BIOREMEDIATION OF CRUDE OIL CONTAMINATED SOIL USING AGRO-WASTE

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ABSTRACT

The two agro-waste (horse dung and unripe plantain peels) used as biostimulants for the bioremediation of crude oil contaminated soil are of biological origin and are practical and safe environmental remediation agents. Microbiological (Gram staining, Catalase test, Citrate test, Starch hydrolysis, Methyl Red test, Motility, Urease test and Sugar fermentation) and physicochemical analysis (pH, Organic matter, Phosphorous, Nitrogen, Cation exchange capacity, Moisture) of the agro-waste and soil (contaminated and uncontaminated soil) were assessed before and after remediation. The agro-wastes were introduced into crude oil contaminated soil in single and combination (10% and 20 % respectively). The treated samples were incubated for 56 days and characterized for their physicochemical properties, microbial counts (which was determined every 14^{th} day) and total petroleum hydrocarbon which was determined before and after the treatments. There was decrease in pH (6.67 to 4.10), organic carbon (15.53 to 6.07 %) and an increase in total phosphorous (10.06 to 15.30 $mgkg^{-1}$) and nitrogen (0.29 to 4.72 %) of the oil contaminated soil remediated with agro-waste. The Total Aerobic Heterotrophic Bacterial Counts increased from $8x10^5$ cfu/g to 8.7×10^7 cfu/g, Hydrocarbon Utilizing Bacterial Counts increased from $2x10^5$ cfu/g to 5.9×10^7 cfu/g, whereas the Total Aerobic Heterotrophic Fungal Counts increased from $2x10^5$ cfu/g to 8.4×10^6 cfu/g, Hydrocarbon Utilizing Fungal Count increased from $3.0x10^4$ cfu/g to 5.4×10^6 cfu/g. The percentage biodegradation of crude oil after the treatment of the contaminated soil with the agro-wastes increased from 2.57 % to 89.05 % after 56 days. Gas Chromatographic and Mass Spectrophotometric analysis of the residual oil revealed that there was reduction in the Total Petroleum Hydrocarbon (TPH) as a result of the rapid degradation by microorganisms. There were significant differences ($P<0.05$) when both agro-wastes (horse dung and unripe plantain peels) were added to the crude oil contaminated soil when compared to the agro-waste when added individually. Both agro-wastes (Horse dung and Unripe plantain peels) are good biostimulants for remediation process however the treatment with the higher quantity of the combined agro-wastes (20 % horse dung (HD) and 20% unripe plantain peel (UPP)) caused the highest increase in the microbial growth, percentage biodegradation and with the least total petroleum hydrocarbons remaining at the end of the remediation process.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Hydrocarbons are the most frequent main energy and fuel resources on the globe due to their tremendous energy output. Petroleum hydrocarbons (PHCs) are essential to society since they are used to generate power, heat homes, and move people throughout the world (Wu *et al*., 2017). Spills that occur during ordinary crude oil production, refining, and distribution, as well as those that occur as a result of an accident, have kept this subject alive because these spills are essentially unavoidable in these processes (Alizadeh *et al*., 2018). Petroleum hydrocarbon pollution can be caused by infrastructure failure, human negligence, or natural disaster (Alvarez *et al*., 2020). It can harm human and environmental health, making this a severe problem on a local and worldwide scale. Petroleum hydrocarbons have a wide range of physiological effects; some injure the central nervous system, while others harm the immune system, liver, kidneys, and lungs (Varjani *et al*., 2017).

Crude oil leaks are caused by vandalism (sabotage, or individuals stealing oil and attempting to claim compensation, as well as cleaning contracts), overburdened facilities, oil storage tanks that unknowingly harm our ecosystem, and neglected pipelines all play a part. When tankers and barges discharge hydrocarbons into the environment, oil spillage occurs not just in oil-producing states, but also in areas prone

to oil spills due to transportation accidents and rupture-prone pipeline networks. Oil products, pipeline overflows, pipeline failures, and storage tank spills are all potential sources of contamination for an oil spill. Oil spills have a negative impact on the soil's chemical and physical properties, leaving it unable to operate efficiently (Ofoegbu *et al*., 2015). The presence of PHC compounds in the environment necessitates the presence of bacteria capable of decomposing them. A significant factor influencing soil and water biodegradation of hydrocarbons by microorganisms is nutrient deficiency, particularly nitrogen and phosphorus. Organic or inorganic nutrient-rich supplements (biostimulation) have been found to be beneficial in bioremediation (Abioye *et al*., 2012).

