ASSESSING THE CAPACITY OF WILD AND MUTANT STRAINS OF BACILLUS SUBTILIS AND PSEUDOMONAS PUTIDA ISOLATED FROM REFINERY EFFLUENT IN THE DEGRADATION OF HYDROCARBONS

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BY

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MARCH, 2019

Declaration

I declare that the work in this dissertation entitled "Assessing the Capacity of Wild and Mutant Strains of *Bacillus subtilis* and *Pseudomonas putida* Isolated from Refinery Effluent in the Degradation of Hydrocarbons"has been carried out by me in the Department of Microbiology, Ahmadu Bello University under the supervision of Prof. D.A. Machido and Prof. S.A. Ado. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

ORJIUDE Josiah Ejike	……………………	……………………
	Signature	Date

Certification

This dissertation report entitled "Assessing the capacity of wild and mutant strains of *Bacillus subtilis* and *Pseudomonas putida* isolated from refinery effluent in the degradation of hydrocarbons"by Ejike Josiah ORJIUDE meets the regulations governing the award of the degree of Master of Science in the Department of Microbiology, Ahmadu Bello University, Zaria and it has been approved for its contributions to knowledge.

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Dedication

This work is dedicated to God Almighty for His infinite mercy, grace and favour throughout the period of this work. To Him alone be all the glory.

Abstract

Petroleum refinery effluents are characterized by the presence of pollutants such as hydrocarbons as such could be of serious environmental consequence if discharged into receiving sites without proper treatment to remove the pollutants. This study assessed the capacity of wild and mutant strains of *Bacillus subtilis* and *Pseudomonas putida* isolated from refinery effluent in the degradation of hydrocarbons present in the effluent. The physicochemical parameters of the raw and treated effluent samples collected from Kaduna Refinery and Petrochemical Company (KRPC) were determined using standard guidelines. With the exception of turbidity (35.3 and 18.2 NTU), BOD (190 and 29.6 mg/L), COD (351.2 and 78.1 mg/L) and Oil and Grease (45.2 and 17.9 mg/L) for the raw and treated effluent respectively, and conductivity (695 µS/cm) for the raw effluent, all other parameters were within the permissible limits set by FMENV. Six (6) and eleven (11) isolates of *Bacillus subtilis* and *Pseudomonas putida*respectively were isolated from the effluent and screened for capacity to utilize and grow on mineral medium containing different concentrations (0.5%, 1%, 1.55% and 2%) of crude oil as the sole source of carbon. Isolates TE8 (B. *subtilis*) and TEC10 (P. putida) had luxuriant growth across the first three concentrations of crude oil and medium growth on medium containing 2% crude oil. Both isolates were treated with nitrous acid and UV-irradiation to generate mutants. Death rate of 59.67% (40.33% survival) and 66.33% (33.67% survival) were observed for *Bacillus subtilis* and *Pseudomonas putida* respectively on treatment with nitrous acid. Also death rate of 51.67% (48.33% survival) and 40% (60%) were observed for *Bacillus subtilis* and *Pseudomonas putida* respectively on exposure to UV- irradiation. The relative efficiency of hydrocarbon degradation by the wild and mutant strains was assessed by evaluating the Hydrocarbon utilizing bacterial (HUB) counts and changes in concentration of oil and grease at

intervals of 3 days for 15 days. The UV-mutant strains had slightly higher HUB counts (2.73×10^8, 1.5×10^8 and 2.391×10^8) than the wild strains (2.73×10^8, 1.32×10^8 and 2.39×10^8) while the nitrous acid mutant strains had the lowest HUB counts (8.35×10^7, 8.13×10^7 and 5.07×10^7) for *P. putida, B. subtilis* and their co-culture respectively. Also, higher oil and grease degradation was observed in the UV-mutant strains with 96.23%, 92.60% and 99.38%, followed by the wild strains with 87.25%, 80.25% and 88.89% and the nitrous acid mutant strains with 80.25%, 68.52% and 81.48% oil and grease degradation for *P. putida, B. subtilis* and their co-culture respectively. It was therefore concluded that while wild strains of *B. subtilis* and *P. putida* are good hydrocarbon degrading agents, irradiation with UV results in increased efficiency and the co-culture of the bacteria were more efficient than the individual culture.

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List of Abbreviations, Glossary and Symbols

P. putida = *Pseudomonas putida*

B. subtilis= *Bacillus subtilis*

PAH = Polycyclic Aromatic Hydrocarbons

BTEX = Benzene, Toluene, Ethyl-benzene and Xylene

NAPL = Non-Aqueous Phase Liquid

BOD = Biological Oxygen Demand

COD = Chemical Oxygen Demand

DO = Dissolved Oxygen

TSS = Total Suspended Solids

TDS = Total Dissolved Solids

NTU = Nephelometric Turbidity Unit

PCR = Polymerase Chain Reaction

RNA = Ribonucleic Acid

DNA = Deoxyribonucleic Acid

nM = Nano Metre

µS/Cm = Micro Siemens per Centimetre

Mg/L = Milligram per Litre

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Petroleum refinery and petrochemical industries play an immense role in national development and improved quality of life. However, pollution effects of the wastes from these industries are causes for worry (Nwaichi *et al*., 2013).Wastewater (effluents) released from petroleum refineries are characterized by the presence of large quantities of petroleum products, polycyclic and aromatic hydrocarbons, phenols, metal derivatives, surface active substances, sulphides, naphthylenic acids and other chemicals (Musa *et al.,* 2015), most of which are known to be mutagenic, carcinogenic and growth inhibitory and by extension can have adverse effects on the ecology of the receiving sites and public health (Nwaichi *et al*., 2013).

Petroleum refinery effluents contain organics and have a characteristic oily nature hence, when discharged into water bodies cause depletion of dissolved oxygen (due to the transformation of the organic components into inorganic compounds by microorganisms), prevent reaeration thereby causing the loss of biodiversity through a decrease in amphipod population that is important in food chain and eutrophication (Nwaichi *et al*., 2013).

Effluents are usually treated before discharge into the receiving sites (Otunkune and Obiukwu, 2005). But due to inefficient treatment systems, the pollutants may not be completely eliminated, consequently polluting water bodies, soils and underground water with potentially serious consequences on the ecosystem (Beg *et al*., 2001; Bay *et al*., 2003; Otunkunefor and Obiukwu, 2005). Though the compositions of effluents are

set by various regulatory agencies, compliance with the legally set toxicant levels for refineries and petrochemical plants in Nigeria has always been very low (Obot *et al.*, 2007). Furthermore, the impact of these toxicants on the quality of the effluent receiving water bodies and soils is rarely investigated (Otunkune and Obiukwu, 2005).

The high demand for petroleum as a source of energy and as a primary raw material for chemical industries in recent years has resulted in an increase in its production worldwide. This dramatic increase in production, refining and distribution of crude oil has brought with it an ever increasing problem of environmental pollution (Ojo, 2006). However, the persistence of petroleum pollution depends on the quantity and characteristics of the hydrocarbon mixture and on the properties of the affected ecosystem (Darsa *et al.*, 2014).

Several techniques including physical, chemical and biological techniques have been developed to resolve the problem of petroleum pollution (Darsa *et al.,* 2014). However, the most promising approach so far is microbial degradation since the physico-chemical methods are rather too expensive, not environmentally friendly and does not result in total elimination of the pollutant (Umanu *et al.,* 2013). Microbial degradation (biodegradation) involves the use of microbes to break down potentially harmful pollutants into harmless products (Hamza *et al.*, 2012). Hence, the ability to isolate high numbers of certain microorganisms from petroleum polluted environment is commonly taken as evidence that such microorganisms are active petroleum degraders (Okerentugba and Ezeronye, 2003). Several researchers have recorded successes in the use of bacteria especially members of the genus *Bacillus* and *Pseudomonas* in degradation of petroleum hydrocarbon (Das and Chandran 2011; Prakash *et al.*, 2014; Adams *et al.*, 2015; Vinothini *et al.*, 2015).

Improvement in the ability of microorganisms to degrade a pollutant could be achieved through modification of the environment or the organism. The organism can be modified through mutagenesis. Various mutagens abound and the exposure of organisms to ultraviolet (UV)-light and treatment with nitrous acid has been employed with relative successes. Such mutants, under optimal growth conditions, could possess enhanced petroleum degradation potential than their parents (Idise *et al.,* 2010).

1.2 Statement of Research Problem

Liquid wastes generated by refineries consists of chemical components such as oil and grease, phenols, Benzene, Toluene, Ethyl benzene and Xylene (BTEX), ammonia, suspended solids, cyanide, sulphide, nitrogen compounds and heavy metals such as Iron (Fe), Cadmium (Cd), Nickel (Ni), Chromium (Cr), Copper (Cu), Molybdenum (Mo), Selenium (S), Vanadium (V) and Zinc (Zn) most of which are known to be toxic (Oaikhena *et al*., 2016).

It has been reported that adherence with legally set toxicant levels for effluents by refineries and petrochemical plants in Nigeria has always been very low(Obot *et al*., 2007). This is because; most of the methods employed for the treatment of wastewater from such plants have been shown to be inefficient. As such, wastewaters may still pose serious dangers, leading to the accumulation of toxic materials at the receiving sites (i.e. bodies of water and soils) with far reaching consequences on the ecosystem (Beg *et al.,* 2001; Bay *et al*., 2003).

The pollutants (hydrocarbons) persist in the environment as the resident microorganisms handle or remedy the problem very slowly.

Such polluted habitats lose their capability to support both plant and animal life and thus constitute public health and socioeconomic hazards (Okerentugba and Ezeronye,

2003). The organic content of the effluent exerts a very high level of biological oxygen demand while its oily nature prevents reaeration of contaminated water bodies. Thus such contaminated water bodies could be rendered anaerobic resulting in massive fish kill and loss of many aquatic lives thereby distorting the natural equilibrium that exists in such aquatic environment. In the same vein, discharge of such effluent into the soil, could drastically alter the physicochemical properties of the soil thereby reducing the productivity of the soil (Salleh *et al.,* 2003; Kayode-Ishola *et al*., 2008; Kiran, 2013; Stanley *et al*., 2015).

1.3 Justification for the Research

The physicochemical methods used to remove hydrocarbon pollutants in general are too complex, expensive, frequently resulting in the introduction of new toxic substances into the environment and seldom ensure total elimination of the pollutants**.** There is therefore, the need to develop an efficient, cost effective, sustainable and eco-friendly approach for removing hydrocarbon from refinery effluent before discharge into receiving sites.

There are several reports to the effect that wild strains of *Bacillus* spand *Pseudomonas* sp are very active in the degradation of petroleum hydrocarbons. But other bacteria capable of utilizing hydrocarbon as the only source of carbon and energy thereby degrading it to harmless products have been reported. However, dependence on the capacity of the wild strains in clean-up processes has been shown to require an extended period of time. There is therefore, the need to conduct investigations with the view to augmenting the degradative capacity of wild strains for faster removal of petroleum contaminants from polluted sites.

It is very necessary to develop an effective means of degrading hydrocarbons in effluents generated from indigenous refineries and petrochemical plants as compliance with the legally set toxicant levels for refineries and petrochemical plants in Nigeria has always been very low.

1.4 Aim and Objectives

1.4.1 Aim

The aim of this study was to assess the capacity of wild and mutant strains of *Bacillus subtilis* and *Pseudomonas putida* isolated from refinery effluent in the degradation of hydrocarbons.

1.4.2 Objectives

The specific objectives of this study were to:

1. determine the physicochemical properties of the raw and treated refinery effluent.
2. isolate and characterize *Bacillus subtilis* and *Pseudomonas putida* from the raw effluent using conventional methods.
3. screen the isolates for capacity to degrade hydrocarbons.
4. generate mutants from the wild isolates using ultraviolet (UV) light and nitrous acid as mutagens.
5. determine the relative efficiency of selected mutant and wild strains in the degradation of hydrocarbons.

CHAPTER TWO

2.0 LITERATURE REVIEW

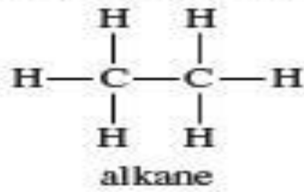
2.1 Petroleum Hydrocarbons

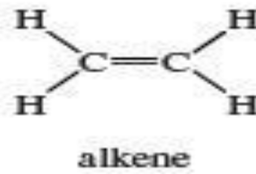
Petroleum hydrocarbons consist of a very large number of compounds that, by definition, are found in crude oil as well as other sources of petroleum such as natural gas, coal and peat (Hentati *et al*., 2013). However, the primary source of hydrocarbon ispetroleum (crude oil) which is a complex, thick, black liquid found in the earth (Okafor, 2007; Olajire and Essien, 2014). Petroleum contains a large number of distinctively different chemicals and usually composed of four main fractions, which includes saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes (Hasanuzzaman *et al*., 2007). On a more specific note, it is composed almost entirely of a mixture of alkanes with some alkenes and smaller amounts of aromatic hydrocarbons (Mbaneme *et al*., 2012).

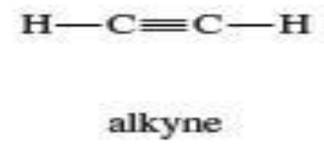
Hydrocarbons are a group of heterogenous organic substances that are primarily composed of carbon and hydrogen molecules (Salleh *et al.,* 2003). They are quite abundant in modern society. Some of the most commonly used hydrocarbons include gasoline, lubricating oil, motor oil, mineral spirits, lighter fluid/naphtha, lamp oil, and kerosene (Bronstein *et al*., 2007).

Structures of representative hydrocarbons

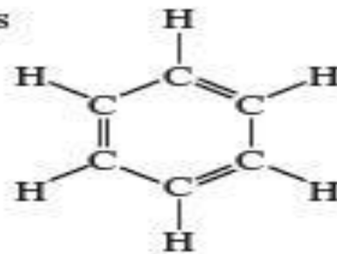
aliphatic hydrocarbons







aromatic hydrocarbons



Unimke *et al*. (2018)

Figure 2.1 Structures of Representative Hydrocarbons

Petroleum is composed of both aliphatic and aromatic compounds. Aliphatic chemical compounds belong to the class of organic compounds in which the atoms are not linked together to form a ring (Van-Hamme *et al.,* 2003; Cao *et al.,* 2009). They are one of the major structural groups of organic molecules and include the alkanes, alkenes and alkynes, and substances derived from them actually or in principle by replacing one or more hydrogen atoms by atoms of other elements or groups of atoms. They are acyclic and non-aromatic carbon compounds (Uzoekwe and Oghosanine, 2011). Thus, aliphatic compounds are opposite to aromatic compounds. In aliphatic compounds, carbon atoms can be joined together in straight chains, branched chains, or non aromatic rings (in which case they are called alicylic). Aliphatic compounds can be saturated (paraffins), joined by single bonds (alkanes), or unsaturated (oleofins), with double bonds (alkenes) or triple bonds (alkynes). Besides hydrogen, other elements can be bound to the carbon chain, the most common being oxygen, nitrogen, sulfur, and chlorine. The simplest aliphatic compound is methane (CH_4) (Hui, 2011).

Aromatics hydrocarbons consist of benzene rings that have multiple double bonds. The term polycyclic aromatic hydrocarbons (PAHs) refer to hydrocarbon with more than one benzene ring. PAHs are formed by incomplete combustion of organic substances. They are highly toxic to organisms due to their carcinogenic and mutagenic potential (Pizzul *et al.,* 2006; Von *et al*., 2014). They are a group of compounds containing carbon and hydrogen composed of two or more fused aromatic rings in linear, angular and cluster arrangements. They are lipophilic in nature and relatively insoluble in water (Johnsen *et al.,* 2005; Kumar *et al.,* 2010).

PAHs are ubiquitous pollutants and are generated from anthropogenic activities such as the burning of fossil fuels, the use of wood preservatives such as creosote and the generation of wastes from coal gasification plants (Ni-Chadhain *et al.,* 2006; Zafra *et al*., 2014). They have been identified as hazardous chemicals by different State and Central Pollution Control Boards because of their toxic, carcinogenic and teratogenic effects on living matter (Ruma *et al.*, 2007; Von *et al*., 2014).

PAHs occur as colourless, white or pale yellow solids with low solubility in water, high melting and boiling points, and low vapour pressure. With an increase in molecular weight, their water solubility and vapour pressure decrease; melting and boiling point increases (Haritash and Kaushik, 2009; Hui, 2011). They are persistent and toxic soil contaminants. Pollution by PAHs is usually found on the sites of gas factories and wood preservation plants.

2.1.1 Petroleum refinery effluent

While petroleum refinery and petrochemical industries play an immense role in national development and improved quality of life, the unwholesome and environmentally unacceptable pollution effects of the waste from these industries are causes for worry (Nwaichi *et al*., 2013). This is because wastes of different kinds are generated during the conversion of crude oil into petroleum products (liquefied petroleum gas, naphtha, kerosene, diesel oil and residual oil) and petrochemical products (polypropylene, polyethylene) (Nwaichi *et al*., 2013). Such wastes can be broadly categorized into oily materials, spent chemicals, spent catalyst and other residuals. These wastes are released to the environment in the form of gases, particles, and liquid effluent (liquid consisting of surface runoff water, sanitary wastewater, solid waste and sludge) (Wong and Hung, 2005).

The waste water released from the petroleum refineries and petrochemical plants (known as effluents) are characterized by the presence of large quantity of crude oil products, polycyclic and aromatic hydrocarbons, phenols, metal derivatives, surface active substances, sulfides, naphthalene acids and other chemicals (Musa *et* al., 2015).

2.1.2 Petroleum hydrocarbon pollution.

Hydrocarbon compounds such as petroleum are essential elements of life. But since they do not naturally occur in the forms most useful to humans, they can be hazardous (Mehrasbi *et al.,* 2003).Nigeria has a vast crude oil and gas deposits and attempts to explore it have left the country with unique vulnerabilities (Nduka and Orisakwe, 2009).The growing concern in recent years with pollution of the soil and aquatic environments by petroleum and petroleum products has resulted in a number of studies designed to provide information on the distribution of naturally occurring hydrocarbons in soils and aquatic environments that serve as the receiving sites and to gain some understanding of possible environmental effects of increasing inputs of petroleum hydrocarbons (Bronstein *et al*., 2007; Unimke *et al*., 2018).

As a result of ineffectiveness of purification systems employed in most petroleum refineries and petrochemical plants in developing nations (Nigeria inclusive), the waste waters generated become seriously polluted, leading to the accumulation of toxic products in the receiving sites with potentially serious consequences on the ecosystem (Beg *et al*, 2003; Aghalino and Eyinla, 2009). Kuehn *et al*. (1995) observed that effluent contaminated with aromatic hydrocarbons produces poor health and lethal toxicity in fishes and other aquatic animals. And earlier, Onwumere and Oladimeji (1990) demonstrated accumulation of heavy metals with accompanying histopathology in

Oreochromnis niloticus exposed to treated petroleum refinery effluent from the Kaduna refining and petrochemical company.

Nigeria is regarded as the greatest gas flaring country in the world, as a result, great amounts of carbon dioxide, sulphur dioxide and nitrous oxides are released into the atmosphere which dissolves in rain water to produce toxic acid rain with resultant damage to vegetation, soil and aquatic life (Atubi, 2009; Atubi, 2011).

Also, oil prospecting in Nigeria has brought with it untold hardship to the environment. The frequency of oil spill and untreated waste water discharge into water bodies and the attendant negative impact on the aquatic and terrestrial ecosystem is well known and well documented over the years (Nduka and Orisakwe, 2009; Prakash *et al*., 2014; Adams *et al*., 2015). It has been demonstrated that oil spillage affects the physical and chemical nature of soils (Minai-Tehran and Herfatmanesh, 2007).

