# BIODEGRADATION OF CRUDE OIL USING GENETICALLY MODIFIED STRAINS OF *BACILLUS CEREUS* AND *PSEUDOMONAS AERUGINOSA* ISOLATED FROM KADUNA REFINERY EFFLUENT

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**MARCH, 2018**

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**A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF DOCTOR OF PHYLOSOPHY INMICROBIOLOGY**

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**MARCH, 2018**

## DECLARATION

I declare that the work in this thesis entitled **"Biodegradation of Crude Oil using Genetically Modified Strains of *Bacillus Cereus* and *Pseudomonas aeruginosa* Isolated from Kaduna Refinery Effluent"** has been carried out by me in the Department of Microbiology, Ahmadu Bello University Zaria, Nigeria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any institution.

**Garba, Umar Chonoko** _____ _____

Name of Student Signature Date

## CERTIFICATION

This thesis titled "**Biodegradation of Crude oil Using Genetically Modified Strains of *Bacillus cereus* and *Pseudomonas aeruginosa* Isolated From Kaduna Refinery Effluents"** by Umar Garba Chonoko meets the regulations governing the award of the degree of Doctor of philosophy of Ahmadu Bello University, and is approved for its scientific contribution to knowledge and literary presentation.

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## DEDICATION

This thesis is dedicated in memory of my late daughter and sister, Fatima Umar Chonoko and Fatima Garba Chonoko. May their souls rest in perfect peace, Ameen.

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**ABSTRACT**

Extensive hydrocarbon extraction and refining activities often result in environmental pollution that has undesired consequences on the biotic and abiotic components of the ecosystem. Biodegradation of hydrocarbons in contaminated systems could be achieved by either physicochemical or biological methods. The present work was undertaken to isolate, identify and assess the hydrocarbon degrading capacity of bacteria associated with effluent samples collected from the Refining and Petrochemical Company at Kaduna. Physicochemical properties of the raw refinery effluents analysed showed that temperature ($27^0$C) and pH (6. 61) were within normal range but biochemical oxygen demand, chemical oxygen demand as well as oil and grease had by far exceeded the Federal Environmental Protection Agency maximum permissible limits. The higher values recorded in the raw effluent samples showed that BOD was 300mg/l, COD 400mg/l, oil and grease 540mg/l as against the FEPA limits of 50, 40 and 10mg/l for BOD, COD, Oil and greases respectively. Physicochemical properties of the crude oil showed that the crude oil obtained from Nigeria had low sulphur contents and is predominantly of the light crude oil category. The effluent samples were further analysed using standard microbiological techniques. *Bacillus cereus* and *Pseudomonas aeruginosa* were isolated from the refinery effluents. A total of 41 and 21 strains of *Bacillus cereus* and *Pseudomonas aeruginosa* respectively were isolated and identified on the basis of their biochemical characteristics from three sites (i.e untreated wastewater channel, waste oil retention pond and outfall of the effluent discharge outside of the refinery). Biochemical characterization confirmed 41 and 21isolates of *Bacillus cereus* and *Pseudomonas aeruginosa* respectively. Further identification of the isolates using Microgen kit revealed that only 17(42%) of the 41 and 9 (43%) of the 21 isolates were actually *B. cereus* and *P. aeruginosa* respectively. The organisms were assessed to determine their biodegrading capacities on crude oil as the sole carbon source using minimal salt medium. The increase in bacterial count (increase in cell number) was used as index of biodegradation. The test on the degrading activity of the isolates on crude oil from effluent samples revealed that *Bacillus cereus* and *Pseudomonas aeruginosa* were the potent degraders of crude oil. The wild strains of *Bacillus cereus* and *Pseudomonas aeruginosa* were UV-irradiated for 30minutes and subjected to nitrous acid treatment. The result of mutagenesis showed that *B. cereus* and *P. aeruginosa* exhibited comparable mean survival rates of 54.74 and 54.29% respectively following 30minutes exposure to UV-radiation. The growth rate of the two bacteria on crude oil supplemented medium was monitored at varying concentrations of crude oil (0.5, 1, 1.5 and 2%) over a period of eight weeks. Crude oil concentrations of 0.5, 1 and 1.5% were more effective for biodegradation compared to 2%. Petroleum product degradation increased from 96.59% for parent *B. cereus* to 99.60% for UV-irradiated nitrous acid treated *B. cereus*, and from 97.58% for parent *P. aeruginosa* to 99.80% for UV-irradiated nitrous acid treated *P. aeruginosa*. Higher crude oil degradation potential was observed for second stage mutants of *P. aeruginosa* than *B. cereus.* In this study, *Pseudomonas aeruginosa* exposed to UV-radiation and nitrous acid mutagenesis exhibited significantly higher potential for crude oil degradation and was thus selected as the best performing mutant strain for degradation. The ability of these isolates to degrade crude oil is clear evidence that their genome harbours the relevant degrading genes.

These bacteria were screened for the presence of the hydrocarbon degrading enzymes (catechol 2, 3 dioxygenase and phenol hydroxylase) by DNA isolation and PCR amplification of gene using specific primers. The organism with catechol 2, 3 dioxygenase enzymes was identified as *Bacillus cereus*, with 216bp amplification using C23O specific primers. The organism with phenol hydroxylase (Pheh) gene was identified as *Pseudomonas aeruginosa* with 870bp amplification using Pheh specific primers. Thus, modification of the organisms with UV-irradiation for 30 min followed by nitrous acid treatment resulted in their increased petroleum product degradation ability and could therefore be used for biodegradation of hydrocarbons in petroleum products polluted environments.

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# CHAPTER ONE

# 1.0 INTRODUCTION

## 1.1 Background of the study

Crude oil is composed of a mixture of aliphatic paraffin, alicyclic and aromatic hydrocarbons, which differ in their susceptibility to microbial attack (Chikere and Azubuike, 2013). It is currently the principal source of energy on which all modern industrial production systems depend (Barathi and Vasudevan, 2016). According to Okey (2003), it will remain the major source of energy for all industrial processes for the next several decades while reliable alternative energy sources are being sampled.

Current technologies employed in the refining of crude petroleum into the diverse petro-products, usually produce large quantities of effluents that are toxic and often resulting in pollution of water bodies and soils into which they are discharged (Asmae *et al*., 2014).

Since water bodies and soils contaminated by refinery effluents often lose their capacity to support plant and animal life, such effluents constitute very serious public health and socio-economic hazards (Olukunle *et al*., 2015). Though several researches are still being conducted on the effluents from Kaduna Refining and Petrochemical Company (KRPC), evidences from studies of similar effluents in the country have shown that, the toxic effects of refinery effluents can be recorded up to 1.5 km distance from the point of discharge (Otokunefor and Obiukwu, 2005).

Traditionally, several physical and chemical methods have been employed in the remediation of environments polluted by refinery effluents. However, such methods have brought many problems. For instance, they are often costly, time consuming and above all, unfriendly to the environment. For these reasons, attention is currently centered on microbial degradation (biodegradation) by the natural microflora as a possible mechanism for the elimination of pollutants of the environment that are found in refinery effluents. For this purpose, either

autochthonous or allochthonous microorganisms or both can be used (Ajayi *et al.,* 2008; Bryan and Voskanyan, 2012).

Biodegradation of hydrocarbons by natural populations of microorganisms represents one of the primary mechanisms of eliminating petroleum from the environment (Malik and Ahmed, 2012).

A wide range of studies have dealt with biotransformation and bioremediation of petroleum hydrocarbons and interest in exploiting crude oil-degrading organisms for environmental clean-up has become central to Petroleum Microbiology. There are so many bacteria and fungi with this ability and these organisms are widely distributed in marine, freshwater and soil habitats (Wake, 2005), but scientists reported that indigenous microorganisms are more efficient for biodegradation of oil pollutants.

The ability to degrade and/or utilize hydrocarbon substrates is exhibited by a wide range of bacteria and fungi (Atlas, 1981; Kaduna Refining and Petrochemical Company, 2004). Prior exposure of a microbial community to hydrocarbons is important in determining how rapidly subsequent hydrocarbon inputs can be degraded. This phenomenon is known as adaptation and is brought about either by induction, depression of enzymes, genetic changes or selective enrichment (Leahy and Colwell, 1990; Okerentugba and Ezeronye, 2003; Asitok and Antai, 2006; Ekpo and Ekpo, 2006; Wuyep, *et al*., 2006).

Improvement in the ability of microorganisms to degrade a pollutant could be achieved through modification of the environment or the organisms. The organisms can be modified through mutagenesis. Various mutants abound and the exposure of organisms to UV- light and treatment with nitrous acid have been employed with relative success (Das and Chandran, 2011). *Pseudomonas* sp and *Bacillus* sp have been reported in previous studies as petroleum degraders (Nwachukwu *et al*., 2001; Okerentugba and Ezeronye, 2003; Anon, 2007).

## 1.2 Statement of the Research Problem

Refining of petroleum produces large amounts of effluents that are toxic and about 5-10% of inputs are released as effluents. Accidental discharge of crude petroleum, field oil and grease from the refinery also contribute to the effluents (Emoyan *et al*., 2005). Liquid refinery effluent usually contains oil and grease, phenolic compounds, suspended solids and organic matter which are toxic to both plant and animal lives of receiving terrestrial and aquatic bodies (Emoyan *et al*., 2005).

Nigeria has vast crude oil and gas deposits. Attempts to explore and exploit them have left the country with unique vulnerabilities (Nduka and Orisakwe, 2009). Rapid urbanization and industrialization of Warri and its environment between 1968 and 1990 created pollution potentials that are as high as some sources of pollution. The rivers, estuaries, creeks and air have been contaminated for decades (Egborge 2001). Refinery and petrochemical plants generate solid wastes and sludge composed of organic and inorganic compounds including heavy metals (Callot and Ocampo, 2000).Wastewater released by petrochemical industries is characterized by the presence of large quantity of polycyclic and aromatic hydrocarbons, phenols, metal derivatives, surface active substances, sulphides, naphthylenic acids and other chemicals (Suleimanov,1995). Due to the ineffectiveness of purification systems, wastewater may become dangerous, leading to the accumulation of toxic products in the receiving water bodies with potentially serious consequences to the ecosystem (Bay *et al.*, 2003).

Physicochemical properties of a refinery effluent in the Niger Delta revealed the presence of phenol of 5.13- 6.38mg/l; oil and grease of 1o.56-15.53mg/l and ammonia nitrogen of 4.37-13.17mg/l. The above concentrations persisted 1.5km downstream from point of discharge with higher concentrations in dry season than in rainy season (Emoyan *et al*., 2005; Otokunefor and Obiukwu, 2005).

### 1.3 Justification for the Study

Biodegradation of crude oil by microorganisms is one of the primary ways of eliminating crude oil from contaminated sites and appears to be the most environmentally friendly method of removing oil pollutants (Nwachukwu, 2000). In recent times, use of microorganisms for removing crude oil pollution from contaminated sites is considered by scientists, because other methods such as surfactant washing and incineration lead to production of more toxic compounds and they are not-economical (Barathi and Vasudevan, 2016).

Petroleum refining results in the production of large amounts of toxic effluents and results in environmental pollution of receiving bodies of water and soils. Such contaminated habitats lose their capacity to support both plant and animal life and thus constitute public health and socioeconomic hazards as well as pose serious aquatic toxicity problems. Mechanical cleaning of the effluent is found to be inefficient in such ecosystems (Okerentugba and Ezeronye., 2003, Asmae *et al*., 2014).

In Nigeria, the Niger-Delta region forms the largest oil producing States and consequently, the environment is exposed to a large proportion of the environmental degradation and health hazards associated with exploration and exploitation of crude oil. The frequency of oil spill and untreated wastewater discharge into water bodies and the attendant negative impact on the aquatic and terrestrial ecosystems has been well known and well documented over the years (Nduka and Orisakwe, 2009). Presence of pollutants in natural waters alters the quality and often pose serious threats to aquatic life. Various studies have shown positive correlation between pollutions from petrochemical and refinery effluents and the health of aquatic organisms (Otukunefor and Obiukwu, 2005). Previous observations suggested a correlation between contaminants of water and sediments with aromatic hydrocarbons from refinery effluents and compromised fish health *(*Kuehn *et al.,* 1995*)*. *Bacillus* sp and *Pseudomonas* sp have been listed amongst petroleum degraders with promising results (Asitok and Antai, 2006; Ekpo and Ekpo, 2006; Anon, 2007).

### 1.4 Aim:

To isolate and genetically modify *Bacillus cereus* and *Pseudomonas aeruginosa* strains for biodegradation of crude oil.

### 1.5 Objectives

The specific objectives of this study were to:

**1.** Determine the physical and chemical properties of the samples of Refinery Effluent.

**2.** Isolate strains of *Bacillus cereus* and *Pseudomonas aeruginosa* from refinery effluents and characterize the isolates using biochemical procedures.

**3.** Determine the capacity of the isolates to utilize crude oil as carbon and energy source for growth

**4.** Mutate the selected strains of *Bacillus cereus* and *Pseudomonas aeruginosa* using UV light and nitrous acid.

**5.** Assess the capacity of the wild and selected mutant strains in biodegradation of crude oil under laboratory conditions.

**6**. Screen and identify the presence of hydrocarbon degrading genes in wild and mutant strains of *Bacillus cereus* and *Pseudomonas aeruginosa.*

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

Extreme environmental sites present harsh climatic conditions such that only certain organisms have the ability to survive. The organisms that are able to tolerate or resist the environmental challenges are called extremophiles. Most of these organisms are prokaryotes. Among them, bacteria represent a large domain of organisms and are present in most habitats on earth, oil spills being one such site (Ekpo and Ekpo, 2006).

Nigeria has a vast crude oil and gas deposits and attempts to explore them have left the country with unique vulnerabilities (Nduka and Orisakwe, 2009).

Crude as well as refined petroleum oil consist of alkanes (*normal* or *iso*), cycloalkanes, aromatics, alkenes, smaller amounts of phenols and naphthenic acids, nitrogen-pyridine, pyrrole, indole and sulfur (alkylthiol, thiophene) containing compounds and partially oxygenated, highly condensed asphaltic fraction (Atlas and Bartha, 1973). Petroleum oil provides a concoction of toluene, xylene, cymene, hexadecane, benzene, ethyl benzene, o-ethyl toluene, styrene, cyclohexane, hexane, petroleum ether organic solvents, hydrocarbons and heavy metals such as vanadium and nickel (Ekpo, 2006). Petroleum consists of groups of components that are: (1) volatile compounds, which are low molecular weight compounds, like methane (natural gas) or propane that is normally gaseous or evaporates very quickly at room temperature; and (2) hydrocarbons that can be divided into four classes: saturates, aromatics, asphaltenes (phenols, fatty acids, ketones, esters and porphyrins), high molecular weight polycyclic aromatic hydrocarbons and resins (pyridines, quinolines, carbazoles, sulfoxides and amides) (Leahy and Colwell, 1990).

Saturated hydrocarbons (alkanes) are the simplest of the hydrocarbon species and are composed entirely of single bonds and are saturated with hydrogen. Saturated hydrocarbons are the basis of petroleum fuels and are found as either linear or branched species. Unsaturated hydrocarbons

such as alkenes and alkynes are also present in petroleum. Cycloalkanes are hydrocarbons containing one or more carbon rings to which hydrogen atoms are attached. An aromatic compound of petroleum oil has the smallest aromatic molecules (one and two-rings) that are both volatile and readily biodegradable but larger aromatic compounds are more resistant to biodegradation. The percentage of polycyclic Aromatic Hydrocarbons in crude oil varies, but the 'priority pollutants' are present at low levels in crude oils. Asphaltenes are examples of high molecular weight PAHs that have additional chemical side chains attached to their aromatic rings (FEPA, 1991; Ekpo, 2006).

Petroleum oil also find its importance in the artificial synthesis of asphalt, lubricant grease, plastics, pesticides, fertilizers, paints, solvents, refrigerants, cleaning fluids, detergents, antifreeze and synthetic fibers. Hence, it is a veritable source of income for the national economy and industries. Due to its importance in energy consumption, it has been consumed in large quantities and invariably released into the environment as noxious waste with global ramification (Osuji and Uwakwe, 2006; Ajayi *et al.*, 2008).

Petroleum-derived contaminants constitute the most prevalent points of origin of pollution in the industrialized world and severely affect the aboriginal microorganisms of the environment.

There are three ways by which oil pollutants enter the environment: direct discharge of effluents and solid wastes, land surface runoff into the coastal zone mainly with rivers and atmospheric fallout of pollutants transferred by the air mass. The amount of natural crude oil seepage was estimated to be 600,000 metric tons per year in Nigeria (Orisakwe, 2009).

The environment contains essential chemicals, which react with the sulfur and nitrogen containing compounds of the spilled petroleum and form the secondary poisonous chemicals. The petroleum impacted soil consists of high concentration of organic solvents and metals, imposing a double stress on the microbial populations. Therefore, the surviving bacteria face extreme conditions for growth and can be considered as extremophiles.

## 2.1 Non-biological degradation of hydrocarbons

Once the petroleum hydrocarbons spill into the environment, the process of "weathering" will take place in which change occurs in the physical, chemical and biological factors of spilled oil. A variety of physical and chemical processes help in the sequential removal of the petroleum oil from the contaminated site. *Evaporation* - the volatile hydrocarbons evaporate quickly into the atmosphere when they reach the water surface. Evaporation of spilled oil will be more at warmer temperature than cold temperature. *Dissolution* - most components of crude oil do not dissolve in water, but some components like heavy polycyclic aromatic hydrocarbons (PAHs), are poorly soluble in water and adhere to soil particles and succumb to microbial degradation over time. *Dispersion* - It is the process by which oil is broken up into small droplets and spread through the water. Hence, microbial degradation is eminent which ultimately results in the removal of hydrocarbons. The main advantage is the conversion of oil slicks into tiny droplets with more surface area thereby facilitating microbial degradation. Often, dispersion prevents the oil from reaching surface where it can evaporate and can increase the exposure of oil to the ecosystem.

*Photo-oxidation* - petroleum components often react with the light and hence broken into simpler substances, which are susceptible to microbial degradation especially PAHs is called photolysis.

## 2.2 Biological degradation of hydrocarbons

Microorganisms alter and metabolize various classes of pollutants carried in the petroleum oil, which help in the sequential removal of the spill. During spills, hydrocarbon degrading microbes increase in their population by utilizing the petroleum as a carbon source. Such microbes can switch "ON" their catabolic/transport/regulator genes or alter their physiological conditions, so that the microbes can either degrade or exclude it from the harmful effects of petroleum oil. Microbes degrade the petroleum in soil by both notic and anoxic conditions. During biodegradation of petroleum oil, microbes prefer to attack n-paraffins (n-alkanes or normal alkanes) followed by acyclic isoprenoids (e.g., norpristane, pristine, phytane, *etc*.), whilst cycloalkanes are the most resistant to microbial attack (Atlas, 1981). This results in the decrease

of saturated and aromatic hydrocarbon content in soil and an increase in oil density, sulphur content, acidity and viscosity of spilled oil (Yousefi *et al.*, 2009). During biodegradation, there will be a generation of acyclic and cyclic, saturated and aromatic carboxylic acids and phenols from hydrocarbons, which subsequently affects the soil parameters. A generalized sequence of petroleum hydrocarbon in order of decreasing biodegradability is represented as follows- n alkanes > branched chain alkanes > branched alkenes > low molecular weight n alkyl aromatics > monoaromatics > cyclic alkanes > aromatics > asphaltenes (Yousefi *et al.*, 2009).

**2.3. Factors affecting biological degradation**

2.3.1 Physical state of oil pollutants

In soil, petroleum oil droplets are dispersed and adhere to particulates which prevent the evaporative losses of volatile hydrocarbons, thus limit its spreading and toxicity to microorganisms. The dispersed oil droplets are susceptible to oxygen, which is essential for biodegradation. Physical status of oil spill and the environmental factors determine the milieu of petroleum degraders. Particulate matter can reduce, by absorption, the effective toxicity of the components of petroleum, but absorption and adsorption of hydrocarbons to humic substances probably contribute to the formation of persistent residues (Espeche *et al*., 1994). Due to increased concentration of hydrocarbons in soil, undispersed oil slicks cause the inhibition of biodegradation by nutrient or oxygen limitation or through toxic effects exerted by volatile hydrocarbons (Fusey and Oudot, 1984). The rates of mineralization of the higher-molecular-weight aromatic hydrocarbons, such as naphthalene and phenanthrene, are related to aqueous solubilities rather than total substrate concentrations (Cowan and Strafford, 2007).

2.3.2 Temperature

Microbial degradation of petroleum compounds in soil depends on the nature of temperature. Low temperature prevents the evaporation of volatile short chain alkanes, thus increases the viscosity and decreases the water solubility of petroleum oil, which in turn affects the rate of degradation by microorganisms. Reduction in the rate of microbial degradation of petroleum

spill at low temperature may be due to the decrease in the activity of enzymes (Leahy and Colwell, 1990).

High temperature (30 - 40ºC) increases the rate of degradation to maximum, which in turn affects the membrane rigidity of microbes and inhibits the degradation of hydrocarbons (Leahy and Colwell, 1990).

2.3.3 Oxygen

Petroleum compounds are highly susceptible to dioxygenase enzyme, which indulges in the incorporation of oxidation and catabolism of aliphatic, cyclic and aromatic hydrocarbons by incorporating oxygen into the substrates (Hong *et al*., 2005). Oxygen availability can vary within soil depending on the rate of microbial oxygen consumption, the type of soil and whether the soil is waterlogged or not. Two types of conditions stimulate the microbial degradation of petroleum compounds in soil. Oil slicks above the surface of the soil can easily undergo degradation by the aerobic conditions. However, oxygen limitation such as anaerobic degradation is possible in aquatic sediments (Hong *et al.*, 2005).

2.3.4 Availability of nutrients

Nutrients are the limiting factors which affects the microbial rate of petroleum hydrocarbons. Petroleum oils spilled into the environment contains low concentrations of inorganic nutrients, which shows high carbon/nitrogen and/or carbon/phosphorus ratios, which are unfavourable for microbial growth (Atlas, 1981).