Many experts have demonstrated that providing organic and inorganic nutrients like nitrogen and phosphorus has a variety of beneficial impacts on microbial activity and/or petroleum hydrocarbon breakdown (Abioye *et al*, 2012).

1.2 Statement of the Research Problem

The presence of polycyclic aromatic hydrocarbons (PAHs) in crude oil can create chronic issues such as mutagenicity and carcinogenicity hence crude oil is a global environmental problem. Due to the presence of poisonous, mutagenic, and carcinogenic PAHs, hydrocarbon spills have an impact on land, air, and water. According to certain studies, persons who work in workplaces with high oil levels for extended periods of time may have liver or renal difficulties, damaged bone marrow and cancer (Abioye *et al*., 2012).

Furthermore, when PAHs enter plant roots, they impact negatively on plant biota. They can also enter the human food chain through animal consumption, posing a concern to human health. Oil has a significant impact on soil fertility, runoff, permeability, and

binding capacity (Selvam *et al*., 2018). It cannot be overstated how important it is to investigate the impact of agro-waste on crude oil biodegradation in soil.

1.3 Justification for the study

There are 3 reasons for soil modification through agro-wastes: (1) Adsorption and absorption of metals and organic compounds, removing them from the environment and preventing contact with plants, animals, and humans; (2) Increasing the amount of beneficial bacteria; and (3) Reducing the amount of harmful bacteria (Lehmann *et al*. 2007; Sohi *et al*. 2015). Because of its capacity to use low-cost equipment, ecologically acceptable or simple methods, and the capacity to reduce waste (horse dung and unripe plantain peel), bioremediation is a promising solution for restoring damaged ecosystems (Clement *et al*., 2012).

1.4 Aim and objectives

The aim of this study was to determine the effect of agro-waste in remediating crude oil contaminated soil.

The objectives of the study were to:

i. Determine the microbiological and physicochemical properties of the soil and agro-waste used for the study.
ii. Characterize the microbial isolates
iii. Determine the potential of the agro-waste in remediating crude oil contaminated soil.
iv. Determine the changes in physicochemical and microbiological properties of soil during the bioremediation of the crude oil.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Petroleum Contaminations

Environmental pollution caused by a varied array of persistent organic and inorganic toxins has increased in lockstep with the growth of the global economy (Gaskin and Bentham, 2010). Petroleum products are one of the most pernicious environmental contaminants in contemporary life (Zang *et al*., 2015; Wang *et al*., 2019,). Saturated hydrocarbons, aromatic hydrocarbons, resins, and asphaltenes comprise it (Liu *et a*l., 2017). Cannibalizing the soil's ecological system has a negative impact on the environment, stifling plant growth, eroding soil structure, and degrading ground water quality (Xu *et al*., 2018). Additionally, these petroleum-derived compounds might have a negative impact on human health (Anyika *et al*., 2015). Petrochemical companies will undoubtedly develop in response to the growing demand for petroleum-based energy (Bierkens and Geerts, 2014). However, in some areas, these businesses contaminate the land and water (Chebbi *et al*., 2017). Hydrocarbon of crude oils and their derivatives, such as gasoline, diesel fuel, heavy oil, motor oil, dregs of fuel and oil, have a significant impact on environmental recovery (Yang *et al*, 2019). Alkanes are one of the easiest petroleum related pollutants to eradicate. However, as the length of a petroleum product's carbon chain increases, its solubility diminishes, making it more susceptible to deterioration (De la Cueva *et al*., 2016). The majority of soil remnants contain PAHs

and other non-hydrocarbon chemicals, which might result in soil salinization and heavy metal poisoning (Wu *et al* 2013). By polymerization, mineralization, or modification, microorganisms are capable of detoxifying harmful organic substances (Shukla *et al*., 2010). These bacteria were discovered to be capable of digesting hydrocarbons, making them the primary carbon source.