The economic and environmental impacts of oil pollution on soil are enormous, causing serious damage to vegetation, soil fertidlity (Nwachukwu and Ugorji, 1995) and soil-borne microorganisms; the toxicity varies depending on the type of oil and additives used during refining and also on the biota of spillage (Reddy and Mathew, 2001). Shamiyan *et al.* (2013) demonstrated the presence of high concentrations of hydrocarbons in aged gasoline contaminated soil with adverse effect on the soil physicochemical properties and fungal population. Also, the presence of pollutants in natural waters alters the quality and often poses serious threats to aquatic life. In line with this, Odinga *et al.* (2015) reported the presence of polycyclic aromatic hydrocarbons in the effluent generated from Port Harcourt Refining Company and an investigation of the effluent on the renal function of albino rats showed a significant increase in the level of the renal markers. Also, various studies have shown positive

correlation between pollutions from petrochemical and refinery effluents and the health of aquatic organisms (Otukunefor and Obiukwu, 2005; Prakash *et al*., 2014; Adams *et al*., 2015). Anaero-Nweke (2016) reported that petroleum refinery effluent had negative impacts on Ekirikana creeks and its adjourning river water qualities as the phytoplankton and benthic organism distribution and abundance of the creeks and its adjourning river water were very poor. Also, it was reported by Otukunefor and Obiukwu (2005) that refinery effluent discharged into the Okirika arm of the Bonny River estuary resulted in the presence of high concentrations of pollutants in the water and sediment. The toxicants were shown to be present in concentrations which may be toxic individually to different aquatic organisms. More so,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*).*

Though petroleum has played an important role in the economy of Nigeria, over the past three decades, the environment (especially the Niger Delta ecosystem) has been subjected to degradation by petroleum and petroleum product spillage and other effluents resulting from operational activities (Adeniyi and Afolabi, 2002). With increase in processed petroleum products (effluents) that are discharged with little regard to aquatic environment, the contents of the effluents exert serious toxicological effects on aquatic environment and humans. Refinery effluent containing oil when discharged into water body can cause depletion of dissolved oxygen due to transformation of organic components into inorganic compounds, loss of biodiversity through a decrease in amphipod population that is important in food chain and eutrophication. Short term toxicity in fishes includes lymphocytosis, epidermal hyperplasia and hemorrhagic septicaemia (Onwurah and Ogugua, 2007; Anaero-Nweke, 2016).

Though, data on ecological risk assessment of effluent discharge in aquatic environment especially environmentally stressed Niger Delta region of Nigeria is scanty, the quality of refinery effluent and its impact on physico-chemical quality of Okrika arm of the Bonny River estuary has been given (Otokunefor and Obiukuwu, 2005). According to Nduka and Orisakwe (2009), dwellers of oil producing areas generally suffer from scarcity of farmlands as their lands have been made unproductive due to constant oil spillages and waste dump. One of the most notable consequences of gross hydrocarbon pollutions had been the loss of mangrove trees. The mangrove was a source of both fuels for the indigenous people and a habitat for the area's biodiversity, but is now unable to be sourced due to the oil toxicity of its habitat. Hence, hydrocarbon pollution poses serious health risks to people when they consume contaminated seafood, as they become accumulated in the tissues of these sea foods (Onuoha, 2007).

Oil in the aquatic environment may be damaging in a variety of ways. These may involve changes in the composition of aquatic communities that affect their ability to survive, permanent damage and, in some cases, massive mortalities. Odour, taste and colour are present in oil polluted water. Oil pollution of water also constitutes a potential health risk to humans who use water for domestic and drinking purposes and consume fish found therein (Helmer, 2006; Atubi, 2009; Anaero-Nweke, 2016).

2.1.3 Effect of petroleum hydrocarbons on living matter.

The uncontrolled disposal of waste into water renders water unsafe for economic use, recreational use and poses a threat to human life and negates the principle of sustainable development. Water borne diseases and water caused health problems are mostly due to incompetent management of water resources. Safe water for all can only be assured when access, sustainability and equality can be guaranteed. Urban areas generally have

a higher coverage of safe water than rural areas (Atubi, 2009). Even within the urban area, there are variations in the quality of water as much of the water get contaminated in many different ways, through industrial effluent and untreated municipal sewage (Oluwande *et al*, 1993; Atubi, 2009; Isife, 2016).

Petroleum refinery effluents pose a serious problem to both aquatic and human life form. Drinking contaminated water can cause various diseases and other health complications (Adeyemi, 2004; Atubi 2011). According to Gore (1993), human beings are made up of water, in roughly the same percentage as water in the surface of the earth. Our tissues and membranes, brains, and hearts, our sweat and tears, all reflect the same recipe for life. Water is essential for the development and maintenance of the dynamics of every ramification of the society (UNDP, 2006). Water is indeed life and thus is the most important natural resource, without which life would be non-existent. Availability of safe and reliable source of water is an essential prerequisite for sustained development (Asonye *et al*, 2007).

Living matter is exposed to petroleum in many ways directly or indirectly. Some by products, formed during petroleum refining and processing which are used for the manufacturing of other products are highly toxic (Abha and Singh, 2012). Constantly, these toxic compounds are inadvertently released into the environment and if this effect is connected to the effect of accidental crude oil spills worldwide, then these combined sources of unrestricted hydrocarbons constitute the major cause of environmental pollution (Nilanjana and Preethy, 2010).

Despite the large number of hydrocarbons found in petroleum products, only a relatively small number of the compounds are well characterized for toxicity. Petroleum hydrocarbon molecules, which have a wide distribution of molecular weights and

boiling points, cause diverse levels of toxicity to the environment. The toxicity of the hydrocarbon molecules and their availability for microbial metabolism depend on their chemical and physical nature. Petroleum is toxic and can be lethal depending upon the nature of the petroleum fraction, the way of exposure to it, and the time of exposure (Singh *et al.,* 2004). Chemicals and dispersants in crude oil can cause a wide range of health effects in people and wildlife, depending on the level of exposure and susceptibility. The highly toxic chemicals contained in crude oil can damage any organ system in the human body like the nervous system, respiratory system, circulatory system, immune system, reproductive system, sensory system, endocrine system, liver, kidney, etc. and consequently can cause a wide range of diseases and disorders (Abha and Singh, 2012). The damage caused by the toxicity of crude oil to organ systems may be immediate or it may take months or years.

Singh *et al*. (2004) studied the toxicity of fuels with different chemical composition on CD-1 mice (A Swiss mice strain that is used as a general purpose stock and an oncological and pharmaceutical research). The objective of the study was to establish a correlation between the physico- chemical properties of the fuel and their biologic effects on mice. The results of the study demonstrated that the automobile derived diesel exhaust particles were more toxic than the exhaust generated by forklift engines. It was also found that the diesel exhaust particles contain ten times more extractable organic matter than the standard exhaust material generated by forklift engines. A similar type of study conducted by Kinawy (2009) revealed that the inhalation of leaded or unleaded (containing aromatics and oxygenated compounds) gasoline vapours by rats impaired the levels of monoamine neurotransmitters and other biochemical parameters in different areas of the rats' brains. Likewise, several behavioural changes causing aggression in rats were observed. Menkes and Fawcett (1997) discussed the toxicities of

lead and manganese added gasoline and the public health hazards due to aromatic and oxygenated compounds in gasoline. The extent of absorption of petroleum components by inhalation, oral, and dermal routes varies significantly because of the wide range of physicochemical properties of these components.

The incorporation of crude oil into the body may affect the reproductive health of humans and to other lives. Obidike *et al*. (2007) observed that when male rats were given an oral crude oil treatment using a drenching tube, degeneration and necrosis of interstitial cell occurred followed by the exudation into the interstices in the testes of rats. The study concluded that exposure of rats to crude oil induces reproductive cytotoxicity confined to the differentiating spermatogonia compartment; likewise it may also harm human reproductive cells. The extent of absorption through the various routes depends on the volatility, solubility, and other properties of the specific component or mixture. The more volatile and soluble the oil fractions (low molecular weight aliphatics and light aromatic compounds) are the faster they can leak into groundwater or vaporize into the air. Therefore, living matter may be easily exposed to these crude oil fractions by breathing the contaminated air and by drinking the contaminated water (Abha and Singh, 2013). As reported by Knox and Gilman (1997), petroleum derived volatiles are one of the causes for the geographically associated childhood cancers.

On the other hand, the non-volatile heavy fractions of crude oil tend to be absorbed by the soil and persist at the site of release, which may harm living beings by skin contact, by intake of contaminated water or food. The heavy fraction of crude oil consists mainly of napthene-aromatics and poly-aromatic compounds that are carcinogenic and long exposure to these compounds often leads to tumours, cancer, and failure of the nervous system (Von *et al*., 2014). The aromatic compounds found in petroleum are an

important group of environmental pollutants. These aromatic compounds are introduced into the environment from various sources such as natural oil seeps, refinery waste products and emissions, oil storage wastes, accidental spills from oil tankers, petrochemical industrial effluents and emissions, and coal tar processing wastes, etc. Petroleum hydrocarbons can rapidly migrate from the site of contamination and adversely affect terrestrial and aquatic ecosystems and humans. Crude oils also contain polar organic compounds that contain N, S, and O atoms in various functional groups. The chemical properties of these NSO compounds, particularly their solubility and toxicity, are of environmental concern. It has been documented, that at the sites where oil spills have occurred, a portion of the polar organic compounds present in the oil had partitioned into the groundwater rendering high concentration of total petroleum hydrocarbons in drinkable water sources. Therefore, drinkable water sources turn out to be unsafe for the human population as well as for the aquatic animals, aquatic plants, and microbes (Abha and Singh, 2013). Fortunately a diverse group of microorganisms like bacteria, fungi, and yeasts may efficiently breakdown crude oil fractions into non-toxic components. There are a large number of studies available on the microbial degradation of crude oil or hydrocarbons.

2.2 Effects of some physico-chemical parameters on water quality and Aquatic life

2.2.1 pH

Most freshwater lakes, streams, and ponds have a normal pH in the range of 6 to 8. Acid deposition has many harmful ecological effects when the pH of most aquatic systems falls below 6 and especially below 5 (Lenntech, 2013). When the pH of an aquatic system approaches 5, certain undesirable species of plankton may proliferate and begin to invade, and the populations of fish such as smallmouth may be eliminated. Below a pH of 5, fish populations begin to disappear, the bottom is covered with undecayed

material, and mosses may dominate near shore areas and at a pH below 4.5, the water is essentially devoid of fish. Aluminium ions (Al^{3+}) attached to minerals in nearby soil can be released into lakes, where they can kill many kinds of fish by stimulating excessive mucus formation. This suffocates the fish by clogging their gills. It can also cause chronic stress that may not kill individual fish, but leads to lower body weight and smaller and makes fish less able to compete for food and habitat (Lenntech, 2013; Ozigis *et al*., 2018).

The most serious chronic effect of increased acidity in surface waters appears to be interference with the fish reproductive cycle. Calcium levels in the female fish may be lowered to the point where she cannot produce eggs or the eggs fail to pass from the ovaries or if fertilized, the eggs and/or larvae develop abnormally (Anaero-Nkwelle, 2013). Extreme pH can kill adult fish and invertebrate life directly and can also damage developing juvenile fish. Water pH level may cause the stripping of a fish of its slime coat and high pH level chaps the skin of fish because of its alkalinity. When the pH of freshwater becomes highly alkaline (e.g. pH 9.6), the effects on fish may include: death, damage to outer surfaces like gills, eyes, and skin and an inability to dispose of metabolic wastes. High pH may also increase the toxicity of other substances. For example, the toxicity of ammonia is ten times more at a pH of 8 than it is at pH 7. It is directly toxic to aquatic life when it appears in alkaline conditions. Low concentration of ammonia is generally permitted for discharge (Okerentugba and Ezeronye, 2003).

2.2.2 Temperature

Aquatic life is greatly affected by changes in temperature. Temperature is one of the factors that determines which organism will thrive and which will diminish in numbers and size. This is because temperature controls the metabolic activities of organisms and

for each organism; there is a thermal death point and an optimum temperature for survival. Poikilotherms, such as fish, are greatly affected by temperature not only in their survival, but growth and reproduction (Science Fair Water, 2013a).

High and low temperatures that are lethal to individual organism of a species determine the distribution and abundance of its population. However, more often the distribution and abundance of populations is determined by less than lethal temperatures interacting with other environmental factors that either tend to favour or not to favour reproduction and growth. Increased water temperature is an important consideration when toxic substances are present in water. Many substances (i.e. cyanides, phenol, xylene, zinc) exhibit increased toxicity at elevated temperatures. These toxicities and other physiological interactions are also influenced by temperature acclimation or history of the species. High temperature of receiving water has the following effects: Higher temperature diminishes the solubility of dissolved oxygen and thus decreases the availability of this essential gas Elevated temperatures increase the metabolism, respiration and oxygen demand of fish and other aquatic life, approximately doubling the respiration for each 10° C. rise in temperature. Hence the demand for oxygen is increased under conditions where oxygen supply is lowered (Abdel-Shafy and Mansour, 2016). The solubility of many toxic substances is increased as well as intensified as the temperature rises, higher temperatures militate against desirable fish life by favouring the growth of sewage fungus and the purification of sludge deposits, and finally, even with adequate dissolved oxygen, there is a maximum temperature that each species of fish or other organism can tolerate (Abdel-Shafy and Mansour, 2016). Higher temperatures result in death of organisms. The maximum temperatures that adult fish can tolerate vary with the species of fish, prior acclimatization, oxygen availability and the synergistic effects of other pollutants (Eunice *et al.*, 2017).

2.2.3 Total dissolved solids

Total Dissolved Solid (TDS) is a measurement of inorganic salts, organic matter and other dissolved materials in water. Water with total dissolved solids concentrations greater than 1000 mg/l is considered to be "brackish". Total dissolved solids cause toxicity through increases in salinity, changes in the ionic composition of the water and toxicity of individual ions. Increases in salinity have been shown to cause shifts in biotic communities, limit biodiversity, exclude less-tolerant species and cause acute or chronic effects at specific life stages (Eunice *et al*., 2017). Phylis and Lawrence (2007) found a significant and negative correlation between concentrations of chlorophyll-A (an estimate of primary production) and concentrations of Na^{+}, Mg^{2+}, SO_4^{2+}, HCO^{3-} and CO_3^{2-} and also reported substantial changes in marsh communities. When TDS increased from 270 to 1170 mg/l, both coontail(*Ceratophyllum demersum*) and cattails (*Typha* spp.) were nearly eliminated. Salinity and aquatic biodiversity are inversely related in lake water. Changes in the ionic composition of water can exclude some species while promoting population growth of others (Phylis and Lawrence, 2007).

2.2.4 Total suspended solids

The term suspended solids refers to the mass (mg) or concentration (mg) of inorganic and organic matter, which is held in the water column of a stream, river, lake or reservoir by turbulence. Total suspended solids are typically comprised of fine particulate matter with a diameter of less than 62 mm, though for the majority of cohesive solids, research has demonstrated that transport frequently occurs in the form of larger aggregated flocs (Anaero-Nkwelle, 2013). All streams carry some suspended solids under natural conditions. However, if concentrations are enhanced through, for example, anthropogenic perturbations, this can lead to alterations to the physical, chemical and biological properties of the water body. Physical alterations caused by

suspended solids (SS) include reduced penetration of light, temperature changes, and infilling of channels and reservoirs when solids are deposited. These physical alterations are associated with undesirable aesthetic effects such as; higher costs of water treatment, reduced navigability of channels and decreased longevity of dams and reservoirs (Bilotta and Brazier, 2008). Chemical alterations caused by Suspended solids include the release of contaminants, such as heavy metals and pesticides, and nutrients such as phosphorus, into the water body from adsorption sites on the sediment. Furthermore, where the suspended solids have a high organic content; their in-situ decomposition can deplete levels of dissolved oxygen in the water, producing a critical oxygen shortage which can lead to fish kills during low-flow conditions (Okerentugba and Ezeronye, 2003).

2.2.5 Turbidity

Turbidity is a measurement of how cloudy water appears. Technically, it is a measure of how much light passes through water, and it is caused by suspended solid particles that scatter light. These particles may be microscopic plankton, stirred up sediment or organic materials, eroded soil, clay, silt, sand, industrial waste, or sewage. Bottom sediment may be stirred up by such actions as waves or currents, bottom-feeding fish, people swimming, or wading, or storm runoff (Science Fair Water, 2013b). Clear water may appear cleaner than turbid water, but it is not necessarily healthier. Water may be clear because it has too little dissolved oxygen, too much acidity or too many contaminants to support aquatic life. Water that is turbid from plankton has both the food and oxygen to support fish and plant life.

However, high turbidity may be a symptom of other water quality problems. Turbidity however has the following effects on water bodies: Turbidity diffuses sunlight and

slows photosynthesis. Plants begin to die, reducing the amount of dissolved oxygen and increasing the acidity (decaying organic material produces carbonic acid, which lowers the pH level). Both of these effects harm aquatic animals (Eunice *et al*., 2017). Turbidity raises water temperature because the suspended particles absorb the sun's heat. Warmer water holds less oxygen, thus increasing, the effects of reduced photosynthesis. In addition, some aquatic animals may not adjust well to the warmer water, particularly during the egg and larval stages (Okerentugba and Ezeronye, 2003; Anaero-Nkwelle, 2013).

. Highly turbid water can clog the gills of fish, stunt their growth, and decrease their resistance to diseases (Abdel-Shafy and Mansour, 2016). The organic materials that may cause turbidity can also serve as breeding grounds for pathogenic bacteria (Eunice *et al*., 2017). When drinking water reservoirs are turbid, the water treatment plant usually filters the water before disinfecting it. Industrial processes and food processing, require clear water (Science Fair water, 2013b).

2.2.6 Conductivity

Conductivity is a measure of the capability of a solution such as water in a stream to pass an electric current. This is an indicator of the concentration of dissolved electrolyte ions in the water. It does not identify the specific ions in the water. However significant increases in conductivity may be an indication that pollution discharges have entered the water. Every creek will have baseline conductivity depending on the local geology and soils. Higher conductivity will result from the presence of various ions including nitrate, phosphate and sodium (Anaero-Nweke, 2013).

2.2.7 Biological oxygen demand

This is the amount of oxygen that would be consumed if all the organic materials in a water sample were oxidized by microorganisms (Food Science and Technology, 2012). Micro-organisms such as bacteria/ protozoa are responsible for decomposing organic matters (Anaero-Nkwelle, 2013). When organic matter such as plant material (leaves, dried grass) sewage or even food waste is present in a water supply, the bacteria starts to break down this waste. In this process much of the available dissolved oxygen is consumed by aerobic bacteria, reducing the requisite amount of oxygen for the other aquatic organisms to live (Food Science and Technology, 2012). It is the most commonly used parameter for determining the oxygen demand on the receiving water of a municipal or industrial discharge. BOD can also be used to evaluate the efficiency of treatment processes, and is an indirect measure of biodegradable organic compounds in water. If there is a large quantity of organic waste in the water supply, there will also be a lot of bacteria present working to decompose this waste through oxidation. In this case, the demand for oxygen will be high to fulfil the demand for oxidation process and aquatic organisms, so the BOD level will be high. As the waste is dispersed through the water, BOD levels will begin to decline (Marcus and Ekpete, 2014).

Phosphates and nitrates are plant nutrients and can help the plant life and algae to grow quickly. Phosphates and nitrates in a body of water can contribute to high BOD levels. Because, when plants grow quickly, they also die quickly. This contributes to the organic waste in the water, which is then decomposed by bacteria. This results in a high BOD level. When BOD levels are high, dissolved oxygen (DO) levels decrease because the demand for oxygen by the bacteria is high as dissolved oxygen in the water. If there is no organic waste present in the water, there would not be as many bacteria present to

decompose it and thus the BOD will tend to be lower and the DO level will tend to be higher (Marcus and Ekpete, 2014; Abdel-Shafy and Mansour, 2016).

