* (2015), who working independently observed that *Pseudomonas* species had higher hydrocarbon degradation efficiency than *Bacillus* species.

The consortium exhibited less capacity to degrade hydrocarbons in both samples than the individual organisms. This may be due to the similarities of the enzyme systems of the bacteria forming the consortium or the antagonistic effects between the two isolates, which reduce their effectiveness (Manal, 2011). This finding agrees with the work of Singh and Lin (2008) and Manal (2011), who reported higher hydrocarbon degradation in the individual cultures than the consortia, but disagrees with the findings of Juhasz (2000), Rahman *et al.* (2002b), Mukred *et al.* (2008) and Sathishkumer *et al.* (2008), Zhang *et al.* (2010), who reported higher hydrocarbon biodegradation by mixed cultures than the pure cultures of individual strains.

The higher rate of hydrocarbon degradation observed in diesel compared to spent engine oil may be due to the type of hydrocarbons that constitute the samples. This agrees with the work of Amund and Akaqngbou (1993), who showed that crude oil fraction with lower amount of saturated hydrocarbons were more resistance to microbial degradation than the fraction containing higher amount of saturated hydrocarbons. Mukred *et al.* (2008) also showed that short and medium chain alkanes undergo higher degradation than the longer chain alkanes.

All the isolates achieved considerable amount of hydrocarbon degradation as the percentage degradation ranged from 75.86% to 98.3% for diesel and 60.8% to 75.03% for spent engine oil after incubation of samples for 18 days. One way analysis of variance revealed that there is significant difference in the rate of percentage degradation of both samples. The reason for this relatively high and progressive biodegradation in the sample might be due to reduction in the concentration of oil in the sample, which does not pose serious challenge to the metabolic activities of the microorganisms. The high rate of biodegradation observed is attributed to the increase in microbial biomass and nutrient availability. From this it can be understood that the efficiency of the organism and its stage of growth should be taken into consideration in clean up or removal of oil from the environment (Darsa *et al.*, 2014). The result is in agreement with the findings of Rahman *et al*. (2002), who reported increase in the rate of biodegradation of crude oil, as the concentration of oil reduces and recorded 78% oil degradation after incubation of samples for 20 days.

pH is an important factor which influences the microbiological metabolic activity and growth of microorganisms. pH of the samples were also monitored and the results revealed that the isolates exhibited optimal growth at pH range of 6.33 to 7.70, which is within the range suitable for microbial growth. This is in agreement with the work of Ogunlaja and Ogunlaja (2007), Nduka

and Orisakwe (2009), Ainon *et al* (2010) and Riskuwa-Shehu and Udeme (2016), who observed optimal growth for bacteria at pH range of 6.0 to 8.0.

A decrease in pH was observed in all the samples after the 18 day period of incubation. pH reduces as biodegradation progresses, this is because utilization of the crude oil as sole carbon and energy source by these microorganisms resulted in their growth with a concomitant production of acid. These acidic metabolic products might account for the decrease in pH of the culture medium (Okerentugba and Ezeronye, 2003).

One way analysis of variance revealed that there is significant difference in the treatment set containing spent engine oil, but there is no significant difference in the treatment sets containing diesel. This may be due to the samples used in the biodegradation experiments which had pH values that are around neutral, which is ideal for metabolic activities. According to Whang *et al*, (2009), microbial growth and diesel biodegradation was found to be at a pH 7.2 and Luo *et al*, (2013) at pH level of 7 *Pseudomonas sp*. strain F4 showed efficient diesel oil degradation potential.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Physicochemical properties of the refiney effluent were generally found to be tolerable. However, some of the parameters were found to be much higher than the permissible limits outlined by the FMEnv, which suggests that the effluent is hazardous to the receiving environment and needs proper treatment before release. Physicochemical analysis also revealed high level of zinc beyond the permissible limits by FMENV.

Three strains of *Pseudomonas putida* and seven strains of *Bacillus megaterium* were isolated from petroleum refinery effluent.

The isolates all showed utilization of hydrocarbons in the screening tests. The most efficient hydrocarbon degraders are *Pseudomonas putida* C15a and *Bacillus megaterium* N9a.