2.3.5 Chemical nature of the oil

Biodegradation rate of petroleum oil depends on the chemical nature of the hydrocarbons in the spilled site. Oil is composed of thousands of different compounds - hydrocarbons in which carbons are arranged in an unbranched chain can degrade quickly, whereas branched hydrocarbons (polycyclic aromatic compounds) are more difficult to biodegrade and persist

longer. Resins and asphaltenes are recalcitrant fractions of petroleum compounds that persist in the soil for millennia (Cowan and Strafford, 2007).

2.3.6 Salinity

The effect of salinity on the degradation of hydrocarbons appears to be complex. On one hand, the presence of salt at a high concentration inhibits microbial growth and subsequent degradation of petroleum contaminants. On the other hand, the presence of petroleum contaminants makes the soil granules hydrophobic and thus hinders salt leaching through water infiltration(Hong *et al*., 2005).

**2.4 Distribution of petroleum hydrocarbon utilizing microbes**

Apart from the bacteria, which are the primary colonizers, fungi (yeasts and filamentous fungi) are the secondary degraders of petroleum compounds in contaminated site. Genera such as *Candida*, *Rhodotorula*, *Saccharomyces*, *Sporobolomyces*, *Amorphoteca*, *Neosartorya*, *Talaromyces, Yarrowia*, *Pichia*, *Graphium* and *Trichosporon* were isolated from petroleum-contaminated soil and proved to be the potential organisms for hydrocarbon degradation (Benkacoker and Ekundayo, 1996).

Isolation of microorganisms from oil polluted sites is commonly taken as evidence that these microorganisms are active degraders of that pollutant (Espeche *et al*., 1994; Okerentugba and Ezeronye, 2003). Several studies reported that mixed cultures carry out more extensive biodegradation of hydrocarbons than pure cultures (Foght *et al*., 1999; Komukai *et al*., 1996; Malik and Ahmad, 2012). There are several advantages relying on indigenous microorganisms rather than adding microorganisms to degrade hydrocarbons. Therefore, it is very useful to screen for new indigenous microorganisms degrading hydrocarbon. Isolation, identification and characterization of potential indigenous biodegrading isolates are helpful in the manipulation, utilization and tracking these potent isolates (Abou-Shanab, 2007).

Refinery and petrochemical plants generate solid wastes and sludge composed of organic and inorganic compounds including heavy metals. Wastewater released by petrochemical industries are characterized by the presence of large quantities of polycyclic aromatic hydrocarbons, phenols, metal derivatives, surface active substances, sulphides, naphthylenic acids and other chemicals (Suleimanov,1995). Due to the ineffectiveness of purification systems, wastewater may become dangerous, leading to the accumulation of toxic products in the receiving water bodies with potentially serious consequences on the ecosystem. Presence of pollutants in natural waters alters the quality and often poses serious threats to aquatic life. (Kuehn *et al.,* 1995; Otukunefor and Obiukwu, 2005).

Petroleum, or crude oil, naturally occurring oily, bituminous liquid is composed of various organic chemicals. It is found in large quantities below the surface of earth and is used as a fuel and as a raw material in the chemical industry. Modern industrial civilization depends on petroleum and its products (Doscher, 2005; Lee, 2009). Crude oil is composed of mixtures of paraffin, alicyclic and aromatic hydrocarbons (Okerentugba and Ezeronye, 2003). The principal forces limiting the biodegradation of polluting petroleum in water bodies are the toxic and resistant components of oil the itself, water temperature, scarcity of mineral nutrients (especially nitrogen and phosphorus), the exhaustion of dissolved oxygen and scarcity of hydrocarbon-degrading microorganisms in previously unpolluted areas (Israel *et al.,* 2007).

## 2.5. Properties of petroleum

### 2.5.1 Chemical composition

Petroleum is made up of both hydrocarbon content and non-hydrocarbon content.

### 2.5.2 Hydrocarbon content

Hydrocarbon consists basically of compounds of only two elements, carbon and hydrogen. These elements form a large variety of complex molecular structures. However, regardless of physical or chemical variations, almost all crude oil ranges from 82 - 87 % carbon by weight and 12 – 15 % hydrogen. The more viscous bitumens generally vary from 80 - 85 % carbon and from 8 – 11 % hydrogen (Doscher, 2005; Israel *et al.,* 2007).

Crude oil can be grouped into three basic chemical series; Paraffins, naphthenes and aromatics. Most crude oils are mixtures of these three series in various and seemingly endless proportions. No two crude oils from different sources are completely identical (Ajayi *et al.*, 2008; Lee, 2009). The paraffin series of hydrocarbons, also called the methane ($CH_4$) series, comprises the most common hydrocarbons in crude oil. It is a saturated straight-chain series that has the general formula $C_nH_{2n}$ in which C is carbon, H is hydrogen and n is an integer. The paraffins, that are liquid at normal temperatures but boil between $40^o$ and $200^oC$, are the major constituents of gasoline. The residues obtained by refining lower-density paraffins are both plastic and paraffin waxes. The naphthene series has the general formula $C_nH_{2n}$ and is a saturated closed-ring series. This series is an important part of all liquid refinery products, but it also forms most of the complex residues form the higher boiling-point ranges. For this reason, the series is generally heavier. The residue of the refinery process is asphalt, and the crude oils in which this series predominates are called asphalt-based crudes. The aromatic series has the general formula $C_nH_{2n-6}$ and is an unsaturated closed – ring series. Its most common member, benzene ($C_6H_6$), is present in all crude oils, but the aromatics as a series generally constitute only a small percentage of most crudes (Ekpo and Ekpo, 2006; Israel *et al.*, 2007; Ajayi *et al.*, 2008).

**2.6 Non – hydrocarbon content**

In addition to the practically infinite mixtures of hydrocarbon compounds that form crude oil, sulfur, nitrogen and oxygen are usually present in small but often important quantities (Asitok and Antai 2006; Lee, 2009).

Sulfur is the third most abundant atomic constituent of crude oils. It is present in the medium and heavy fractions of crude oils. In the low and medium molecular ranges, sulfur is associated only with carbon and hydrogen, while in the heavier fractions it is frequently incorporated in the large polycyclic hydrocarbon that also contains nitrogen and oxygen. Generally, the higher the specific gravity of the crude oil, the greater is its sulfur content. Excess sulfur is removed from crude oil during refining. The oxygen content of crude is usually less than 2 % by weight and is present

as part of heavier hydrocarbon compounds in most cases. Nitrogen is present in almost all crude oil, usually in quantities of less than 0.1 percent by weight. Many metallic elements like vanadium and nickel are found in crude oils (Ajayi *et al*., 2008).

Crude oil also may contain a small amount of decay – resistant organic remains, such as siliceous skeletal fragments, wood, spores, resin, coal and various other remnants of former life (Doscher, 2005; Lee, 2009; Bayoumi *et al*., 2011).

**2.7. Physical properties of crude oil**

Oil consists of a closely related series of complex hydrocarbon compounds that range from gasoline to heavy solids. The various mixtures that constitute crude oil can be separated by distillation under increasing temperature into such components as (from light to heavy) gasoline, kerosene, gas oil, lubricating oil, residual fuel oil, bitumen and paraffin (Israel *et al*., 2007).

Crude oils vary greatly in their composition. Because they consist of mixtures of thousands of hydrocarbon compounds, their physical properties such as color, specific gravity and viscosity also vary widely (Doscher, 2005; Lee, 2009).

2.7.1 Specific gravity

Crude oil is immiscible with and lighter than water hence it floats. Crude oils are generally classified as heavy oils, medium and light oils on the basis of specific gravity at standard conditions and relative mobility (Ojo, 2006; Lee, 2009).

Tar sands contain immobile oil, which does not flow into a well bore, heavy crude oils have enough mobility that, given time, they can be obtained through a well bore in response to enhanced recovery methods. The widely used American Petroleum Institute (API) gravity scale is based on pure water, with an arbitrarily assigned API gravity of $10^0$. Liquids lighter than water, such as oil, have API gravities numerically greater than $10^0$. Crude oils below $20^0$ API gravity are usually considered heavy whereas the conventional crudes with API gravities between $20^0$ and $25^0$ are regarded as medium, with light oils ranging above $25^0$ (NNPC, 2002; Doscher, 2005; Lee, 2009).

2.7.2 Boiling and freezing points

Because oil is always at a temperature above the boiling point of some of its compounds, the more volatile constituents constantly escape into the atmosphere unless confined (Oboh *et al*., 2004). It is impossible to refer to a common boiling point for crude oil because of the widely differing boiling points of its numerous compounds, some of which may boil at temperatures too high to be measured. By the same token, it is impossible to refer to a common freezing point for a crude oil because the individual compounds solidify at different temperatures. However, pour point is the temperature below which crude oil becomes plastics and will not flow. Pour points range from $32^0$ to below – $57^0$C (Doscher, 2005).

**2.8. Growth conditions for petroleum degraders**

These include biotic and abiotic conditions.

2.8.1. Abiotic conditions
These include the following:

2.8.2. Composition of the oil or hydrocarbons

Petroleum oil consists of a mixture of compounds that have varying degrees of susceptibility to microbial attack. Generally, this order decreases as follows: N-alkanes – branched alkanes low molecular weight aromatics cyclic alkanes (Oboh *et al*., 2004).

2.8.3 Physical state of the oil or hydrocarbons
The state of the oil affects its availability to microbial attack. The various states include slicks (thin or thick) when the oil occur as patches of oil on the water surface; emulsion – when the oil appears as oil-in water emulsions; tarballs/Scums when there are large aggregates of weathered and undegraded oils which restricts accessibility by microorganisms because of their limited surface area and when the oil is in a mobile state, that is, migration, which could be vertical or horizontal. However, artificial dispersants have been studied as a means of increasing surface area and hence biodegradability of oil slicks and evidences of biosurfactants production by petroleum degraders abound (Oboh *et al*., 2004; Ojumu *et al*., 2005; Ojo, 2006).

2.8.4 Concentration of the oil or hydrocarbons

The rate of uptake and mineralization of organic compounds by microbial population in the aquatic environment are proportional to the concentration of the compound. High concentration of hydrocarbons can be associated with heavy, undispersed oil slicks in water or oil spills on land that cause inhibition or toxic effects exerted by volatile hydrocarbons (Margesin and Schinner, 2001; Anon, 2008).

2.8.5 Temperature

This influences petroleum biodegradation by its effect on physical nature and chemical composition of the oil. At low temperature, oil viscosity is high, volatilization is reduced, water solubility is increased thereby reducing the availability of the oil for microbial degradation and thus delaying biodegradation. Its effects on the rate of metabolism of hydrocarbons by microorganisms and the effects on the composition of the microbial community as well as climate and season expectedly select different populations of petroleum degraders (Harry, 2005).

2.8.6 Oxygen

The initial step in the catabolism of hydrocarbons by bacteria and fungi involves the oxidation of the substrate by oxygenation for which molecular oxygen is required. Thus aerobic conditions are necessary for microbial oxidation of hydrocarbons in the environment. The availability of oxygen decreases with depth in water (Grishchenkov *et al*., 2000; Mrayyan and Battikhi, 2004).

2.8.7 Nutrients

The release of hydrocarbons into aquatic environments which contain low concentrations of inorganic nutrients, often produces excessively high C: N or C: P or both, which are unfavorable for microbial growth. The addition of fertilizers to adjust C: N: P stimulates biodegradation of crude oil in aquatic and terrestrial environments (Nwachukwu, 2000; Pellerin *et al*., 2004; Adoki, 2007; Nwaogu *et al*., 2008).

**2.9 Biotic factors**

These include the composition of the microbial community population of petroleum degraders and adaptations of the organisms from prior exposure of a microbial community to petroleum oil. This could either be from anthropogenic sources accidental oils spills, petroleum exploration, transportation activities and waste oil disposal or from natural sources – seeps or plants-derived hydrocarbons. These lead to increase in hydrocarbon – oxidizing potential of microbial community through induction and/or repression of specific enzymes (Adoki, 2007; Nwaogu *et al*, 2008).

Genetic changes often result in new metabolic capabilities and/or selective enrichment of an organism able to transform the compound (s) of interest. Plasmids may play an important role in genetic adaptation of an organism as it represents a highly mobile form of DNA transferable through conjugation or transformation (Margesin and Schinner, 2001; Anon., 2007).

Seeding can be through the introduction of allochthonous microorganisms into the natural environment, for the purpose of increasing the rate or extent or both of the biodegradation of pollutants. The rationale of this being the autochthonous microorganisms may not be capable of degrading a wide range of potential substrates present in complex mixtures like petroleum (Okerentugba and Ezenroye, 2003).

The criteria for effective seed organisms' ability to degrade most petroleum components are genetic stability, viability of the organisms during storage, rapid growth following storage, high degree of enzymatic activity and growth ability in the environment, ability to compete with indigenous (autochthonous) microorganisms, non-pathogenecity and inability to produce toxic metabolites (Bryan and Voskanyan, 2012).

### 2.9.1 Turbidity

This is a measure of the cloudiness of water. The cloudier the water, the greater the turbidity. Turbidity in water is caused by suspended matter such as clay, silt, organic matter and plankton as well as other microscopic organisms that interfere with the passage of light through the water. Turbidity is closely related to total suspended solids (TSS), but also includes planktons and other organisms. Turbidity itself is not a major health concern, but high turbidity can

interfere with disinfection and provides a medium for microbial growth. It may also indicate the presence of microbes. High turbidity can be caused by soil erosion, urban runoff and high flow rates (Anon, 2000).

One of the primary factors affecting turbidity is total suspended solids and as such the factors affecting TSS will also affect turbidity. In addition, organic matter contributes to turbidty (Das and Chandron, 2011).

The flow rate of a water body is a primary factor influencing turbidity concentrations. Fast running water can carry more particles and larger-sized sediment. Heavy rains can pick up sand, silt, clay and organic particles from the land to surface water. A change in flow rate can also affect turbidity; if the speed or direction of the water current increases, particulate matter from bottom sediments may be re-suspended. The effluent from Wastewater Treatment Plants (WWTPs) can add suspended solids and organic material to a stream. The wastewater from houses contains food residues, human wastes, and other solid materials that are deposited in the drains. Most of the solids and organic materials are removed from the water at the WWTP before being discharged in to the stream, but treatment cannot eliminate everything (Okey, 2003).

As plants and animals present in a water body die and decay, suspended organic particles are released and can contribute to turbidity. Bottom-feeding fishes (such as carp) can stir up sediments as they remove vegetation. These sediments can contribute to turbidity. Algal blooms can contribute to turbidity. Algal production is enhanced when nutrients are released from bottom sediments during seasonal turnovers and changes in water current. As flood waters recede, they will bring along inorganic and organic particles from the land surface, and contribute this to the stream (Wake, 2005).

2.9.2 *Electrical conductivity*

This is the reciprocal of the a-c resistance in ohms measured between opposite faces of a centimeter cube of an aqueous solution at a specified temperature (WHO, 2002). The unit of electrical conductivity is Siemens per centimeter (S/cm) at a specified temperature, normally 25°C. Specific Conductance (SC) is thus a measure of how well water can conduct an electrical current. Conductivity increases with increasing amount and mobility of ions. These ions, which come from the breakdown of compounds, conduct electricity because they are negatively or positively charged when dissolved in water. Therefore, SC is an indirect measure of the presence of dissolved solids such as chloride, nitrate, sulfate, phosphate, sodium, magnesium, calcium, and iron, and can be used as an indicator of water pollution (WHO, 2002).

2.9.3 pH

The term "pH" was originally derived from the French terms "pouvoir hydrogene," In English, this means "hydrogen power" (Murphy, 2007a). pH represents the effective concentration (activity) of hydrogen ions ($H^+$) in water. Very

high (greater than 9.5) or very low (less than 4.5) pH values are unsuitable for most aquatic organisms. Young fish and immature stages of aquatic insects are extremely sensitive to pH levels below 5 and may die at these low pH values. High pH levels (9-14) can harm fish by denaturing cellular membranes. Changes in pH can also affect aquatic life indirectly by altering other aspects of water chemistry. Low pH levels accelerate the release of metals from rocks or sediments in the stream. These metals can affect a fish's metabolism and the fish's ability to take water in through the gills and can thus kill fish fry (WHO, 2002).

Carbon dioxide ($CO_2$) enters a water body from a variety of sources, including the atmosphere, runoffs from land, release from bacteria in the water, and respiration by aquatic organisms. This dissolved $CO_2$ forms a weak acid. Natural, unpolluted rainwater can be as acidic as pH 5.6, because it absorbs $CO_2$ as it falls through the air. Because plants take in $CO_2$ during the day and release it during the night, pH levels in water can change from daytime to night (Murphy, 2007b).

Air pollution from car exhausts and power plant emissions increases the concentrations of nitrogen oxides ($NO_2$, $NO_3$) and sulfur dioxide ($SO_2$) in the air. These pollutants can travel far from their place of origin and react in the atmosphere to form nitric acid ($HNO_3$) and sulfuric acid ($H_2SO_4$). These acids can affect the pH of streams by combining with moisture in the air and falling to the earth as acid rain or snow (Olukunle *et al*., 2015).

When acid waters (waters with low pH values) come into contact with certain chemicals and metals, they often make them more toxic than normal. As an example, fish that usually withstands pH values as low as 4.8 will die at pH 5.5 if the water contains 0.9 mg/L of iron. If an acid water environment is mixed with small amounts of aluminum, lead or mercury, a similar problem - one far exceeding the usual dangers of these substances, will be observed (WHO, 2002).

2.9.4 Solids

The solid wastes include dissolved and suspended solids. Dissolved solids are the materials that will pass through a filter paper while suspended solids are those that do not. The suspended solids are further divided into settleable and nonsettleable solids, depending on how many milligrams of the solids will settle out of 1 liter of wastewater in 1 hour. All these classes of solids can be divided into volatile or fixed solids, the volatile solids generally being organic materials and the fixed solids being inorganic or mineral matter (Anon, 2009).

2.9.5 *Total solids*

The term "total solids" refers to matter suspended or dissolved in water or wastewater, and is related to both specific conductance and turbidity. Total Solids include both total suspended solids, the portion of total solids retained by a filter (usually with a pore size of 0.45 micrometers), and total dissolved solids, the portion that passes through a filter (American Public Health Association (APHA,1998).

*2.9.6 Total Suspended Solids (TSS)*

These are solids in water that can be trapped by a filter. TSS can include a wide variety of materials such as silt, decaying plant and animal matter, industrial wastes and sewage. High concentrations of suspended solids can cause many problems for stream health and aquatic life (APHA, 1998). High TSS can block light from reaching submerged vegetation. As the amount of light passing through the water is reduced, photosynthesis slows down. Reduced rates of photosynthesis cause less dissolved oxygen to be released into the water by plants. If light is completely blocked from bottom dwelling plants, the plants will stop producing oxygen and will die. As the plants are decomposed, bacteria will use up even more oxygen from the water. Low dissolved oxygen can lead to fish kills (WHO, 2002).

High TSS can also cause an increase in surface water temperature, because the suspended particles absorb heat from sunlight. This can cause dissolved oxygen levels to fall even further (because warmer waters can hold less DO), and can harm aquatic life in many other ways. High TSS in a water body can often mean higher concentrations of bacteria, nutrients, pesticides and metals in the water (Anon, 2009).

2.9.7 Total dissolved solids (TDS)

Total Dissolved Solids (TDS) are solids in water that can pass through a filter (usually with a pore size of 0.45 micrometers). TDS is a measure of the amount of material dissolved in water. This material can include carbonate, bicarbonate, chloride, sulfate, phosphate, nitrate, calcium, magnesium, sodium, organic ions and other ions. A certain level of these ions in water is necessary for aquatic life. Changes in TDS concentrations can be harmful because the density of the water determines the flow of water in and out of an organism’s cells. However, if TDS concentrations are too high or too low, the growth of many aquatic lives can be limited, and death may occur (Anon, 2000). Similar to TSS, high concentrations of TDS may also reduce water clarity, contribute to a decrease in photosynthesis, combine with toxic compounds and heavy metals, and lead to an increase in water temperature (Anon, 2009).

**2.10 Chemical parameters of wastewater**

*Dissolved Oxygen (DO):* Dissolved Oxygen (DO) is found in microscopic bubbles of oxygen that are mixed in the water and occur between water molecules (WHO, 2002). DO is a very important indicator of a water body's ability to support aquatic life. Oxygen enters the water by absorption directly from the atmosphere or by aquatic plant and algae photosynthesis. Oxygen is removed from the water by respiration and decomposition of organic matter. Very high DO concentrations can also be harmful to aquatic life (Anon. 2000). When dissolved oxygen concentrations drop, major changes in the types and amounts of aquatic organisms found living in the water can occur. Waters that have low dissolved oxygen sometimes smell bad because of waste products produced by organisms that live in low oxygen environments. Very low DO concentrations can result in mobilization of trace metals (WHO, 2002; Anon, 2009).

**2.11** ***Factors affecting dissolved oxygen***

*2.11.1 Volume and velocity of water flowing in the water body*

In fast-moving streams, rushing water is aerated by bubbles as it churns over rocks and falls down hundreds of tiny waterfalls. These streams, if unpolluted, are usually saturated with oxygen. In slow, stagnant waters, oxygen only enters the top layer of water, and deeper water is often low in DO concentration due to decomposition of organic matter by bacteria that live on or near the bottom of the reservoir (Murphy, 2007a).

*2.11.2 Climate/season*

The colder the water, the more oxygen can be dissolved in the water. During dry seasons, water levels decrease and the flow rate of a river slows down. As the water moves slower, it mixes less with the air, and the DO concentration decreases. During rainy seasons, oxygen concentrations tend to be higher because the rain interacts with oxygen in the air as it falls. More sunlight and warmer temperatures also bring increased activity levels in plant and animal life; depending on what organisms are present. This may increase or decrease the DO concentration (Anon, 2009).

*The type and number of organisms in the water body:* During photosynthesis, plants release oxygen into the water. During respiration, plants remove oxygen from the water. Bacteria and fungi use oxygen as they decompose dead organic matter in the stream. The type of organisms present (plant, bacteria, fungi) affects the DO concentration in a water body (Anon, 2009).

Nutrients are food for algae, and water with high amounts of nutrients can produce algae in large quantities. When these algae die, bacteria decompose them, and use up oxygen. This process is called eutrophication. Nitrate and phosphate are nutrients. Nitrate is found in sewage discharge, fertilizer runoff, and leakage from septic systems. Phosphate is found in fertilizer and some detergents.