At high BOD levels, organisms such as macro invertebrates that are more tolerant of lower dissolved oxygen (i.e. leeches and sludge worms) may appear and become numerous. Organisms that need higher oxygen levels (i.e. caddis fly larvae and mayfly nymphs) may not survive. If increased levels of BOD lower the concentration of DO in a water body, there is a potential for effects on the water body itself, and the aquatic life. When the dissolved oxygen concentration falls below 5 mg/L, species intolerant of low oxygen levels become stressed. Eventually, species sensitive to low dissolved oxygen levels are replaced by species that are more tolerant of adverse conditions, significantly reducing the diversity of aquatic life in a given body of water (Eunice *et al*., 2017). If dissolved oxygen levels fall below 2 mg/L for more than even a few hours, fish kills can result. At levels below 1 mg/L, anaerobic bacteria replace the aerobic bacteria. As the anaerobic bacteria break down organic matter, foul smelling hydrogen sulfide can be produced (Anaero-Nkwelle, 2013).

2.2.8 Oil and grease

The oil in the refinery effluent can affect marine organisms in a number of different ways. Animals coated by even small amounts of oil may be unable to swim or fly properly, maintain their body temperature, feed or even reproduce. Oil can also cover beaches and other vital habitats, making it difficult for animals to find uncontaminated food, nesting and resting places (Anaero-Nweke, 2013). Some animals are more vulnerable to oil than others. For example, young may be less able to deal with either coatings or exposure to toxic substances than adults due to their size, underdeveloped immune systems and behaviour’s. Marine mammals, seabirds (especially penguins) and

sea turtles are all particularly vulnerable to oil on surface waters as they spend considerable amounts of time on the surface feeding, breathing and resting (Marcus and Ekpete, 2014). Turtles and marine mammals are vulnerable to floating oil at all life stages as they do not appear to avoid oil slicks and they must inhale large amounts of air prior to diving. Turtles also feed in convergence zones, areas where air flows and currents meet, which tend to collect floating oil. It can kill them directly through coating and asphyxiation, contact poisoning, or through exposure to water-soluble components. It can also cause the destruction of more sensitive juveniles or of the food organisms therefore wiping out a population. Oil is capable of causing sub-lethal and stress effects, carcinogenic and mutagenic effects and can affect the behaviour of individuals (Eunice *et al*., 2017).

2.2.9 Nitrates and phosphates

Nitrates and phosphates in water bodies may have considerable effect on the water quality. Nitrogen in its various forms is considered to be the limiting nutrient in marine water. Therefore an increase in nitrogen compound should lead to phytoplankton blooms and when blooms occur, water conditions (such as reduced water clarity and dissolved oxygen) may become unfavourable for aquatic organisms. Inorganic phosphates are also rapidly taken up by algae and other aquatic plants although phosphates are usually not the limiting nutrient in marine waters. Ecological alterations result from biotic response to nutrient inputs, most noticeable are the massive algal blooms (Eunice *et al*., 2017; Ozigis *et al*., 2018), that at times the algae are known to cover heavily 50% of the pelagic surface.

An international environmental research centre (Royal Swedish Academy of Sciences) through satellite remote-sensing technology has detected an important source of nutrient that is killing Lake Victoria, the world’s largest fresh water lake. It reported that these

nutrients are feeding a carpet of water hyacinth that is rapidly choking the life out of the lake. Also a Kenyan-based international centre for research in Agro-forestry discovered that the satellite imagery had discovered a plume of colouring water. The plume was showing a flow of sediment causing eutrophication (the process by which a body of water become enriched in dissolved nutrients that stimulate the growth of aquatic plants), the satellite imagery also showed that the nutrients were not coming solely from agricultural runoff as previously suspected, but also largely from low-lying deforestation riparian zones and other areas surrounding the lake that are not in private hands. The discovery further stated that the over supplies of nutrients and untreated sewage may have led to massive fish die-off, toxic algae blooms and the rampant spread of the aggressive flotation of weed water hyacinth. The hyacinth starves fish and plantation of oxygen and sunlight and also reduces the diversities of important aquatic plants. The hyacinth is also blocking water-ways traffic; it also causes water to stagnate, making the chocked shoreline a breeding ground for mosquitoes (Marcus and Ekpete, 2014; Ozigis *et al*., 2018).

2.2.10 Sulphides

Hydrogen sulfide (H_2S) results from septic conditions during the collection and treatment of wastewater. Hydrogen sulfide has long been recognized as a major problem for municipal and industrial wastewater systems. This colourless gas, known for its rotten egg smell, is produced by the biological reduction of sulphates and the decomposition of organic material. It forms at virtually every point in a system from interceptors, force mains, and lift stations, to holding tanks, mechanical dewatering equipment and drying beds (Mirzoyan and Schreier, 2014).

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Sulphides can be removed by certain group of bacteria (Wake, 2005) but have the opposite relationship with pH. The toxicity of sulphides increases with decreasing pH (Wake, 2005). Beyond its nuisance odour, hydrogen sulfide also poses a serious problem for the structural integrity of the collection system and has been reported to be toxic to most aquatic lives including microorganisms as it interferes with the normal metabolic processes of microorganisms except a small population of iron utilizing bacteria that possess the metabolic capacity to utilize sulphides (Anaero-Nkwelle, 2013).

Millions of dollars are lost to corrosion caused by the sulphuric acid formed from the interaction of H_2S with moisture. Of greatest concern are the safety hazards associated with H_2S. Hydrogen sulfide gas is acutely toxic and a leading cause of death among workers in sanitary sewer systems. Although disagreeably pungent at first, it quickly deadens the sense of smell and a worker may not be aware that it is there. Even at low concentrations in air, exposure to hydrogen sulfide has been linked to fatigue, headaches, eye irritation, sore throats and other health problems (Mirzoyan and Schreier, 2014).

2.3 Biodegradation and Bioremediation

Biodegradation is the process by which living organisms degrade or transform complex and hazardous organic contaminants to simple inorganic components, such as CO_2, H_2O and NO_3 (Nandal *et al.,* 2015). It employs the use of biological agents, mainly microorganisms, e.g. bacteria, fungi or yeasts to clean up contaminated soil and water by breaking down the complex compounds while obtaining nutrient and energy in the process (Hui, 2011). Biodegradation of oil is a natural process that slowly over the course of weeks, months or years, removes the oil spilled from the environment.

However, rapid removal of spilled oil from shorelines and wetlands is necessary in order to minimize potential environmental damage to these sensitive habitats (Hui, 2011).

The biodegradation of petroleum and other hydrocarbons in the environment is a complex process, the quantitative and qualitative aspects of which depend on the nature and amount of the oil or hydrocarbons present, the ambient and seasonal environmental conditions, and the composition of the autochthonous microbial community (Atlas, 1981; Leahy and Colwell, 1990). Microbial degradation of oil has been shown to occur by attack on aliphatic or light aromatic fractions of the oil, with high-molecular-weight aromatics, resins, and asphaltenes considered being recalcitrant or exhibiting only very low rates of biodegradation, although some studies have reported their removal at high rates under optimal conditions (Hui, 2011).

Biodegradation of complex hydrocarbons, particularly pollutants that are made up of many different compounds, such as crude oil or petroleum, usually requires the cooperation of more than one single species. Individual microorganisms can metabolize only a limited range of hydrocarbon substrates, so the mixed cultures with overall broad enzymatic capacities are required to increase the rate of petroleum biodegradation (Ajao *et al.*, 2013).

2.3.1 Bioremediation

The use of microbes in modern bioremediation is credited, in part, to George Robinson (US Microbics, 2003). The investigator used microbes to consume an oil spill along the coast of Santa Barbara, California in the late 1960s. Since the 1980s bioremediation of oil spills and other hazardous wastes has received more consideration (Shannon and Unterman, 1993). Bioremediation is a process which uses microorganisms and their

products to remove contaminants from the environment (USEPA, 2012). In particular, rnicroorganisms autochthonous to the polluted environments play a key role in bioremediation of such environments as biogeochemical agents to transform complex organic compounds into simple inorganic compounds or into their constituent elements. This process is termed mineralization (Adams *et al*., 2015). Bioremediation technology uses microorganisms to reduce, eliminate, contain, or transform to benign contaminants present in soils, sediments, water, and air. Bioremediation is described as the use of microorganisms to destroy or immobilize waste materials (Shanahan, 2004). This process of detoxification targets the harmful chemicals by mineralization, transformation, or alteration (Shannon and Unterman, 1993). For centuries, civilizations have used natural bioremediation in wastewater treatment, but intentional use for the reduction of hazardous wastes is a more recent development (Adams *et al*., 2015).

Bioremediation involves the production of energy in a redox reaction within microbial cells. These reactions include respiration and other biological functions needed for cell maintenance and reproduction. A delivery system that provides one or more of the following is generally required: an energy source (electron donor), an electron acceptor, and nutrients. Different types of microbial electron acceptor classes can be involved in bioremediation, such as oxygen-, nitrate-, manganese-, iron (III), sulphate, or carbon dioxide-reducing, and their corresponding redox potentials. Redox potentials provide an indication of the relative dominance of the electron acceptor classes.

The presence of microorganisms with the appropriate metabolic capabilities is the most important requirement for oil spill bioremediation according to Venosa *et al.* (2002). The communities which are exposed to hydrocarbons become adapted, exhibiting selective enrichment and genetic changes (Leahy and Colwell 1990; Adams *et al*.,

2015). The adapted microbial communities can respond to the presence of hydrocarbon pollutants within hours and exhibit higher biodegradation rates than communities with no history of hydrocarbon contamination (Leahy and Colwell, 1990) So, the ability to isolate high numbers of certain oil degrading microorganisms from an environment is commonly taken as evidencethat those microorganisms are the most active oil degraders of that environment (Okerentugba and Ezeronye, 2003) and can be used in the bioremediation of petroleum polluted sites. Since crude oil is made of a mixture of compounds, and since individual microorganisms metabolize only a limited range of hydrocarbon substrates, biodegradation of petroleum hydrocarbon requires mixture of different bacterial groups or consortia functioning to degrade a wider range of hydrocarbons (Bordenave, 2007). This process depends on nutrient availability and the optimum presence of other factors that support biological functions.

2.3.2 Mechanism of Oil Biodegradation by Bacteria

The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions. Figure 2.2 shows the main principle of aerobic degradation of hydrocarbons (Nilanjana and Preethy, 2010). The initial intracellular attack of organic pollutants is an oxidative process and the activation as well as incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases. Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, for example, the tricarboxylic acid cycle. Biosynthesis of cell biomass occurs from the central precursor metabolites, for example, acetyl-CoA, succinate, pyruvate. Sugars required for various biosyntheses and growth are synthesized by gluconeogenesis (Nilanjana and Preethy, 2010).

The degradation of petroleum hydrocarbons can be mediated by specific enzyme system. Figure 2.3 shows the initial attack on xenobiotics by oxygenases. Other mechanisms involved are attachment of microbial cells to the substrates and production of biosurfactants (Nilanjana and Preethy, 2010).

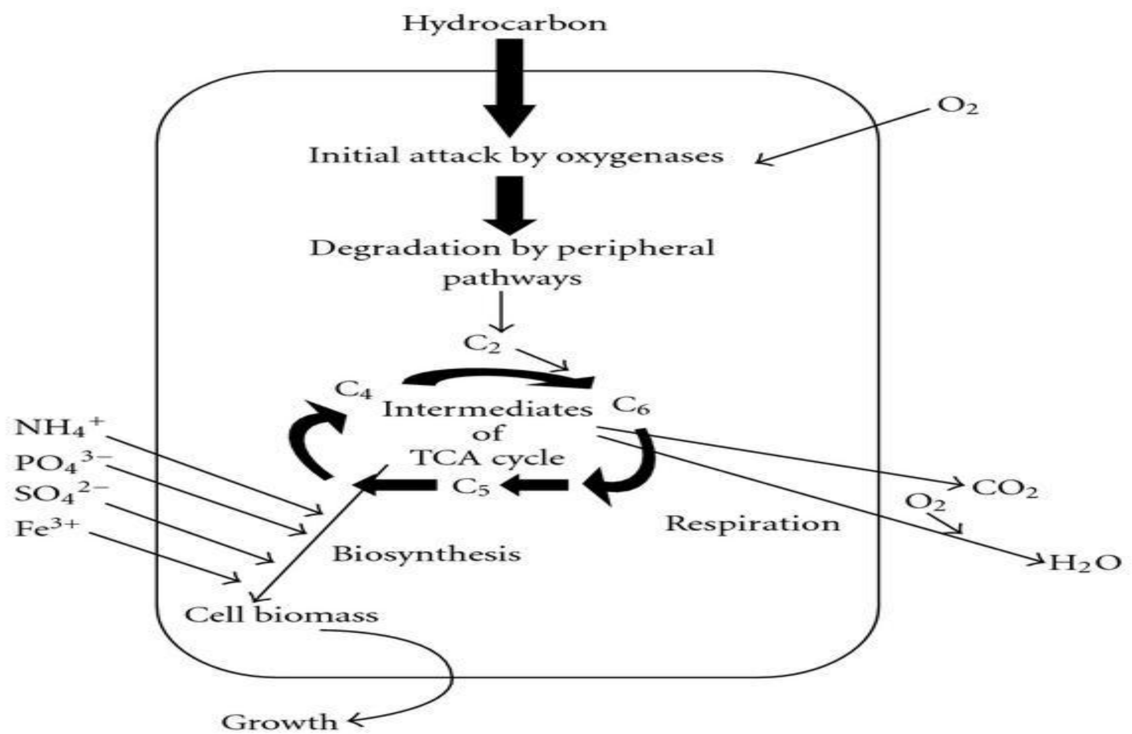

Figure 2.2 Main principle of aerobic degradation of hydrocarbons by microorganisms.

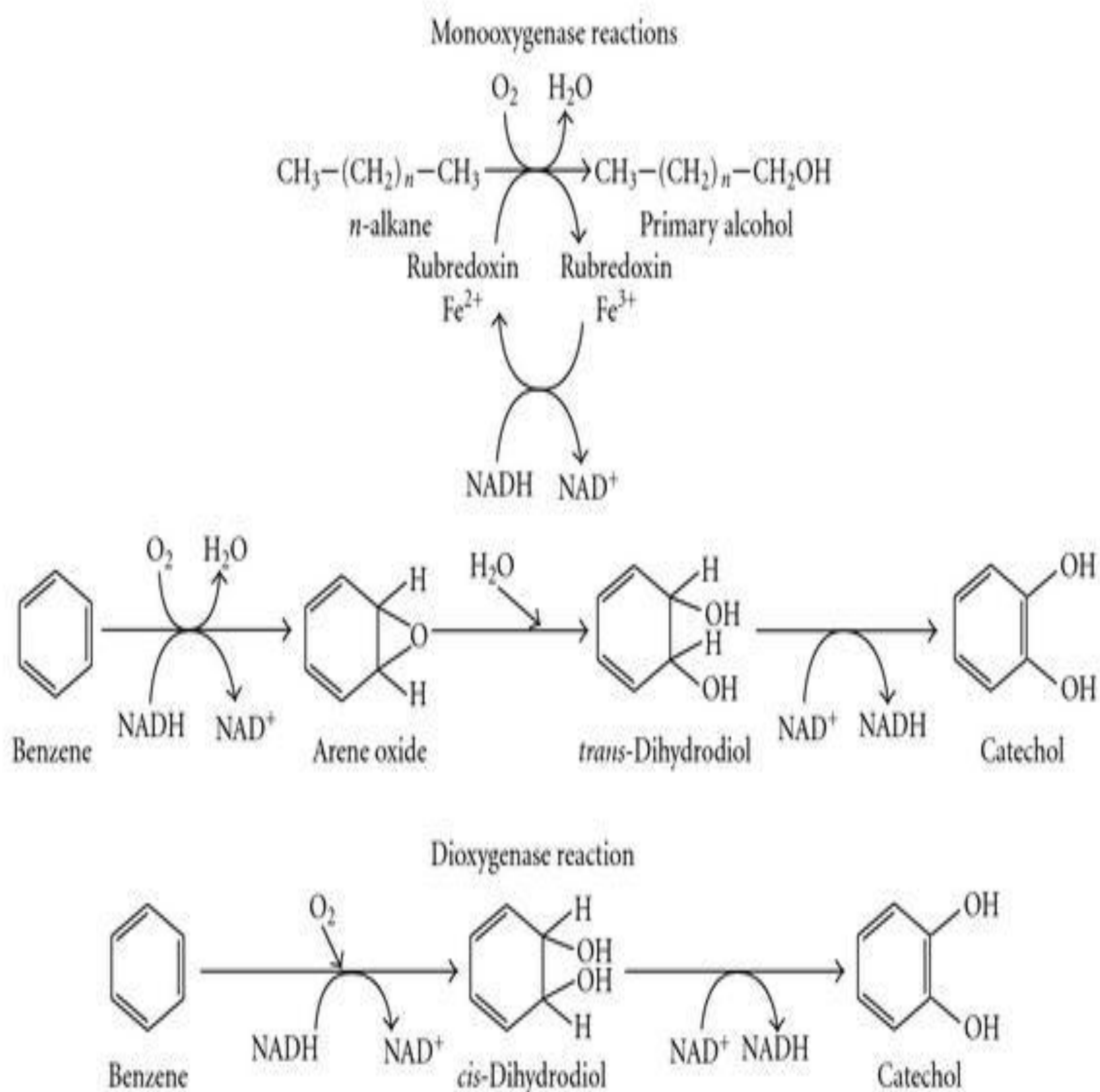

Figure 2.3 Enzymatic reactions involved in the processes of hydrocarbons degradation.

2.3.3 Enzymes Participating in Degradation of Hydrocarbons

Cytochrome P450 alkane hydroxylases constitute a super family of ubiquitous Heme-thiolate Monooxygenases which play an important role in the microbial degradation of oil, chlorinated hydrocarbons, fuel additives, and many other compounds. Depending on the chain length, enzyme systems are required to introduce oxygen in the substrate to initiate biodegradation. Higher eukaryotes generally contain several different P450 families that consist of large number of individual P450 forms that may contribute as an ensemble of isoforms to the metabolic conversion of given substrate. In microorganisms such P450 multiplicity can only be found in few species (Nilanjana and Preethy, 2010). Cytochrome P450 enzyme systems was found to be involved in biodegradation of petroleum hydrocarbons (Nilanjana and Preethy, 2010). The capability of several yeast species to use n-alkanes and other aliphatic hydrocarbons as a sole source of carbon and energy is mediated by the existence of multiple microsomal Cytochrome P450 forms. These cytochrome P450 enzymes had been isolated from yeast species such as Candida maltosa, Candida tropicalis, and Candida apicola (Nilanjana and Preethy, 2010). The diversity of alkaneoxygenase systems in prokaryotes and eukaryotes that are actively participating in the degradation of alkanes under aerobic conditions like Cytochrome P450 enzymes, integral membrane di-iron alkane hydroxylases (e.g., alkB), soluble di-iron methane monooxygenases, and membrane-bound copper containing methane monooxygenases have been discussed by Van Beilen and Funhoff (Nilanjana and Preethy, 2010).

2.3.4 Factors affecting bioremediation

2.3.4.1 Contaminant concentrations

Contaminant concentrations directly influence microbial activity. When concentrations are too high, thecontaminants may have toxic effects on the microorganisms present in the environment. In contrast, low contaminant concentration may prevent induction of bacterial degradation enzymes (Adams *et al*., 2015). According to Zahed *et al.* (2010), bioremediation is not recommended for crude oil concentrations of 2000 mg/L or higher, lower concentration of crude oil demonstrated more efficient hydrocarbon removal.

2.3.4.2 Contaminant bioavailability

The bioavailability of the contaminants depends on the degree to which they sorb to solids or are sequestered by molecules in contaminated media, are diffused in macropores of soil or sediment, and other factors such as whether contaminants are present in Non-Aqueous Phase Liquid (NAPL) form. Bioavailability for microbial reactions is lower for contaminants that are more strongly sorbed to solids, enclosed in matrices of molecules in contaminated media, more widely diffused in macropores of soil and sediments, or are present in NAPL form (Intercontinental Centre for Soil and Contaminated Sites, 2006).