Hydrocarbon Utilization bacteria counts, optical density and pH were used in the study as measures of hydrocarbon degradation by the isolates. Diesel (98.3%) was shown to be degraded better than spent engine oil (75.03%). The growth rate of the organisms correlated with the rate of oil degradation, suggesting an increase in log number of cells with increase in degradation efficiency. For the diesel, highest percentage degradation occurred in the treatment containing *Pseudomonas putida* (98.3%), followed by *Bacillus megaterium* (81.03%) and then the consortium (68.97%) with their corresponding highest population density of 1.85 x 10^7CFU/ml, 1.35 x 10^7CFU/ml and 6.1 x 10^6CFU/ml, respectively. In spent engine oil, *Pseudomonas putida* (75.03%) also showed highest percentage degradation, followed by *Bacillus megaterium* (66.22%) and then the consortium (60.86%) with their corresponding highest population density of 1.35 x 10^7CFU/ml, 1.05 x 10^7CFU/ml and 5.6 x 10^6CFU/ml, respectively at pH range of 6.33-7.70.

For both samples, there was no growth recorded for the control treatments. As the incubation progresses, the available carbon source got depleted, hence, the bacterial growth in all the treatments experienced a drop on the 18 day of incubation.

6.2 Recommendations

1. Species of *Bacillus* and *Pseudomonas*, which are autochthonous to petroleum-contaminated environments, should be utilized for bioremediation of polluted sites.
2. Molecular methods should also be employed in identifying bacteria that are important in hydrocarbon degradation beside the culture technique used in this study.
3. Effects of nitrogen at different concentrations to get the best C: N ratios for optimum biodegradation should be investigated since availability of nutrients affects the rate of biodegradation.
4. Enforcement of regulations for release of hazardous effluents from refineries should be ensured by government in order to limit the indiscriminate discharge of such effluents into the environment.
5. Studies on the degradation of hydrocarbons under different pH conditions should be investigated to ascertain the optimum pH for bioremediation.

6.3 Contribution to knowledge

1. The study showed that the refinery effluent contains high amount of lead (0.456mg/l), cadmium (0.007mg/l), dissolved oxygen (200mg/l), biological oxygen demand (100mg/l), chemical oxygen demand (1200mg/l), total dissolved solids (950mg/l) and turbidity (179 NTU) above the permissible limit.(FMEnv, 1991)

2. Out of the strains of *Pseudomonas putida* isolated, strain C15a degraded (98.3%) diesel and 75.03% spent engine oil while among the *Bacillus megaterium* strains isolated, N9a degraded 81.03% diesel and 66.22% spent engine oil.
3. *Pseudomonas putida* (98.3%, 75.03%) showed highest percentage degradation ability, followed by *Bacillus megaterium* (81.03%, 66.22%) and the consortium (68.97%, 60.86%) had the lowest, in both diesel and spent engine oil, respectively.

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APPENDIX I

Collection of spent engine oil from a service station during oil change for vehicles