2.2 Adverse Effect of Petroleum Hydrocarbons Contamination on Soil

Soil is a three-component substance (solid, liquid and gas), with the primary application in the field being in relation to its mechanics (Abousnina *et al*., 2015). Oil-contaminated soil alters the makeup of its phases and can result in the failure of geotechnical projects due to altered mechanical characteristics of sand. Khamehchiyan *et al*., (2007) observed a decline in the strength and permeability of sandy and clayey soils. According to Singh *et al*, (2008), these traits have shifted. Once petroleum hydrocarbons enter an ecosystem, they primarily cause biological damage by impeding the transport of water, nutrients, oxygen, and light, as well as by affecting soil fertility, plant growth, and germination. Polycyclic aromatic hydrocarbons are prevalent in crude oil-contaminated environmental systems due to their complex benzene ring and higher boiling point (Atashgahi *et al*., 2018). Researchers analysed soil from contaminated and uncontaminated oil sites to investigate the effect of crude oil residuals on soil chemistry at oil sites. They discovered that polluted areas had higher levels of carbon, pH, and nitrogen, whereas uncontaminated areas had lower levels of carbon and pH (Wang *et al*., 2019). Nickel and vanadium contamination of oil mixes, as well as elevated salt levels in oilfield output water, have been proven to have an effect on soil ecology (Odukkathil *et al*., 2016). Additionally, despite relatively high levels of organic carbon, electrical conductivity, phosphate availability, and nitrogen levels were found to be

fairly low in crude oil-contaminated soil. Aliphatic hydrocarbon oil films and slicks hinder soil oxygen and nutrient exchange, as well as soil structure and microbiology.

2.3 Crude Oil

Volatile gasoline, petroleum, kerosene, oil lubrication, and solid asphalt residue are examples of aliphatic and aromatic hydrocarbon mixtures. It is quite complicated. The release of crude oil into the environment causes a number of issues. In relatively high quantities, the presence of several hazardous compounds such as polycyclic aromatic hydrocarbons, benzene and its substituted and cyclo alkane rings is physically, chemically, and medically damaging to soil. This oil has the potential for long-term sub-acute toxicological consequences (reduced development and reproduction, poor health, and low recruitment rates), which can alter population dynamics and disrupt the tropics and the ecology of ecosystem-based communities (Bejarano *et al*., 2010).

Physical, chemical, and biological alternatives are used in remediation activities that ultimately result in the eradication of these petroleum hydrocarbons from the environment (Okoh *et al*., 2006). The most often utilized approaches for clean-up are physical and chemical (Ikhajiagbe *et al*., 2011). Physicochemical approaches, on the other hand, have limitations in that they may not always result in complete pollutant elimination (Vidali, 2001). Due to this constraint, a substantial body of literature suggests that bioremediation approaches may be used in lieu of or in addition to these methods (Joo *et al*., 2008). This is because they are inexpensive, environmentally friendly, and straightforward to deploy (Mandri *et al*., 2007; Adams and Guzmán-Osorio, 2008; Agarry *et al*., 2015). The method necessitates a longer term of treatment (Agarry and Ogunleye, 2012). Despite the great benefits of bioremediation,

this approach is today used in just around 5% of all soil treatments (Vasilyeva *et al*., 2020). The high toxicity of soil to microorganisms and plants is one rationale for the lengthy processing time for soil bioremediation. This characteristic typically restricts its application in extremely contaminated soils. This problem can be alleviated by modifying the soil with natural adsorbents (Vasilyeva *et al*., 2020).

In many natural ecosystems, biological and adsorption processes coexist. Industrial pollutants are eliminated from the environment via a mixture of adsorption and biodegradation (Agarry *et al*., 2015). As a result, hybrid techniques such as concurrent adsorption and biodegradation are becoming more prevalent (Aina *et al*., 2018).

2.4 Bioremediation

Bioremediation is the process of degrading and converting toxins found in the environment using living organisms and microorganisms. Bioremediation is based on microorganisms' ability to degrade hydrocarbons into components that can be consumed as nutrients or safely released to the environment (Alegbeleye *et al*., 2017,). It is a simple and effective technique for aerobic mineralisation of hydrocarbons in the presence of carbon dioxide and water. Microbial bioremediation can decompose inorganic pollutants (metals, metalloids, and tiny molecules such as ammonia) and organic contaminants because microorganisms such as macro and micronutrients require the bulk of these xenobiocides (organic contaminants) and metals (inorganic pollutants) (Chowdhury and Sreeparna, 2011). The number and species of bacteria present, as well as the concentration of hydrocarbons and environmental variables that contribute to microbial breakdown (pH, temperature, nutrition, oxygen content, and humidity) are not exempted. As a result, environmental factors must be addressed in order for bioremediation to be effective, allowing microorganisms to multiply rapidly and degrade contaminants (De la Cueva *et al*., 2016).

2.4.1 Biostimulation

Bioremediation of highly hazardous waste from oil refineries, which contains various types of alphatic, aromatic, other complex hydrocarbons, and heavy metals, is a global technological problem (Roy *et al*., 2018). In situ biological remediation with indigenous microorganisms is severely hampered by inadequate nutrition levels and/or physicochemical conditions such as temperature, pH, humidity, and nutrient availability prevalent in contaminated locations (Bisht *et al*., 2015).