*2.11.3 Organic Wastes*

Organic wastes are the remains of any living or once-living organism. Organic wastes that can enter a body of water include leaves, grass clippings, dead plants or animals, animal droppings, and sewage. Organic waste is decomposed by bacteria; these bacteria remove dissolved oxygen from the water when they breathe. If more food (organic waste) is available for the bacteria, more bacteria will grow and use oxygen, and the DO concentration will drop. Directly downstream from where sewage effluent is discharged to a river, DO content often decreases, because of the increase in growth rate of bacteria that consume the organic matter contained in the effluent (Murphy, 2007a).

**2.12 Biochemical oxygen demand and chemical oxygen demand**

This is the amount of oxygen used by microorganisms in the process of breaking down organic matter in water. The more organic matter there is (as in sewage), the greater the number of microbes. The more microbes there are, the greater the need of oxygen to support them; consequently, less oxygen is available for higher animals such as fishes. The BOD is therefore a reliable gauge of the organic pollution of a body of water. One of the main reasons for treating sewage or wastewater prior to its return to a water resource is to lower its BOD (Anon, 2009).

The composition of wastewater is analysed using several physical, chemical and biological measurements. The concentration of organic matter is measured by the BOD and COD analysis. The $BOD_5$ is the amount of oxygen used over a five-day period by microorganisms as they decompose the organic matter in sewage at a temperature of 20°C. Similarly, the COD is the amount of oxygen required to oxidize the organic matter by use of strong oxidizing agent e.g dichromate in an acid solution and to convert it to carbon dioxide and water. The value of COD is always higher than that of BOD because many organic substances can be oxidized chemically but cannot be oxidized biologically. Commonly, BOD is used to test the strength of untreated and treated municipal and biodegradable industrial wastewaters. COD is used to test the strength of wastewater that is either not biodegradable or contains compounds that inhibit activities of microorganisms (WHO, 2002; Anon, 2009).

**2.13 *Nitrogen***

Nitrogen is required by all organisms for the basic processes of life to make proteins, to grow,

and to reproduce. Nitrogen is very common and found in many forms in the environment.

Inorganic forms include nitrate ($NO_3$), nitrite ($NO_2$), ammonia ($NH_3$), and nitrogen gas ($N_2$).

These compounds contribute to alkalinity of water bodies (Osuji and Anita, 2006).

### 2.13.1 *Common forms of nitrogen in water*

#### *2.13.1.1 Nitrate and nitrite*

Nitrate ($NO_3$) is highly soluble (dissolves easily) in water and is stable over a wide range of environmental conditions. It is easily transported into streams and ground waters. Nitrates feed planktons (microscopic plants and animals that live in water), aquatic plants and algae, which are then eaten by fish. Nitrite ($NO_2$) is relatively short-lived in water because it is quickly converted to nitrate by bacteria. Excessive concentrations of nitrate and/or nitrite in water can be harmful to humans and wildlife. Nitrate is broken down in our animal intestines to become nitrite. Nitrite reacts with hemoglobin in human blood to produce methemoglobin, which limits the ability of red blood cells to carry oxygen. This condition is called methemoglobinemia or "blue baby" syndrome (because the nose and tips of ears can appear blue from lack of oxygen). It is especially serious for infants, because they lack the enzyme necessary to correct this condition. High nitrate and nitrite levels can also cause methemoglobinemia in livestock and other animals. If excessive amounts of phosphorus and nitrates are added to the water, algae and other aquatic plants can be produced in large quantities, which could lead to eutrophication (WHO, 2002; Anon, 2009).

#### *2.13.1.2 Ammonia*

Ammonia, another inorganic form of nitrogen, is the least stable form of nitrogen in water. Ammonia is easily transformed to nitrate in waters that contain oxygen and can be transformed to nitrogen gas in waters that are low in oxygen. Ammonia is found in water in two forms - the ammonium ion ($NH_4^+$), and dissolved, unionized (no electrical charge) ammonia gas ($NH_3$). Total ammonia is the sum of ammonium and unionized ammonia. The dominant form depends on the pH and temperature of the water (WHO, 2002).

***2.14 Factors affecting nitrate and nitrite concentrations:***

*2.14.1 Wastewater and septic system effluent*

Human waste is significant contributor of nitrogen to water. Ammonia, nitrite and nitrate are decomposition products from urea and protein, which are in human wastes. Ammonia is an ingredient in many household cleaning products and is sometimes used to remove carbonate from hard water. Therefore, these nitrogen species go down *the drains in our houses and businesses, and can enter streams from* wastewater treatment plant (WWTPs) effluent, illegal sanitary sewer connections and poorly functioning septic systems (Anon, 2009).

*2.14.2 Fertilizer runoff*

Fertilizer is a major influence on nitrogen concentrations in the environment. Commercial nitrogen fertilizers are applied either as ammonia or nitrate, but ammonia is rapidly converted to nitrate in the soil. Animal manure is also used as a nitrogen fertilizer in some areas. Organic nitrogen and urea in the manure are converted to ammonia and, ultimately, to nitrate in the soil. Nitrate that is not used by plants washes from farmlands and residential as well as commercial lawns into storm drains and nearby streams or seeps into groundwater (Oyekunle and Famakin, 2004).

*2.14.3 Animal waste*

A significant amount of nitrogen is released in the wastes produced by animals. This can be a serious problem in waters near cattle feedlots, hog farms, dairies and barnyards. Excretions of aquatic organisms are very rich in ammonia, a decay product of animal proteins, but the amount of nitrogen they add to waters is usually small. Through the process of nitrification, ammonia is oxidized to nitrite and then to nitrate in water (Murphy, 2007a).

*2.14.4 Fossil fuels*

The burning of fossil fuels such as gasoline and coal in cars, trucks and power plants produces many by-products. Coal and petroleum generally contain about 1 percent nitrogen. Part of the nitrogen is converted to the gas nitrous oxide (NO) during the burning of the fuel. Nitrous oxide is converted by sunlight and photochemical processes in air to nitrogen oxide gases. Nitrogen oxide gases are a major contributor to acid rain (Nehb, 2006).

*2.14.5 Industrial discharge*

Many industries use nitrogen during processing. Nitrite is sometimes used as a corrosion inhibitor in industrial process water. Ammonia is also used in cleaning water supplies and to remove carbonate from hard water. Water from industries is usually discharged to a wastewater treatment plant (WWTP), and may end up in a downstream water body (WHO, 2002; Anon, 2009).

2.14.6 Phosphate

Phosphorus is a nutrient required by all organisms for the basic processes of life. Phosphorus is a natural element found in rocks, soils and organic material (Anon, 2009). Phosphorus clings tightly to soil particles and is used by plants, so its concentrations in clean waters are generally very low. However, phosphorus is used extensively in

fertilizer and other chemicals, so it can be found in higher concentrations in areas of human activity. Many seemingly harmless activities added together can cause phosphorus overloads (Pellerin *et al*., 2004).

2.14.7 *Forms of Phosphorus*

Phosphorus exists in water in cither a particulate phase or a dissolved phase. Particulate matter includes living and dead planktons, precipitates of phosphorus, phosphorus adsorbed to particulates, and amorphous phosphorus. The dissolved phase includes inorganic phosphorus and organic phosphorus. Phosphorus in natural waters is usually found in the form of phosphates ($PO4^{-3}$). Phosphates can be in inorganic form (including orthophosphates and polyphosphates), or organic form (organically-bound phosphates) (Abdulkareem and Kovo, 2006).

*2.14.8 Organic phosphate*

This is phosphate that is bound to plant or animal tissue. Organic phosphates are formed primarily by biological processes. They are contributed to sewage by body wastes and food residues, and also may be formed from orthophosphates in biological treatment processes or by receiving water biota. Organic phosphates may occur as a result of the breakdown of organic pesticides, which contain phosphates. They may exist in solution, as loose fragments, or in the bodies of aquatic organisms (Onyema *et al*., 2010).

*2.14.9 Inorganic phosphate*

This is phosphate that is not associated with organic material. Types of inorganic phosphates include orthophosphate and polyphosphates. Orthophosphate is sometimes referred to as "reactive phosphorus." Orthophosphate is the most stable kind of phosphate and is the form used by plants. Orthophosphate is produced by natural processes and is found in sewage. Polyphosphates (also known as metaphosphates or condensed phosphates) are strong complexing agents for some metal ions. Polyphosphates are used for treating boiler waters and in detergents. In water, polyphosphates are unstable and will eventually convert to orthophosphate (WHO, 2002).

Phosphates are not toxic to people or animals unless they are present in very high levels. Digestive problems could occur from extremely high levels of phosphate. In fresh water lakes and rivers, phosphorus is often found to be the growth-limiting nutrient, because it occurs in the least amount relative to the needs of plants. If excessive amounts of phosphorus and nitrogen are added to the water, algae and other aquatic plants can be produced in large quantities and these may lead to eutrophication. Dissolved oxygen concentrations can drop and the loss of oxygen in the bottom waters can free phosphorus-previously trapped in the sediments, further increasing the available phosphorus (Anon, 2009).

**2.15** ***Factors affecting phosphorus concentrations***

*2.15.1 Industrial discharge*

Polyphosphates are often added to water to prevent iron oxides or calcium carbonates from forming. If this water is released into streams or lakes, polyphosphates can enter the water body and will convert to orthophosphate (Margesin and Schinner, 2001).

*2.15.2 Phosphate mining*

Phosphate mining, concentrating and processing are sources of phosphate to rivers in some areas. The most common phosphorus-containing mineral is apatite ($Ca_5F\ (P0_4)_3$) (Madu *et al.*, 2011).

*2.15.3 Synthetic materials*

Organophosphates are commonly used as construction materials, flame retardant and plasticizers. Reduced forms of phosphorus are present in certain synthetic organic chemicals, including some that are used in insecticides (Anon, 2009).

*2.15.4 Eutrophication*

This is a process that results from accumulation of nutrients in lakes or other water bodies. Eutrophication is a natural process, but can be greatly accelerated by human activities that increase the rate at which nutrients enter the water. In fresh water lakes and rivers, phosphorus is often the growth limiting nutrient, because it occurs in the least amount relative to the needs of plants. "Eutrophic" waters are characterized by high nutrient concentrations, resulting in high productivity of plant growth. Some scientists have categorized trophic status according to phosphorus concentration. Lakes with phosphorus concentrations below 0.010 mg/L are classified as oligotrophic, phosphorus concentrations between 0.010 and 0.020 mg/L are indicative of mesotrophic lakes, and eutrophic lakes have phosphorus concentrations exceeding 0.020 mg/L (WHO, 2002).

*2.15.5 Sulphate*

Sulfate is the oxy-anion of sulphur in the +6 oxidation state. Sulfate also **imparts** a **bitter or salty taste to** water (Margesin and Schinner, 2001).

*2.15.6* ***Oil and grease***

The presence of oil and grease in domestic and industrial wastewater is of concern to the public because of its deleterious aesthetic effect and its impact on aquatic life. Regulations and standards have been established that require monitoring of oil and grease in water and waste water. Problems caused by their presence include interference with such vital processes as the mass transfer of oxygen from air to water (essential in river are aeration), blockage of pipes, fouling of plant and animal life, odor and taste problems, alteration of balance in aquatic ecosystem due to migration of species away from areas of pollution and possible eutrophication of receiving water bodies (Anon, 2009).

*2.15.7* Lead

Heavy metals are toxic to man and fish (to a degree varying greatly from metal to metal). They are easily accumulabled in fish and other tissues and hence liable to enter the food chain. Some metals form complexes with organic materials (so-called 'Organometallics'), the resulting compounds being very toxic indeed, perhaps far more than the un-complexed elements (WHO, 2002; Murphy, 2007a, b; Anon, 2009).

*2.15.8* Chromium (VI)

Its salts are used extensively in metal finishing and plating applications, in anodizing aluminum and in the manufacture of paints, dyes, explosives and ceramics. Chromium may be present in wastewater from these industries and may also be discharged from chromate-treated cooling waters. Chromium is an essential dietary requirement - in limited amounts - and its deficiency can disrupt glucose metabolism (Margesin and Schinner, 2001; Ukpaka, 2011).

**2.16 Mutation**

Mutation is an heritable material, that is, DNA replication when the chemical structure of genes undergoes random modifications. Once a change has occurred, the altered genes continue to replicate in their changes form unless another mutation occurs (Liu, 2005). Most mutations harm an organism. If a mutation occurs in gene sequence that codes for a particular protein, the mutation may result in a change in the amino acid sequence directly by the gene. This change, in turn, may affect the function of the protein (Anon, 2007). There are two types of mutation basically- base pair (nucleotide pair) substitutions and frame shift mutation. These could either occur spontaneously or could be induced.

2.16.1 Base pair substitutions

These could be either transitions, which are either purine to purine (A, T) or pyrimidine (C,G) or transversions (Purine to primidine or pyimidine to purine ) base pair substitution could lead to silent mutation (sense mutation) when the change in the product of the same amino acid missenses mutation when the change in the codon leads to the base pair result in any of the stop codons, which then results in termination of protein synthesis (Anon, 2007).

2.16.2 Frame shift mutation

This results when there is insertion or depletion of one more (not in multiples of three) nucleotides in the coding region (exon)of a gene. These cause alteration of the reading frame (anon, 2007). Codons are groups of three nucleotides and thus there are three possible reading frames for each gene although only one is used. This type of mutation changes all the amino acids downstream and very like creates a malfunctioned product since it may differ greatly from the normal protein. Reading frames other than the correct often contain stop codons which will truncate the mutant protein prematurely (Prescott *et al.*, 2008).

2.16.3 Spontaneous mutation

This occurs as a result of natural processes in cells. They differ from induced mutations as they have a very low frequency of occurrence ($10^{-12}$) and occur through DNA replication errors

(SanMartin, 2011).

Polymerase accuracy (presence of proof reading) is usually carried out by $3^1$-$5^1$ exonuclease to remove incorrectly paired bases. Base alteration often results into automerization, which is the ability to exist in two forms of between which they interconvert. Base degradation, which is the deamination of C to U, also occurs at a significant rate cells. There can also be spontaneous frame shift mutation (Wuyep *et al*., 2006).

2.16.4 Mutagens

Mutagen is a natural or human-made agent (physical or chemical) which can alter the structure or sequence of DNA (Bryan and Voskanyan, 2012). The different types of mutagens according to their mechanisms of action include:

(a) Chemical mutagens: On the basis of mode of action, chemical mutagens can be grouped into five:

(i) Base analogs: These are chemicals, which closely resemble nucleic acid base and are erroneously inserted into newly synthesized DNA strands such as bromouracil and amino purine (Ado, 2004).

(ii) Chemicals, which alter structure and pairing properties of base: These could be nitrous acid, which causes cytosine (C) to uracil (U), adenine (A) to hypoxanthine and adenine (A) to tyrosine (T) transitions. There are chemical agents that react with bases and add methyl or ethyl groups. These include nitrosoguanidine, methylmethane sulfonate (MMS) and ethylmethane sulfonate (EMS). The alkylated base degrades to yield a baseless site, which is muagenic and recombinogenic, or mispair to result in mutations upon DNA replication.

(iii) Intercalating agents: These are flat, multiple ring molecules, which interact with bases of DNA and insert between them. This insertion causes a ‘stretching’ of the DNA duplex and the DNA polymerase is ‘fooled’ into inserting an extra base opposite an intercalated molecule thereby resulting in frame shifts. Intercalating agents include acridine orange, proflavin and

ethidium bromide (Chikere and Azubuike, 2013).

(iv) Agents altering DNA structure: These may be large molecules, which bind to bases in DNA and cause them to be non-coding. These include N-acetoxy-2- aceetylamine fluorine (NAAAF). An example is peroxide (Chikere and Azubuike, 2013)

(v) Radiation: Radiation was the first mutagenic agent known. Effects on genes were first reported in the 1920's. Visible light and other forms of radiation are all types of electromagnetic (EM) radiations, which consist of both electric and magnetic waves. The length of EM waves (wavelength) varies and is inversely propotional to the energy they contain. This is the basis of the EM spectrum. Long waves (such as AM radio) contain least energy while short wave (such as FM radio, TV, microwaves, infrared, ultraviolet (UV) and gamma radiation) contain higher energy (VanHamme *et al*., 2003).

Measurement of radiation: Units used for ionizing radiation of all types are rems (roentgen equivalent man). One rem of any ionizing radiation produces similar biologic effects. Previously used unit was the rad (radiation absorbed dose). The dose rate is taken into account as the same number of rems given in a brief. Intense exposure (high dose rate) causes burns and skin damage as against a long – term weak exposure (low dose rate), which would only increase the risk of mutation and cancer (Anon, 2009).

The sources of radiation: This could be natural (e.g cosmic rays from the sun and outer space); radioactive elements in soil and terrestrial products (wood, stone) and in the atmosphere (radon). They produce the so-called background radiation and one's exposure varies with geographic location. Artificial sources include medical testing equipment such as diagnostic X-rays and like equipment, nuclear testing and power plants and various other products such as television sets, smoke detectors and airport X-rays equipment. Generally, a person's overall total average exposure from all sources is about 350m rem/year (Orisakwe, 2009).

Biological effects of radiation: Ionizing radiation produces a range of damage of cells and

organisms primarily due to the production of free radicals of water (the hydroxyl or OH radical). Free radicals possess unpaired electrons and are chemically very reactive and will interact with DNA, proteins, lipids in cell membranes, e.t.c. The severity of the effects depends upon the received – sublethal dose (100 – 250 rems), lethal dose (350 – 450 rems) and supralethal dose (> 450 rems) (Osuji and Uwakwe, 2006).

Genetic effects of radiation: Ionizing radiation produces a range of effect on DNA both through free radical effects and direct action through one or both strands, damage to/loss of bases (mutation) and cross linking of DNA to itself or proteins. Ultraviolet (UV) radiation is less energetic and therefore non-ionizing but its wavelengths are preferentially absorbed by base of DNA and by aromatic amino acids of proteins. purine and pyrimidine bases of nucleic acids absorb UV irradiation strongly and the absorption peak for DNA and RNA is 260mm. Killing of cells by UV irradiation is due primarily to its action on DNA, so that UV - irradiation at 260nm is most effective as a lethal agent (Nwaogu *et al*., 2008). UV light may cause two adjacent pyrimidine residues (cytosine or thymine) to form a dimer (Ado, 2004). In a normal cell, the dimer can then trigger the repairing process. However, if it becomes non-functional, the pyrimidine dimer may lead to mutation (Nduka and Orisakwe, 2009).

**2.17 DNA Repairs System**

As DNA damage occurs spontaneously and also as a result of ubiquitous environmental agents, most organisms possess some capacity to repair the DNA. DNA is the only macromolecule, which is repaired by cells (Prescott *et al*., 2008).

There are 3 mechanisms for DNA repairs – Damage reversal in which simple, enzymatic action restores normal structure without breaking backbone structure of the DNA. These are photo reactivation, where photolyase enzymes catalyze the splitting of pyrimidine dimers in the presence of light; ligation of single strands breaks where DNA ligase repairs simple breaks in one strand caused by X-rays and some chemicals like proxides and damage removal which

involves cutting and replacing a damaged or misappropriate base or section of nucleotides (SanMartin, 2011, Akpan, 2005).

Base excision repair occurs when a damaged or inappropriate base is removed from its sugar linkage and replaced. The enzymes involved are glycosylases which cut the sugar base bond (Ado, 2004,). Mismatch repair is a process, which occurs after DNA replication as a check on its accuracy. It is carried out by a group of proteins, which scan the DNA for incorrectly paired bases (or unpaired bases). The incorrect nucleotide is removed and the DNA polymerase replaces it with the right sequence (Prescott *et al.*, 2008).

Damage tolerance is not truly a repair but a way of coping with damage so that life can go on. For example, recombination (daughter-strand gap) repair, which promotes recombination to fix the daughter-strand gas and not the dimer as a way to cope with the problems of non-coding lesion persisting in DNA polymerase blocked at a dimmer, which then changes its specificity such that it can insert any nucleotide opposite the dimmer and thus continue replication in a kind of `mutate or die` situation (Prescott *et al*., 2008).

### 2.18 *Bacillus* species

*Bacillus* species belong to the family Bacillaceae with the distinguishing feature of production of endospores, which are highly refractive resting structures formed within the bacterial cells. *Bacillus* species are characterized as Gram-positive, rod-shaped, aerobic or facultative, endospores-forming bacteria. They are highly ubiquitous in nature and can degrade most substrates derived from plant and animal sources, including hydrocarbons (Nduka and Orisakwe, 2009).

#### 2.18.1 Nutrition and growth of *Bacillus* species

Most *Bacillus* species are versatile, chemohetetrophs, capable of respiration using a variety of simple organic compounds such as sugars, amino acids and organic acids. In some cases, they also ferment carbohydrates in a mixed reaction that typically produces glycerol and butanediol. The majority are mesophiles, with temperature optimum between 30 and 45$^{o}$C, but the genus

also contains a number of thermophilic species with temperature as high as 65$^{o}$C (Prescott *et al.,* 2008).

2.18.2 Endospores of *Bacillus* species

They are so named because they are formed intracellularly and they do not form normally during active growth and cell division. Rather, their differentiation begins when a population of vegetative cells passes out of the exponential phase of growth, usually as a result of nutrient depletion. Typically, one endospore is formed per vegetative cell. Mature spores have no detectable metabolism, a state described as cryptobiotic (Nwachukwu, 2000).

2.18.3 Ecology of *Bacillus species*

Due to the resistance of their endospores to environmental stress as well as their long-term survival under adverse conditions, most aerobic spore formers are ubiquitous and can be isolated from a wide variety of sources. The primary habitat of aerobic endospore-forming bacilli is the soil. The great Russian microbiologist Winogradsky, considered them as "normal flora" of the soil. They may play a biodegradative role in whatever they contaminate (Prescott *et al.,* 2008).

2.18.4 Characteristics of some species of Bacilli

(i*) B. acidocaldarious*: This organism is a thermoacidophile with temperature growth range of 45-65$^{0}$C and pH range of 2-6. It is found in hot acidic environments and its spores have surprisingly weak thermal resistance.

(ii) *B. alkalophilus*: It is an alkalophile, tolerant to alkaline conditions and does not grow at pH 7 but capable of growth at pH >10.