2.3.4.3 Site characteristics

This has a significant impact on the effectiveness of any bioremediation strategy. Site environmental conditions important to consider for bioremediation applications include pH with an optimum in the range of 6-8 (ICSS, 2006), temperature, water content, nutrient availability, and redox potential. (Environmental Security Technology Certification Programme, 2007).

2.3.4.4 Redox Potential and oxygen content

Redox potential is influenced by the presence of electron acceptors such as nitrate, manganese oxides, iron oxides and sulphate (ICSS, 2006). Oxidation-reduction potential provides the measurement of the electron density of a system. Biological energy obtained from the oxidation of compounds in which electron acceptors are transferred to various more oxidized compounds referred to as electron acceptors. A low electron density (Eh greater than 50mV) indicates oxidizing, aerobic conditions, whereas high electron density (Eh less than 50mV) indicates reducing, anaerobic conditions. High positive Eh values (+100 mV to +400mV) indicate well aerated conditions that are optimal for biodegradation (Eskander and Saleh, 2017)

2.3.4.5 Nutrients

Nutrients are needed for microbial cell growth and division (ESTCP, 2007). Suitable amounts of trace nutrients for microbial growth are usually present, but nutrients can be added in a useable form or via an organic substrate amendment which also serves as an electron donor, to stimulate bioremediation. The nutrient status of a soil directly impacts microbial activity and biodegradation. A group of nutrient elements or organic compounds are required as a source of carbon or electron acceptor. Inorganic nutrients including exchangeable cations, nitrates and phosphates are important for bioremediation. Aerobic organisms utilize elemental oxygen as their ultimate electron acceptor. Anaerobes utilize nitrates, sulphates, CO_2 and ferrous metals as electron acceptors and often have unique reaction mechanisms in the deep surface, where the

anaerobic pathway is likely to be meritorious to degrade some resistant compounds (Yang *et al*., 2009; Hui, 2011).

2.3.4.6 Moisture content

Microbial growth requires an optimum presence of water in the environmental matrix. For optimum growth and proliferation, microorganisms require 12% to 25% of moisture (Mukherjee and Das, 2005).Moisture is required for all biological processes to help transport nutrients, foods, and waste products in and out of the microorganisms. For oceans, lakes, and other surface water environments, this poses no immediate problems. For soil environments, some moisture must be present for degradation to occur. Too much water can impede the re aeration of the soil, and the process may turn anaerobic. The optimum ratio of moisture will depend on the climate and soil type. Ratios range from 30-90% in one study and 12-32% in others and the aerobic biodegradation of hydrocarbons in soils is greatest in ranges of 50-70% of the soil water holding capacity whereas waves and tidal actions are useful in supplying aerated sea water to beaches and marshes, rainfall is useful in the biodegradation of inland soils by supplying moisture and useful dissolved oxygen to the microbes (Sihang *et al*., 2014)

2.3.4.7 Temperature

Temperature is the most important factoraffecting biodegradation as it directly affects the rate of microbial metabolism and consequently microbial activity in the environment (Iranzo *et al.,* 2001). The biodegradation rate, to an extent rises with increasing temperature and slows with decreasing temperature (ESTCP 2007). This is because the biological enzymes involved in the degradation pathway have temperatures at which their activity is optimum (Iranzo *et al.,* 2001). Hydrocarbon biodegradation can occur

over a wide range of temperatures and the rate of biodegradation generally decreases with decreasing temperature. The highest degradation rates generally occur in the range of 30 to 40°C in soil environments, 20 to 30°C in some freshwater environments, and 15 to 20°C in marine environments (Kiama, 2015). Also, temperature influences petroleum biodegradation by its effect on the physical nature and chemical composition of the oil, rate of hydrocarbon metabolism by microorganisms, and composition of the microbial community (Venosa and Zhu, 2003).

2.3.5 Types of bioremediation

Feasibility of bioremediation depends on the location of contaminants. Approaches for implementation of bioremediation depend on whether the impacted soil to be treated is intact in the environment or it is to be excavated for treatment in an offsite facility. If on site, the term *in-situ* remediation suffices and if offsite, it is described as *ex-situ.* Some authors (Kumar, *et al.,* 2011; Orji *et al.,* 2012) have used this to describe the type of bioremediation. However, it is necessary to determine what exactly is done *in-situ and ex-situ* and use same to describe the types of bioremediation.

2.3.5.1 Biostimulation

Hydrocarbon biodegradation in soil can be limited by many factors, including nutrients, pH, temperature, moisture, oxygen, soil properties and contaminant presence (Bundy *et al.,* 2002; Al-Sulaimani *et al.,* 2010). Biostimulation involves the modification of the environment to stimulate existing bacteria capable of bioremediation. This can be done by addition of various forms of limiting nutrients and electron acceptors, such as phosphorus, nitrogen, oxygen, or carbon (e.g. in the form of molasses), which are otherwise available in quantities low enough to constrain microbial activity (Adams *et al*., 2015). It was described by Perfumo *et al.* (2007) that the addition of nutrients,

oxygen or other electron donors and acceptors to the coordinated site increases the population or activity of naturally occurring microorganisms available for bioremediation.

Margesin and Schinner (2001) defined biostimulation as a type of natural remediation that can improve pollutant degradation by optimizing conditions such as aeration, addition of nutrients, pH and temperature control. They opined that biostimulation can be considered as an appropriate remediation technique for petroleum pollutants removal in soil and requires the evaluation of both the intrinsic degradation capacities of the autochthonous microflora and the environmental parameters involved in the kinetics of the in situ process. The primary advantage of biostimulation is that bioremediation will be undertaken by already present native microorganisms that are well-suited to the subsurface environment, and are well distributed spatially within the subsurface. The primary challenge is that the delivery of additives in a manner that allows the additives to be readily available to subsurface microorganisms is based on the local geology of the subsurface. Tight, impermeable subsurface lithology (tight clays or other fine-grained material) make it difficult to spread additives throughout the affected area. Fractures in the subsurface create preferential pathways in the subsurface which additives preferentially follow, preventing even distribution of additives. Addition of nutrients might also promote the growth of heterotrophic microorganisms which are not innate degraders of total petroleum hydrocarbon thereby creating a competition among the resident micro-flora (Adams *et al*., 2015).

2.3.5.2 Bioaugmentation

Since the 1970s, bioaugmentation, or the addition of oil-degrading microorganisms to supplement the indigenous populations, has been proposed as an alternate strategy for

the bioremediation of oil contaminated environments. The rationale for this approach is that indigenous microbial populations may not be capable of degrading the wide range of potential substrates present in complex mixtures such as petroleum (Leahy and Colwell, 1990; Adams *et al*., 2015) or that they may be in a stressed state as a result of the recent exposure to the spill. Other conditions under which bioaugmentation may be considered are when the indigenous hydrocarbon-degrading population is low, the speed of decontamination is the primary factor, and when seeding may reduce the lag period to start the bioremediation process (Adams *et al*., 2015). For this approach to be successful in the field, the seed microorganisms must be able to degrade most petroleum components, maintain genetic stability and viability during storage, survive in foreign and hostile environments, effectively compete with indigenous microorganisms, and move through the pores of the sediment to the contaminants (Adams *et al*., 2015).

Different microbial species have different enzymatic abilities and preferences for the degradation of oil compounds. Some microorganisms degrade linear, branched, or cyclic alkanes. Others prefer mono- or polynuclear aromatics, and others jointly degrade both alkanes and aromatics (Unimke *et al*., 2018). The study of microbes in bioremediation systems makes possible the selection of microorganisms with potential for the degradation and production of compounds with biotechnological applications in the oil and petrochemical industry. Successful bioaugmentation treatments depend on the use of inocula consisting of microbial strains or microbial consortia that have been well adapted to the site to be decontaminated. Foreign microorganisms (those in inocula) have been applied successfully but their efficiency depends on ability to compete with indigenous microorganisms, predators and various abiotic factors (Prakash *et al*., 2014). Factors affecting proliferation of microorganisms used for bioaugmentation including the chemical structure and concentration of pollutants, the

availability of the contaminant to the microorganisms, the size and nature of the microbial population and the physical environment should be taken into consideration when screening for microorganisms to be applied (Adams *et al*., 2015; Unimke *et al*., 2018).

2.3.6 Genera of bacteria involved in hydrocarbon degradation

Various genera of bacteria have been reported to be involved in the oil degrading process. These hydrocarbonocastic bacteria are ubiquitous in nature. According to Matsuoka *et al.* (2009), *Burkholderia arboris* secretes lipase to aid biodegradation process while *Candida cylindracea* is responsible in glycerol assimilation. On the other hand, Kabuto *et al*. (2008) reported that *Rhodococcus* species and *Gordonia* species used n-alkane and c-alkane as sole carbon and energy source and the strains degraded more than 27% of car engine based oil (1% addition).

According to Sayavedra-soto *et al.* (2006), *Rhodococcus* strain NTU-1 carried out the degradation of alkane via a hydroxylase, while *Bacillus fusiformis* L-1 and *Ochrobactrum* species would not degrade alkanes butaided flocculation by forming more rigid bacterial aggregates that enhanced thetrapping of alkanes. They also reported that on batch culture of these three bacteria,transformation and removal of the linear and branched alkanes was achievedwithin 66 hours with more than 95% efficiency.

Kasai *et al.* (2002) observed that polycyclic aromatic hydrocarbon (PAH) degradation proceeds parallel with the growth of *Cycloclasticus* cells on the surfaces of the oil polluted gravel. They reported that bacteria belonging to the genus *Cycloclasticus* play an important role in the degradation of petroleum PAHs in the marine environment. Other marine bacteria that have previously been reported to be PAH degraders include

members of the genera *Flavobacterium, Marinobacter, Moraxella, Pseudomonas, Sphingomonas* and *Vibrio*(Kasai *et al*., 2002).

Iyer *et al.* (2006) reported that *Enterobacter cloacae* produced exo-polysaccharides that can emulsify hexane, benzene, xylene, kerosene, paraffin wax, cotton seed oil, coconut oil, jojoba oil, castor oil, groundnut oil and sunflower oil. Based on the study from Haritash and Kaushik (2009), enzymes involved in the degradation of PAHs are oxygenase, dehydrogenase and lignolytic enzymes.

2.3.5 Recent strategies for bioremediation

The use of certain genetically engineered microorganisms to influence their ability to utilize specific contaminants such as hydrocarbons and pesticides is gaining grounds. This technique had an early mention in the late 1980s and early 1990s. The ability to 'engineer' microorganisms to improve degradative properties is based a possibility to explore genetic diversity and metabolic versatility of microorganisms. The blueprint necessary for gene encoding for biodegradative enzymes is present in chromosomal and extra chromosomal DNA of such microbes. Recombinant DNA techniques explore the ability of an organism to metabolize a xenobiotic by detecting the presence of degradative genes and transforming them into appropriate hosts through a suitable vector within a controlled setting (Das and Chandran, 2011). This technology explores Polymerase Chain Reaction (PCR), anti-sense RNA technique, site directed mutagenesis, electroporation and particle bombardment techniques. The first step in Genetically Modified Microorganism (GMM) construction is selection of suitable gene(s), next, the DNA fragment to be cloned is inserted into a vector and introduced into host cells. The modified bacteria are called recombinant cells. The next step is production of multiple gene copies and selection of cells containing recombinant DNA.

The final step includes screening for clones with desired DNA inserts and biological properties. Since the possibility of conferring new properties into existing organisms abound, researchers have studied the ability of modified organisms to degrade petroleum hydrocarbons or hydrocarbon based compounds (Unimke *et al*., 2017). Additionally, they studied transconjugant occurrence, their identification and plasmid persistence. Both inoculated donors were detectable and they transferred plasmid pJP4 to indigenous recipients to different extents. In the first experiment 2,4-D was degraded significantly faster (28 days) in soil inoculated with *R. eutropha JMP134* as compared with soil inoculated with *E. coli D11* (49 days). Interestingly, a greater number of transconjugants was detected in *E. coli D11*-inoculated soil and they were members of the *Burkholderia* and *Ralstonia* genera. After re-amendment, 2,4-D was degraded more rapidly in the soil with *E. coli* D11 inoculants than in *R. eutropha* JMP134-inoculated treatments (Das and Chadran, 2011). These results indicated that choice of donor microorganisms is a crucial factor to be considered for bioaugmentation approach.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of Refinery effluent Samples

Raw and treated refinery effluent samples were collected from Kaduna Refining and Petrochemical Company (KRPC), Kaduna Nigeria, by using a clean cup to draw the samples from the effluent ponds into new clean 1 litre plastic containers (Oaikhena *et al*., 2016). The samples were transported at ambient temperature to the Department of Microbiology, Ahmadu Bello University Zaria for analysis.

Already Profiled Escravos light crude oil was obtained from the Department of Microbiology, Ahmadu Bello University Zaria.

3.2 Determination of the Physico-chemical Properties of the Raw and Treated Refinery Effluents

The physicochemical parameters that were assayed included:

3.2.1 pH and temperature

The pH and temperature of the effluents were determined at the point of collection using the HANNA combo tester (H198130, Denver, USA); a water proof tester that accurately measures pH and temperature in a single test. Briefly, following the manufacturer's instructions, the electrodes connected to each meter were submerged in

a clean bucket containing the sample. The values read for each parameter by the tester were observed and recorded.

3.2.2 Electrical conductivity

The conductivity of the sample was determined using a conductivity cell. The temperature of the sample was first adjusted to about 20ºC and a portion of the sample was used to wash the conductivity cell and then the sample was filled completely ensuring that no air bubbles adhered to the electrode and the readings were recorded.

3.2.3 Total dissolved Solids

One hundred millilitre (100ml) of the sample was filtered using 0.45mm filter paper and placed in a pre-weighed crucible and dried in an oven at 103^0C for 2 hours. The dish was allowed cool briefly and dried in a desicator for 1hour 30 minutes. The weight of empty crucible was subtracted from the weight of crucible after drying to give weight of total residue. The total dissolved solids were calculated using the formula (APHA, 1995):

$$\text{Total dissolved solid (mg/l)} = \frac{\text{Weight of total residue}}{\text{Volume of sample}} \times 1000$$

3.2.4 Total suspended solids

An evaporating dish was weighed empty and 100ml of the sample was dispensed into it, evaporated to dryness in a drying oven at 103°C for 3hours, allowed to cool and dried in a desicator. The dish was reweighed and the total suspended solids were obtained using the formula:

$$\text{Total suspended solids (mg/l)} = \frac{\text{Weight of suspended solid}}{\text{Volume of sample analyzed}} \times 1000$$

3.2.5 Dissolved oxygen and biochemical oxygen demand

Dissolved oxygen and biological oxygen demand was determined using HANNA instrument (HANNA 3200, Denver USA). Dissolved oxygen was determined by inserting the instrument into the sample at the point of collection while the BOD was carried out by transferring the sample into a BOD bottle and incubated for five days at 25^0C. The cell of the instrument was inserted into the incubated sample and the level of residual oxygen was recorded. The value of residual DO was then subtracted from the initial DO value to obtain the BODof the samples (Radojavic and Bashkin, 1999).

3.2.6 Nitrates

The sample was adjusted to pH 7.0 and 100ml of the sample was placed in a beaker and allowed to evaporate to dryness in a water bath. The resulting residue was dissolved using a glass rod with 2ml disulphonic acid reagent and transferred to Nessler's tube. Six millilitres (6ml) of ammonium hydroxide (NH_4OH) and EDTA was added drop-wise wise till the residue dissolved. A blank was also prepared the same way using distilled water instead of the sample. The absorbance of the colour developed was then read at 410nm with a light path of 1cm using spectrophotometer. The nitrate concentration was estimated using a standard curve. The nitrate concentration in the samples was calculated using the formula (APHA, 1995).

$$\text{Nitrate (mg/l)} = \frac{\text{Nitrate (N)} \times 1000}{\text{Volume of sample analyzed.}}$$

3.2.7 Sulphates

One hundred millilitres (100ml) of the sample was dispensed into 250ml Erlenmeyer flask containing 5ml of conditioning reagent and mixed using a magnetic stirrer. Ten grams of barium chloride crystals was added while the solution was still being stirred.

The turbidity was measured for 30seconds after stirring at interval of 4 minutes using turbidity meter (Hach 2100N, USA). A blank was run with no barium chloride added. The sulphate concentration was calculated using the formula below and sulphate concentration was estimated by reading off the standard curve (APHA, 1995).

Mg /l of sulphate (SO_4) = $MgSO_4 \times 1000$/ ml of sample taken

3.2.8 Phosphates

Preliminary sample treatment

About 0.1ml of phenolphthalein indicator was added to 100ml of the sample and concentrated hydrochloric acid solution was added drop wise until the colour turns pink.

Colour development

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

$$\text{Mg/l } PO_4 = \frac{PO_4 \times 1000}{\text{Volume of sample analyzed.}}$$

3.3 Microbiological Investigations

3.3.1 Isolation of *Bacillus subtilis* and *Pseudomonas putida* from the raw refinery effluent

Preparation of Mineral Salt Medium

Hydrocarbon utilizing strains of *Bacillussubtilis* and *Pseudomonas putida* were isolated from the effluent sample using mineral salt medium (MSM) with the following composition per litre: KH_2PO_4 (2.0g), $NaNO_3$ (2.0g), NaCl (0.8g), KCl (0.8g), $Na_2HPO_4.12H_2O$ (2.0g), $MgSO_4$ (0.2g), $FeSO_4.7H_2O$ (0.001g). The salts above were dissolved in 1(one) litre of distilled water and sterilized by autoclaving at 121^0C for 15 minutes (Eniola *et al.,* 2014; Musa *et al.,* 2015).

Inoculation of sample

This was carried out by following the method reported by Naga *et al*. (2016) with slight modification. The effluent sample was shaken vigorously and 200ml was dispensed into a beaker and allowed to set at room temperature on a thoroughly disinfected laboratory work bench for 4 hours to concentrate the sample by sedimentation. The supernatant was discarded leaving the sediment to about 20ml volume followed by vigorous shaking to resuspend the sediment. Ten (10) ml of the concentrated effluent sample was dispensed into 250ml conical flask containing 90 ml of mineral salt medium supplemented with 1% (v/v) of crude oil. The preparation was incubated at ambient temperature on a shaker at 150rpm for 14 days. About 50 ml of the culture was dispensed into a sterile 100ml conical flask and heat shocked in a water bath at 80^0C for

1 hour to eliminate all non-spore formers. Both the heat shocked and non-heat shocked samples were serially diluted to a factor of five and 0.1 ml from each dilutions of the heat shocked and non-heat shocked culture were respectively inoculated unto the surface of nutrient agar (for B. *subtilis*) and centrimide agar (for P. putida) prepared according to manufacturer's instruction. The resulting bacterial colonies were examined for size, shape, margin consistency and pigmentation. Distinctive colonies were sub-cultured unto nutrient agar plates by streaking for purification. The resulting pure colonies were subcultured unto a nutrient agar slant medium for further studies and preserved at 4-5^{0}C (Ekhaise and Nkwelle, 2011).

Identification of the Isolates

The isolates were identified with a combination of microscopic and biochemical techniques. For the microscopic identification, the isolates were gram stained and examined microscopically to determine their gram reaction and cellular morphology. Spore staining test was carried out on isolates showing gram positive reaction and rod morphology. Then the spore forming gram positive rod isolates and those that showed gram negative reaction and rod morphology were transferred unto nutrient agar slant medium and preserved in the refrigerator at 4-5^{0}C for further identification tests.