The diversity, metabolic capabilities, and response (change in community composition) of indigenous microbial communities to biostimulatory medications are critical for the advancement of bioremediation technology. Technological improvements in both culture and molecular approaches have led to the discovery of a wide array of aerobic and anaerobic bacteria and archaea assemblies, competent of hydrocarbon degradation, nitrate/sulphide/iron reduction, fermentation and metabolism of heavy metal pollutants (Adams *et al*., 2015; Yu *et al*., 2016).

Enhanced experience and knowledge of bacterial communities in contaminated environments is crucial to the development and optimization of bioremediation solutions (Liu *et al*., 2017).

Infusion of nutrients and acceptors (such as P, C, N, and O_2) is commonly used as a biostimulation procedure and a viable technique for recovering from oil-contaminated and nutrient-deficient areas (Li *et al*., 2017). Based on these constraints, a method for in situ bioremediation of hydrocarbon degradation by indigenous species using selective nutrient inputs was developed (Wu *et al*., 2016).

2.5 Petroleum Hydrocarbon Degrading Microorganisms

Numerous studies on petroleum hydrocarbon degradation have demonstrated that a range of microorganisms, most notably fungi and bacteria can digest oil hydrocarbons and use them exclusively for carbon metabolism and energy (Lin *et al*., 2010). The abundance and catabolic activity of soil microorganisms are critical for hydrocarbon breakdown in the soil. Microorganisms must be capable of catabolism in order to biodegrade hydrocarbons efficiently. This includes genetically modifying selected organisms to attack pollutants, enhancing the metabolic capacity of bacteria, and inducing specific enzymes (Karanja *et al*., 2019).

In soil, water, and loam, bacteria are the most active oil degraders, and they are the major degraders of a wide range of activities (Koshlaf and Ball, 2017).

Joshi and Pandey (2011) in their study discovered that *Proteus* and *Bacillus* species are effective at bioremediating crude oil. In addition, bioremediation of used motor and diesel oil utilising an efficient BM consortium A2457, which included *Fusarium* spp, *Phanerochaete* spp, *Chrysosporium* spp, *Cuuninghamella* species, *Penicillium chrysogenum* species, and *Aspergillus niger* species, has been described (Mao *et al*., 2012). To expedite the biodegradation process, a microbe consortium or material assistance may be used to aid in the process (Zhang *et al*., 2015). Addition of cow dung to oil-contaminated soils has been established by Omotayo *et al.* (2012). Cow dung protects soil structures, enhances nutrient intake by indigenous microorganisms, and further exploits crude oil bacteria that are not cultivable but are vital for hydrocarbon decomposition (Ikuesan *et al*., 2016). According to a study, the introduction of acclimated microbial consortia in activated materials (soil and cow dung) proved effective in bioremediating crude oil-contaminated agricultural soil samples in Ondo State, Nigeria (Adeleke *et al*., 2016).

2.6 Environmental Factors Affecting Biodegradation of Petroleum Hydrocarbons

Multiple studies have been conducted to investigate a number of environmental conditions that influence the rate of hydrocarbon biodegradation (Gutierrez *et al*., 2015). According to the findings of Liu *et al*., bacterial populations had the greatest influence on temperature during oil degradation, accounting for 57 percent of the variance in the data collected during the experiment. Temperatures ranging from 4 ^{o}C 24 ^{0}C are particularly beneficial for psychrophilic and mesophilic organisms (Xu *et al*., 2014).

The majority of the strains studied by Sarkar *et al*., (2017) demonstrated a wide range of temperature tolerance (5–40 ^{o}C). Bacillus bacteria are the most temperature tolerant (5–45 ^{o}C), followed by Pseudomonas strains (5–40 ^{o}C), according to the data. As a rule, temperature-dependent degradation rates rise; these broad trends mirror the reaction rates required by oil biodegradation enzymes, which typically double at 10 °C to meet the enzyme's optimum effectiveness (Boufadel *et al*., 2016).