(iii*) B. azotoformans*: Are Gram negative aerobes, which utilize $NO_3$, $NO_2$, $SO_4$ or fumarate as a final electron acceptor. It is a vigorous denitrifying bacterium found in soils, which converts $NO_3$, $NO_2$ and $N_2O$ to large amounts of $N_2$.

(iv) *B. brevis*: This has been isolated chiefly from soils and foods. It requires a mixture of amino acids without vitamins for growth.

(v) *B. cereus*: The spores of *B. cereus* are widespread in soil and air. The organisms are usually observed multiplying in foods such as cooked rice and may cause food poisoning. It produces antibiotics.

(vi) *B. circulans*: Some strains are cellulolytic. It has a distinct rhizoid colony.

(vii) *B. fastidiosis*: It uses only uric acid, allantoic acid as an energy source. It is isolated from soil and from poultry litter.

(viii) *B. globisporous*: It forms spherical spores and is found in soil and river water

(ix) *B. lentus*: It is nutritionally-versatile and isolated from soil, food and spices.

(x) *B. macerans*: Most strains fix $N_2$ under anaerobic conditions. It degrades pectin and plant polysaccharides. Some strains are moderately thermophilic.

(xi) *B. mageterium*: "Magaterium" means "big beast", it has the largest cell diameter of any aerobic spore former (1.2-1.5 micrometers). It grows in minimal medium without any added growth factors. Spores are common in soil. It is the subject of many basic studies of Gram-positive bacteria in the laboratory.

(xii*) B. pasteurii*: It converts urea to ammonium carbonate more actively than any known bacterium. It requires all alkaline medium (pH 9) for growth and is isolated from soil, water, sewage and encrustations and urinals.

(xiii*) B. pumilus*: The spores are ubiquitous and occur in soil more frequently that those of *B. subtilis.*

(xiv) *B.sphaericus*: It is isolated from soil, marine and fresh water sediments, milk and foods.

(xv) *B. stereothermophilus*: It grows at 65$^{o}$C, tolerant to acid and occurs in soil, hot springs, desert sand, artic waters, ocean sediments, food and compost.

(xvi) *B. subtilis*: It grows as a unicellular rod, seldom as chains and degrades pectin and polysaccharides in plant tissues. Some strains cause rots in live potato tubers. It grows in a minimal defined medium with no added growth factors. Endospores are widespread. It is the

causative agent of ropy (slimy) bread. This bacterium is the "*E. coli*" of Gram-positive bacteria. Much of the information we have on the biology, biochemistry and genetics of the Gram-positive cell, indeed, of bacteria in general, has been derived from the study of *B. subtilis* (Emoyan *et al*., 2005).

2.18.5 *Bacilllus* as a petroleum degrader

*Bacillus* sp has been reported by previous studies as a petroleum degrader (Nwachukwu *et al*., 2001: Okerentugba and Ezeronye, 2003; Ojumu *et al*., 2005).

**2.19 *Pseudomonas* Species**

*Pseudomonas aeruriginosa* is a Gram-negative, aerobic rod belonging to the bacterial family Pseudomonadaceae. The family includes other genera, which together with certain other organisms constitute the bacteria informally known as pseudomonads. These bacteria are common inhabitants of soil and water (Emory *et al*., 2001).

2.19.1 Characteristics *Pseudomonas* Species

*Pseudomonas aeruginosa* is a Gram-negative rod measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm. Almost all strains are motile be means of single polar flagellum. The bacterium is ubiquitous in soil and water, and on surfaces in contact with soil and water. Its metabolism is respiratory and never fermentative, but it grows in the absence of $O_2$ if $NO_3$ is available as a respiratory electron acceptor. The typical *Pseudomonas* bacterium in nature might be found in a biofilm, attached to some surface or substrate (Prescott *et al*., 2008).

2.19.2 Growth and nutrition of *Pseudomonas* Species

*Pseudomonas aeruginosa* has very simple nutritional requirements (Okey, 2003). It is often observed "growing in distilled water", which is evidence of its minimal nutritional needs. It possesses the metabolic versatility for which pseudomonades' are so renowned. Organic growth factors are not required, and it can use more than seventy-five organic compounds for growth. Its optimum temperature for growth is 37$^{o}$C and is able to grow at temperatures as high as 42$^{o}$C. It has a predilection for growth in moist environments, which is probably a reflection of its natural

existence in soil and water (Cowan and Strafford, 2007).

2.19.3 Colonial morphology

Its isolate may produce three colony types. Natural isolates from soil or water typically produce a small, rough colony. Clinical samples, in general, yield one or another of two smooth colony types. One type has fried-egg appearance, which is large, smooth, with flat edges and an elevated appearance. Another type, frequently obtained from respiratory and urinary tract secretions, has mucoid colonies, are presumed to play a role in colonization and virulence. The strains produce two types of soluble pigments: the florescent pigment, pyoverdin and the blue pigment, pyocyanin (Nwachikwu, 2000; Nwachukwu *et al*., 2001).

2.19.4 Other attributes of *Pseudomonas* Species

These include genetic, where genetic exchange is by transduction and conjugation and it also possesses an inherent (natural) drugresistance plasmids (Asitok and Antai, 2006).

It possesses adaptability to minimal nutritional requirements possessing widespread metabolic diversity and can be isolated from a variety of habitats (Nwachukwu, 2000); motility/chemotaxis usually is expressed by means of flagella (Das and Mukharjee, 2007). *Pseudomonas aeruginosa* has been reported in previous studies as a petroleum degrader (Ojuma *et al*., 2005; Ekpo and Ekpo, 2006).

Crude oil degraders have been reported to be endowed with enzyme systems, nutritional capabilities and metabolic activities, which enable them to withstand adverse environmental conditions. The degrading ability demonstrated by the microorganisms is a clear indication that they can be used in hydrocarbon degradation (Olukunle *et al*., 2015).

Some microorganisms can utilize the hydrocarbons as sole carbon sources for getting their energy and metabolic activities (Jyothi *et al*., 2012). Biodegradation is a complex process that depends on the nature of petroleum and also on the amount of the hydrocarbons present (Das *et al*.,2011). The microbes can utilize the hydrocarbons depending on the chemical nature of the

compounds within the petroleum mixture (Adeline *et al*., 2009). Hydrocarbon degrading microorganisms usually exist in very low abundance in aquatic environments (Sivaraman *et al*., 2011). Biodegradation by bacteria is considered as the most active process in petroleum degradation and they are the primary degraders of spilled oil (Brooijmans *et al*.,2009; Rahman *et al*.,2011) and this is specially carried out largely by diverse bacterial populations, mostly by *Pseudomonas* species (Dubey *et al*.,2009; Boboye *et al*.,2010).

There are so many known consortia of microorganisms, which can degrade mineral oil hydrocarbons under laboratory or field conditions (Olukunle *et al*., 2015).

# CHAPTER THREE

# 3.0 MATERIALS AND METHODS

## 3.1 Study area

The study area is Kaduna Refining and Petrochemical Company (KRPC), Kaduna. KRPC is located to the west off Kachia Road in Kaduna. It occupies a land area of about two square kilometers (KRPC, 2004). The wastewater treatment plant (WWTP) of the KRPC is located to the west end of the company, behind the NNPC Clinic, from the main entrance to the refinery. There is a circular road that passes through the back of the product loading bay and joins an earth road beside the rail track. The Romi stream flows from the west end of the refinery to the east, running through the back of the Tanker's Park (TP) to the above earth road (Fig. 1).

## 3.2. Effluent sampling points

Effluent samples were collected from three effluent points located in the Kaduna Refining and Petrochemical Company (KRPC) (Fig.1). Points A and B were located in the wastewater treatment plant while point C was located outside the company complex. Point A is the untreated wastewater channel, which retains the untreated effluent for sometime before flowing into point B for treatment (Plate A). Point B, which is about 100m from point A, is the waste oil retention pond where the untreated wastewater from point A flows into. Here, caustic soda is added during treatment to neutralize the acid. Booms and skimmers are placed in this point to trap oils from the wastewater (Plate B).

Point C, located outside the refinery, is the discharge point, which has a distance of 600m from point B. It discharges the refinery effluent directly into the railway bridge stream; a tributary, which flows directly into Romi river, which is a tributary of river Kaduna (Plate C).

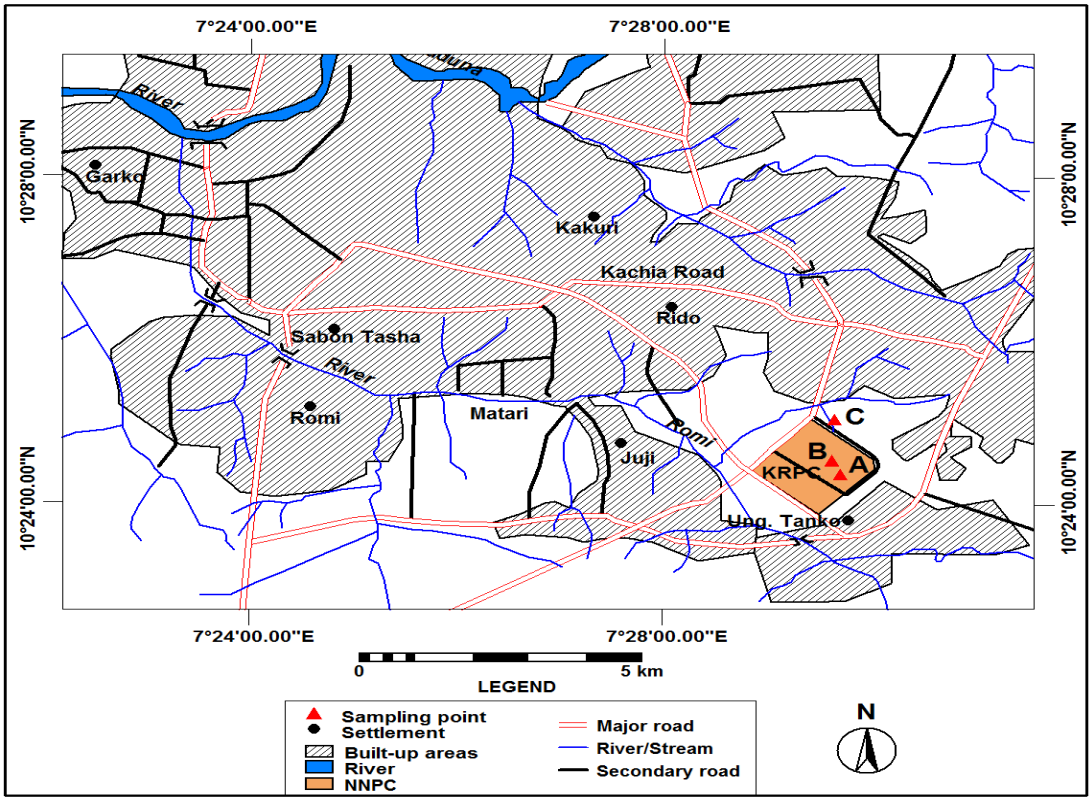



FIG. 1 : PART OF KADUNA SOUTH SHOWING THE STUDY SITE AND THE SAMPLING POINTS

Source : Adapted and modified from Google map, 2013, Digitized in GIS Lab. Geography Dept. A.B.U. Zaria



Plate A. Untreated wastewater channel of KRPC Kaduna (Sampling point A).



Plate B. Waste oil retention pond of KRPC Kaduna (Sampling point B).

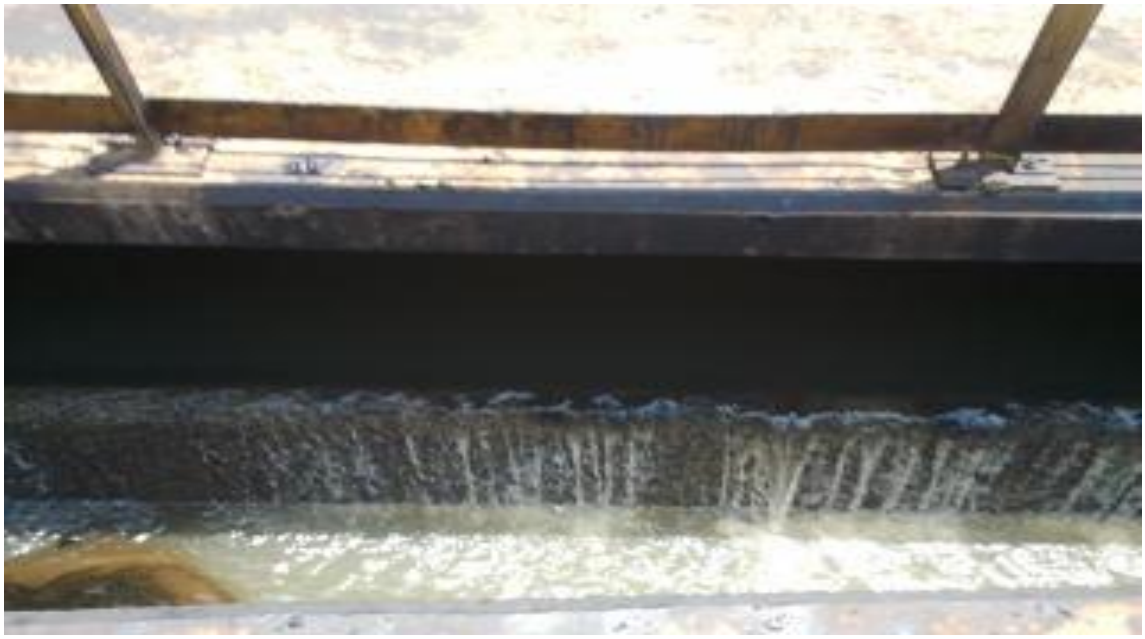


Plate C. Outfall of the effluent discharge outside of KRPC (Sampling point C).

## 3.3 Collection of Samples

3.3.1 Collection and handling of refinery effluents and crude oil

The Refinery effluent samples were collected in sterile wide mouth bottles. The containers were first rinsed with the effluents at the points of collection. The samples were collected by lowering the bottles (with a weighted rope tied to its neck) into the bottom of the well mixed section of the water body, 30 cm deep, and allowed to over flow before withdrawing. Small air space was left in the bottle to allow mixing of sample at the time of analysis. After collection, the body of the containers was rinsed thoroughly with water.

Three hundred milliliters of Crude oil was collected from Fuels Laboratory of KRPC, Kaduna. To avoid deterioration, the samples were transported in ice chest to the laboratory, Department of Microbiology, Ahmadu Bello University, Zaria immediately after collection.

Information on the physicochemical properties of the sample of crude oil obtained showed that the API gravity of the crude oil is low which classifies it as light crude oil. The low levels of sulphur content indicate that it is sweet crude. Other parameters like viscosity, water content, pour point, % Nitrogen, salt and acid contents were relatively low which implies that, it has the ability to flow rapidly during spillage and possess very low corrosion potentials (Appendix vi).

## 3.4 Determination of physicochemical parameters of the refinery effluents

3.4.1 Determination of physical parameters

*3.4.1.1Temperature*

Temperature was determined *in-situ* using thermometer with the range of 0 -100$^0$C. The Thermometer was dipped in the effluent sample at an inclined position for 5 minutes to allow for equilibrium and the temperature of the sample was recorded. American standard methods (2002).

*3.4.1.2 pH*

This was determined *in-situ* and in accordance with the method in ASTM (2002) using a Horiba M - 13 pH meter. The pH meter and associated electrode were standardized with Phosphate buffer solutions (pH 5 and 10) once monthly in accordance with the Manufacturer's instructions. The electrode was dipped in the effluent sample at an inclined position for 5 minutes to

allow for equilibrium and the values displayed were read and recorded.

*3.4.1.3 Turbidity*

This was determined using ASTM (2002) method as adopted by the Chemical Laboratory, KRPC, Kaduna. This was carried out using HANNA Instruments LP 2000 turbidity meter. The turbidity meter was standardized with formazin and calibrated once monthly in accordance with the manufacturer's instructions. One milliliter of the effluent sample was placed in a cuvette, it was shook and the sides of the cuvette were cleaned with cotton wool and placed in cuvette apartment and closed up with the apartment lid. Turbidity values were read from the meter as displayed directly at 254nm wavelength.

*3.4.1.4 Electrical conductivity*

This was determined using ASTM (2002) as adopted by the Chemical Laboratory, KRPC, Kaduna. It was carried out using HANNA Instruments HI 9932 microprocessor conductivity meter. The meter was standardized once monthly in accordance with the manufacturer's instructions. The cell was rinsed with the portion of the effluent sample and there after filled with the effluent sample and fitted. Upon a press of the test button, the readout on screen was automatically displayed and values were recorded and expressed in micro Siemens per centimeter ($\mu/cm^3$).

*3.4.1.5 Solids (Total Suspended Solids and Total Dissolved Solids*

These were determined using ASTM (2002) as adopted by the Chemical Laboratory, KRPC, Kaduna. The dry weights of two 100ml beakers were determined. Twenty milliliters of the unfiltered effluent sample was added to one of the beakers and twenty milliliters of the filtered effluent sample was added to the second beaker. The weights of the two beakers and their contents were then determined. The effluent samples contained in beakers were gently heated until completely dried, allowed to cool in a desiccator and their weights were again determined. This was used to calculate *total suspended solids and total dissolved solids as follows:*

Total suspended Solids (TSS) was calculated using the relation:

$$\frac{\text{weight of residue}}{\text{weight of sample}} \times 10^6 \text{ of the unfiltered sample}$$

Total dissolved solids (TDS) was calculated as:

$$\frac{\text{weight of residue}}{\text{weight of sample}} \times 10^6 \text{ of the filtered sample}$$

**3.5 Determination of Chemical parameters**

3.5.1 *Dissolved Oxygen and Biochemical Oxygen Demand*

These were determined using ASTM (2002) as adopted by the Chemical Laboratory, KRPC,

Kaduna. The reduction of the DO in a water sample, which has been kept for 5 days at a constant temperature in a tightly closed vessel after sampling, was measured and calculated. The diluents water (aeration water) was prepared by measuring one liter of distilled water in a Winchester bottle and aerating it for one hour. To the aerated water in the bottle was added 1ml each of solution A (Phosphate buffer solution), solution B (MgS04 solution), solution C ($CaCl_2$ solution) and solution D ($FeCl_2$ solution). The aerated water was then used for the determination of dissolved oxygen by titrating 0.25N sodium thiosulphate ($Na_2S_2O_3$) solution against the sample in a 400ml beaker until a pale yellow color was obtained.

Two (2mls) of starch solution were added to obtain blue colour with continued titration until the colour disappeared. The DO was calculated using the formula:

$$\text{DO in mg/1} = \frac{T \times 0.2 \times 1000}{\text{Volume of Winchester bottle}}$$

1ml of 0.025N $Na_2S_20_3$ = 0.2mg of oxygen (or 0.2mg of DO).

$$\text{BOD in mg/1} = \frac{(D_1 - D_2) \times T}{V}$$

Where T = Titer value, $D_1$ = DO of the sample before incubation in mg/1

$D_2$ = DO of the sample after incubation in mg/1

3.5.2 C*hemical oxygen demand*

This was determined using Reflux digestion and titration method in 1ASTM (2002) as adopted by the Chemical Laboratory, KRPC, Kaduna. Fifty milliliters of distilled water was measured into a reflux flask as a blank and on another reflux flask, measured five milliliters of the effluent sample and made up to fifty milliliters with distilled water. The reflux flasks (i.e. sample and blank) were placed in an ice-bath. To each flask was added one gram of powdered mercuric sulphate, five milliliters of concentrated 95% sulphuric acid ($H_2SO_4$) and several beads or boiling stones. Both flasks were mixed well to complete dissolution. With the Reflux flasks still in the ice-bath, twenty-five milliliters of 0.25N standard potassium dichromate solution ($K_2Cr_2O_7$), one gram of silver sulphate and twenty milliliters of concentrated 95% sulphuric acid ($H_2SO_4$) were slowly added to the sample and blank. The reflux flasks were attached to the condenser and started the flow of cold water. Heat was then applied to the flasks and refluxed for two hours. After refluxing, the flasks were allowed to cool and the condensers were washed

down with twenty-five milliliters of water before removing the flasks. The acid solution was diluted to three hundred milliliters with distilled water and allowed to cool to room temperature. To each flask was added eight drops of phenanthroline ferrous sulphate solution ($FeS0_4$ ($NH_4$) S04.6H2O) as indicator and titrated the excess dichromate against 0.25N ferrous ammonium sulphate solution until there was a sharp color change from blue-green to reddish blue. In the event of the solution immediately turning a reddish brown upon the addition of the indicator, the analysis was repeated on a smaller aliquot. For effluent samples of low COD (10 – 50mg/l), 0.025N $K_2Cr_2O_7$ and $FeSO_4$ (NFI4) **$SO4.6H_2O$** were used while for effluent samples with high COD (> 50mg/l),0.25N $K_2Cr_2O_7$ and $FeSO_4$**(NH4)** $SO_4.6H_2O$ were used. The COD was calculated using the formula:

$$\text{COD in mg/1} = \frac{(A - B) \times N \times 8000}{S}$$

Where: A = Titer value of $FeSO_4$ ($NH_4$) $SO_4.6H_2O$ of the blank in ml.

B = Titer value of $FeSO_4$ ($NH_4$) $SO_4.6H_2O$ of the effluent sample in ml

N = Normality of the ($FeS0_4$**($NH_4$)** $SO_4.6H_2O$ and

S = effluent sample used for the test in ml.

3.5.3 *Oil and Grease*

The oil and grease content of the refinery effluent was determined using ASTM (2002) as described by the Global Environmental Consultants Limited, Warri. Thirty millilitres of carbon tetrachloride ($CCl_4$) was added to one litre of the effluent sample to dissolve the hydrocarbons and shaken vigorously for two minutes. The mixture was poured into a separating funnel and shaken intermittently for three minutes and expelled the air by releasing the valves of the funnel. The funnel was then rinsed with twenty millilitres of carbon tetrachloride ($CCl_4$). The resulting oil was drained into a fifty millilitres volumetric flask through a funnel containing cotton wool to which had been added anhydrous sodium sulphate ($Na_2SO4.7H_2O$) to remove traces of water from the oil. The separating funnel was then rinsed with ten millilitres of $CCI_4$ to remove all the oils. The Infra-red Spectrophotometer was calibrated at 420nm using dry Forcados and dry crude oil with concentrations between 100 and 500 mg/1 and obtained a standard curve. To determine oil and grease concentration, the Optical Density of the extracted oil was determined at 420nm.