Biochemical Identification

The isolates were biochemically identified following the scheme described by Cowan and Steel (2003) which include:

Motility test

This test was carried out by inoculating the motility medium with the colonies of the gram positive and negative isolates in separate test tubes. A stab was made with a straight wire to a depth of about one third the total volume of the medium. The culture

was then incubated at 35°C for 24hours. If the culture turns cloudy (turbid) after incubation, it means the organism is motile but if growth is restricted to the line of inoculation and the rest of the culture remains clear, then the organism is non-motile (Cowans and Steel, 2003).

Citrate utilization test

This test was carried out by inoculating the colonies of the gram positive and negative rod isolates on Simmons' citrate agar slant and the inoculated slantswere incubated at 35°C for 48-72 hours and examined for development of a deep blue colour which indicated a positive reaction (Cowans and Steel, 2003).

Urease test

Urease test was carried out by inoculating urea agar slant with the colonies of the gram positive and negative isolatesand the slant was incubated at 35°C for 48-72 hours and examined for the development of bright pink or red colour which indicated a positive reaction (Cowans and Steel, 2003).

Nitrate reduction test

This was carried out by inoculating Nitrate Broth lightly with colonies of the gram positive and negative rod isolates and incubated at 37°C for 24-48hrs. One (1 ml) of reagent A (sulphanilic acid) was added followed by 1 ml of reagent B (α-napthol amine) and examined. A deep red colour showed the presence of nitrite and thus, indicating that nitrate has been reduced.

To tubes didn't show a red colour within 5 min, powdered zinc (up to 5 mg/ml of culture) was added and allowed to stand and then examined for red colour which indicated that nitrate was present in the medium (i.e. not reduced by the organism) and

absence of red colour indicated nitrate was absent in the medium (i.e. reduced by the organism to nitrite, which in turn was itself reduced) (Cowans and Steel, 2003).

Starch hydrolysis test

This was carried out by inoculating colonies of the gram positive and negative rod isolates on nutrient agar containing 0.2% soluble starch and the plates were incubated at 30°C for 24hours. After incubation, the plates were flooded with Lugol's iodine and examined for zone of hydrolysis (Cowans and Steel, 2003).

O-nitro-phenyl-D-galactopyranoside (ONPG) test

This was carried out by inoculating separate tubes of ONPG broth with colonies of the gram positive and negative rod isolates and incubated at 37°C for 48 hours and examined for β-galactosidase activity which was indicated by the appearance of a yellow colour due to the production of o-nitrophenol (Cowans and Steel, 2003).

Carbohydrate fermentation test

A series of test tubes containing sterile nutrient broth with 1% each of membrane-filter-sterilized (0.45μm) single fermentable sugar was inoculated with the test organisms.. The sugar fermentation tubes were incubated at 35°C for 24 hours. At the end of the incubation period, all tubes were examined for acid following the addition of methyl red indicator and then compared with the control for interpretation (Cowans and Steel, 2003). A total of eight (8) sugars were used for the fermentation tests, these include; Glucose, Cellobiose, Galactose, Mannose, Melibiose, Raffinose, Salicin and Xylose. This was done for only the gram positive rod isolates.

Oxidase test

This was carried out by placing a piece of filter paper in a clean Petri dish and 2-3 drops of freshlyprepared oxidase reagent was added. Using a glass rod, a colony of the test organism was smeared on the filter paper and examined for the development of a blue-purple colour within a few seconds which indicated a positive test and absence of blue-purple colour within 10 seconds indicated a negative test.

Indole test

Indole test was carried out by inoculating colonies of the gram positive rod isolates into 1% peptone water and then the inoculated peptone was incubated at 37°C for 24 hours. After 24 hours of incubation, 3 drops of Kovac's reagent was added and shaken and examined. A positive reaction was indicated by the development of a red colour in the reagent layer above the broth while a negative reaction was indicated by a yellow colour (Cowans and Steel, 2003).

Methyl Red - Voges-Proskauer (MR-VP) test

This test was carried out by inoculating 5ml of MR-VP broth with colonies of the gram positive rod isolates and then the inoculated broth was incubated at 35°C for 48-72 hours. After 24 or 48 hours of incubation, about 1ml of the cultured broth was transferred to a small test tube to which 2-3 drops of methyl red indicator was added and examined. Formation of red colour on addition of the indicator signified a positive methyl red test and a yellow colour signified a negative test.

To the rest of the broth, 2 drops of 40% potassium hydroxide was added followed by 2 drops of 5% α-Naphthol in ethanol. The tube was shaken and placed in a slope and examined. Development of a red colour starting from the liquid – air interface within 1

hour indicated a VP positive test while no colour change indicated VP negative test (Cowans and Steel, 2003).

Isolates confirmed to be *Bacillus subtilis* and *Pseudomonas putida* on the basis of biochemical characteristics were authenticated using the Microgen kits; *Bacillus*-ID kit for *Bacillus subtilis* and GNA and GNB Enterobacteriaceae-ID kit for *Pseudomonas putida*. The kits were obtained from Microgen Bioproducts Company, United Kingdom (UK). Isolates thus authenticated were stored in the refrigerator for further studies.

3.4 Standardization of inoculum

A suspension of the wild strains of *Bacillus subtilis* and *Pseudomonas putida* isolated was prepared separately in normal saline and compared with a 1.0 McFarland standard. Colonies were added to the normal saline until it was as turbid as the McFarland standard to obtain a bacterial population density of 3.0×10^{8}cfu/ml (Ekhaise and Nkwelle, 2011).

3.5 Screening the Capacity of *Bacillus subtilis* and *Pseudomonas putida* to Degrade Hydrocarbons.

Mineral salt agar medium was prepared as previously described into different conical flasks and enriched separately with 0.5%, 1%, 1.5% and 2% crude oil and sterilized by autoclaving. The medium were dispensed into petri dishes and allowed to solidify after which they were inoculated with 18hours culture of the *Bacillus subtilis* and *Pseudomonas putida* isolates separately by streaking. The preparation was incubated at ambient temperature for 48 hours. The capacity of the isolates to degrade hydrocarbons was determined by the ability of the organisms to grow on the medium which were categorized as luxuriant growth, medium growth, scanty growth and no growth (Ekhaise

and Nkwelle, 2011). Isolates that grew well on the various concentration of the crude oil media was subjected to mutation.

3.6 Induction of Mutation

3.6.1 Induction of mutation using ultraviolet (UV) irradiation.

Five (5) ml of the standard suspensions (in phosphate buffer) of the wild strains were aseptically transferred into different sterile Petri plates and exposed to the light of a UV at 254nm wavelength in a dark room for 10, 20 and 30 minutes at a distance 6cm from the UV light source. The irradiated cells were transferred into a sterile centrifuge tube and treated with caffeine (0.2w/v) and stored in the dark overnight to avoid photo-reactivation. The tubes were centrifuged at 3000rpm, the supernatant was discarded and the sediment resuspended in normal saline and washed thrice. Then, 1ml of the irradiated cell suspension was serially diluted in normal saline and plated by transferring 0.1ml unto the surface of a sterile nutrient agar plate using spread plate method and incubated at 37^0C for 24 hours (Idise *et al.,* 2010).

3.6.2 Induction of mutation using nitrous acid

Standard suspension of the isolates was prepared and acetate buffer (0.2M, pH 4.5) was also prepared in accordance with the procedure reported by Idise *et al.* (2010). To 50ml of the standard suspension in 150ml conical flask, 50 ml of acetate buffer was added and treated with 1.5ml of 2.0M nitrous acid. This was allowed to stand at ambient temperature for 20 minutes. The reaction was terminated by serial dilution with Tris HCl. The treated cells were centrifuged at 1500rpm for 5 minutes and resuspended in acetate buffer and washed thrice to remove all traces of nitrous acid. The treated cells were then inoculated using spread plate technique on nutrient agar and incubated at 37^0C for 24 hours (Ikram *et al*., 2009; Idise *et al.,* 2010).

Plates with about 50% survival rate after treatment were used for further studies. The resulting mutant strains of *Bacillus subtilis* and *Pseudomonas putida* were stored in nutrient agar slant. Then, hydrocarbon degradation capacity test was carried out for the selected wild and mutant isolates of *Bacillus subtilis* and *Pseudomonas putida*.

3.7 Determination of Hydrocarbon Degrading Capacity of the Wild and Mutant *Bacillus subtilis* and *Pseudomonas putida*

Mineral salt medium was prepared as previously described but using raw (untreated) effluent as the solvent instead of distilled water. About 99ml of the effluent medium was dispensed into four (4) 250ml Erlenmeyer flask and sterilized by autoclaving. Two of the flasks were inoculated separately with 1 ml (1.5×10^{8}cfu/ml) suspension of the selected isolates of *Bacillus subtilis* and *Pseudomonas putida*, the third flask was inoculated with a co-culture of the both isolates while the fourth was not inoculated, serving as a control (Okerentugba and Ezeronye, 2003). This was done for both the wild and mutant strains of the isolates. The set-up was incubated at ambient temperature on a rotary shaker at 150rpm for 3, 6, 9, 12 and 15 days respectively. After each interval of incubation, a set of the experiment was analysed for growth and residual hydrocarbons (Anupama and Padma, 2009).

The bacterial growth was determined by measuring the optical density using spectrophotometer (Ekhaise and Nkwelle, 2011), while the residual oil and grease (hydrocarbons) was determined using gravimetric method (Umanu *et al*., 2013). Here, the oil and grease was extracted by mixing 50ml of culture broth with 50ml of petroleum ether in a separating funnel and shaken vigorously to get a single emulsified layer. Acetone was added to it and shaken gently to break theemulsification, which resulted in three layers. Top layer was a mixture of petroleum ether, oil and acetone; clumping cells made up the middle layer and the bottom aqueous layer contained

acetone, water and biosurfactant in soluble form. The two lower layers were discarded while the top layer containing a mixture of petroleum ether, residual hydrocarbons and acetone was collected in a clean pre-weighed vial. The extracted oil was passed through anhydrous sodium sulphate to remove moisture. The resulting petroleum ether and acetone was evaporated on a water bath and dried briefly in a desiccator and weighed. The residual oil and grease after biodegradation was determined using the formula (Anupama and Padma, 2009).

$$\text{Oil and Grease (mg/l)} = \frac{\text{A-B} \times 1000}{\text{Volume of sample analyzed}}$$

Where;
A = Weight of vial + residual oil and grease

B = Weight of empty vial

3.8 Statistical Analysis of Data

Data obtained in this study were subjected to analysis of variance (ANOVA) test at 95% confidence interval (0.05 level of significance) using Statistical Package for Social Science (SPSS) version 17 and were presented in tables charts and graphs where necessary.

CHAPTER FOUR

4.0 RESULTS

4.1 Physicochemical Parameters of Raw and Treated Refinery Effluent

Improvement in quality was observed between the raw effluent and that undergoing treatment (biologically treated). The pH, Temperature, Total Dissolved Solids (TDS), Nitrate, Sulphate and Phosphate of both effluent samples were within the permissible limits stipulated by the Federal Ministry of Environment (FMENV) for refinery effluents in Nigeria while other parameters analyzed for the raw effluent were above the permissible limits. The Turbidity, Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and oil and grease content of the effluent undergoing treatment were above the maximum permissible limits as shown in Table 4.1.

4.2 Isolation and Characterization of *Bacillus subtilis* and *Pseudomonas putida* from the Raw and Treated Refinery Effluent

The isolates were identified using a combination of cultural characteristics, microscopy and biochemical reactions as presented in Tables 4.2 and 4.3 for *Bacillus subtilis* and *Pseudomonas putida* respectively.

All the isolates (B. *subtilis*) were Gram positive, rod shaped and endospore formers. On the basis of cultural characteristics, all the isolates had creamy colonies and were circular in shape. With the exception of the isolates TE3, RE12 and RE17 that had convex shaped morphology, all the other isolates were flat. Isolates TE4, RE12 and

RE20 formed large colonies on nutrient agar while isolates TE6, TE10 and RE19 formed tiny colonies on the same medium (Table 4.2).

All the isolates reacted positively to starch hydrolysis, motility, citrate, VP, nitrate reduction Catalase test and fermented all the sugars tested, with the exception of RE17 and RE18 that were both negative to starch hydrolysis and citrate tests, RE19 and RE20 negative to starch hydrolysis, TE1, TE6 and TE10 negative to nitrate reduction test, R15 and R18 negative to glucose, Salicin and galactose fermentation, TE1, RE12 and RE13 to salicin and galactose fermentation, TE6 to glucose and Galactose, TE2 and TE4 to Salicin, TE8 to galactose and RE17, RE19 and RE20 to glucose fermentation. All the isolates were negative to indole, methyl red and urease tests with the exception of isolates TE6 and TE10, RE17, RE18, RE20 which reacted positively to methyl red and urease respectively.

Table 4.3 depicts the gram negative bacteria isolated from the sample. All the isolates were rod shaped and had small, greenish, and circular colonies on centrimide agar. With the exception of isolates TEC3, TEC11, TEC12, REC13, REC19 and REC20 that were flat all the other isolates were convex-shaped.

All the isolates were motile, citrate and oxidase positive and fermented glucose and arabinose but not sucrose. TEC4, TEC5, TEC6, TEC10, REC13, REC15 and REC16 were urease negative while the others were positive. TEC3, TEC4, TEC5, TEC11, TEC12, REC13, REC14, REC15, and REC16 were positive to nitrate reduction, fructose and Xylose fermentation test while others were negative except REC17 that is positive to only fructose fermentation.

Table 4.1: Physicochemical Properties of Raw and Treated Petroleum Refinery Effluent

Physicochemical parameters	Raw Effluent	Biologically Treated Effluent	percentage Reduction (%)	FMENV Standard
pH	6.86	6.58	4.08	6.0-9.0
Temperature (^{o}C)	29.9	20.7	30.77	≤ 40
Conductivity (µS/cm)	695	374	46.19	≤ 400
Dissolved Solids (mg/L)	354	216	38.98	≤ 500
Suspended Solids (mg/L)	35	14	60	≤ 50
Turbidity (NTU)	35.3	18.2	48.44	≤ 10
Dissolved Oxygen (mg/L)	160	5.7	96.44	≥ 10
Biochemical Oxygen Demand (mg/L)	190.5	29.6	84.46	≤ 10
Chemical Oxygen Demand (mg/L)	351.2	78.1	77.76	≤ 10
Oil and Grease (mg/L)	45.2	17.9	60.40	≤ 10
Nitrate (mg/L)	0.04	0.03	25	≤ 10
Sulphate (mg/L)	18.06	9.169	49.23	≤ 50
Phosphate (mg/L)	2.80	0.01	99.64	

BOD=Biological Oxygen Demand, TDS=Total Dissolved solids, COD=Chemical Oxygen Demand, FEMNV=Federal Ministry of Environment, Nigeria, µS/cm=Micro siemens per centimetre, mg/l=Milligram per litre.

Table 4.2: Cultural, Microscopic and Biochemical Characteristics of Gram Positive Isolates

Isolate Code	Colonial Morphology	Gram Reaction	ES	BIOCHEMICAL CHARACTERISTICS														Tentative Identity
				SH	M	C	U	I	Cat	MR	VP	NR	G	Sal	Gal	Xyl	Ara	
TE1	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	-	+	-	-	+	+	*B.* species
TE2	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	-	+	+	+	*B. subtilis*
TE3	SCCC	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	+	+	+	+	*B. subtilis*
TE4	LCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	-	+	+	+	*B. subtilis*
TE6	TCCF	G+ve rods	+(oval)	+	+	+	-	-	+	+	+	-	-	+	-	+	+	*B.* species
TE7	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	-	+	+	+	*B. subtilis*
TE8	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	+	-	+	+	*B. subtilis*
TE9	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	+	+	+	+	*B. subtilis*
TE10	TCCF	G+ve rods	+(oval)	+	+	+	+	-	+	-	+	-	+	+	+	+	+	*B.* species
RE12	LCCC	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	-	-	+	+	*B. subtilis*
RE13	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	-	-	+	+	*B.* subtilis
RE14	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	+	-	+	+	*B. subtilis*
RE15	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	-	-	-	+	+	*B. subtilis*
RE16	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	-	+	+	+	*B. subtilis*
RE17	SCCC	G+ve rods	+(oval)	-	+	-	+	-	+	-	+	+	-	+	+	+	+	*B.* species
RE18	SCCF	G+ve rods	+(oval)	-	+	-	+	-	+	-	+	+	-	-	-	+	+	*B.* species
RE19	TCCF	G+ve rods	+(oval)	-	+	+	-	-	+	-	+	+	-	+	+	+	+	*B.* species
RE20	LCCF	G+ve rods	+(oval)	-	+	+	+	-	+	-	+	+	-	+	+	+	+	*B.* species

KEY: TE; Treated Effluent, RE: Raw Effluent, SCCF: Small Cream Circular Flat, SCCC; Small Cream Circular Convex, LCCF; Large Cream Circular Convex, TCCF, Tiny Cream Circular Flat, LCCC; Large Cream Circular Convex, ES; Endospore, SH; Starch Hydrolysis, M; Motility, C; Citrate, U; Urease, I; Indole, Cat; Catalase, MR; Methyl Red, VP; Vogues Proskeur, NR; Nitrate Reduction, Sal; Salicin, Gal; Galactose, Xyl; Xylose, Ara; Arabinose

Table 4.3: Cultural, Microscopic and Biochemical Characteristics of Gram Negative Isolates

Isolate Code	Colonial Morphology	Gram Reaction	BIOCHEMICAL CHARACTERISTICS										Tentative Identity
			M	C	U	OX	NR	Glu	Ara	Fruc	Xyl	Suc	
TEC1	SGCC	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.
TEC2	SGCC	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.
TEC3	SGCF	G-ve rods	+	+	+	+	+	+	+	+	+	-	*Pseudomonas* spp.
TEC4	SGCC	G-ve rods	+	+	-	+	+	+	+	+	+	-	*Pseudomonas* spp.
TEC5	SGCC	G-ve rods	+	+	-	+	+	+	+	+	+	-	*Pseudomonas* spp.
TEC6	SGCC	G-ve rods	+	+	-	+	-	+	+	-	-	-	*Pseudomonas* spp.
TEC7	SGCC	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.
TEC8	SGCC	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.
TEC9	SGCC	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.
TEC10	SGCC	G-ve rods	+	+	-	+	-	+	+	-	-	-	*Pseudomonas* spp.
TEC11	SGCF	G-ve rods	+	+	+	+	+	+	+	+	+	-	*Pseudomonas* spp.
TEC12	SGCF	G-ve rods	+	+	+	+	+	+	+	+	+	-	*Pseudomonas* spp.
REC13	SGCC	G-ve rods	+	+	-	+	+	+	+	+	+	-	*Pseudomonas* spp.
REC14	SGCC	G-ve rods	+	+	+	+	+	+	+	+	+	-	*Pseudomonas* spp.
REC15	SGCC	G-ve rods	+	+	-	+	+	+	+	+	+	-	*Pseudomonas* spp.
REC16	SGCC	G-ve rods	+	+	-	+	+	+	+	+	+	-	*Pseudomonas* spp.
REC17	SGCC	G-ve rods	+	+	+	+	-	+	+	+	-	-	*Pseudomonas* spp.
REC18	SGCF	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.
REC19	SGCF	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.
REC20	SGCF	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.

KEY: TE; Treated Effluent, RE: Raw Effluent, SGCF: Small Green Circular Flat, SGCC; Small Green Circular Convex, M; Motility, C; Citrate, U; Urease, NR; Nitrate Reduction, Glu; Glucose Ara; Arabinose, Fruc; Fructose, Xyl; Xylose, Suc; Sucrose.

4.2.1: Identification of Isolates using Microgen® *Bacillus and Enterobacteriaceae* (GNA+GNB)Kits.

The Microgen® kit identification for *Bacillus subtilis* and *Pseudomonas putida* is presented in Table 4.4. Six (6) and eleven (11) isolates were identified as *Bacillus subtilis* and *Pseudomonas putida* respectively.