Using Arctic saltwater obtained from Alaska's Chukchi Sea and incubated at 1 °C, McFarlin *et al*. (2014) presented a study on the biodegradation of Alaska North Slope crude oil in Arctic saltwater. When McGenity *et al*. (2012) carried out their review, they discovered a substantial amount of evidence of crude marine oil biodegradation. Teramoto *et al*., (2011) identified and described *Oleibacter* sp as well as microorganisms of the order Oceanospirillales, which are psychrophilic bacteria that breakdown hydrocarbons (Hazen *et al*., 2012). Apart from the obvious biological consequences, higher temperatures may reduce liquid surface voltage and enhance the likelihood of oil spreading across warmer rather than very cold water. Sarkar *et al*. (2018) reported that numerous bacterial strains from thirty genera-associated species. At pH 3.0, *Bacillus*, *Pseudomonas*, and *Microvirgula* thrived; at pH 10,0. *Enterobacter* and *Kocuria* strains flourished, followed by a large number of *Bacillus* and *Pseudomonas*

strains. It was discovered by Olama *et al*. (2013) that it is possible to establish the best environmental and physiological parameters for maximum hydrocarbon breakdown by a *Bacillus cereus* strain through tests. According to their findings, the pH 7 was the best pH for the maximum degradation of diesel oil, with higher or lower pH values of *Bacillus cereus* (producing 85.99 % and 83.84 % aliphatic and aromatic hydrocarbons, respectively) being used to achieve maximum degradation (Wang *et al*., 2012).

According to *Liu et al*., the influence of air pressure on crude oil bioremediation in the deep sea horizon has been examined (2017). An increase in oil degradation was noticed at a depth of 1500 metres when a hydrostatic pressure of 15 MPa was applied. At this pressure, Sharma *et al*. (2016) discovered that the alkane-degrading *Rhodococcus* grew well, whereas the aromatic-degrading *Sphingobium* was significantly injured, but still kept the ability to degrade naphthalene. Scoma *et al*. (2016) also discovered that pressure significantly hindered the growth of two *Alcanivorax* species, indicating that pressure may have an impact on the organisation of microbial communities following an oil spill.

2.7 Horse Dung

Horse manure alone provides virtually all of the carbon and nitrogen that compost microorganisms require. In manure and organic matter bedding, carbon is the most prevalent element (45 to 55 %). According to a well-known "thumb rule," the best beginning carbon to nitrogen ratio (C:N ratio) for composting is between 25 and 35:1. (Smith *et al*., 2016). Microbial manure frequently contains a high proportion of nitrogen (C : N) to carbon, indicating that decaying bacteria utilize nitrogen to meet their development requirements (Chen *et al*., 2016). Due to the N immobilization net in horse manure, this is not the fertilizer you desire. However, due to its beneficial effects on the soil structure and carbon content, it may be regarded a beneficial soil conditioner.

Composting is a biological process that reduces the volume, wetness, and odor of manure while enhancing its homogeneity, stability, and concentration and eradicating pathogens, parasites, and flies (Bernal et al., 2016; Keskinen *et al*., 2020).

2.8 Unripe Plantain

In tropical regions such as Africa, South America, Central America, and Asia, the plantain fruit (*Musa parasidiasca*) is mostly farmed and consumed. It is a member of the *Musaceae* family and the *Plantaginaceae* natural order, and it is native to South America (Auta *et al*., 2015). One of the oldest fruits cultivated in Central and West Africa, it is also a staple meal in Sub-Saharan Africa, particularly in Nigeria. It is one of the most popular fruits in the world. In Nigeria alone, about 2.11 million metric tonnes of the fruit are produced each year, demonstrating the importance of the fruit in Nigerian agriculture (Stephen *et al*., 2015). Plantain accounts up more than 10 % of the daily calorie intake of a population of over 70 million people on the African continent, according to the World Health Organization (Baiyeri *et al*., 2011). Meals produced from the plantain fruit are referred to by a variety of names in Nigeria, depending on the method of preparation used to prepare them. Among its many names are "Amala" (when prepared with hot water and served with various soups), "Dodo" (fried ripe fruit), and "Kpekere" (thinly sliced, deep-fried plantain fruit that is partly ripe or unripe). The peels, on the other hand, are typically disposed of irresponsibly in landfills, drainage systems, and along roadside embankments, causing environmental damage (Auta *et al*., 2015; Baiyeri *et al*., 2011). Plantain is similar to unripe bananas in appearance, but is larger and has a starchy rather than sweet flesh. It is often consumed unripe and requires boiling. Peels, the primary by product of banana processing comprises around 30 % of the fruit. This by product poses an environmental hazard due to its high nitrogen and phosphorus concentration and susceptibility to microbial alteration (Gilver *et al*., 2017).