*3.5.4* Chloride

These were determined titrimetrically. Twenty five (25ml) of the effluent sample was measured in a beaker and a drop of potassium chromate indicator added. The solution was titrated against $AgNO_3$ solution until the appearance of a brick red colour as the end point. The amount of chlorine (mg/l) was obtained as:

$$\frac{Cl \quad A \times 0.3 \times 1000}{V}$$

Where: A = Volume of $AgNO_3$ titrated V = Volume of effluent sample (APHA, 1998).

*3.5.5 Nitrate*

This was determined using the automatic cadmium reduction procedure of ASTM (2002) using HATCHDR/2010 Spectrophotometer (HATCH DR prop, Germany) as adopted by the Chemical Laboratory, KRPC, Kaduna. Using the stored program for medium range (MR) nitrate nitrogen **($NO^-_3N$)** at 400nm, one clean sample cell was filled with twenty-five millilitres of the sample. Another sample cell was filled with twenty-five millilitres of distilled water (as BLANK). To each sample cell was added one milliliter of brucine-sulfanilic acid, covered with a stopper and shaken for one minute. These were allowed to stand for five minutes and readings were recorded and expressed.

*3.5.6 Sulphate*

This was determined in accordance with the method of ASTM (2002) using HATCHDR /2010 Spectrophotometer as adopted by the Chemical Laboratory, KRPC, Kaduna. Using the stored program for sulphate **($SO_4^{2-}$)** at 450nm, one clean sample cell was filled with twenty-five millilitres of distilled water (Blank) and another clean sample cell was filled with twenty-five millilitres of the filtered effluent (test) sample. To each sample was added five millilitres of conditioning reagent (a solution containing glycerin and sodium chloride, which was added to stabilize the suspension and minimize interferences)**. A quantity** (0.3g) of barium chloride crystals and swirled to dissolve. On **dissolution,** white precipitates were developed in samples containing sulphate. Both sample cells were allowed to stand for five minutes and readings taken. The conditioning reagent above was prepared by adding 30ml concentrated hydrochloric acid, 300ml reagent water, 100ml 95% ethanol and 75g sodium chloride in a container. To this was added 50ml glycerol and mixed properly (ASTM, 2002).

3.5.7 *Phosphate*

This was determined in accordance with the procedure in American Public Health Association APHA (1998) as adopted by the Chemical Laboratory, KRPC, Kaduna. Fifty millilitres of filtered effluent sample was introduced into a clean, dry, 125ml capacity Erlenmeyer flask. Two

millilitres of molybdate reagent was added and mixed by swirling. Two millilitres of sulfonic acid reagent was added and mixed. The content in flask was allowed to stand for 5mins for color development which was then determined using a colorimeter in accordance with the manufacturer's instructions. Values were then obtained from a standard curve previously determined with known concentrations of orthophosphate. The instrument was calibrated in accordance with the manufacturer's instructions.

3.5.8 *Lead*

Lead concentration was determined using Unicam 929 Atomic Absorption Spectrophotometer (AAS) in accordance with ASTM (2002) for Lead as adopted by the Global Environmental Consultants, Warri, Nigeria. A representative portion of the well mixed effluent sample (100ml) was transferred into a beaker and five millilitres of concentrated 95% nitric acid ($HNO_3$) was added. The solution was evaporated to dryness on a hot plate, making sure that the sample did not boil. The beaker was allowed to cool and another five millilitres of concentrated 95% nitric acid ($HNo_3$) was added. The beaker was covered with a watch glass and returned to the hot plate. A gentle refluxing action of the solution was set by increasing the temperature of the hot plate. Heating was continued with addition of acid ($HN0_3$) as necessary until digestion was completed to obtain a light colored residue. The residue was dissolved by addition of 1.2ml concentrated $HNO_3$, washed with distilled water and filtered to remove silicate and other insoluble materials. The volume of the solution was adjusted to 100ml in a volumetric flask. A reagent blank determination was carried out and the samples as well as the blank were analyzed for the heavy metal with the Flame AAS. The concentration of the heavy metal was obtained using the following relation:

Concentration of the element in mg/1 = A - B

Where: A = Concentration of the element (mg/1)

B = Concentration of the lead in the blank (mg/1)

3.5.9 C*hromium (VI)*

This was determined using the colorimetric method of ASTM (2002) as adopted by the Global Environmental Consultants, Warri, Nigeria. A standard curve of Chromium (VI) at 540nm was prepared. Ten millilitres of the effluent sample was measured into a twenty-five millilitres volumetric flask. One millilitre of 3M concentrated 95% sulphuric acid ($H_2SO_4$) and five millilitres of 25% diphenylcarbazide solution were added to the sample, mixed and allowed to stand for five minutes for full color development. This was made up to twenty-five millilitres with distilled water and mixed properly. Five millilitres was transferred into a one-cm absorption cell and the absorbance at 540nm was determined using distilled water as the blank. The absorbance reading of the sample was corrected by

subtracting the absorbance of the blank. Using the corrected absorbance value, the concentration of Cr (VI) in mg/1 was extrapolated from the standard curve and obtained using the following relation:

$$Cr\ (mg/1) = A \times B$$

Where A = Effluent sample volume

B = Volume of portion from the 100ml digested sample

### 3.6 Isolation of *Bacillus* and *Pseudomonas* species

Twenty (20ml) of the raw refinery effluent sample was transferred into sterile test tube and centrifuge at 200 rpm for 5 minutes. The content was removed and the supernatant discarded. The remaining content (debris) was aseptically transferred onto the surface of freshly prepared mannitol egg yolk polymyxin agar and centrimide agar for the isolation of *Bacillus* and *Pseudomonas* species respectively. The inocula were spread evenly using a sterile bent glass rod. The inoculated plates were incubated in an incubator at $37^0$C and observed for bacterial growth after 24hours (Osuji and Uwakwe, 2006).

Colonies observed on the incubated plates with characteristic brown colour, creamy, large spreading, finely wrinkled and slimy were picked and purified by repeated streaking on freshly prepared mannitol egg yolk polymyxin agar until presumptive pure colonies of *Bacillus* species were obtained (Plate D). Similarly, the observed colonies on the incubated plates with characteristic fluorescent green colour, large, opaque, irregular colonies with earthy odour were picked and purified by repeated streaking on freshly prepared centrimide agar until presumptive pure colonies of *Pseudomonas* species were obtained (Plate E). Purified bacterial isolates were transferred onto sterile nutrient agar slants and stored at $4^0$C for further identification.

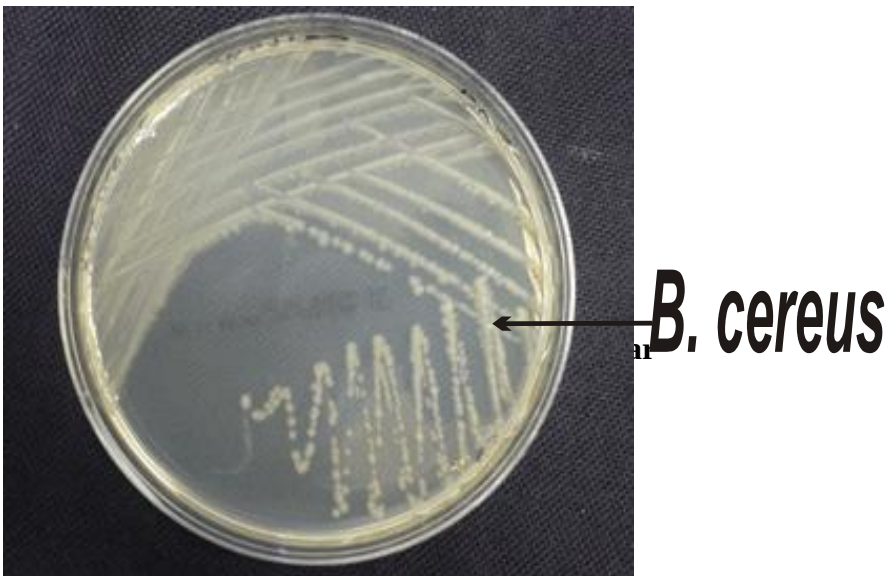
### 3.7 Characterization of the bacterial isolates

Isolates presumed to be *Bacillus* and *Pseudomonas* species based on their cultural characteristics were further characterized by a combination of microscopic, physiological and biochemical tests described by Cowan and Steel (2004).

3.7.1. Microscopic Identification (Procedures)

3.7.1.1 *Gram's staining*

A smear of pure culture of the isolates from stored labeled slant was prepared on a grease-free slide and heat fixed. The prepared slide was flooded with crystal violet and allowed to stand for 1 minute.


B. cereus



The stain was poured off and the slide placed at an angle downwards and then flooded with Gram' iodine and allowed to stand for 1 minute. It was washed off with water. The smear was decolorized with 95% alcohol and rinsed immediately with water. The smear was then counterstained with safranin for one minute and washed with water. The slide was allowed to air dry and observed with oil immersion under x10 objective lens of the microscope. Gram- positive bacteria retained purple colour of the primary dye (crystal violet) while Gram- negative bacteria retained the pink colour of the secondary dye (safranin).

3.7.1.2 *Spore Staining*

A heat fixed smear of pure culture presumed to be *Bacillus* sp was prepared on a clean sterile glass slide and heat fixed. A small piece of blotting paper (adsorbent paper) was placed over the smear and the slide was placed on wire gauze on a ring stand. The slide was gently heated on a Bunsen burner till it started to evaporate. As the paper began to dry, two drops of malachite green were added to keep it moist. The slide was removed from the rack after five minutes using clothspin. The blotting paper was removed and the slide was allowed to cool to room temperature for two minutes. The slide was rinsed with tap water (to wash the malachite green from both sides of the slide) and stained with safranin for two minutes. The smear was again rinsed with tap water to remove the secondary stain and the slide was blotted/air dried. Vegetative cells appeared pink/red while the spores appeared green.

3.7.2 Physiological characterization

3.7.2.1 *Motility Test*

A tube containing motility medium was inoculated by making a fine stab with a needle containing the test organism to a depth of 2cm to the bottom of the tube. It was incubated in an incubator at $37^0$C for 24hours. The line of inoculation was sharply defined and the rest of the medium was somewhat cloudy for motile organisms. In the case of non motile organisms, the growth was restricted to the line of inoculation which became sharply visible.

3.7.2.2 *Aerobic growth*

Nutrient agar plates were prepared according to manufacturer's instructions. A loopful of pure culture of the organism from stored agar slants was inoculated and incubated in an incubator at $37^0$C for 24hours. The growth of the organisms on the surface of the nutrient agar was recorded as aerobic growth.

3.7.2.3 *Anaerobic Growth*

Nutrient agar plates were prepared as described according to manufacturer's instructions and inoculated with a loopful of pure culture of the isolate from stored agar slants and incubated in anaerobic jar containing anaerobic Gaspak at $37^0$C for 24h and observed for growth after incubation. Growth of the organisms on the agar surface of the nutrient agar was recorded as an anaerobic growth.

3.7.2.4 *Fluorescence under UV Light*

Inoculated plates with growth on nutrient agar earlier obtained under aerobic and anaerobic growths were viewed under UV light at 254nm for fluorescence. The observed colonies showing fluorescence under UV Light at 254nm were typical of *Pseudomonas aeruginosa.*

3.7.3 Biochemical characterization

3.7.3.1 *Catalase test*

A drop of 3% hydrogen peroxide was placed on a sterile glass slide. Using a sterile wire loop, a bit of growth from stored labeled slant was emulsified on the hydrogen peroxide. Positive test was indicated by bubbling and frothing. Absence of bubbling and frothing was indicative of a negative test. Colonies showing bubbling and frothing were picked and preserved as presumptive *Bacillus cereus* and *Pseudomonas aeruginosa*.

3.7.3.2 *Oxidase test*

A piece of filter paper was wetted with a few drops of dilute solution (1%) of oxidase reagent (tetramethyl-p-phenylene diamine dihydrochloride). Using a sterile wire loop, a bit of growth from stored labeled slant was smeared on the wet piece of paper. The development of an intense purple colour by the cells in the smears within 30 seconds indicated a positive test. Absence of purple colour was indicative of a negative test. Colonies that developed an intense purple colour within 30 seconds were picked and preserved as presumptive *Bacillus cereus* and *Pseudomonas aeruginosa*.

3.7.3.3 *Indole*

A loopful of growth from stored labeled slants was inoculated in 5ml peptone water and incubated at $37^0$C for 24h. After incubation, Kovac's indole reagent was added and gently shaken. Development of red colour in the reagent layer above the broth within 1 minute indicated positive reaction. Retention of the yellow colour of the indole reagent indicated a negative indole reaction. Colonies that Developed red colour in the reagent layer above the broth within 1 minute were picked and preserved as presumptive *Pseudomonas aeruginosa* while those that do not develop red colour were discarded.

3.7.3.4 *Starch Hydrolysis*

Plate of starch agar was prepared according to manufacturer's instructions and inoculated by making a streak with a loopful of growth from stored labeled slant and incubated at $37^0$C for 48h. The surface of the plate was flooded with iodine solution after 48h. Positive result was indicated by the appearance of a clear zone underneath the growth. Colonies showing clear zone underneath the growth were picked and preserved as presumptive *Bacillus cereus* while those that do not develop a clear zone were discarded.

3.7.3.5 *Gelatin Liquefaction*

A nutrient gelatin tube was prepared and inoculated by making a streak with a loopful of growth from stored labeled slants and incubated at $35^0$C for 24h. Following incubation, the tube was removed from the incubator and placed in a refrigerator for 1h and examined for liquefaction by tilting the tube. If the nutrient gelatin solidified during the cooling period, the gelatin liquefaction did not occur. The tube was re-incubated again and re-examined for liquefaction each day for 7 days. A positive test was indicated by the occurrence of liquefaction within 7 days and negative test was indicated by the absence of liquefaction within 7 days. Colonies showing the occurrence of liquefaction within 7 days were picked and preserved as presumptive *Bacillus cereus* and *Pseudomonas aeruginosa*. While colonies with absence of liquefaction within 7 days were discarded.

**3.8 Confirmation of identity of isolates**

The isolates identified using conventional biochemical tests were further confirmed by microgen identification system. Colonies from pure culture of isolates identified as *Bacillus cereus* and *Pseudomonas aeruginosa* were separately emulsified in a vial containing suspending medium (5ml) and mixed thoroughly using a vortex mixer suspension equivalent to Mac Farland 2.0 standard. The adhesive strip sealing the micro wells was carefully pealed out. Using a sterile Pasteur pipette, 4 drops (100-125 µl) of the standardized bacterial suspension were inoculated into the wells of both micro wells strips. Well 21 of the *Bacillus* srip was overlayed with 4 drops of mineral oil and the adhesive strip sealed back to cover the wells and was incubated in an incubator at $30^0$C for 24h. After 24h of incubation, the results of wells 1-18 (carbohydrates) were observed and recorded with reference to the control well. Appearance of yellow colour as compared to the control well was recorded positive. The arginine, ONPG and citrate results were read against the colour chart and recorded. The adhesive strip was sealed back and re-incubated at $30^0$C for further 24h (to make up 48 h). After 48h of incubation, 2 drops of kovac's reagent were added to well 19 and result recorded after 60 seconds. Formation of pink colour indicated a

positive result. One drop each of the VP1 and VP11 reagents was added to well 23 and read after 30 minutes. Formation of pink colour indicated a positive result. Another one (1) drop each of nitrate A and nitrate B reagents were added to well 20 and result read after 60 seconds. Formation of red colour indicates positive result. Isolates that formed red colour at the end of the test were picked as confirmed *Bacillus cereus* and *Pseudomonas aeruginosa*.

**3.9 Media preparation**

3.9.1 Preparation of Nutrient agar

Seven grams (7g) of nutrient agar powder was carefully weighed using weighing balance and placed into a clean conical flask containing 250ml of sterile distilled water, the mixture was vigorously shooked and the homogenous mixture was sterilized by autoclaving at 121$^{o}$C for 15 minutes. It was allowed to cool to a temperature of 50$^{o}$C and dispensed into sterile petridishes.

3.9.2 Preparation of Centrimide agar (For the isolation of *Pseudomonas aeruginosa*)

Centrimide agar powder (11.33g) was carefully weighed using weighing balance and placed into a clean conical flask containing 250ml of sterile distilled water. The solution was mixed well to ensure dissolution and added 2.5ml glycerol. The preparation was sterilized in an autoclave at 121$^{0}$C for 15 minutes. It was allowed to cool to a temperature of 50$^{0}$C and dispensed into sterile petridishes.

3.9.3 Preparation of eggyolk polymyxin agar (For the isolation of *Bacillus cereus*)

Nutrient agar was prepared as described in section 3.6.1, it was allowed to cool to 45$^{0}$C in a water bath and 100ml concentrated egg-yolk emulsion (Oxoid) was added and shaken thoroughly to mix. One (1ml) of sterile polymixin B sulphate (500,000iu) was aseptically added using sterile syringe and the mixture shaken to ensure dissolution. It was sterilized in an autoclave at 121$^{0}$C for 15 minutes. The preparation was allowed to cool to a temperature of 50$^{0}$C and dispensed in to sterile petri dishes.

3.9.4 Preparation of Mc Farland turbidity standard

McFarland turbidity standard was prepared by adding 0.5ml of 1.175% (wt/vol) barium chloride dehydrate ($BaCl_2.2H_20$) solution to 99.5ml of 1% (vol/vol) sulfuric acid ($H_2S0_4$). The turbidity standard was then poured into test tubes identical to those used to prepare the inoculum suspension and sealed with wax to prevent evaporation.

### 3.10 Determination of the ability of *B. cereus* and *P. aeruginosa* to utilize crude oil for growth

The isolates were inoculated on Minimal Basal Medium ($KH_2PO_4$ 2g/l, $Na_2HPO_4$ 3g/l, $NH_4NO_3$1g/l, KCl 1g/l, $MgSO_4$ 4mg/l, $CaCl_2$ 0.2mg/l, $FeSO_4$ 0.2mg/l, $MnCl_2$ 0.2mg/l, Agar 15g, Distilled water 1litre) supplemented with 0.5, 1, 1.5 and 2% crude oil and incubated at $37^0$C for 24h. Growth of the organisms was considered as indicative of the capacity of the isolates to utilize crude oil as source of carbon and energy (Asmae *et al.*, 2014).

### 3.11 Generation of mutants of *B. cereus* and *P. aeruginosa*

3.11.1 By UV irradiation at 254nm

The method used by Ado (2004) was employed where wild strains of *Bacillus cereus* and *Pseudomonas aeruginosa* were grown on nutrient broth for 24hours and their cell counts determined using Mac Ferland standard ($1.0x10^6$ cells/ml). Ten milliliters of *B. cereus* and *P. aeruginosa* respectively were placed in six sterile Petri dishes in a 1mm thick layer and exposed to UV irradiation at 254nm for 30minutes at a distance of 6cm from the cells. After irradiation, the plates were immediately transferred to the dark to prevent photo reactivation. To avoid dark repair, post irradiation manipulations were carried out. The cells were pretreated with 0.2% (w/v) caffeine for 5 hours and centrifuged at 1500rpm for five minutes to remove the caffeine and the cells re-suspended in 0.85% (w/v) saline and re-centrifuged and supernatant discarded. The treated organisms were serially diluted with sterile 0.85% (w/v) saline and 0.1ml of each of the dilutions was plated on nutrient agar and incubated at $30^0$C for 24hours and colony counts determined afterwards.

3.11.2 By nitrous acid ($HNO_2$) exposure

The method employed by Idise *et al* (2010) was used where 0.15ml of filter sterilized, freshly prepared, aqueous 2.0M $NaNO_2$ was added to 5ml of a cell suspension in acetate buffer (0.2M, pH4.4). After 20 minutes of exposure, the reaction was determined by serial dilution in Tris/HCl buffer (0.2M, pH 8.0). Aliquots of 0.1ml of the diluted suspension were plated on nutrient agar and incubated at $30^0$C for 24hours and colony counts determined afterwards.

3.11.3 Mutation using combination of ultraviolet light and nitrous acid

The wild strains of *Bacillus cereus* and *Pseudomonas aeruginosa* were subjected to a combination of UV irradiation and nitrous acid treatment as described in section 3.12.1 and 3.12.2. The treated organisms were serially diluted with sterile 0.85% (w/v) saline. Aliquots of 0.1ml of the diluted suspensions were plated on nutrient agar and incubated at $30^0$C for 24hours and colony counts determined (Asmae *et al.*, 2014).

### 3.12 Assessment of the capacity of mutant strains of *B. cereus* and *P. aeruginosa* to degrade crude oil

Petroleum-hydrocarbon degradation by the isolates obtained was assessed using mineral salt medium (100ml) with the following composition: ($KH_2PO_4$ 2g/l, $Na_2HPO_4$ 3g/l, $NH_4NO_3$1g/l, KCl 1g/l, $MgSO_4$ 4mg/l, $CaCl_2$ 0.2mg/l, $FeSO_4$ 0.2mg/l, $MnCl_2$ 0.2mg/l, Agar 15g, Distilled water 1litre) supplemented with 0.5, 1, 1.5 and 2%, crude oil contained in 150ml Erlenmeyer flasks. The pH of the medium was adjusted to 7.0 using dilute hydrochloric acid solution and autoclaved at $121^0$C for 15minutes. One mililiter suspension of each isolate (Mc Farland's standard 7 was used to standardize the inoculum size) was added to the sterile medium. The flasks were continuously aerated during the incubation period in a gyratory shaker incubator (New BrunsWick Scientific Shaker Incubator) programmed at 2000 rpm. Un-inoculated flasks containing the same set of medium were used as control. Seven days after the start of the incubation, each flask was sampled for the determination of optical density ($OD_{600nm}$) using PG T70 U.V/VIS Spectrophotometer. This was repeated at weekly interval for 8weeks. Increase in level of optical density (bacterial cell density) of the test isolates was used as the evaluation

criterion for the isolates adaptation to the medium used and their ability to degrade crude oil being tested (Asmae *et al.*, 2014).