APPENDIX II

GNA and GNB Enterobacteriacae-ID kit for *Pseudomonas putida*

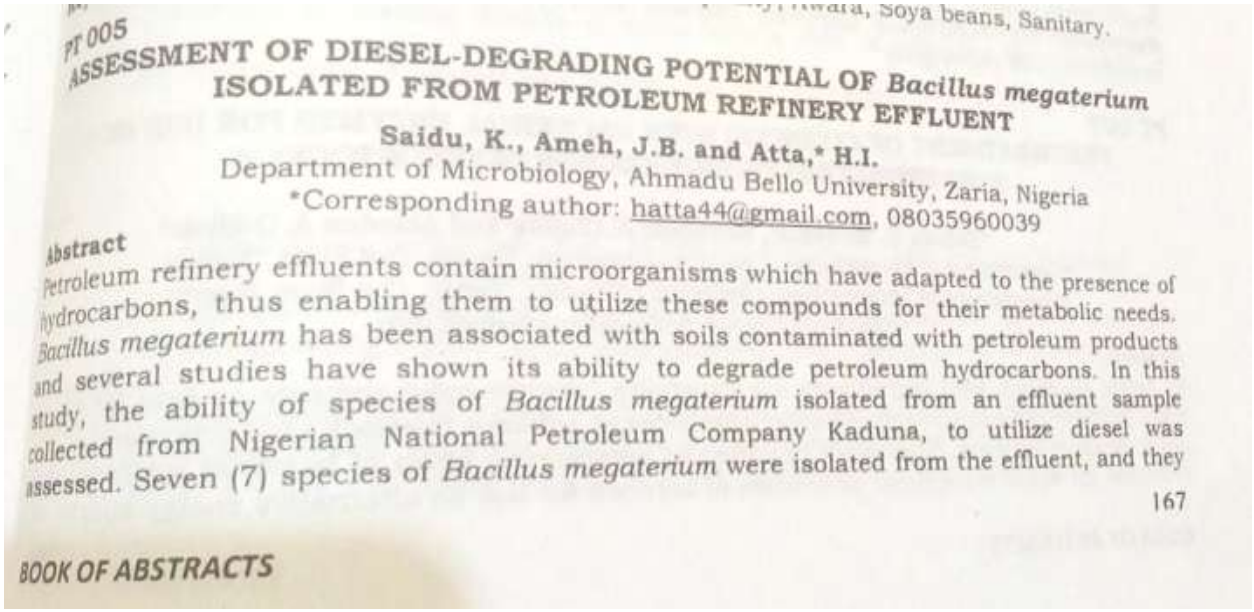
APPENDIX III

Bacillus-ID kit for *Bacillus megaterium*

APPENDIX IV

Set-up for biodegradation studies

APPENDIX V: Publication from conference proceedings of 41st Annual Scientific Conference and General Meeting of the Nigerian Society for Microbiology



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PT 005

ASSESSMENT OF DIESEL-DEGRADING POTENTIAL OF *Bacillus megaterium* ISOLATED FROM PETROLEUM REFINERY EFFLUENT

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Abstract
Petroleum refinery effluents contain microorganisms which have adapted to the presence of hydrocarbons, thus enabling them to utilize these compounds for their metabolic needs. *Bacillus megaterium* has been associated with soils contaminated with petroleum products and several studies have shown its ability to degrade petroleum hydrocarbons. In this study, the ability of species of *Bacillus megaterium* isolated from an effluent sample collected from Nigerian National Petroleum Company Kaduna, to utilize diesel was assessed. Seven (7) species of *Bacillus megaterium* were isolated from the effluent, and they

were identified using biochemical and phenotypic testing (Microgen kit). The isolates were then screened for their ability to utilize diesel using sterile mineral media containing each of the isolate, and two uninoculated controls (one containing only the mineral media and the other containing the mineral media and diesel). The optical density of the liquid culture was determined using a spectrophotometer after a period of seven days, and it was used as a measure of utilization of diesel by the isolates. Of the seven isolates, only three (43%) showed an increase in cell growth as shown by the optical density, while the remaining the remaining four showed a decrease in optical density. The isolate with profile number, 60070223, showed the highest increase in optical density (48%), and thus highest diesel utilization. This implies that even among the same species, there might be a varying degree in metabolic abilities as factors such as plasmids and other genetic elements play a major role in the resulting phenotype of the organism. The isolate of *Bacillus megaterium* showing the highest diesel utilization is a good candidate forthe bioremediation of diesel-contaminated soils.

Keywords: Assessment, Diesel, Potential, *Bacillus megaterium*, Petroleum, Refinery Effluent

APPENDIX VI: Publication from UMYU Journal of MicrobiologyResearch (UJMR), 2019 (In press).

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Diesel-Degrading Potential of *Pseudomonas putida* Isolated from Effluent of a Petroleum Refinery in Nigeria

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Abstract

The contamination of soil and groundwater by hazardous chemicals has become a major concern due to the associated risks to human health and the environment. The ability of Pseudomonas putida isolated from a petroleum refinery effluent to degrade diesel was assessed in this study. The effluent sample was collected from the treatment plant in the Kaduna Refining and Petrochemical Company (K.R.P.C), Nigeria. The physicochemical properties and heavy metal content of the effluent was determined, and three strains of Pseudomonas putida were identified among the bacteria isolated using conventional biochemical and phenotypic tests. The strain showing the highest degradation potential after screening was selected for the final biodegradation studies. The ability of the selected strain of Pseudomonas putida (C15a) to utilize the hydrocarbons in diesel was assessed over a period of eighteen days, and monitored on a three-day interval by evaluating the pH, hydrocarbon utilizing bacterial count and oil and grease content. It was observed that the organism was able to utilize diesel for its metabolic needs as shown by the increase in hydrocarbon utilizing bacterial (HUB) count and corresponding decrease in oil and grease content as well as pH. The highest hydrocarbon utilizing bacterial count was observed at day 15 (1.85 x 10^7 CFU/mL) with highest hydrocarbon degradation occurring at day 18 (98.3%). The strain of Pseudomonas putida (C15a) isolated in this study can be used as a candidate for further bioremediation studies on petroleum.

Keywords: *Petroleum, biodegradation, Pseudomonas putida, diesel, effluent*