Plantain fruits are consumed at various stages of maturity, and the amount of peels produced is predicted to increase as processing industries utilizing green and mature bananas develop. Banana peel flour may enable the development of novel goods with standardized compositions for a variety of industrial and residential applications (Emaga *et al*., 2011). It has evidently been established that the peel of the plantain, which accounts for around 40 % of the entire weight of the fruit (Gilver *et al*., 2017), has potential as a promising raw material with industrial applications, notably in the agro-based industries. Peels have been investigated for use as organic fertilisers in regions such as Somalia, where they have the potential to enhance soils and increase agricultural production and harvests. The peels of fruits and vegetables, when combined with other waste products, have been found to be a viable substitute for corn starch in the diets of snails and pigs (Okareh *et al*., 2015). An extensive body of research has found that a range of plant parts, particularly the peel of the fruit, exhibit antifungal and antibacterial properties that can be used to treat a number of human illnesses (Auta *et al*., 2015). Eight human pathogenic microorganisms, five bacteria, and three fungi have been treated with ethanol extracts from the peels, and the peels themselves have been proven to be effective against a wide range of human pathogens (Ighodaro. 2012). Incorporating peel extracts into pharmacological and therapeutic compositions has been recommended. Several of the plant's principal pharmacological qualities, including peels, include antiulcer, analgesic, wound healing, hair growth stimulant, and hemostatic action, among others (Akinsanmi *et al*., 2015). Plantain peel has received little attention in the food and medical industries; nonetheless, its pharmacological applications have piqued the interest of many researchers. As a result, the nutraceutical potential of unripe and ripe plantain peels was investigated in this study. This was accomplished by screening multiple solvent extracts of the peels for phytochemical

content and evaluating the proximate and mineral contents of the extracts in the laboratory (Baiyeri *et al*., 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of Samples

The soil sample (24 kg of 20 cm surface soil) was collected from the biological garden of the Federal University of Technology Minna, Niger State, Nigeria and packaged in sterile polyethene bags. After 48 hours of drying, the soil samples were sieved through 2 mm sieve.

Four liters of Crude Oil (Bonny Light Crude, BLC) were obtained from the Nigerian National Petroleum Corporation (NNPC) refinery in Alesa-Eleme, Rivers State.

Horse dung was collected from a barn in Bosso, Minna, Niger State, Nigeria, and unripe plantain peels were collected from plantain vendors in Mobil, Berger, and Kure Markets, in Minna, Niger State.

3.2 Pollution of Soil with Crude Oil

Twenty four (24) pots containing 1000 g of soil in each pot were polluted and randomly assigned to receive 5 percent crude oil (w/w)(50 g) (CRD). The crude oil was completely blended into the soil using a hand trowel.

3.3 Stimulation of Crude oil Contaminated Soil with Agro-waste

After 48 hours of crude oil contamination, the agro-waste was added to the crude oil-contaminated soil at a ratio of 50 g crude oil (5 %):100g (10%) of each agro-waste and 50 g crude oil (5 %):200 g (20 %) of each agro-waste, respectively, while the negative control treatment was contaminated with crude oil and twenty percent of sodium azide in the soil.

3.4 Experimental Design

Eight treatments were included in the experimental design (crude oil and agro-waste), 24 clay pots were soaked in water overnight and each pot was filled with 1000 g of soil. The experimental design is summarized in Table 3.1.

Table 3.1: Experimental Design Used for Bioremediation of Crude Oil Contaminated Soil

Design	Treatment
A	1000 g of Soil + 5 % (50 g) (w/w) crude oil (Control)
B	1000 g of Soil + 5 % (50 g) (w/w) crude oil + 100 g of horse dung agro-waste
C	1000 g of Soil + 5 % (50 g) (w/w) crude oil + 100 g of unripe plantain peel agro-waste
D	1000 g of Soil + 5 % (50 g) (w/w) crude oil + 100 g of horse dung + 100 g of unripe plantain peel agro-waste
E	1000 g of Soil + 5 % (50 g) (w/w) crude oil + 200 g of horse dung agro-waste
F	1000 g of Soil + 5 % (50 g) (w/w) crude oil + 200 g of unripe plantain peel agro-waste
G	1000 g of Soil + 5 % (50 g) (w/w) crude oil + 200 g of horse dung agro-

	waste +200 g of unripe plantain peel agro-waste
H	1000 g of autoclaved soil +5 % (50 g) (w/w) crude oil +2 % (20 g) of sodium azide (Negative control).