### 3.13 Determination of rate of crude oil degradation by wild and selected mutant strains of *Bacillus cereus* and *Pseudomonas aeruginosa*

The method used by Latha *et al,* (2012) was employed where one milliliter of standardized inoculum from best performing wild and mutant strains of *Bacillus cereus* and *Pseudomonas aeruginosa* each was separately inoculated in to 100ml of mineral salt medium ($KH_2PO_4$ 2g/l, $Na_2HPO_4$ 3g/l, $NH_4NO_3$1g/l, KCL 1g/l, $MgSO_4$ 4mg/l, $Cacl_2$ 0.2mg/l, $FeSO_4$ 0.2mg/l, $Mncl_2$ 0.2mg/l, Agar 15g, Distilled water 1litre) contained in 150ml Erlenmeyer flasks. To each flask was introduced 1ml of crude oil and corked with cotton wool. The experiment was made in replicates. The control flasks contained 100mls of mineral salt medium plus 1ml of crude oil but without any of the organisms. The flasks were incubated at $30^0$C for 8 weeks in a gyratory shaker (New BrunsWick Scientific Shaker Incubator) programmed at 2000rpm. At the start of the experiment (week 1), the weight of 1ml of crude oil was pre -determined using weighing balance (Bench Top Practum 64 – 1s Sartorius) and recorded as initial weight. At the end of week 8, one flask of each representative isolate was removed and used to determine the amount of crude oil left. The amount of residual crude oil was weighed after extracting the oil from the medium and evaporating it to dryness in rotary evaporator at 40°C under reduced pressure. The weight of extracted oil (final weight) was deducted from the initial weight. Thus the amount of crude oil degraded was calculated as follows:

Initial weight of crude oil – Weight of extracted crude oil (final weight).

The % degradation was calculated as follows:

$$\% \text{ degradation} = \frac{\text{Amount of crude oil degraded}}{\text{Initial weight of crude oil}} \times 100$$

### 3.14 Detection of crude oil degrading genes in wild and selected mutant strains of *Bacillus cereus* and *Pseudomonas aeruginosa*

Pure cultures of the wild and mutant strains of *Bacillus cereus* and *Pseudomonas aeruginosa* were subjected to amplification of 16s ribosomal RNA gene using polymerase chain reaction (PCR) in avian influenza laboratory of Veterinary Teaching Hospital, Ahmadu Bello University Zaria, Nigeria.

3.14.1 PCR Procedures

*3.14.1.1 DNA extraction*

The DNA extraction from pure cultures of *Bacillus cereus* and *Pseudomonas aeruginosa* was carried out using the QIAamp tissue kit spin columns (QIAGEN, Hilden, Germany). DNA was extracted from 1ml of the bacterial culture. The culture was pelleted by centrifuging at 12,000rpm for 2 minutes. The pellet was treated with lysis solution and proteinase k and incubated at $60^0$C for 30 minutes. Nucleic acids were precipitated with isopropanol by centrifuging at 10, 000rpm for 10 minutes washed with 1ml of a 70% (v/v) ethanol solution and dissolved in 0.1 ml of a TE buffer (Sandhu *et al*., 2009; Jyothi *et al.*, 2012).

3.14.1.2 DNA Amplification

The genus specific PCR mixture consisting of the parent DNA and the gene specific primers (Appendix I) was subjected to an initial denaturation temperature of $94^0$C for 5 minutes. This was followed by annealing the genes specific primers at $55^0$C for 60 seconds. The extention process was allowed to take place at $72^0$C for 60 seconds. This cycle was repeated 35 times to obtain sufficient quantity of the targeted gene (Sandhu *et al*., 2009; Jyothi *et al.*, 2012). The purity and quantity of the DNA were examined by recording its UV absorption spectrum.

3.14.1.3 Detection of hydrocarbon degrading genes in *Bacillus cereus* and *Pseudomonas aeruginosa*

The detection of genes encoding degradation of long chain hydrocarbon in *Bacillus cereus* and *Pseudomonas aeruginosa* was carried out by agarose gel electrophoresis as described by Sandhu *et al* (2009) and Jyothi *et al* (2012). One percent (1%) (w/v) agarose gel was made by adding 1 g

of agarose to 100 ml of 1X TBE buffer solubilize by heating at boiling temperature, then the agarose was left to cool at 55°C before pouring in a tray to solidify. A comb was placed near one edge of gel and the gel was left to harden. 1X TBE was poured into gel tank and the gel tray was placed horizontally in electrophoresis tank, 3µl of loading dye was mixed with 10 µl DNA sample, and then samples were added carefully to individual wells. Power was turned on at 45 Volts for 15minutes and 85 Volts for 5 hours to run DNA. Agarose gel was stained with ethidium bromide by immersing them in distilled water containing the dye of final concentration of 0.5µg/ml for 45 minutes. DNA bands were visualized using U.V illumination at 366nm wavelength on U.V transilluminator. Catechol 2, 3-dioxygenase gene was detected at the 216bp position in genome of the wild and mutant strains of *B. cereus* while phenol hydroxylase (pheh) gene was confirmed in the genomes of both the wild and mutant strains of *Pseudomonas aeruginosa* at 870bp location

# CHAPTER FOUR

## 4.0: RESULTS

### 4.1 Physico-chemical properties of raw and treated refinery effluent samples

The physico-chemical analysis of the samples of refinery effluents revealed only slight differences between the temperature and pH of the raw and treated samples (Table 4.1). It was observed that the levels of turbidity, total dissolved solids (TDS), total suspended solids (TSS), dissolved oxygen (DO), biological oxygen demand (BOD), chemical oxygen demand (COD) as well as oil and greases (OandG) were relatively high in the raw refinery effluents as expected of all untreated industrial wastewaters. However, marked decreases were observed in the Turbidity, Dissolved oxygen (DO), chemical oxygen demand (COD), biological oxygen demand (BOD) and oil and greases following treatment of the raw effluents (Table 4.1). On the other hand, increases in the total nitrogen (N) and phosphate contents were observed in the treated effluents (Table 4.1).

### 4.2 Cultural, morphological and biochemical characteristics of the bacterial isolates

The results of characterization of isolates are presented in Tables 4.2. The isolates were identified as *Bacillus species* and *Pseudomonas species* based on cultural, morphological and biochemical characteristics.

### 4.3 Biochemical characterization and identities of *Bacillus cereus* and *Pseudomonas aeruginosa* using Microgen test kits

The result of biochemical characterization using Microgen kit presented in Table 4.3 confirmed the isolates as *Bacillus cereus* and *Pseudomonas aeruginosa*. However the test revealed that only 17(42%) of the 41 and 9 (43%) of the 21 isolates were actually *B. cereus* and *P. aeruginosa* respectively (Table 4.3).

**Table 4.1: Physico-chemical properties of raw, treated and discharge point refinery effluent used as source of the bacterial isolates**

| Parameters | Effluents analysed | | | Recommended limits (FEPA) |
|---|---|---|---|---|
| | Raw | Treated | Discharge | |
| Temperature 0$^{o}$C | 29$^{o}$C | 27$^{o}$C | 28$^{o}$C | 40$^{o}$C |
| pH | 6.21 | 6. 61 | 6.63 | 6-9 |
| Turbidity (NTU) | 70.1 | 6.33 | 6.28 | 5.82 |
| Conductivity (µs/cm) | 103.2 | 97.2 | 96.6 | 400 |
| Total Dissolved Solids (mg/l) | 260.0 | 180.0 | 178.4 | 2000 |
| Total Suspended Solids (mg/l) | 210.0 | 200.0 | 193.0 | 30 |
| Dissolved Oxygen (mg/l) | 150.0 | 2.3 | 3.6 | 4.0 |
| Biochemical Oxygen Demand (mg/l) | 300.0 | 1.3 | 2.50 | 50 |
| Chemical Oxygen Demand (mg/l) | 400.0 | 170.0 | 168.0 | 40 |
| Oil and Grease (mg/l) | 540.0 | 40.0 | 23.0 | 10 |
| Chloride (mg/l) | 12.50 | 8.0 | 7.2 | 10 |
| Total Nitrogen (mg/l) | 1.15 | 1.97 | 2.40 | 10 |
| Phosphate (mg/l) | 5.56 | 6.96 | 6.98 | 5.0 |
| Lead | 8.13 | 0.01 | 0.01 | 0.03 |
| Chromium | 5.04 | 0.01 | 0.01 | 0.01 |

**FEPA** = Federal Environmental Protection Agency (1991)

**Table 4.2 Cultural, morphological and biochemical characteristics of the bacterial isolates from effluent**

| Characteristic/Test | Isolate 1 | Isolate 2 |
|---|---|---|
| Colony Shape | Spherical | Round Spherical |
| Colony Colour | Brown | Fluorescent greenish |
| Shape of cell | Long Rod | Short Rod |
| Motility | + | + |
| Gram's reaction | + | - |
| Presence of endospore | + | - |
| Aerobic growth | + | + |
| Anaerobic growth | + | - |
| Fluorescent Pigmentation | - | + |
| Catalase | + | + |
| Oxidase | + | + |
| Indole | - | + |
| Starch hydrolysis | + | - |
| Gelatin liquefaction | + | + |
| Voges proskauer | + | - |
| Identity | *Bacillus cereus* | *Pseudomonas aeruginosa* |

**KEY:** **+** = Positive; **-** = Negative

**Table 4.3 Biochemical profiles and identities of *Bacillus cereus* and *Pseudomonas aeruginosa* using Microgen *Bacillus* Strip (BAC 1 & 2), GNA and GNB-ID system**

| | ***Bacillus* Strip 1 (BAC 1)** | | | | | | | | | | | | | ***Bacillus* Strip 2 (BAC 2)** | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Isolate Code | | | | | | | | | | | | | | | | | | | | | | | | | | Octal code | Identity |
| | **ARA** | **CEL** | **INO** | **MAN** | **MNS** | **RAF** | **RHA** | **SAL** | **SOR** | **SUC** | **TRE** | **XYL** | **ADO** | **GAL** | **MDM** | **MDG** | **INU** | **MLZ** | **IND** | **ONPG** | **ARG** | **CIT** | **VP** | **NIT** | | |
| 01 | - | - | - | - | - | - | - | + | - | + | + | - | - | - | - | - | - | - | - | - | + | - | + | + | 00260013 | *Bacillus cereus* (99.11%) |

| | **G N A Wells** | | | | | | | | | | | | **G N B Wells** | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 02 | **Lysine** | **Ornithin** | **H2S** | **Glucose** | **Mannitol** | **Xylose** | **ONPG** | **Indole** | **Urease** | **V. P.** | **Citrate** | **TDA** | **Gelatine** | **Malonate** | **Inositol** | **Sorbitol** | **Rhamnos** | **Sucrose** | **Lactose** | **Arabinos** | **Adonitol** | **Raffinose** | **Salicin** | **Arginine** | 00510022 | *Pseudomonas aeruginosa* (87.09%) |
| | - | - | - | - | - | - | + | - | + | - | - | + | - | - | - | - | - | - | - | - | + | - | + | - | | |

### 4.4 Strains of *Bacillus cereus* and *Pseudomonas aeruginosa* obtained from the refinery effluents

A total of 41 and 21 strains of *Bacillus cereus* and *Pseudomonas aeruginosa* respectively identified on the basis of their biochemical characteristics were obtained from samples of refinery effluents collected from three sites (Table 4.4). Of the 41 strains of *B. cereus*, 17, 11 and 13 strains were isolated from sampling sites A, B, and C respectively. As of the 21 strains of *P. aeruginosa*, 7, 5 and 9 were obtained from the corresponding sites (Table 4.4).

### 4.5 Ability of wild strains of *Bacillus cereus* and *Pseudomonas aeruginosa* to utilize crude oil as carbon and energy source for cell growth

The results of test conducted to determine the ability of the wild strains of *Bacillus cereus* and *Pseudomonas aeruginosa* obtained from the refinery effluents to degrade and utilize crude oil as source of carbon and energy for growth is presented in Table 4.5. It was observed that all the strains of the two bacterial species tested were able to degrade crude oil when provided in the growth medium at concentrations ranging from 0.5 to 1.5%v/v. However, it was noted that, only 12 of the 17 strains of *Bacillus cereus* grew in medium containing 2.0%v/v of crude oil. Similarly, only 6 of the 9 strains of *Pseudomonas aeruginosa* grew at this concentration (Table 4.5).

### 4.6 Responses of the wild strains of *Bacillus cereus* and *Pseudomonas aeruginosa* to mutagens

The responses of *B. cereus* and *P. aeruginosa* to 30 minutes exposure to UV- radiation are shown in table 4.6. It was observed that *B. cereus* and *P. aeruginosa* exhibited comparable mean survival rate of 54.74 and 54.29% respectively following 30 minutes exposure to UV- radiation (Table 4.6). Exposing the same strains of these bacterial species to Nitrous acid on the other hand, elicited 62.99 and 68.98% increases in the survival rates of *B. cereus* and *P. aeruginosa* respectively (Table 4.7).The mutant strains of *Bacillus cereus* and *Pseudomonas aeruginosa* were subjected to a combination of mutagenes (UV light and nitrous acid) and exhibited survival rates of 27.2 and 20.1% respectively (Table 4.8)

**Table 4.4 Isolates of *Bacillus cereus* and *Pseudomonas aeruginosa* obtained from the refinery effluents**

| Sampling points | No. of Isolates obtained based on Biochemical Identity | | No. (% ) of Isolates confirmed using Microgen Test Kits | |
|---|---|---|---|---|
| | Bc | Pa | Bc | Pa |
| A | 17 | 07 | 06(35) | 04(57) |
| B | 11 | 05 | 04(36) | 02(40) |
| C | 13 | 09 | 07(54) | 03(33) |
| Total | 41 | 21 | 17(42) | 09(43) |

Key: Bc =*Bacillus cereus,* Pa = *Pseudomonas aeruginosa*

**Table 4.5 Growth of the wild strains of *Bacillus cereus* and *Pseudomonas aeruginosa* on crude oil supplemented mineral medium**

| Concentration of crude oil tested (% v/v) | *B. cereus (N=17)* No.+ve | *P. aeruginosa (N=9)* No. +ve |
|---|---|---|
| 0.5 | 17 | 9 |
| 1.0 | 17 | 9 |
| 1.5 | 17 | 9 |
| 2.0 | 12 | 6 |

Key: N= Number of strains tested

**Table 4.6: Effects of UV radiation on the survival rate of *Bacillus cereus* and *Pseudomonas aeruginosa***

| Organism | Initial TVC (cfu/ml) | Final TVC (cfu/ml) | Reduction in TVC | % Kill | % Survival |
|---|---|---|---|---|---|
| *B. cereus* | $2.85x10^{13}$ | $1.56x10^{13}$ | $1.29x10^{13}$ | 45.26 | 54.74 |
| *P. aeruginosa* | $4.20x10^{13}$ | $2.28x10^{13}$ | $1.92x10^{13}$ | 45.71 | 54.29 |

Key: TVC = Total Viable Count, cfu/ml = Colony Forming Units per milliliter

**Table 4.7: Effects of nitrous acid on the survival rate of *Bacillus cereus* and *Pseudomonas Aeruginosa***

| Organism | Initial TVC (cfu/ml) | Final TVC (cfu/ml) | Reduction in TVC | Kill (%) | Survival (%) |
|---|---|---|---|---|---|
| *B. cereus* | $2.54x10^{13}$ | $1.60x10^{13}$ | $0.94x10^{13}$ | 37.01 | 62.99 |
| *P. aeruginosa* | $2.74x10^{13}$ | $1.89x10^{13}$ | $0.85x10^{13}$ | 31.02 | 68.98 |

Key: TVC = Total viable count, cfu/ml = Colony Forming Units per milliliter

**Table 4.8: Effects of combination of nitrous acid and UV light on the survival rate of second stage mutants of *Bacillus cereus* and *Pseudomonas aeruginosa***

| Bacterial Isolates | Initial TVC (cfu/ml) | Final TVC (cfu/ml) | Reduction in TVC | Kill (%) | Survival rates (%) |
|---|---|---|---|---|---|
| *B. cereus* | $5.98x10^{13}$ | $1.20x10^{13}$ | $4.78x10^{13}$ | 80 | 20 |
| *P. aeruginosa* | $9.10x10^{13}$ | $1.27x10^{13}$ | $7.83x10^{13}$ | 86 | 14 |

Key: TVC = Total viable count, cfu/ml = Colony Forming Units per milliliter

### 4.7 Effects of the mutagenesis on the capacity of *Bacillus cereus* and *Pseudomonas aeruginosa* to utilize crude oil

In this study, increase in cell population (cfu/ml) with time in a medium containing crude oil as the sole source of carbon and energy was used as the index by which the ability of the tested strains of *B. cereus* and *P. aeruginosa* to biodegrade crude oil was assessed. Thus, the growth dynamic of both the wild and mutant strains of these bacteria was monitored at varying concentrations of crude oil over a period of 8 weeks.

Results obtained from the study reveals that both the wild type and the mutant strains can degrade crude oil and grow when concentration of crude oil in the growth environment was 0.5% v/v. In the case of *Bacillus cereus,* the growth of the wild strains remained consistently lower than what were recorded for the mutant strains derived from exposure to UV radiations, Nitrous acid (NA) and combination of both mutagens throughout the 8 weeks period of the study (Figure 1). However, it was noted that, mutants derived from exposure to Nitrous acid exhibited higher growth rates than those obtained from exposure to UV radiations. On the other hand, the growth of mutants obtained following exposure to combination of these mutagens had their growth retarded during the first 3 weeks. However, by the $5^{th}$ week, their growth rates increased sharply to levels above that of both the UV and Nitrous acid derived mutants (Figure 4.1).

Similarly, both the wild and mutant strains of *Pseudomonas aeruginosa* exhibited the ability to degrade crude oil when present in the growth environment at concentration of 0.5%v/v (Fig 4.2). However, the wild strains of this bacterium grew at slightly higher rates than the UV-radiated and UV radiated-nitrous acid (UVNA) derived mutants during the first 3 weeks of the study. But these same mutants grew at much higher rates than the wild strains between the $5^{th}$ and the $8^{th}$ week of the study. On the other hand, the UV derived mutant grew at consistently higher rate than the wild type throughout the study period but at a lower rate compared to the nitrous acid (NA) and UV radiated-nitrous acid (UVNA) derived mutants between the $4^{th}$ and $8^{th}$ week period of the study (Fig 4.2).

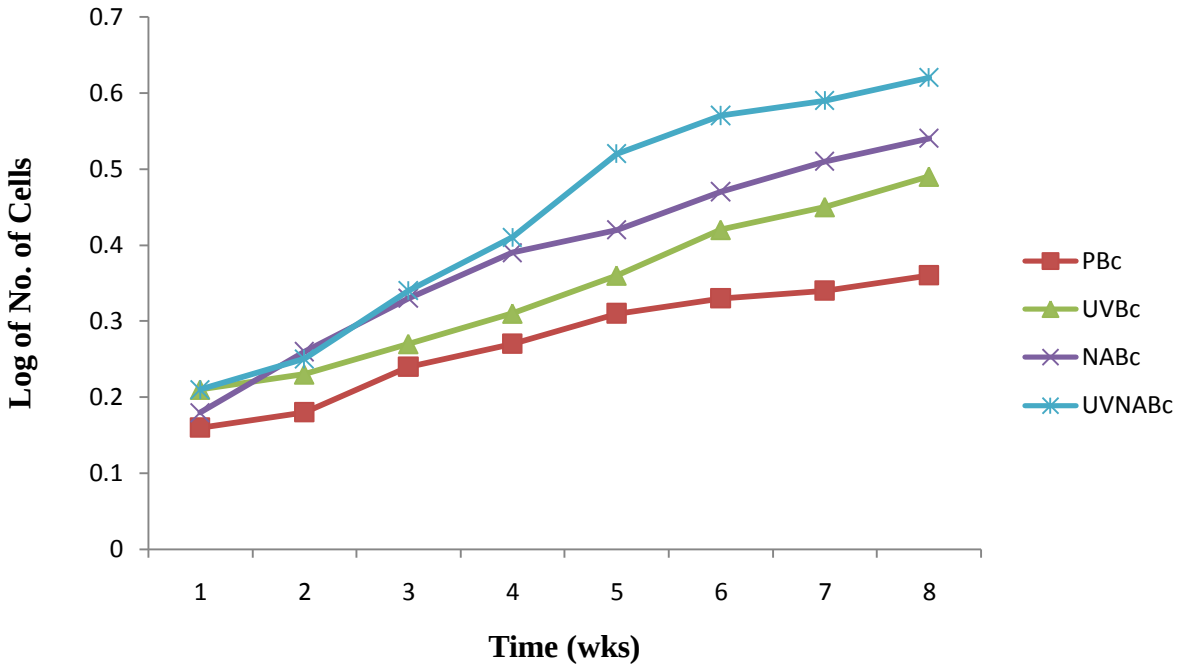



**Figure 4.1: Effects of UV and Nitrous acid mutagenesis on the capacity of *Bacillus cereus* to degrade crude oil at 0.5% V/V**

**Key:** PBc = Parent *Bacillus cereus*, UVBc = UV radiated *Bacillus cereus*, NABc = Nitrous acid modified *Bacillus cereus*, UVNABc = UV- radiated nitrous acid modified *Bacillus cereus*

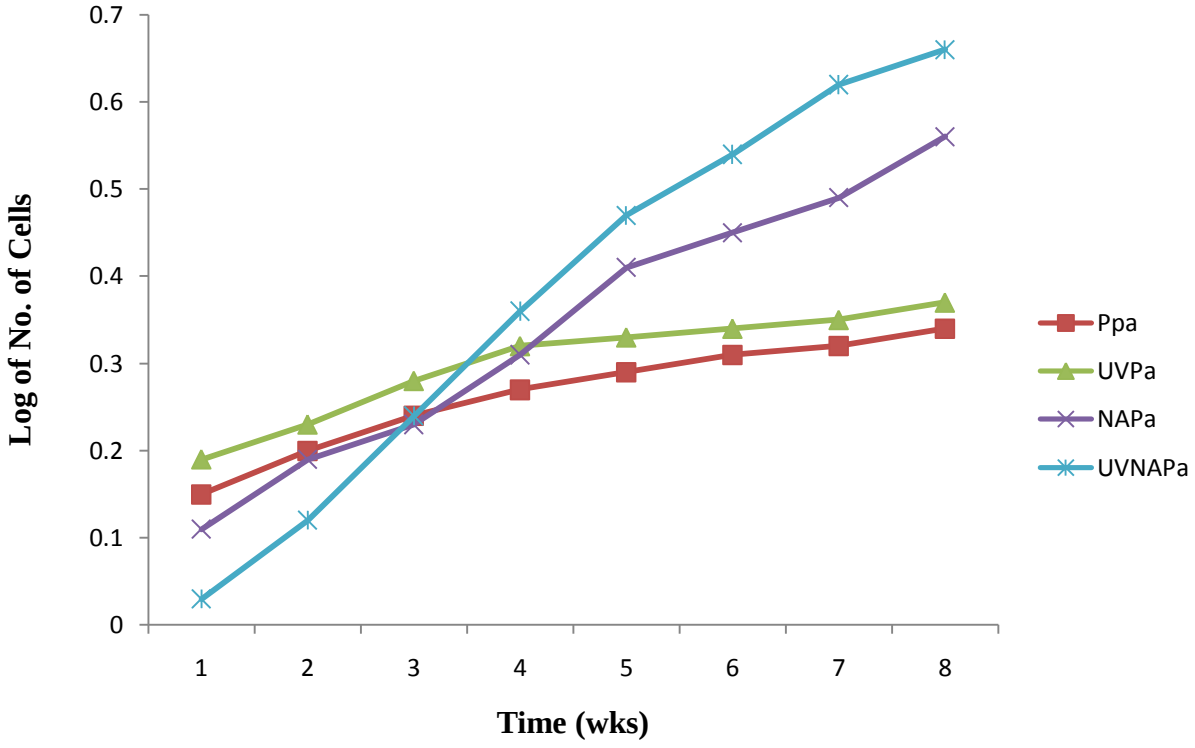



**Figure 4.2: Effects of UV and Nitrous acid mutagenesis on the capacity of *Pseudomonas aeruginosa* to degrade crude oil at 0.5% V/V**

**Key:** PPa = Parent *Pseudomonas aeruginosa*, UVPa = UV modified *Pseudomonas aeruginosa*, NAPa = Nitrous acid modified *Pseudomonas aeruginosa*, UVNAPa = UV- radiated Nitrous acid modified *Pseudomonas aeruginosa*

Increasing the concentration of the crude oil to 1.0% elicited higher growth rates of the NA and UVNA derived mutants of *B. cereus* compared to both the wild type and UV derived mutant strains (Fig 4.3). However, the growth of the nitrous acid (NA) derived mutants was retarded during the first 2 weeks of the study.