4.3: Crude Oil Utilization by the Isolates of *Bacillus subtilis* and *Pseudomonas putida*

The ability of wild strains of *Bacillus subtilis* and *Pseudomonas putida* isolates to grow on mineral medium containing varying concentrations of crude oil as the sole carbon source is presented in Table 4.5.

Bacillus subtilis isolate TE8 and *Pseudomonas putida* TEC10 showed luxuriant growth in Mineral medium containing 0.5%, 1% and 1.5% concentrations of crude oil but moderate growth on that containing 2% crude oil. All the isolates of *Bacillus subtilis* and *Pseudomonas putida* showed luxuriant growth on medium containing 0.5% crude oil with the exception of isolate TEC2 which had luxuriant growth on 1% crude oil medium, all the other isolates medium and scanty growth on medium containing 1% and 1.5% crude oil respectively. Also, isolate RE14, REC17 and REC18 showed no evidence of growth on the mineral medium containing 2% crude oil while all the other isolates of both *Bacillus subtilis* and *Pseudomonas putida* showed scanty growth.

Table 4.4: Identity of *Bacillus subtilis* and *Pseudomonas putida* Isolates Based on Microgen Identification Kit

Isolate's Code	Prophile Number	Probability (%)	Identity
TE2	73710427	99.68	*B. subtilis*
TE3	74370622	95.75	*B. subtilis*
TE7	74370622	95.75	*B. subtilis*
TE8	74370622	95.75	*B. subtilis*
TE9	74370624	75.75	*B. subtilis*
RE12	74370622	95.75	*B. subtilis*
TEC1	640722001	90.50	*P. putida*
TEC2	640522001	90.50	*P. putida*
TEC6	640522001	90.50	*P. putida*
TEC7	640522001	90.50	*P. putida*
TEC8	640722001	90.50	*P. putida*
TEC9	640522001	90.50	*P. putida*
TEC10	640522001	90.50	*P. putida*
REC17	640522001	90.50	*P. putida*
REC18	640722001	90.50	*P. putida*
REC19	640722001	90.50	*P. putida*
REC20	640522001	90.50	*P. putida*

KEY: **TE**, Treated Effluent, **RE**; Raw Effluent, **TEC;** Treated Effluent (Inoculated on centrimide agar), **REC;** Raw Effluent (Inoculated on centrimide agar)

Table 4.5: Growth of *Bacillus subtilis* and *Pseudomonas putida* Isolates on Mineral Medium Containing Crude Oil

Isolate's code	organism	Crude Oil Concentration (%)			
		0.5	1.0	1.5	2.0
TE2	*B. subtilis*	+++	++	+	+
TE3	*B. subtilis*	+++	++	+	+
TE7	*B. subtilis*	+++	++	+	+
TE8	*B. subtilis*	+++	+++	+++	++
TE9	*B. subtilis*	+++	++	+	+
RE14	*B. subtilis*	+++	++	+	-
TEC1	*P. putida*	+++	++	+	+
TEC2	*P. putida*	+++	+++	+	+
TEC6	*P. putida*	+++	++	+	+
TEC7	*P. putida*	++	++	+	+
TEC8	*P. putida*	++	++	+	+
TEC9	*P. putida*	+++	++	+	+
TEC10	*P. putida*	+++	+++	+++	++
REC19	*P. putida*	+++	++	+	+
REC20	*P. putida*	+++	++	+	+
REC17	*P. putida*	++	++	+	-
REC18	*P. putida*	++	++	+	-

KEY: **TE**, Treated Effluent, **RE**; Raw Effluent, **TEC;** Treated Effluent, **REC;** Raw Effluent

+ = Scanty growth**,** ++ = Medium growth, +++ = Luxuriant growth,**-** = No growth

4.4: Nitrous Acid and UV- Light Modification of Selected of *Bacillus subtilis* and *Pseudomonas putida* Strains

The effects of treatment with nitrous acid and UV-Irradiation on the selected strains (strains which had best growth in all the concentrations of crude oil tested)of *Bacillus subtilis* and *Pseudomonas putida* are presented in Table 4.6 and 4.7 respectively. Treatment with nitrous acid for 20 minutes caused the death of 59.67% of the *Bacillus subtilis* with 40.33% survival thus recording a higher survival rate than the *Pseudomonas putida* where the treatment caused the death of 66.33% of the cell population with a survival rate of 33.67% (Table 4.6).

Exposure to UV-radiation for 30 minutes resulted in the death of 51.67% of the population of *Bacillus subtilis* exposed with 48.33% of the population surviving the treatment. The population of *Pseudomonas putida* exposed to similar treatment recorded a 40% percentage death rate while 60% of the cell population survived (Table 4.7).

Table 4.6: Effects of 20 minutes Nitrous Acid Treatment on *Bacillus subtilis and* *Pseudomonas putida*

Organism	ITVC (cfu/ml)	FTVC (cfu/ml)	% Kill	%Survival
B. *subtilis*	3.00×10^8	1.21×10^8	59.67	40.33
P. putida	3.00×10^8	1.01×10^8	66.33	33.67

KEY: ITVC; Initial Total Viable Count, FTVC; Final Total Viable Count.

Table 4.7: Effects of 30 minutes UV-Irradiation on *Bacillus subtilis and Pseudomonas putida*

Organism	TVC (cfu/ml)	FTVC (cfu/ml)	%Kill	%Survival
B. subtilis	3.00×10^8	1.45×10^8	51.67	48.33
P. putida	3.00×10^8	1.80×10^8	40.00	60.00

KEY: ITVC; Initial Total Viable Count, FTVC; Final Total Viable Counts

4.5 Evaluation of the Hydrocarbon Degradation potential of the Wild and Mutant *Bacillus subtilis* and *Pseudomonas putida*

Biodegradation potential of wild and mutants strains of *Bacillus subtilis* and *Pseudomonas putida* was evaluated by assessing the growth pattern of the isolates and measurement of residual oil and grease in effluents inoculated with the test isolates.

4.5.1 Growth Pattern

The wild strain of *B. subtilis* (B), nitrous acid mutant (BNA) and UV mutant (BUV) strains of *B. subtilis* exhibited different growth patterns as shown in Fig.4.1. The highest population densities of 1.634×10^{8}cfu/ml and 1.33×10^{8}cfu/ml were recorded for B and BUV respectively, at day 12. While the population density of B remained relatively stationary to day 15, BUV declined to 1.492×10^{8}cfu/ml. Growth rate was slowest in BNA which attained highest population density of 9.03×10^{7}cfu/ml at day 12 followed by a slight decline in population to 8.13×10^{7}cfu/ml.

The growth patterns of the wild strain of *P. putida* (P), UV-mutant strain (PUV) and nitrous acid mutant strain (PNA) are presented in Fig. 4.2. Highest population density was observed in PUV followed by P while PNA had the lowest population density. P and PNA attained highest population density of 2.93×10^{8}cfu/ml and 9.03×10^{8}cfu/ml respectively at day 12 which was followed by a death phase. Highest population density (3.77×10^{8}cfu/ml) was observed in PUV at day 9 after which a steady decrease in population was observed in the remaining days of incubation.

Different growth patterns were observed in the mixed cultures of the wild strains of *B. subtilis* and *P. putida* (B+P), UV-mutants (BUV+PUV) and nitrous acid mutants (BNA+PNA) during the degradation of hydrocarbons in raw refinery effluent as shown in Fig. 4.3. Though, the bacterial population of the cultures increased gradually from

day 0 to 9, faster growth rate was observed in BUV+PUV. B+P and BUV+PUV attained highest population densities (3.181×10^8 and 2.192×10^8cfu/ml respectively) at day 9 while BNA+PNA attained its highest population density (1.15×10^8cfu/ml) at day 12. A decline in population was observed in all the cultures after day 9. B+P showed a sharp decline fron days 9 to 12 and remained stationary. BUV+PUV and BNA+PNA showed a sharp and steady death phase in the remaining period of incubation.

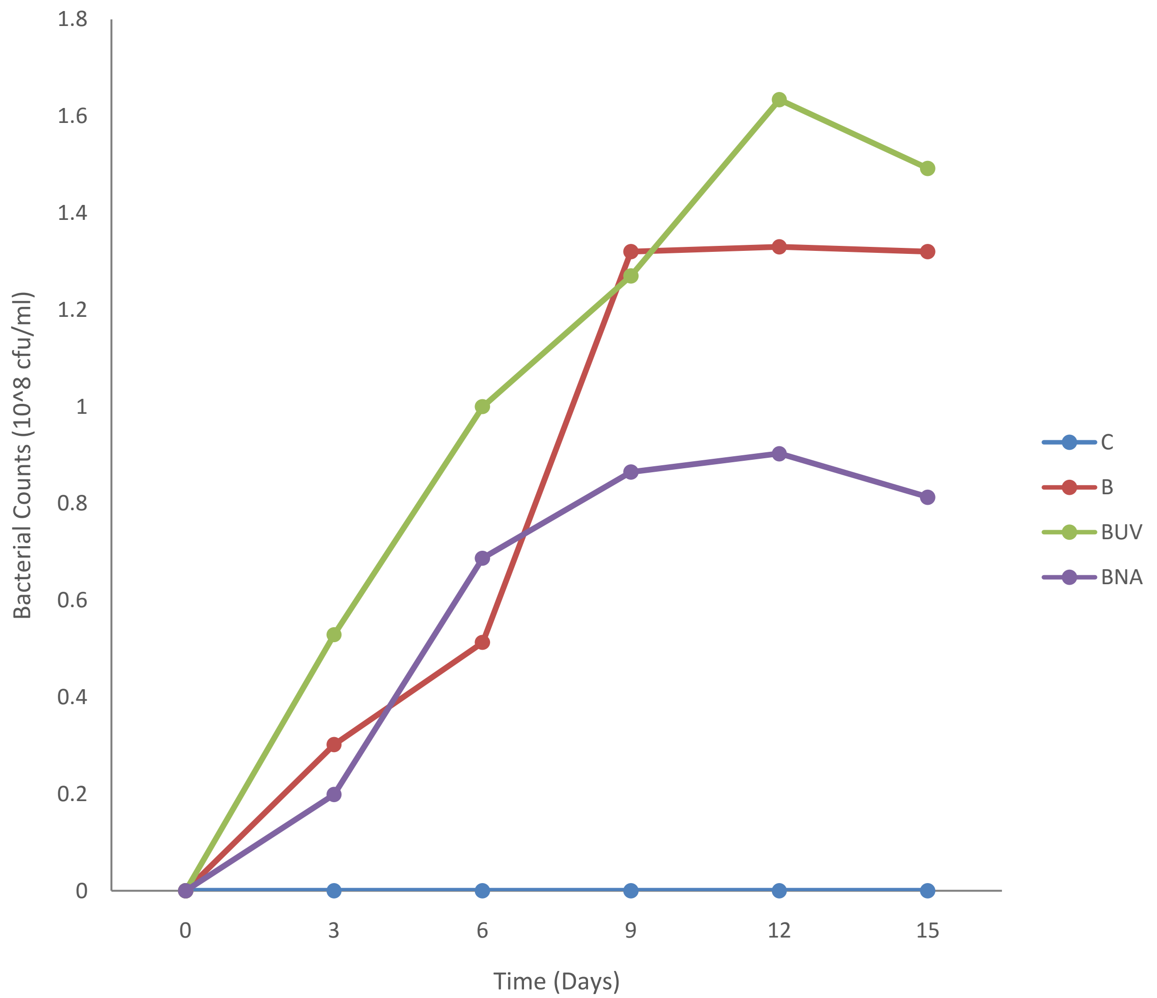

Figure 4.1: Changes in Bacterial Counts during Hydrocarbon Degradation by Wild and Mutant Strains of *Bacillus subtilis*

C: uninoculated,**B:** *Bacillus subtilis*, **BUV:** UV-Irradiated *Bacillus subtilis*, **BNA:** Nitrous Acid Treated *Bacillus subtilis*.

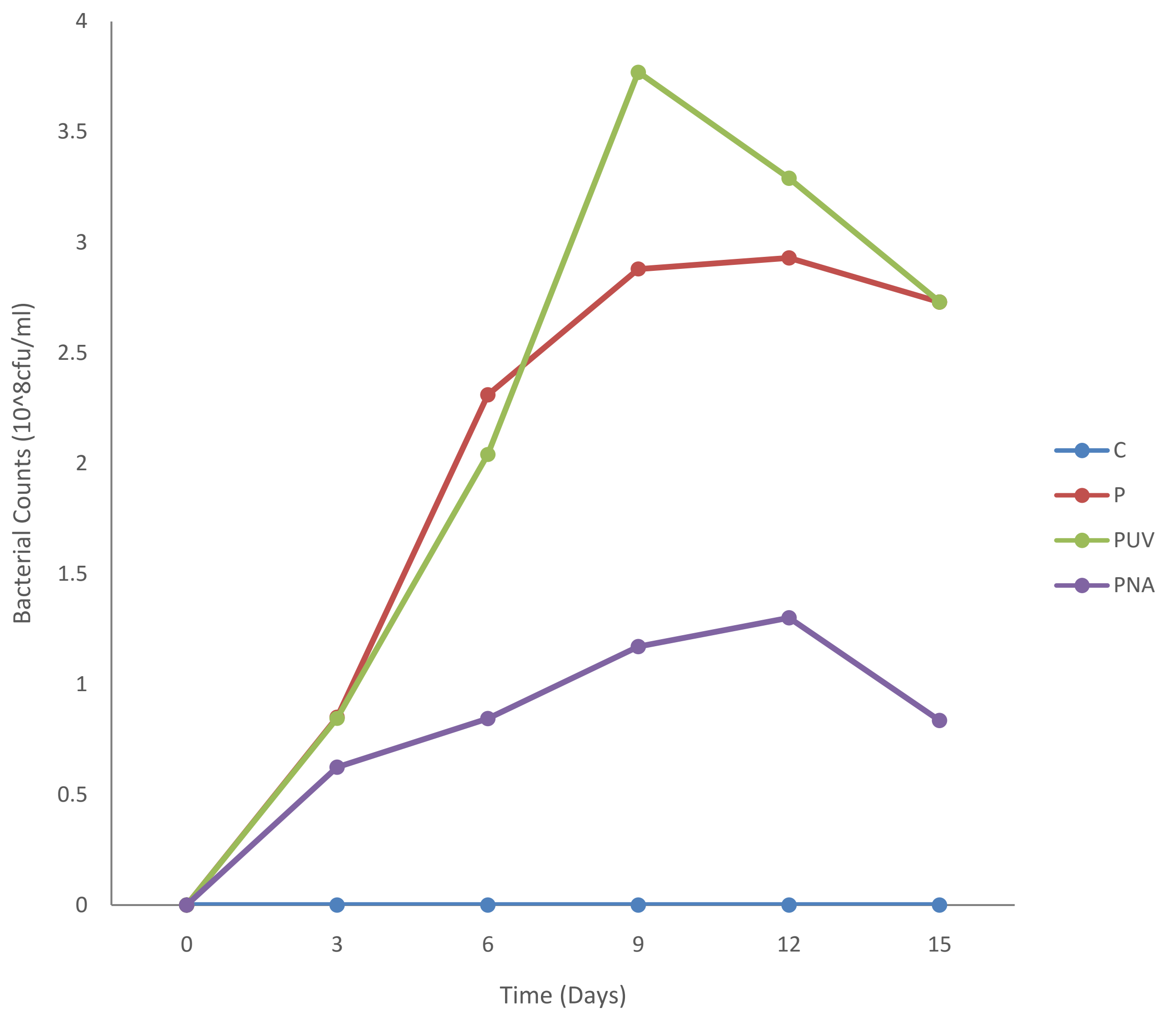

Figure 4.2: Changes in Bacterial Counts during Hydrocarbon Degradation by Wild and Mutant Strains of *Pseudomonas putida.*

C: uninoculated, **P:***Pseudomonas putida,* **PUV:** UV-Irradiated *Pseudomonas putida,* **PNA:** Nitrous Acid Treated *Pseudomonas putida***.**

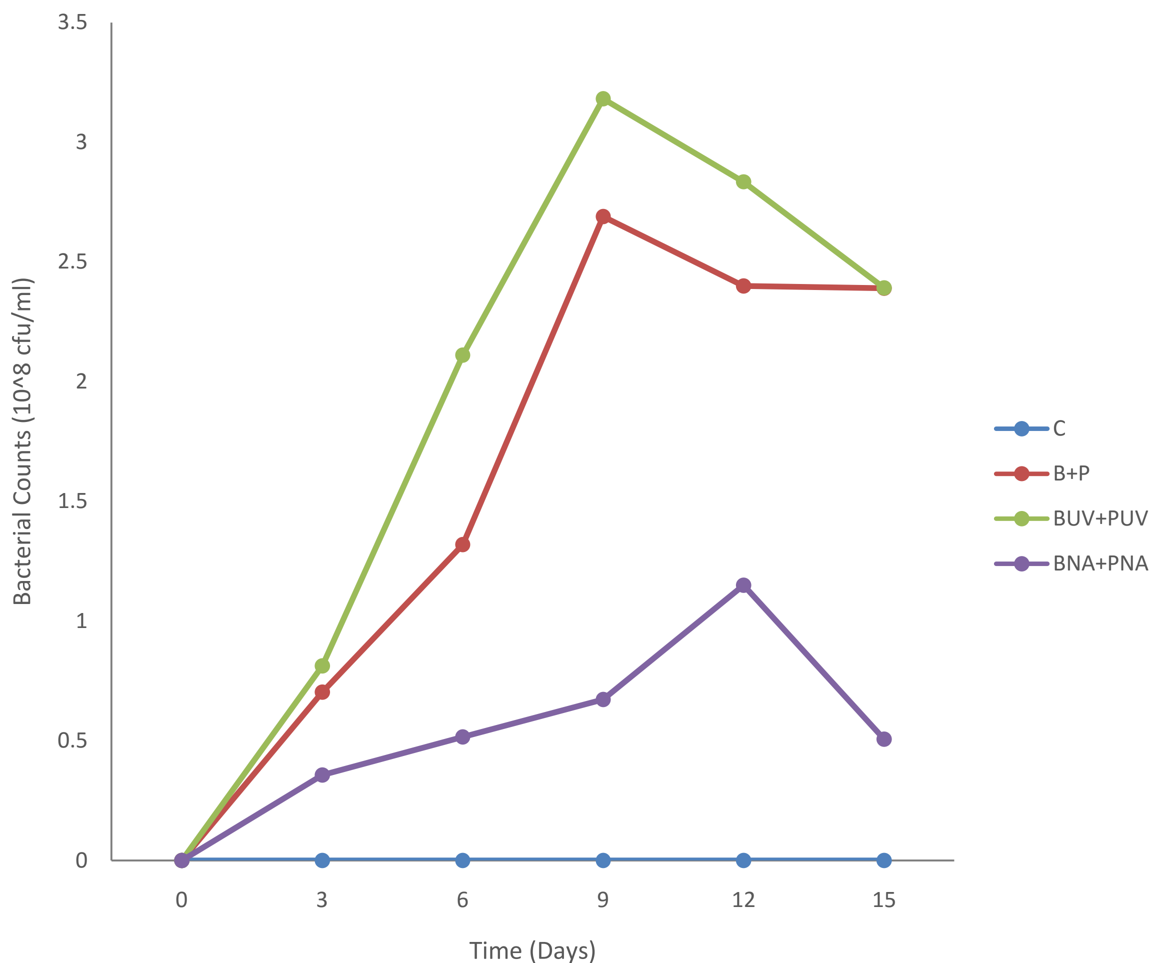

Figure 4.3: Changes in Bacterial Counts during Hydrocarbon Degradation by the Co-culture of Wild and Mutant Strains of *Bacillus subtilis* and *Pseudomonas putida.*

C: uninoculated, **B+P:** *Bacillus subtilis* + *Pseudomonas putida,***BUV+PUV:** UV-Irradiated *Bacillus subtilis* + UV-Irradiated *Pseudomonas putid*a, **BNA+PNA:** Nitrous Acid Treated *Bacillus subtilis* + Nitrous Acid Treated *Pseudomonas putida.*

4.5.2 Oil and Grease Degradation

The oil and grease degradation capacity of the wild and mutant strains of *B. subtilis and P. putida* are presented in figures 4.4 - 4.6. Fig. 4.4 shows the percentage amount of oil

and grease degraded by the wild strain of *B. subtilis* (B), UV-mutant strain (BUV) and nitrous acid mutant strain (BNA). Consistent pattern in oil and grease degradation was observed in BNA through out the period of incubation. B and BUV showed higher but similar degradation pattern with BNA from day 0 to 6 which was followed by a sharp increase in rate of degradation between day 6 and 9 while slower rate of degradation was observed in the remaining period of incubation. BUV showed highest percentage amount of oil and grease degraded followed by B while BNA showed the least degradation capacity.