3.5 Aerobic Heterotrophic Bacterial and Fungal Counts in Uncontaminated Soil

One gram of uncontaminated soil was suspended in 9 ml of sterile distilled water, ten folds serial dilution was done and 0.1 ml of 10^5 dilutions for each sample was plated on nutrient agar for bacteria and potato dextrose agar for fungi. The nutrient agar plates were incubated at 37 ^oC for 48 hours while the potato dextrose agar plates were incubated at room temperature (28$\pm$2 ^oC) for 72 hours. The number of viable bacteria and fungi were counted and presented as colony forming units per gram (Jorfi *et al*., 2013).

3.6 Aerobic Heterotrophic Bacterial and Fungal Counts in Agro-waste (Horse Dung and Unripe Plantain Peel)

One gram of the agro-waste (Horse dung and unripe plantain peel) was suspended in 9 ml of sterile distilled water, ten folds serial dilution was done and 0.1 ml of 10^5 dilutions for each sample was plated on nutrient agar for bacteria and potato dextrose agar for fungi. The nutrient agar plates were incubated at 37 ^oC for 48 hours while the potato dextrose agar plates were incubated at room temperature (28$\pm$2 ^oC) for 72 hours. The number of viable bacteria and fungi were counted and presented as colony forming units per gram (Jorfi *et al*., 2013).

3.7 Aerobic Heterotrophic Bacterial and Fungal Counts in Soil Contaminated with Crude Oil

One gram of crude oil contaminated soil was suspended in 9 ml of sterile distilled water, ten folds serial dilution was done and 0.1 ml of 10^5 dilutions for each sample was plated on nutrient agar for bacteria and potato dextrose agar for fungi. The nutrient agar plates were incubated at 37 ^oC for 48 hours while the potato dextrose agar plates were incubated at room temperature (28$\pm$2 ^oC) for 72 hours. The number of viable bacteria and fungi were counted and presented as colony forming units per gram (Jorfi *et al*., 2013).

3.8 Isolation and Characterization of Crude Oil Degrading Bacteria and Fungi

One gram of crude oil contaminated soil sample from the different treatments was serially diluted and 0.1 ml was plated on oil agar. The oil agar consisted of mineral salt medium which contains 1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 1.2 g KH_2PO_4, 0.01 g $FeSO_4.7H_2O$, 0.1 g NaCl, 1% crude oil (as the only carbon source) in one liter of distilled water and 20g agar agar as the solidifying agent and incubated at 28$\pm$2 ^oC for 24-48 hours for bacteria and 28$\pm$2 ^oC for 48-72 hours for fungi. Nystatin (anti fungi) was introduced in the agar plate to strictly grow bacteria while Chloraphenicol and Penicillin (anti bacteria) were introduced in the agar plate to strictly grow fungi. Colonies of different hydrocarbon utilizing bacteria / fungi were picked randomly using a sterile inoculating wire loop and sub cultured for purification by streaking on nutrient agar plates and potato dextrose agar plate for bacteria and fungi respectively. The nutrient agar plates were incubated at 28$\pm$2 ^oC for 24 hours while the potato dextrose agar plates were incubated at 28$\pm$2 ^oC for 72 hours. The isolates were sub cultured repeatedly. All isolates were subjected to biochemical tests. The bacteria

were identified by comparing their characteristics with those of known taxa (Jorfi *et al*., 2013).

3.9 Identification and Characterization of Bacterial Isolates

The biochemical characterization of the bacterial isolates were identified based on Bergy's Manual of Systemic Bacteriology (Garrity, 2012).

3.9.1 Gram staining

A smear of bacterial isolates was passed through flame to fix, flooded with crystal violet and allowed to stand for 60 seconds, the crystal violet was poured away and slide flooded with Grams iodine and allowed to stand for 60 seconds and rinsed with water after which decolorisation was done using 95 % ethyl alcohol and rinsed with water, counterstaining was done by flooding smear with safranin and allowed to stand for 30 seconds before rinsing with water. The slides were viewed using oil immersion objective lens of light microscope. Gram postive organisms appeared purple while gram negative was pink.

3.9.2 Catalase test

The smear of the bacterial isolate was aseptically placed on a clean glass slide using a sterilized wire loop. A drop of hydrogen peroxide was added on each smear and observed for bubble formation which indicates positive reaction.

3.9.3 Citrate utilization test

Simmon citrate medium was dispensed into a clean test tube and sterilized in an autoclave at 121 ^{o}C for 15 minutes. Using a sterile straight wire, the slopes were streaked and stabbed to the bottom of the butt with the bacterial isolates and incubated

for 48 hours at 37 ºC. Bright blue colour indicates a positive citrate reaction. No change in colour indicates a negative citrate reaction.