On the other hand, all the three mutant strains of *Pseudomonas aeruginosa* grew at much higher rates than the wild strain at 1.0% crude oil concentration (Fig 4.4). However, the UV-radiated, nitrous acid (UVNA) derived mutants had the highest growth rates followed by the UV derived and the nitrous acid (NA) derived mutants in that order (Fig 4.4).

Further increase in the concentration of crude oil in the growth medium to 1.5% did not alter the growth rates of both the nitrous acid (NA) derived and UV-radiated-nitrous acid (UVNA) derived mutants of *B. cereus* which remained higher than that of the wild strain (Fig. 4.5). But, the growth of the UV- derived strains of this bacterium was decreased to a level much lower than even that of the wild strain during the first 6 weeks of the study (Fig. 4.5).

On the other hand, all the three mutant strains of *P. aeruginosa* exhibited much higher growth rates than the wild strain at 1.5% crude oil concentration. The highest growth rate was recorded for the UVNA derived mutants followed by the NA derived and UV derived mutants in that order (Fig 4.6).

When grown in a medium containing 2.0% crude oil, the growth of wild *B. cereus* remained lower than the UV-derived and nitrous acid modified mutants. But the mutant strains derived from exposure to the two mutagens had higher growth rates (Figs. 4.7).

Similarly, the growth of mutants following exposure to the combination of both UV-radiation and nitrous acid mutagenesis was much higher than the nitrous acid modified strains followed by the UV- radiated strains. However, the wild strains were observed as having their growth retarded (Fig. 4.8).

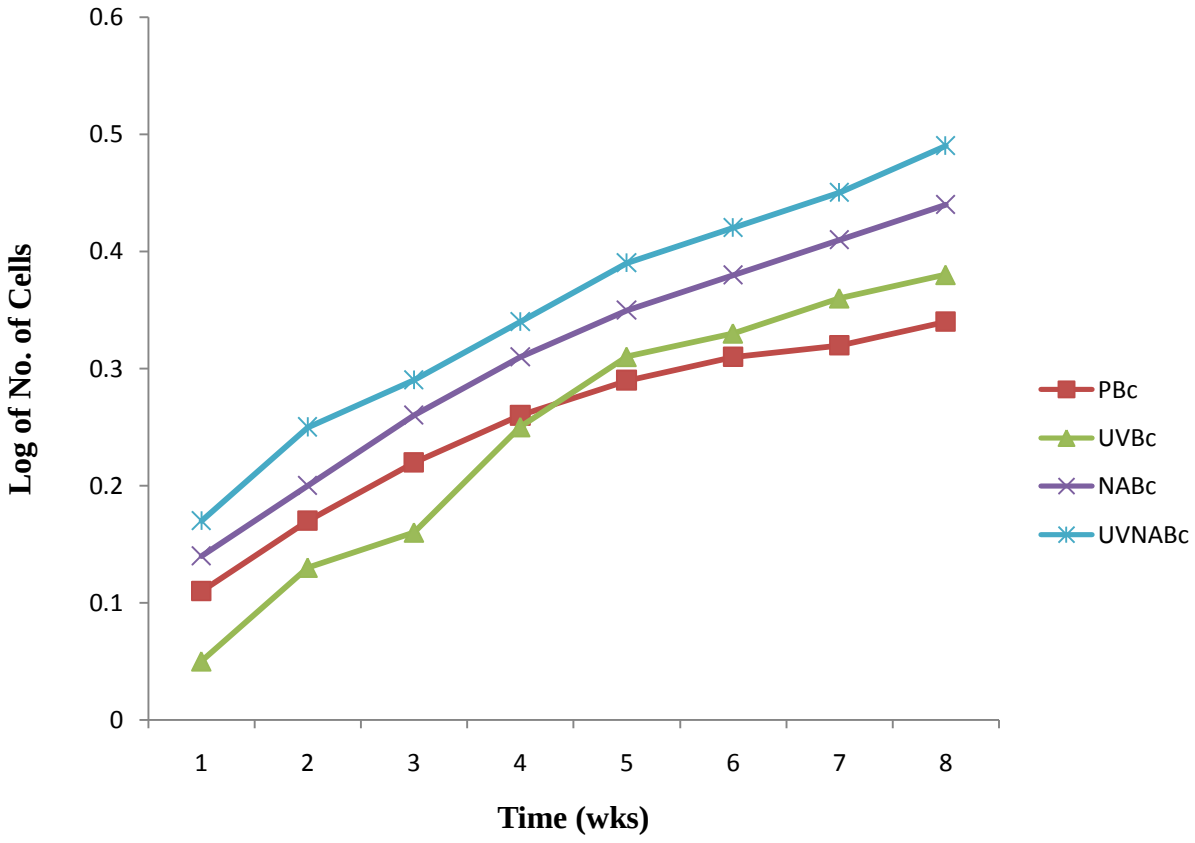



**Figure 4.3: Effects of UV and Nitrous acid mutagenesis on the capacity of *Bacillus cereus* to degrade crude oil at 1% V/V**

**Key:** PBc = Parent *Bacillus cereus*, UVBc = UV- radiated *Bacillus cereus* NABc = Nitrous acid modified *Bacillus cereus*, UVNABc = UV- radiated nitrous acid modified *Bacillus cereus*

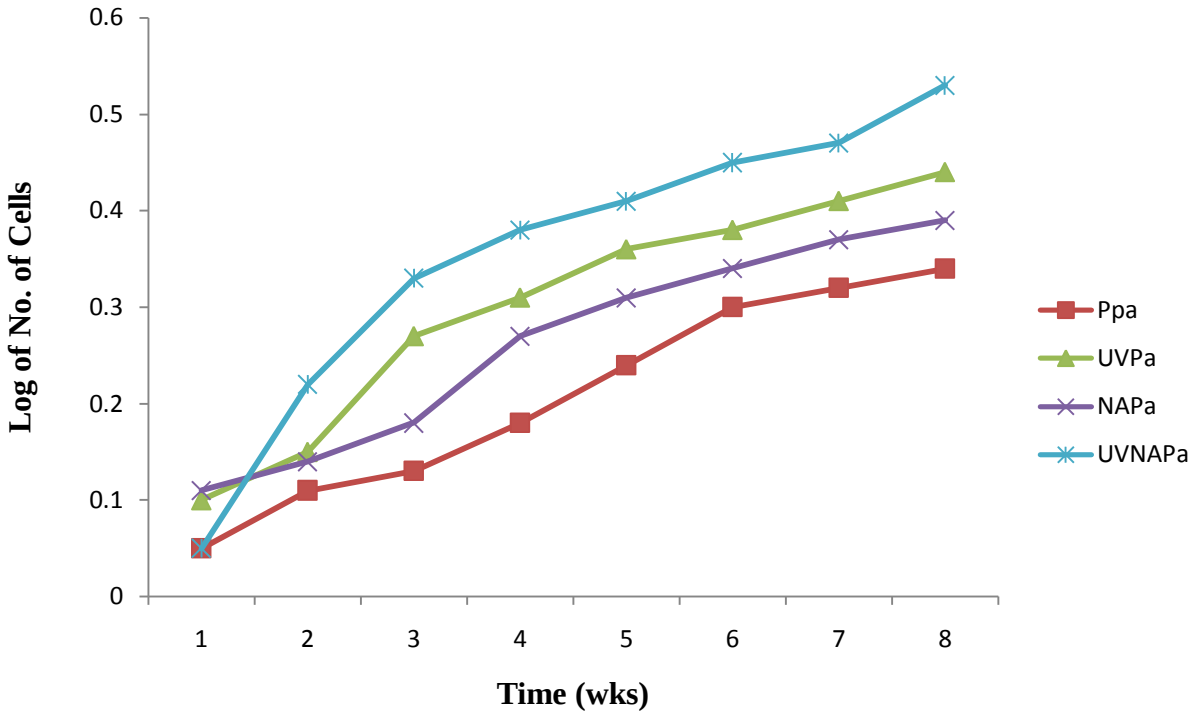



**Figure 4.4: Effects of UV and Nitrous acid mutagenesis on the capacity of *Pseudomonas aeruginosa* to degrade crude oil at 1% (v/v)**

**Key:** PPa = Parent *Pseudomonas aeruginosa*, UVPa = UV - radiated *Pseudomonas aeruginosa*, NAPa = Nitrous acid modified *Pseudomonas aeruginosa*, UVNAPa = UV- radiated+ Nitrous acid modified *Pseudomonas aeruginosa*

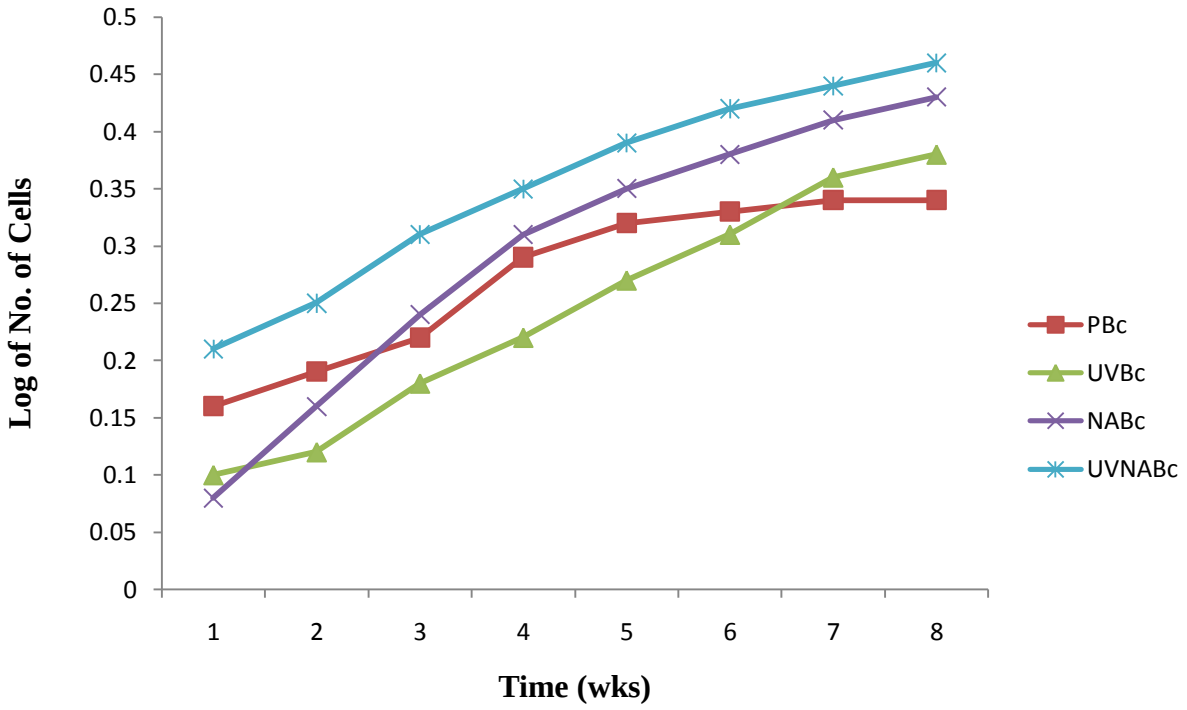



**Figure 4.5: Effects of UV and Nitrous acid mutagenesis on the capacity of *Bacillus cereus* to degrade crude oil at 1.5% V/V**

**Key:** PBc = Parent *Bacillus cereus*, UVBc = UV - radiated *Bacillus cereus* NABc = Nitrous acid modified *Bacillus cereus*, UVNABc = UV- radiated nitrous acid modified *Bacillus cereus*

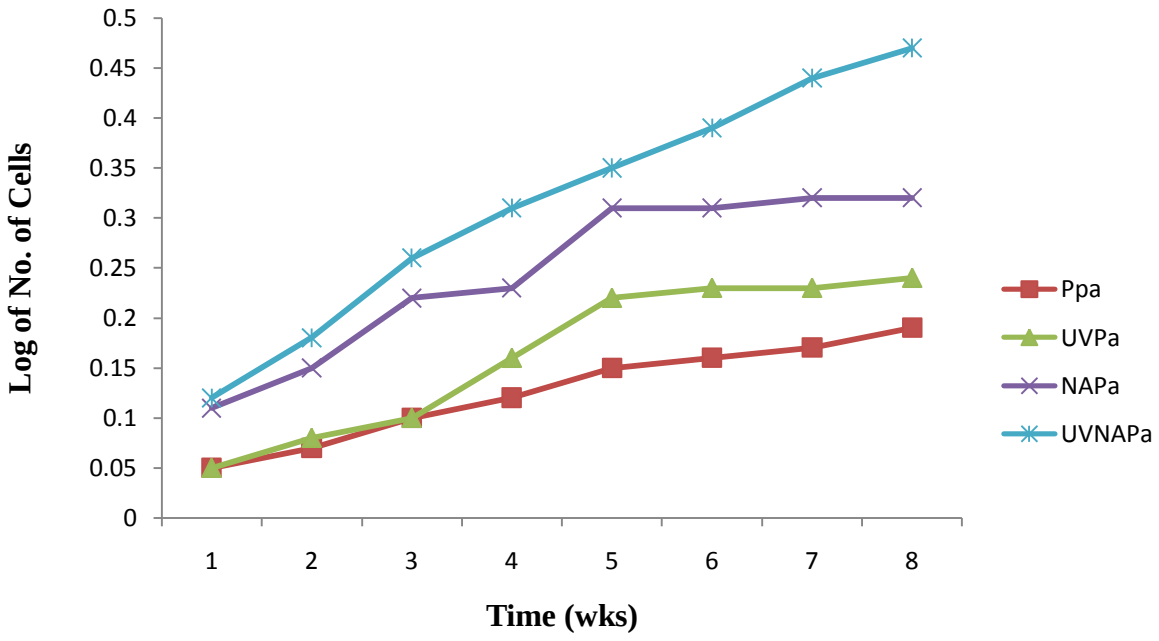



**Figure 4.6: Effects of UV and Nitrous acid mutagenesis on the capacity of *Pseudomonas aeruginosa* to degrade crude oil at 1.5% V/V**

**Key:** PPa = Parent *Pseudomonas aeruginosa*, UVPa = UV - radiated *Pseudomonas aeruginosa* NAPa = Nitrous acid modified *Pseudomonas aeruginosa*, UVNAPa = UV- radiated nitrous acid modified *Pseudomonas aeruginosa*

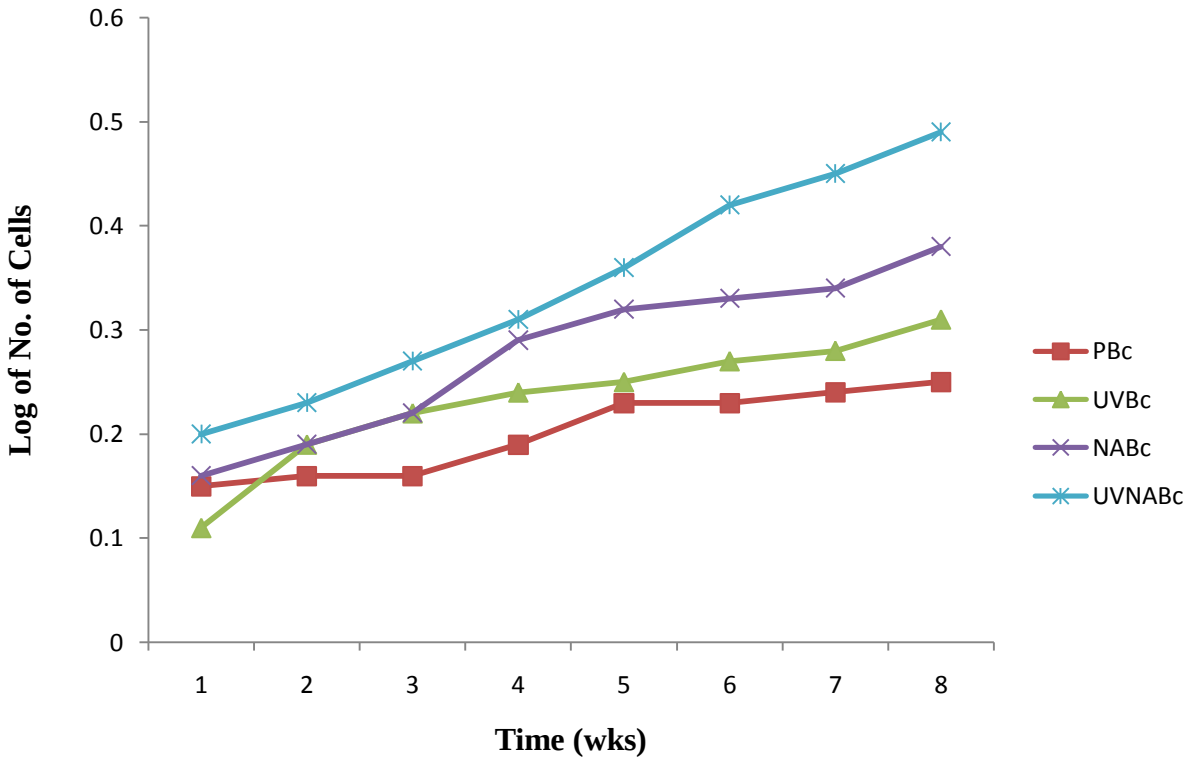



**Figure 4.7: Effects of UV and Nitrous acid mutagenesis on the capacity of *Bacillus cereus* to degrade crude oil at 2% V/V**

**Key:** PBc = Parent *Bacillus cereus*, UVBc = UV - radiated *Bacillus cereus* NABc = nitrous acid modified *Bacillus cereus*, UVNABc = UV- radiated nitrous acid modified *Bacillus cereus*

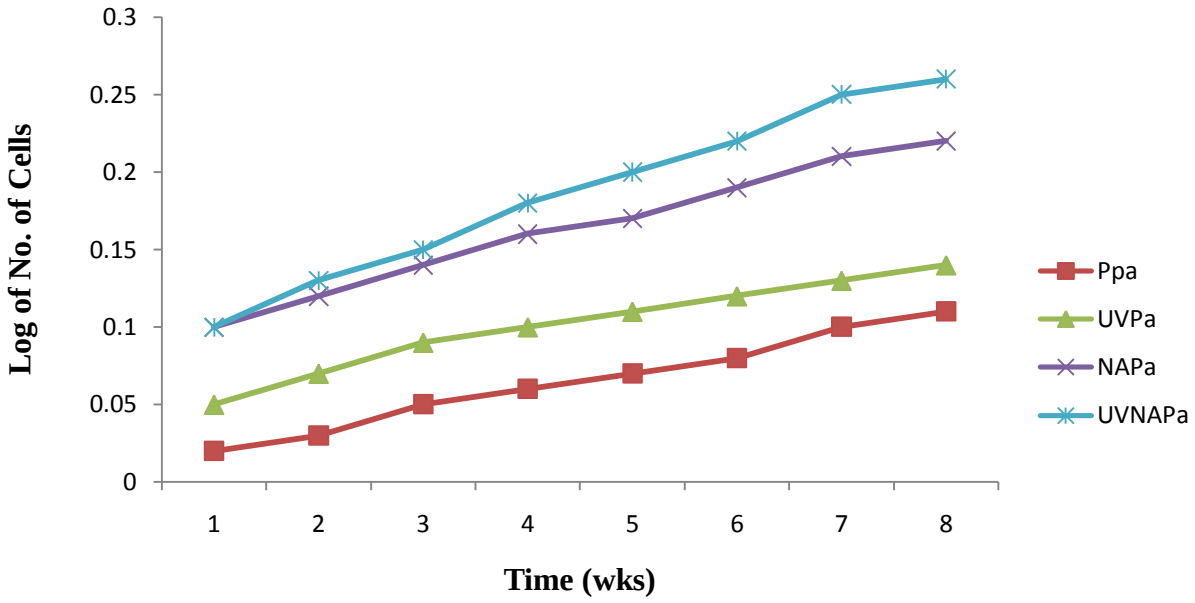



**Figure 4.8: Effects of UV and Nitrous acid mutagenesis on the capacity of *Pseudomonas aeruginosa* to degrade crude oil at 2% V/V**

**Key:** PPa = Parent *Pseudomonas aeruginosa*, UVPa = UV - radiated *Pseudomonas aeruginosa* NAPa = Nitrous acid modified *Pseudomonas aeruginosa*, UVNAPa = UV- radiated nitrous acid modified *Pseudomonas aeruginosa*

### 4.8 Rate of hydrocarbon degradation by wild and mutant strains of *B. cereus* and *P. aeruginoas*a

The rate of hydrocarbon degradation by wild and mutant strains of *B. cereus* and *P. aeruginosa* was monitored using gravimetric analysis for the period of 8 weeks. It was observed that the second stage mutant strains of *B. cereus* and *P. aeruginosa* degraded crude oil much higher than the parents and the first stage mutants. This further confirmed the potential of the mutant strains in crude oil degradation as compared to the parent strains. Parent strains of *B. cereus* and *P. aeruginosa* degraded crude oil at 96.59 and 97.58% respectively while the second stage mutant strains of *B. cereus* and *P. aeruginosa* exhibited 99.60 and 99.80% crude oil degradation respectively (Table 4.9).