The nitrous acid strain of *P. putida* (PNA), showed a steady degradation rate through the period of incubation. The wild strain (P) showed similar rate from day 0 to day 6 after which a sharp increase in degradation rate was observed between day 6 and 9 followed by a lower rate which remained steady through the remaining incubation period. Much lower rate was observed in the UV- mutant strain (PUV) from day 0 to day 6 after which a very sharp increase was observed between day 6 and 9. PUV showed the highest degradation capacity followed by P as shown in Fig. 4.5

The rate of oil and grease degradation by co-culture of the nitrous acid mutants of *B. subtilis* and *P. putida* (BNA+PNA) was relatively steady all through the period of incubation. The co-culture of the wild strains (B+P) showed a higher degradation rate from day 0 to day 9 but a reduction in rate of degradation was observed and was steady to the end of the incubation period. The co-culture of the UV-mutants (BUV+PUV) started with a lower rate which greatly increased between day 6 and 9. BUV+PUV showed the highest degradation capacity followed by B+P as shown in Fig. 4.6

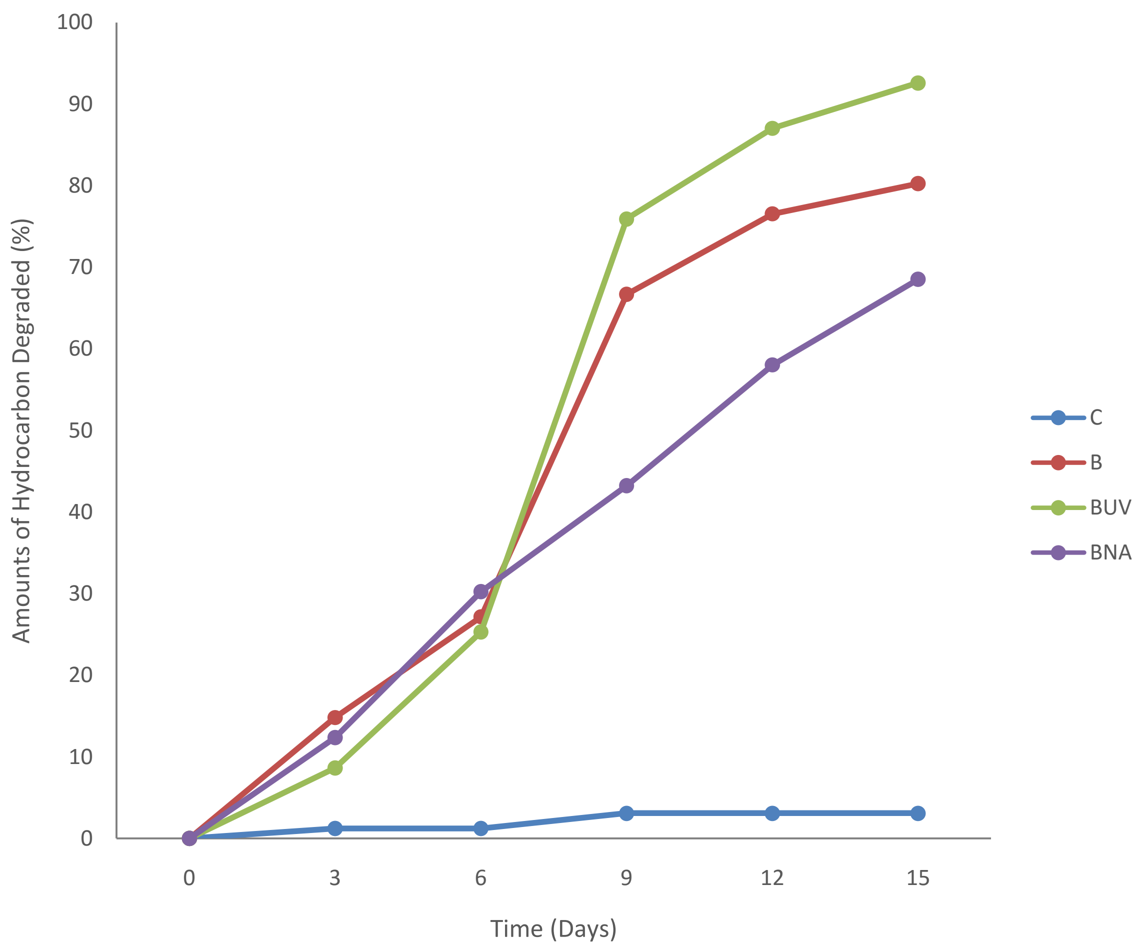

Figure 4.4: Percentage Amount of Hydrocarbons Degraded by Wild and Mutant Strains of *Bacillus subtilis*

C: uninoculated,**B:** *Bacillus subtilis*, **BUV:** UV-Irradiated *Bacillus subtilis*, **BNA:** Nitrous Acid Treated *Bacillus subtilis*.

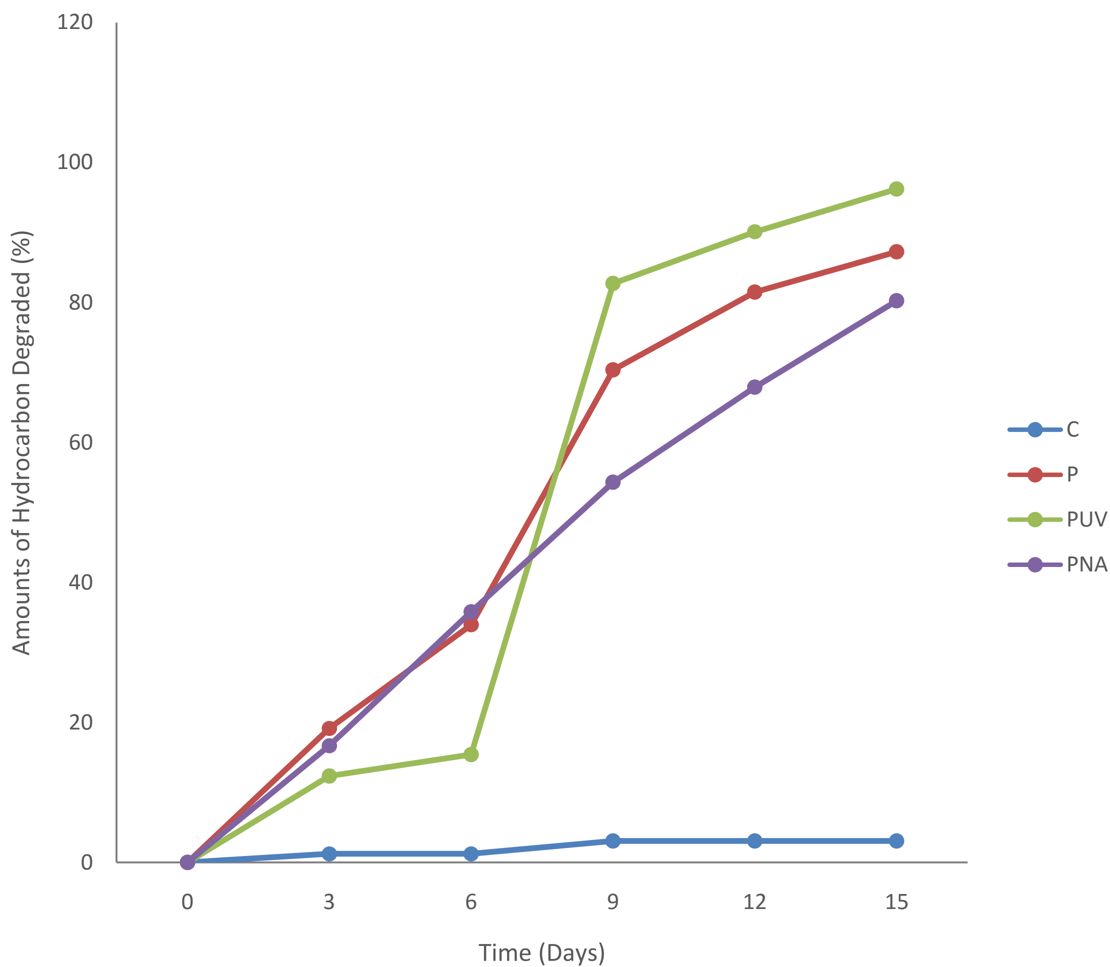

Figure 4.5: Percentage Amount of Hydrocarbons Degraded by Wild and Mutant Strains of *Pseudomonas putida*

C: uninoculated, **P:***Pseudomonas putida,* **PUV:** UV-Irradiated *Pseudomonas putida,* **PNA:** Nitrous Acid Treated *Pseudomonas putida.*

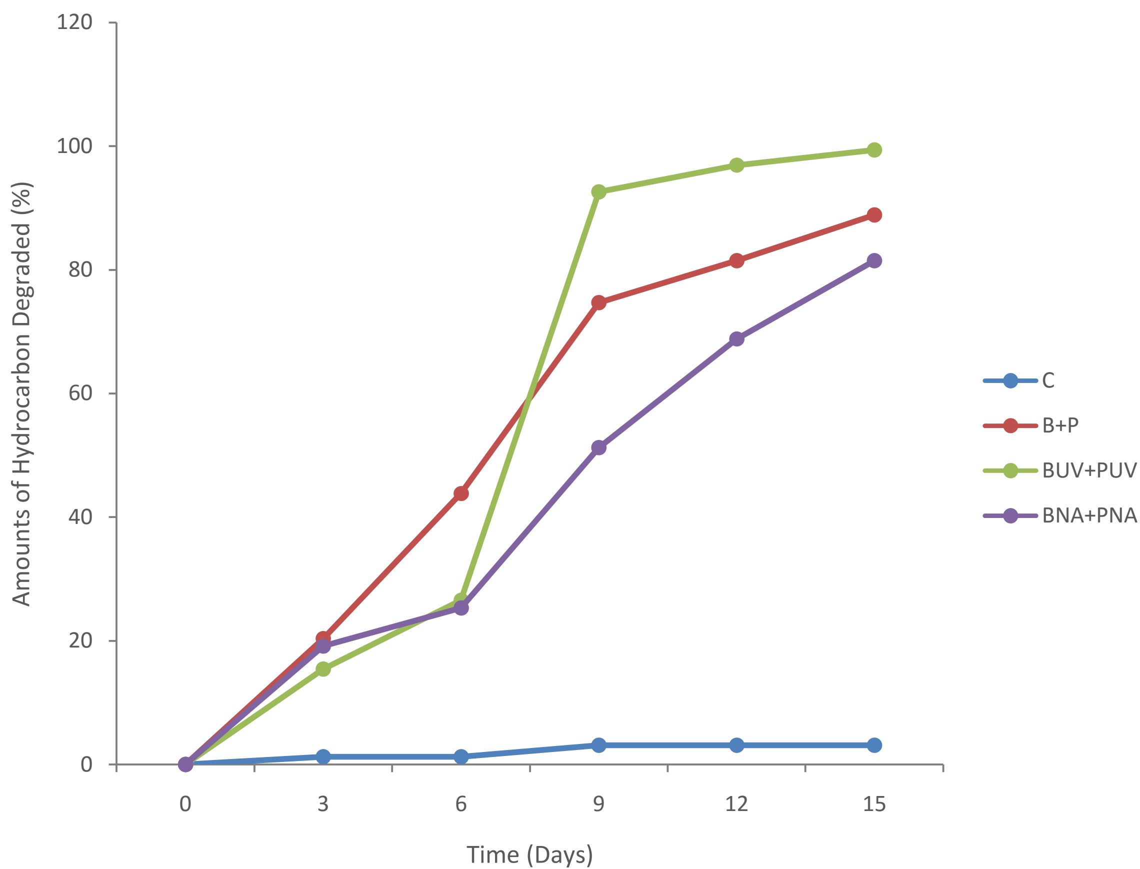

Figure 4.6: Percentage Amount of Hydrocarbons Degraded by the Co-culture of Wild and Mutant Strains of *Bacillus subtilis* and *Pseudomonas putida*

C: uninoculated, **B+P:** *Bacillus subtilis + Pseudomonas putida,***BUV+PUV:** UV-Irradiated *Bacillus subtilis* + UV-Irradiated *Pseudomonas putid*a, **BNA+PNA:** Nitrous Acid Treated *Bacillus subtilis +* Nitrous Acid Treated *Pseudomonas putida.*

CHAPTER FIVE

5.0 DISCUSSION

Physicochemical Parameters of the Refinery Effluent

The raw refinery effluent was grossly polluted as most of the physicochemical parameters analysed were found in concentrations considered to be deleterious. Therefore it will be unsafe to discharge such effluent into receiving sites without adequate treatment to reduce the pollutant levels. However some of the parameters (pH, temperature, suspended solids, nitrate, sulphate and phosphate) were found to be within the permissible limits set by the Federal Ministry of Environment for (FEMNV) refinery effluent in Nigeria.

The occurrence of certain physicochemical parameters such as temperature, total suspended solids, total dissolved solids, dissolved oxygen, nitrate, sulphate and phosphate at levels considered not hazardous to the environment based on FEMNV standard could be attributed to several factors which includes season of the year and time of sampling, process water used and other chemicals used in the refining process (Otukunefor and Obiukwu, 2005).

The high amounts of dissolved oxygen observed in the raw effluent could be as a result of the use of highly oxygenated process water. The fact that the effluents were coming fresh from the refining tower could also suggest that active microbial degradation of the organics that would have drastically reduced the level of the dissolved oxygen had not set in (Attioghe *et al*., 2007). The time of sampling (morning hours during dry and cold season) and the process water used could be the reason for the low amounts of suspended solids, dissolved solids and low temperature recorded. This is because rainfall plays an important role in increasing the suspended and dissolved solids of

wastewater due to constant washing of top soil materials into the effluent pond while temperature of wastewater is usually a function of the prevailing environmental temperature (Uzoekwe and Oghosanine, 2011; Marcus and Ekpete, 2014; Unimke *et al*., 2018).

Though the concentrations of these parameters appear to be low and within the acceptable limits, the need to treat prior to discharge into receiving sites cannot be overemphasized as continuous discharge could result in accumulation of these substances which may become hazardous especially if the receiving sites are soils or water bodies that are stagnant or with a very low flowrate.

These findings agree with the findings of Otukunefor and Obiukwu (2005); Obot *et al*. (2007) and Marcus and Ekpete (2014) who reported values of temperature, TDS, phosphate, nitrate and sulphate similar to that obtained in this work and within the FEMNV standard. However, this finding disagrees with the report of Uzoekwe and Oghosanine (2011) and Ajao *et al*. (2013) who reported a much higher value of TDS and DO (higher than the FEMNV standard) respectively.

The turbidity, BOD, COD and Oil and Grease content of the effluent samples were very high and well above the permissible limits set by FMENV for refinery effluents in Nigeria. The high turbidity could be attributed to the cumulative effects of the suspended and dissolved solids contained in the effluent. The high BOD value could be as a result of the presence of pollutants of organic origin especially hydrocarbons. The COD value obtained, in the same vein is a measure of oxygen demand exerted by both the organic component and reduced inorganic component of the effluent while the high value of oil and grease could have resulted from aggregate of oil that escaped the

refining process into the effluent receiving ponds (Obot *et* al., 2007; Uzoekwe and Oghosanine, 2011; Anaero-Nkwelle, 2013).

The reduction in the values of these parameters in the biologically treated effluent (effluent undergoing treatment) (Table 4.1) could be attributed to the activity of the autochthonous microorganisms in the biological treatment plant in a bid to digest the organic materials to obtain energy.

Based on the results obtained, the effluent is a potential source of environmental hazard and as such must be treated thoroughly before discharge to avoid drastic alteration of the physicochemical properties of the receiving sites. High or increased turbidity of the effluent receiving water body could reduce the rate of aeration from the atmosphere and penetration of sunlight which can contribute to a continuous build up of oxygen deficit with consequent devastating effect on the aquatic organisms. Also, proliferation of aquatic organisms that require sunlight to thrive could be reduced drastically as penetration of sunlight become very minimal (Attioghe *et al*., 2007; Unimke *et al*., 2018).

BOD test is useful in determining the relative organic wasteloading of water. High value therefore indicates the presence of large amount of organic pollutants andrelatively higher level of microbial activities with consequent depletion of oxygen content (Uzoekwe and Oghosanine, 2011). The high values of BOD observed in this study are causes for worry because the continuous discharge of waste water containing organic matterinto water bodies could result in depletion of oxygen as microorganisms engage in biochemical decomposition of the organic pollutants (Attiogbe *et al*., 2007). This problem leads to the inadequatemaintenance of higher life forms. In addition, oxygen availability is important because the endproducts of chemical and biochemical reactions

in anaerobic systems often produce aestheticallydispleasing colours, tastes and odours in water (Attiogbe *et al*., 2007). The findings in this work are in agreement with those of Otukunefor and Obiukwu (2005); Uzoekwe and Oghosanine (2011); Ajao *et al*. (2014) and Marcus and Ekpete (2014) who also reported high values of BOD (much higher than the FEMNV standard) in raw petroleum refinery effluents

Joel andAmajuoyi (2009) reported that high level of contamination with oil and grease poses a greatconcern and long term threat to all forms of life.Oil and grease are sticky in nature; they tend to aggregate, clogging drain pipes and sewer lines,causing unpleasant odours and corroding sewer lines under anaerobic conditions(Chen *et al*., 1999; Xu and Zhu, 2004). They also interfere with unit operations in municipalwastewater treatment plants because they float as a layer on top of the water. They alsostick onto pipes and walls consequently blocking strainers and filters (Xu and Zhu, 2004). Though, the amount of oil and grease observed in the treated effluent was within the permissible limit it can still pose serious threat to receiving environments as such amount (8.9mg/l) is capable of exerting a BOD that may stress the environment. This result is in agreement with the of findings of Ajao *et al*. (2014) who reported oil and grease content of raw effluent to be 43.2mg/l in Kaduna refinery but disagree with the work of Otukunefor and Obiukwu (2005) who reported the oil and grease content of treated effluent from Port-Harcourt refinery to be 13.52mg/l.

Isolation of Hydrocarbon Utilizing*Bacillus subtilis* and *Pseudomonas putida*

Eleven (11) and six (6) isolates of *P. putida* and *B. subtilis* were isolated from the refinery effluent samples after 14 days of enrichment in mineral medium containing 1% (v/v) of crude oil (Table 4.4). The occurrence of *B. subtilis* and *P. putida* from refinery effluent portrays that these organisms were able to adapt and thrive in environments

polluted with petroleum hydrocarbons. Also, their ability to survive in mineral medium enriched with 1% (v/v) of crude oil as the sole source of carbon and energy during two weeks of enrichment technique is important and suggests the synthesis of enzymes involved in crude oil metabolism (Okoh *et al*., 2001). These findings showed that *B. subtilis* and *P. putida* were able to adapt to hydrocarbon polluted sites and that enrichment technique is useful in isolation of various culturable oil degrading bacteria from contaminated sites. The findings are also in agreement with the work of other researchers such as Okerentugba and Ezeronye (2003); Bako *et al.* (2008); Idise *et al*. (2010); Moneke and Nwangwu (2011); Salam and Obayori (2013); Chikere and Ekwuaba (2014) and Liyange and Manage (2016) who isolated either *Bacillus* or *Pseudomonas* species or both from petroleum polluted sites. The mechanisms employed by the autochthonous microorganisms to achieve this feat includes synthesis of inducible enzymes, mutations such as single nucleotide change or DNA re-arrangement that results in degradation of the compound and acquisition of genetic information from closely related or phylogenetically distinct population within the hydrocarbon-challenged community (Salam *et al*., 2011).