### 4.9 Detection of hydrocarbon degrading genes in *B. cereus* and *P. aeruginosa*

The molecular investigations conducted in this study confirmed the existence of hydrocarbon degrading genes in genomic DNA of wild and mutant strains of *B. cereus* and *P. aeruginosa.* The catechol 2, 3-dioxygenase gene was detected at the 216bp position in genome of the wild and mutant strains of *B. cereus* as expected of all crude oil degrading *Bacillus species* (plate F). Similarly, the phenol hydroxylase (pheh) gene was confirmed to be present in the genomes of both the wild and mutant strains of *Pseudomonas aeruginosa* at the expected 870bp location (Plate G).

**Table 4.9 Rate of hydrocarbon degradation by wild and mutant strains of *B. cereus* and *P. aeruginoas*a**

| Bacterial Isolates | Initial TPH (ppm) | Final TPH (ppm) | Amount and % degraded (ppm) |
|---|---|---|---|
| PBc | 8910 | 303.83 | 8606.17 (96.59) |
| UVBc | 8910 | 50.87 | 8859.13 (99.43) |
| NABc | 8910 | 31.84 | 8872.25 (99.58) |
| UVNABc | 8910 | 31.84 | 8878.16 (99.60) |
| Ppa | 8910 | 215.62 | 8694.38 (97.58) |
| UVPa | 8910 | 39.20 | 8870.79 (99.56) |
| NAPa | 8910 | 33.48 | 8876.52 (99.62) |
| UVNAPa | 8910 | 17.82 | 8892.18 (99.80) |
| CONTROL | 8910 | 7658 | 1252 (14.05) |

**Key:** TPH = Total Petroleum Hydrocarbon, ppm = Parts per million, PBc = Parent *Bacillus cereus*, UVBc = UV- radiated *Bacillus cereus* NABc = Nitrous acid modified *Bacillus cereus*, UVNABc = UV- radiated nitrous acid modified *Bacillus cereus*, PPa = Parent *Pseudomonas aeruginosa*, UVPa = UV - radiated *Pseudomonas aeruginosa*, NAPa = Nitrous acid modified *Pseudomonas aeruginosa*, UVNAPa = UV- radiated Nitrous acid modified *Pseudomonas aeruginosa*

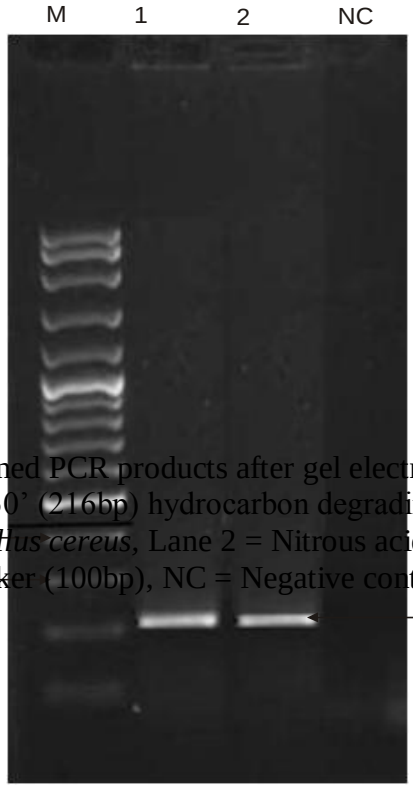



PLATE F: Ethidium bromide- stained PCR products after gel electrophoresis for the catechol 2, 3 dioxygenase 'C230' (216bp) hydrocarbon degrading genes in *Bacillus cereus.*

**Key:** Lane 1 = UV - radiated *Bacillus cereus,* Lane 2 = Nitrous acid modified *Bacillus cereus,* M = DNA molecular size marker (100bp), NC = Negative control.

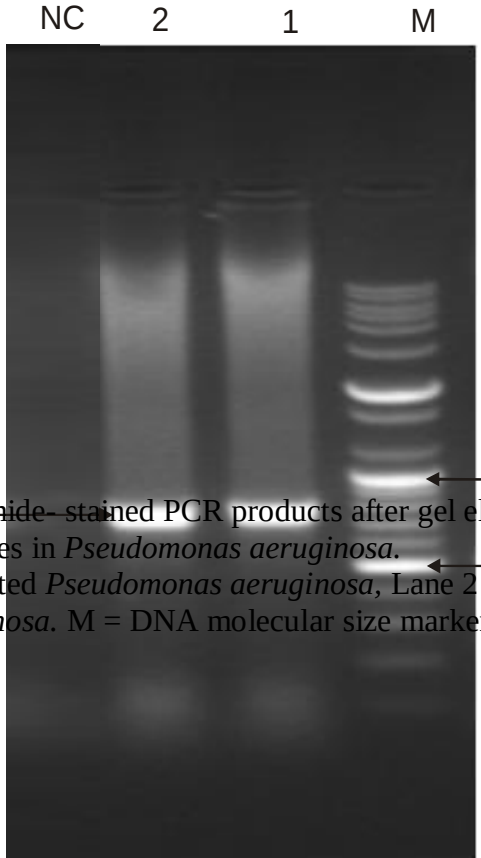



PLATE G: Ethidium bromide- stained PCR products after gel electrophoresis for the *pheh* (870bp), genes in *Pseudomonas aeruginosa.*

**Key:** Lane 1 = UV - radiated *Pseudomonas aeruginosa,* Lane 2 = Nitrous acid modified *Pseudomonas aeruginosa.* M = DNA molecular size marker (100bp), NC = Negative control.

It is also note worthy that, the positions of these genes in the genomes of first and second stage mutant strains of both *B. cereus* and *Pseudomonas aeruginosa* were not altered following exposure to both UV and NA (Plate I and II).

# CHAPTER FIVE

## 5.0 DISCUSSION

Biodegradation of crude oil by microorganisms appears to be a natural process by which the bulk of the polluting oil is used as an organic carbon source, causing the breakdown of hydrocarbons to lower molecular weight compounds or transformed into the other organic compounds such as biosurfactants (Zhang *et al*., 2005). Crude oil degradation by *Bacillus cereus* and *Pseudomonas aeruginosa* used in this study was enhanced by genetic improvement of the organisms using mutation technique. This technique has been widely employed to obtain strains with higher potential for hydrocarbon degradation (Debajit and Yadav, 2014). This was achieved by a combination of physical and chemical mutagenesis using ultraviolet irradiation and nitrous acid.

**Physicochemical Properties of the Samples of raw Refinery Effluent**

The observations made in this study indicated that all the physicochemical properties of the raw refinery effluents, with the exception of temperature and pH exceeded the FEPA (1991) Permissible limits. For instance, the high levels of biological oxygen demand, chemical oxygen demand as well as oil and greases could pose a serious challenge to the survival and growth of microorganisms in effluents (Obayori *et al*. 2009; Barathi *et al*.2010). Under such conditions, only microorganisms with the requisite adaptations could survive and grow in it. Organisms capable of surviving and growing in an environment provided by the raw refinery effluents, therefore, could be adapted to the general toxic properties of the refinery effluents (Barathi *et al*. 2010). In addition, such organisms could be able to depend on hydrocarbons as their sole source of carbon and energy for cellular growth (Debajit *et al*. 2014). It is conceivable therefore that such an environment as provided by the refinery effluents, is most likely to be populated by hydrocarbon degrading strains of bacteria (Okerentugba and Ezeronye 2003; Ojo, 2006; Edise *et al*. 2010; Obinna *et al*. 2014). This assertion is strongly supported by the drastic reduction in the level of DO, BOD, COD as well as oil and grease observed following effluent treatment strategy

employed by the Refinery (Table 4.1).The concentrations of oil and grease in the effluent at the raw, treated and point of discharge were found to be higher than the maximum permissible limit of 10 mg/l for the raw effluent. In effect, the level of effluent treatment at the point of discharge may be considered ineffective. Petroleum hydrocarbons have been observed to be toxic to aquatic life and can accumulate in the membrane lipid bilayers of microorganisms thereby interfering with their structural and functional properties (Obinna *et al*., 2014). Phosphate, nitrate and chloride contents showed reduced concentrations (Table 4.1). Phosphates and nitrates are components of agricultural fertilizers commonly used by farmers for agricultural purposes. These fertilizers may be leached into the rivers during the rainy season and thus account for the increased concentrations in the receiving water body. Phosphate level in the effluent has been observed to be associated with the refinery operations (Poul *et al*., 2016).

**Isolation and characterization of strains of *Bacillus cereus* and *Pseudomonas aeruginosa* from refinery effluents using biochemical procedures**

The isolation of hydrocarbon utilizing strains of *B. cereus* and *P. aeruginosa* from the refinery effluents is considered as evidence that they are active hydrocarbon degraders in that environment (Chikere and Ekwubu 2014). This is not unexpected in view of many earlier reports to the effect that, consistent exposure of non- hydrocarbon degrading strains indigenous to hydrocarbon polluted sites often results in the emergence of hydrocarbon degrading strains (Sekine *et al*., 2006; Nilanjana and Prethy, 2014). To be able to survive such an environment, these bacteria must have developed enzymatic and physiological responses that allowed them to utilize the hydrocarbons as substrates (Ghazali *et al*., 2004; Ojo, 2006). It also indicates that the refinery effluents contained appreciable populations of indigenous active hydrocarbon degrading strains of these bacteria (Chikere and Ekwuabo, 2014). The process by which a non-hydrocarbon degrading strain could be converted to a degrading strain has been attributed to the process of induction of genes which code for enzymes required for hydrocarbon degradation (Prescott *et al*.

2008). The observations made in this study strongly suggest that refinery effluents could provide a rich source of wild strains of hydrocarbon degrading bacteria with the requisite genes suitable for modification studies. However, the proper identity of such isolates should be thoroughly confirmed using techniques other than their biochemical profiles (Table 4.3).

**Capacity of the isolates to utilize crude oil as carbon and energy source for growth**

The screening test revealed the ability of the isolated strains of *B. cereus* and *P. aeruginosa* to grow in mineral medium containing 0.5-2.0% crude oil as sole source of carbon and energy for cell growth (Table 4.6). These findings suggest that the screened *B. cereus* and *P. aeruginosa* are active hydrocarbon degraders in the environment. According to some earlier reports (Yin, 2008; Joel 2011; Asmae *et al*. 2014), the ability of strains of *B. cereus and P. aeruginosa* to grow in crude oil supplemented medium lies in their possession of genes that encode enzymes required for the degradation of several categories of hydrocarbons. Hydrocarbonutilizing bacteria have been shown to produce bioemulsifiers and biosurfactants, which greatly enhance bioavailability by transporting hydrocarbons in to the cell through efficient uptake mechanisms (Mahmoud *et al*. 2008; Das and Mukherjee, 2011) This observation strongly suggests that the wild strains of *B. cereus* and *P. aeruginosa* isolated in this study have the potential for application in cleanup of crude oil contaminated sites. Further more, the capacity of these isolates in crude oil degradation could be improved through mutagenesis (API, 2011; Asmae *et al*., 2014).

**Mutation of the selected wild strains of *Bacillus cereus* and *Pseudomonas aeruginosa* using UV light and nitrous acid**

Mutagenesis produced mutants with high potential to degrade crude oil. This indicates the efficiency of the mutagens as tools of genetic improvement of crude oil degrading bacteria. The nitrous acid treated strains of *B. cereus* and *P. aeruginosa* when subjected to another round of mutagenesis using UV light for 30 minutes, yielded second stage mutants (UVNABc and UVNAPa) with increased crude oil degrading ability as compared with the wild strains. The results are in agreement with the findings of Idise *et al*. (2010) who isolated a second stage

mutant (*P. aeruginosa*) with a reduced population by 83% and with survival rate of 17%. The chemical mutagen used in this study ($HNO_2$) has been reported to cause mutation by acting on the nucleic acid base of resting DNA in such a way that the specific pairing with other bases is changed, for instance adenine is deaminated to form hypoxanthine which then pairs with cytosine instead of thymine. It can also deaminate cytosine to form uracil (VanHamme *et al*., 2003). Ultraviolet light on the other hand is known to cause mutation by dimerization of pyrimidines such as thymine nucleotides resulting in thymine dimmers. The dimerization is caused by linking the 4, 5 unsaturated bonds of adjacent pyrimidines to form cyclobutane ring. This causes distortion of the DNA double strand, which interferes with replication because dymerization of thymine on opposite strands causes cross linkage of the strands and thereby preventing their separation (Prescott *et al*., 2008).

**Assessment of the capacity of the selected wild and mutant strains of *B. cereus* and *P. aeruginosa* in biodegradation of crude oil under laboratory conditions**

The degradation of crude oil by wild and mutant strains of *B. cereus* and *P. aeruginosa* indicated that the mutants were better degraders than their wild or parent strains. It was observed in the second stage mutation that *Pseudomonas aeruginosa* had the highest degradation potential than *Bacillus cereus*. The growth dynamics observed might either be due to their constitutive nature of hydrocarbon assimilating capabilities or adaptation of the strains as a result of previous exposure to exogenous hydrocarbons (Adebusoye *et al.,* 2007; Wang *et al*., 2012). This further confirmed that second stage mutants of *Bacillus cereus* and *Pseudomonas aeruginosa* possess highest potential for crude oil degradation. The rapid crude oil degradation exhibited by *Pseudomonas aeruginosa* in the second stage of mutagenesis could be due to the fact that some microorganisms naturally have the potential to utilize crude oil as source of carbon and energy (Rahman *et al*., 2003; Latha *et al*., 2012). It was also reported by Latha *et al.* (2012) that crude oil contains hydrocarbons and does not resist attack by microorganisms. Ferreira *et al.* ( 2012) reported that an increase in hydrocarbon degradation corresponds to an increase in cell number

during the degradation process demonstrating the microbial ability to utilize hydrocarbon as soul source of carbon and energy. According to Boboye *et al*.(2010), significant increase in biodegradation of hydrocarbon by *Bacillus cereus* and *Pseudomonas aeruginosa* was found with respect to time i.e., from 0 hour to 15 days. However, *Pseudomonas aeruginosa* possessed higher potential than *Bacillus cereus.* This agrees with previous reports (Asitok and Antai, 2006; Kim and Crowley 2007; Anon, 2008). Cunliffe and Kertesz (2006), reported that individual bacteria can metabolize only a limited range of hydrocarbon substrates; hence assemblages of the mixed populations with overall broad enzymatic capacities would be required to achieve considerable biodegradation of petroleum hydrocarbons.

**Screening for the crude oil degrading wild and mutant strains of *Bacillus cereus* and *Pseudomonas aeruginosa* for crude oil degrading genes.**

In this study, hydrocarbondegrading genes [catechol 2, 3 - dioxygenase and phenol hydroxylase (pheh)] were detected in the genomic DNA of wild and mutant strains of *B. cereus* and *P. aeruginosa* at 216 and 870bp locations respectively. According to some reports (VanBeilen *et al*. 2007; Berlendis *et al*., 2010) genes encoding enzymes required for hydrocarbon degradation are carried on transposons or plasmids. More recently, Asmae *et al*. (2014) reported that hydrocarbon degradation is mediated by oxygenases encoded by *alk* genes which are under the control of *alk* operons. However, genetic modification of wild strains of hydrocarbon degrading bacteria through mutagenesis should be able to target the appropriate genes irrespective of their locations within the cell (Ilori *et al*., 2006; Bryan and Voskanyan, 2012). Asmae *et al* (2014) further reported that 100 strains of *Pseudomonas aeruginosa* grew in long chain n-alkenes at concentrations ranging from 0.1 – 0.5% of crudeoil. Thus suggesting that many strains of *Pseudomonas aeruginosa* possess genes responsible for the degradation of long chain alkenes. Many biodegradation studies reported on diesel were carried out using lesser diesel concentrations ranging from 0.5-3% and generally unfavourable at higher concentrations

(Adeline *et al*., 2009). Strains of *P. aeruginosa* previously exposed to alkanes as carbon source normally retain the genes required for their biodegradation (Smith *et al*., 2003; Rojo, 2010). Asmae *et al* (2014) also emphasized the likelihood that *alk B1* gene of *P. aeruginosa* is carried on the chromosome. Raji *et al*. (2016) reported that C23O genes are among the functional genes present in bacteria dwelling in environments contaminated with petroleum. Members of the *Pseudomonas* and *Bacillus* species were reported to possess *nah H* and *alkJ* genes that encode key enzymes (which encode catechol 2, 3-dioxygenase and phenol hydroxlase) for the degradation of both aromatic and aliphatic hydrocarbons (Nikolopoulou and Kalogerakis, 2009; Asmae *et al.*, 2014). Isolates of *Pseudomonas and Bacillus* species with high hydrocarbon degrading ability contained either one or both genes (Nikolopoulou and Kalogerakis, 2009).

# CHAPTER SIX

## 6.0 CONCLUSION AND RECOMMENDATIONS

### 6.1 Conclusion

Biodegradation is one of the most rapidly growing areas of biotechnology, which has been used for the clean up of pollutants. This is because of its low costs and its public acceptability. Crude oil degradation by environmental microflorae involves microorganisms having specialized metabolic capacities. In polluted environments, specialized microorganisms are abundant because of the adaptation of the microflorae to pollutants. It is evident from this study that crude oil degrading bacteria are ubiquitous in refinery effluents and they can be isolated from hydrocarbon polluted sites and waste water.

Evidently, *Bacillus cereus* and *Pseudomonas aeruginosa* could be isolated from effluents and used effectively in the degradation of crude oil in crude oil contaminated sites. Both mutants could thus be employed, either alone or in combination in the degradation of crude oil as well as environments polluted with 2% (v/v) crude oil.

In this study, the growth of the bacteria in question was determined by increase in the logarithm of number of cells and the results showed that the isolates grew maximally on the Crude oil supplied as the sole source of carbon and energy.

Mutational treatments of 30 min UV-radiation followed by nitrous acid treatment (20 min) produced mutants that possessed higher crude oil biodegradation potential than their wild/parents strains.

The degradation process was observed to gradually increase with the peak value at week 8. The crude oil concentrations at 0.5, 1and 1.5% were found to be most effective for biodegradation as compared to 2% concentration.

In this study, *Pseudomonas* aeruginosa exposed to UV - radiation and nitrous acid (second stage mutants) demonstrated significantly higher potential to degrade crude oil.

The study revealed that hydrocarbon degrading genes [catechol 2, 3 - dioxygenase and phenol hydroxylase (pheh)] genes were present in the genomes of both the wild and mutant strains of *B. cereus* and *Pseudomonas aeruginosa* at 216 and 870bp locations.

## 6.2 Recommendations

**1**. Based on this research, the need for further treatment action on Kaduna refinery effluents is necessary in order to reduce the contaminant concentrations to acceptable levels.

**2.** The second stage mutants of *Bacillus cereus* and *Pseudomonas aeruginosa* be employed in the biodegradation of refinery effluents to acceptable levels prior to discharge in to the receiving environment.

**3**. The introduction of these strains in to the wastewater treatment process of KRPC could be a plausible solution to the environmental pollution problem therein in the discharge of petroleum refinery effluent

**4**. The modified strains could also be employed in the biodegradation of environments polluted with 0.5 – 2% (v/v) petroleum products with increased efficiency.

**5.** Further studies should be carried out on the genes or biosurfactants genes responsible for the degradation ability of these organisms for strain improvement and prospective use for biotechnological exploitation.

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**Appendix I:**

**Choice of primers for detecting petroleum hydrocarbon degrading genes**

PCR primers and cycling conditions for the detection of catechol 2, 3-dioxygenase and phenol hydroxylase (Pheh) genes encoding degradation of long chain hydrocarbon in *Bacillus cereus* and *Pseudomonas aeruginosa* respectively, as designed by Sandhu *et al*., (2009) and Jyothi *et al.*, (2012).

| Gene | Primer sequence (5' - 3') | PCR cycling conditions | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| C2,3,O | C2,3,O - F CGACCTGATC(AT)(CG)CATGACCGA<br>C2,3,O - R T(CT)AGGTCA(GT)(AC)ACGGTCA | 94$^{o}$C, 5 min→ (94$^{o}$C, 45 sec→55$^{o}$C, 60 sec→72$^{o}$C, 60 sec) 35 cycles→ 72$^{o}$C, 60 sec | 216 | Jyothi *et al.*, (2012) |
| Pheh | Pheh-3F CGKATGACSTACGGCTGATGGGCG<br>Pheh-3R ACGTCCTGTTCGATGATCTCCTTGATCCGC | 95$^{o}$C, 10 min→ (95$^{o}$C, 60 sec→60$^{o}$C, 60 sec→72$^{o}$C, 60 sec) 35 cycles→ 72$^{o}$C, 5 min | 870 | Sandhu *et al.*, (2009) |

**Appendix II:**
**Physico-chemical properties of the crude oil sample**

| Parameters | Values Obtained | |
|---|---|---|
| Specific Gravity (60/60°F) | 0.8500 | |
| API Gravity (60/60°F) | | 39.31 |
| Sulfur (wt %) | | 0.04 |
| Viscosity ($40^0$C) | 5.23 | |
| Water Contents (Vol %) | 0.05 | |
| Pour point $^0$C | 1.67 | |
| % Salt Content | | 0.39 |
| % Nitrogen | 0.03 | |
| Acid No. Mg, KOH/g | 0.05 | |