Higher numbers of hydrocarbon utilizing *B. subtilis* and *P. putida* were isolated from the treated effluent than the raw effluent. This could suggest that the bacteria community in the treated effluent was more adapted to petroleum hydrocarbons probably due to longer exposure which enhanced better adaptation and colonization of the biological treatment plant unlike the raw effluent that came fresh from the refining process as a result, the bacterial community may not have completely adapted to the environments.

Utilization of Crude Oil as Source of Carbon by the Wild Strains of *B. subtilis* and *P. putida*

The isolated strains of *B. subtilis* and *P. putida* were tested for their ability to grow on solid mineral salts medium (MSM) with crude oil as the sole carbon source. Visual detection of bacterial growth was taken as an indication of ability to utilize crude oil. This measurement is rapid and simple being used by several workers (Ijah, 1998; Sepahi *et al*., 2008; Idise *et al*., 2010). It gives a preliminary screening of the degradation ability of all isolates within a short time. The results presented in Table 4.5 show different levels of growth as luxuriant, medium and scanty on mineral salt agar medium enriched with different concentrations of crude oil. The difference in the ability of the various strains of *Bacillus subtilis* and *Pseudomonas putida* to grow on mineral medium containing varying concentrations of crude oil may be attributed to better adaptation with corresponding better developed enzyme system for hydrocarbon metabolism of certain strains than others and the presence of different catabolic genes involved in hydrocarbon degradation in the bacterial species (Majid *et al*., 2008; Onuoha *et al*., 2011).

Evaluation of Hydrocarbon Degradation by the Wild and Mutant of *B. subtilis* and *P. putida*

The present findings revealed that both the wild and mutant strains of the isolates (*B. subtilis* and *P. putida*)have varying hydrocarbon degradation capacity. *P. putida* showed higher ability to degrade hydrocarbons than *Bacillus subtilis* and such difference in the rate of hydrocarbon degradation may be due to the presence of different catabolic genes and better developed enzyme systems involved in hydrocarbon degradation in the bacterial species (Majid *et al*., 2008; Onuoha *et al*., 2011). This is in agreement with the findings of Anene and Chika (2011); Moneke and Nwangwu (2011); Nwanyawu and Abu (2012) and Vinothini *et al.* (2015) who working independently observed that

Pseudomonas species had higher hydrocarbon degradation efficiency than *Bacillus* species.

While all the strains tested degraded considerable amounts of hydrocarbons, highest hydrocarbon degradation was observed in the UV-mutant strains of both isolates. The nitrous acid mutant strains showed lower degradation capacity compared to the wild strains. The observed difference in the degradative capability of the wild and mutant strains could be attributed to the changes in the genetic make-up or catabolic genes of the isolates that may have occurred in a bid to survive the exposure to mutagens. While the UV-irradiation resulted in increased hydrocarbon degradation, treatment with nitrous acid resulted in decreased ability to degrade hydrocarbons. This finding agrees with the findings by Idise *et al.* (2010) who reported that UV-irradiated nitrous acid treated strains of *Bacillus cereus* and *Pseudomonas putida* showed higher hydrocarbon degradation than the wild strains Similarly, the findings agree with that ofAjao *et al.* (2013) who reported higher activity of cathecol-2,3-dioxygenase enzyme in UV-irradiated strains of *Pseudomonas* species than their parent counterparts.

Mutation does not always result in improved efficiency of organisms. The phenotypic expression of mutated genes could be either on the positive (favourable) or negative (unfavourable) note depending on the specific changes exerted on the nucleotide sequence of the exposed organism (Idise *et al.,* 2010). This could however be seen as the reason the nitrous acid mutant strains of both isolates had lower hydrocarbon degradation capacity than the wild strains.

The co-culture of both the wild and mutant strains of the isolates exhibited higher capacity to degrade hydrocarbons than the individual organisms. This could suggest that individual organisms could only metabolize limited range of hydrocarbons (Adebusoye

et al. 2007). Many researchers such as (Salam *et al*., 2011) also reported higher hydrocarbon degradation efficiency in bacterial consortium than individual cultures. This has led to the assertion that mixed culture exhibited superior degradative potential than pure culture strains (Leahy and Colwell, 1990; Adebusoye *et al.,* 2007; Ajao *et al*., 2014). In a culture, some species utilize intermediates of degradation of the original hydrocarbon produced by other members of the culture leading to a complete degradation of the oil (Atlas, 1981; Ajao *et al*., 2014). Several reports have confirmed microbial consortia as better degraders than the individual organism. Thus, a mixed culture is a better inoculum for bioremediation. The observation of diauxic growth phenomenon in the complex mixture of petroleum is expected as petroleum consists of linear as well as aromatic compounds which usually required different enzymes and biodegradation pathways (Idise *et al.,* 2010).

These findings showed that the isolates achieved a great feat in hydrocarbon degradation as the percentage degradation ranged from 68.52% to 99.38% after incubation of samples for 15 days. With the exception of the nitrous acid mutant strain of *Bacillus subtilis*, all the other strains tested brought the concentration of oil and grease (46mg/l) in the raw refinery effluent to within the permissible limit set by the Federal Ministry of Environment (FMENV) with the co-culture of the UV-mutant strains of the *Bacillus subtilis* and *Pseudomonas putida* reducing the concentration of oil and grease from 46mg/l to as low as 0.29mg/l. In similar studies, Ajao *et al*. (2014) reported 90% oil and grease degradation after 20 days of incubation, Idise *et al*. (2010) reported 98.25 oil degradation, Rahman *et al*. (2002) also showed degradation of oil up to 78% after incubation of samples for 20 days inoculated with the bacterial consortium. Singh *et al*. (2013) also demonstrated 70% degradation of oil from the wastewater after 10 days of incubation.

Biodegradation of petroleum hydrocarbon generally relies upon the cooperation of more than a single bacterial species. This is particularly true when complete mineralization of the hydrocarbons to CO_2 and H_20 is desired. A pure bacterial culture may not have the metabolic capability to readily degrade certain compounds or to have the biomass necessary to degrade the compounds rapidly enough. Consortium or mixed populations with overall broad enzymatic capacities may be required to achieve total degradation of the petroleum. The higher biodegradation rate and microbial activity observed in the mixed culture may be related to shift in importance of one metabolic pathway over another possibly as a result of microbial synergism. The assumption that, more than one hydrocarbon compound degradation pathway exists in different microbial species and therefore, it is possible that individual bacteria able to degrade more than one aromatic substrate will have more than one pathway for their metabolism (Ajao *et al*., 2013).

The utilization of the oil resulted in increase in the population densities of the various bacterial strains. The growth rate of the organisms correlated with the rate of oil degradation, suggesting that the break down of the hydrocarbons resulted in the provision of utilizable compounds required for their growth. Ajao *et al.* (2013) also reported an increase in log number of cells with increase in degradation efficiency. The trends observed in the growth of the various isolates were similar with each attaining its highest population density either at day 9 or day 12 of the incubation period and this coincides with the period during which highest amount of oil was degraded. Though the highest hydrocarbon degradation capacity was observed in the co-culture of the UV-mutant strains, the UV-mutant strain of *P. putida* had the highest population density of 3.77×10^{8}cfu/ml. A decrease in population occurred between day 12 and 15 in all the strains. This could be attributed to decrease in the amount of utilizable hydrocarbons consequently resulting in competition, build up of toxic substances such as organic

acids and other metabolic products that may be unfavourable to the growth of the organisms. Other researchers such as Nwanyanwu and Abu (2012); Esedafe *et al*. (2015) and Asadirad *et al.* (2016) reported that that bacterial isolates growing in mineral medium containing various hydrocarbons as the carbon source increased in population with increase in the rate of degradation but decreased progressively as the incubation continued

The observation of diauxic growth phenomenon in the complex mixture of petroleum is expected as petroleum consists of linear as well as aromatic compounds which usually require different enzymes and biodegradation pathways (Ajao *et al*., 2014).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Based on the observations made in this study, the following conclusions were drawn:

The raw petroleum refinery effluent was grossly polluted especially with pollutants of organic origin.

The petroleum refinery effluent is a good source for *Bacillus subtilis* and *Pseudomonas putida* with hydrocarbon degrading capacity

Isolates of *Bacillus subtilis and Pseudomonas putida* from refinery effluent undergoing biological treatment were better developed for hydrocarbon degradation than those from raw refinery effluents

Mutants of *Bacillus subtilis* and *Pseudomonas putida* with improved hydrocarbon degradation can be generated by treatment with UV-irradiation.

While wild strains of *Bacillus subtilis* and *Pseudomonas putida* had a good hydrocarbon degrading efficiency, modification by irradiation with UV- light at 254nm for 30 minutes resulted in increased efficiency.

6.2 Recommendations

1. Bacteria with good hydrocarbon degrading potential should be sought for in petroleum effluents biological treatment plants

2. Regulatory agencies in Nigeria should set up reference laboratories to monitor the quality of effluent generated in refineries to ascertain the level of compliance to the legally set toxicant levels

3. Irradiation with UV should be employed for improving the hydrocarbon degrading efficiency of bacterial isolates

4. UV mutants of *Bacillus subtilis* and *Pseudomonas putida* should be used to augment the native microbial community in biological treatment plant to enhance hydrocarbon stabilization.

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APPENDICIES

Appendix 1: Cultural Characteristics of Hydrocarbon Utilizing Bacteria

(a) Cultural Characteristics of Colonies on Nutrient Agar

Isolate's code	Size	Pigmentation	Form	Elevation
TE1	small	cream	circular	flat
TE2	small	cream	circular	flat
TE3	small	cream	circular	convex
TE4	large	cream	circular	flat
TE5	large	cream	circular	convex
TE6	tiny	cream	circular	flat
TE7	small	cream	circular	flat
TE8	small	cream	circular	flat
TE9	small	cream	circular	flat
TE10	tiny	cream	circular	flat
TE11	large	cream	circular	convex
RE12	small	cream	circular	flat
RE13	large	cream	circular	flat
RE14	small	cream	circular	flat
RE15	small	cream	circular	flat
RE16	small	cream	circular	flat

RE17	small	cream	circular	convex
RE18	small	cream	circular	flat
RE19	tiny	cream	circular	flat
RE20	large	cream	circular	flat

KEY: TE; Treated Effluent on nutrient agar, RE; Raw Effluent on nutrient agar

(b) Cultural Characteristics of Colonies on Centrimide Agar

Isolate's code	Size	Pigmentation	Form	Elevation
TEC1	small	green	circular	convex
TEC2	small	green	circular	convex
TEC3	small	green	circular	flat
TEC4	small	green	circular	convex
TEC5	small	green	circular	convex
TEC6	small	green	circular	convex
TEC7	small	green	circular	convex
TEC8	small	green	circular	convex
TEC9	small	green	circular	convex
TEC10	small	green	circular	convex
TEC11	small	green	circular	flat
TEC12	small	green	circular	flat
TEC13	small	green	circular	convex
REC14	small	green	circular	flat
REC15	small	green	circular	convex
REC16	small	green	circular	convex
REC17	small	green	circular	convex
REC18	small	green	circular	flat
REC19	small	green	circular	flat
REC20	small	green	circular	flat

TEC; Treated Effluent on centrimide agar, **REC**; Raw Effluent on centrimide agar.

Appendix 2: Growth of Hydrocarbon Utilizing Bacteria on Mineral Medium Containing 1% Crude Oil.

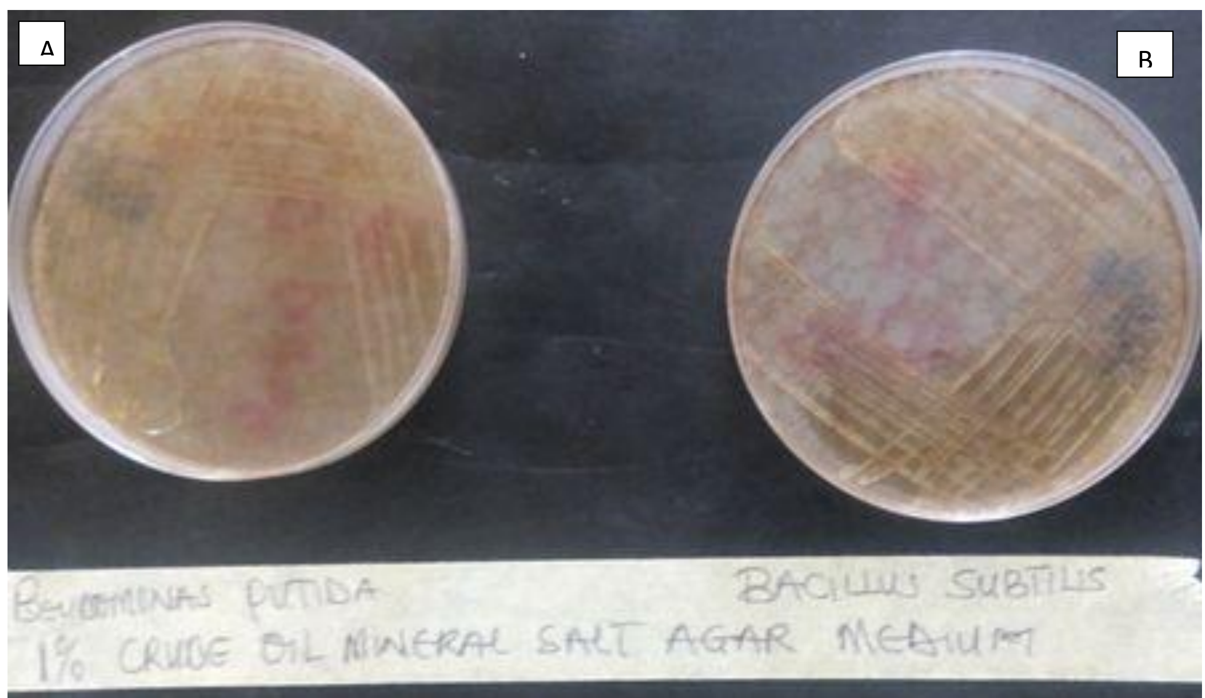

Plate A: Pseudomonas *putida* and Plate B: Bacillus *subtilis* in1% Crude oil Mineral Salt Agar Medium

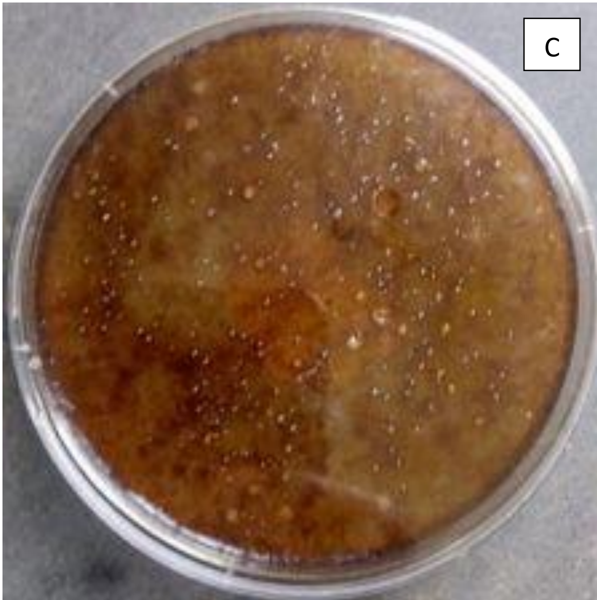

Plate C: Co-culture of Pseudomonas *putida*and Bacillus *subtilis* in1% Crude oil Mineral Salt Agar Medium

Appendix 3: Breakdown of Effluent by Wild and Mutant Strains of *B. subtilis* and *P. putida.*

Six (6) days Culture of the various Isolates in Raw Effluent Medium.

KEY: C; Control flask, **B;** Parent *Bacillus subtilis* culture, **P;** Parent *Pseudomonas putida* culture, **B+P;** Co-culture of the parent organisms, **BUV;** UV-light mutated *Bacillus subtilis*, **PUV;** UV-light mutated *Pseudomonas putida*, **BUV+PUV;** Co-culture of the UV-light mutants, **BNA;** Nitrous acid mutated *Bacillus subtilis*, **PNA;** Nitrous acid mutated *Pseudomonas putida*, **BNA+PNA;** Co-culture of the nitrous acid mutants.

Twelve (12) days Culture of the various Isolates in Raw Effluent Medium.

KEY: C; Control flask, **B;** Parent *Bacillus subtilis* culture, **P;** Parent *Pseudomonas putida* culture, **B+P;** Co-culture of the parent organisms, **BUV;** UV-light mutated *Bacillus subtilis*, **PUV;** UV-light mutated *Pseudomonas putida*, **BUV+PUV;** Co-culture of the UV-light mutants, **BNA;** Nitrous acid mutated *Bacillus subtilis*, **PNA;** Nitrous acid mutated *Pseudomonas putida*, **BNA+PNA;** Co-culture of the nitrous acid mutants.

Appendix 4: One Way ANOVA of Residual Concentration of Oil During Biodegradation.

	Residual Concentration of Oil (Mg/L)			
Time (Days)	Control	B	P	B+P
0	46.00	46.00	46.00	46.00
3	45.43±0.41	39.19±0.08*	37.20±0.21**	36.63±0.55***
6	45.43±0.38	33.51±3.12*	30.38±0.33*	25.84±0.20***
9	44.57±0.16	15.33±0.32*	13.63±0.17**	11.64±0.26***
12	44.57±0.38	10.79±0.35*	8.57±0.40**	8.52±0.20***
15	44.57±0.38	9.09±0.66*	5.87±0.50**	5.05±0.07***

(*) indicate significance difference in degradation compared to control
(**) indicate significance difference in degradation when compared to B
(***) indicate significance difference in degradation when compared to B

Time (Days)	Residual Concentration of Oil (Mg/L)			
	Control	BNA	PNA	BNA+PNA
0	46.00	46.00	46.00	46.00
3	45.43±0.41	40.33±1.26*	38.33±0.56*	37.20±0.31**
6	45.43±0.38	32.09±0.94*	29.53±0.39*	34.36±0.36**
9	44.57±0.16	26.12±1.32*	21.01±0.10**	20.43±0.77***
12	44.57±0.38	19.31±0.73*	14.77±0.17**	14.35±0.68***
15	44.57±0.38	14.48±0.45*	9.09±0.11**	8.52±0.10***

(*) indicate significance difference in degradation compared to control
(**) indicate significance difference in degradation when compared to BNA
(***) indicate significance difference in degradation when compared to BNA

Time (Days)	Residual Concentration of Oil (Mg/L)			
	Control	BUV	PUV	BUV+PUV
0	46.00	46.00	46.00	46.00
3	45.43±0.41	42.03±1.89*	40.32±0.17*	38.90±0.85**
6	45.43±0.38	34.36±0.17*	38.90±0.34**	33.79±0.02**
9	44.57±0.16	11.07±0.02*	7.49±0.20**	3.41±0.11***
12	44.57±0.38	5.96±0.33*	4.54±0.61**	1.42±0.10***
15	44.57±0.38	3.40±0.12*	1.73±0.02**	0.29±0.02***

(*) indicate significance difference in degradation compared to control
(**) indicate significance difference in degradation when compared to BNA
(***) indicate significance difference in degradation when compared to BNA