3.9.4 Coagulase test

A drop of physiological saline was placed on a clean glass slide to make a smear of the bacterial isolate. A drop of human plasma was added to the suspension and mixed gently. The formation of a clump by the organism indicates positive result for coagulase.

3.9.5 Starch hydrolysis

Twelve grams of nutrient agar was weighed into a 500 ml conical flask; 1.75 g of soluble starch was added into the conical flask. Five hundred milliliter (500 ml) of distilled water was added to the mixture, pre-heated and sterilized by autoclaving at 121 ºC for 15 minutes. The medium was allowed to cool to 40 ºC and aseptically poured into sterile Petri dishes and allowed to solidify. Each test bacterial isolate was inoculated by streaking, while the duplicate sets of nutrient agar plates was left uninnoculated (control) and incubated at 37 ºC for 24 hours. After incubation, the plates were flooded with 10 ml of Gram's iodine and observed for colour change. A clear zone shown around the colonies of the test organism confirmed a positive result, while blue-black colouration with Gram's iodine indicated a negative result.

3.9.6 Methyl red test

Glucose phosphate broth was prepared, dispensed into test tubes and sterilized by autoclaving at 121 ºC for 15 minutes. The sterile medium was inoculated with bacterial culture and incubated at 37 ºC for 48 hours. Four drops of methyl red indicator was

added and gently mixed. Positive test was indicated by bright red color while negative test was indicated by yellow color.

3.9.7 Voges proskauer test

One milliliter (1 ml) of 40 % KOH and 3 ml of 5 % alpha–naphtol was added to the test organism in peptone water and incubated at 37 ^{o}C for 48 hours and shaken gently. Pinkish coloration indicates positive to Voges proskauer test.

3.9.8 Motility test

A drop of bacterial suspension was placed at the center of a cover slip, soft paraffin was applied over the corners of the cover slip. A glass slide was gently placed over the cover slip and held upside down, it was in such a manner that the bacteria was hanging between the cover slip and glass slide. Examination under the light microscope was done under x10 and x40 objective lens (Cheesbrough, 2000).

3.9.9 Sugar fermentation

Peptone water and sugars (glucose, lactose, maltose, arabinose, xylose and mannitol) was dispensed into conical flask, to which 0.1 g of bromocresol purple was added. The solutions were dispensed into test tubes and Durham tube was introduced in an inverted position into each test tube and sterilized appropriately. After sterilization the medium was allowed to cool. Each sterile medium tube was inoculated with bacterial culture and afterwards incubated at 35 ^{o}C for 48 hours. After incubation the tubes were observed for acid production by change in color from purple to pink or any color different from that of the control. The tubes were also observed for gas production in the inverted Durham tubes.

3.9.10 Urease test:

Urea agar slants were prepared and inoculated with the test organism and incubated at 37 ^{o}C for 24 hours and examined every 12 hours. Positive result showed a colour change from light red or pink while a negative result shows no colour change.

3.10 lsolation and Identification of Fungal Isolates

One gram of the homogenized soil sample was measured into 9 ml of sterile distilled water in a test tube and swirled gently. One ml of the sample was pipetted and serially diluted up to 10^{-3} dilution. Zero point one ml of the sample from the 10^{-2} and 10^{-3} dilutions were transferred onto the surface of a freshly prepared mineral salt agar using the spread plate technique. A Whatman No.1 filter paper saturated with sterilized crude oil was placed inside the lid of the plates. The plates were incubated at 28 ^{o}C for 7 days. Each distinct colony on oil degrading enumeration plates were purified by repeated sub culturing onto the surface of a freshly prepared Sabouraud Dextrose Agar (SDA) (Merck, Germany) plates to obtain pure cultures of the isolates. The pure cultures were maintained on SDA slants. The isolates were screened for used crude oil biodegradation potentials on mineral salt broth using the method of Olajide *et al*. (2010) with determination of pH and total viable count at time intervals as biodegradation indices.

The cultural characteristics of the pure isolates on Potato Dextrose Agar Medium (PDA) were noted, and the microscopic features were observed using the wet mount and the micro slide culture technique with reference to the Manual of Fungal Atlas Akpoveta *et al*. (2011).

3.11 Physicochemical Analysis

The physicochemical analysis of the soil (contaminated and uncontaminated) and agro wastes (horse dung and unripe plantain peels) were analysed.

3.11.1 Determination of pH

The pH of the soil was determined before and after contamination. Ten grams of soil sample was weighed into an extraction cup, 10ml distilled water was added to the soil sample and allowed to stand for 15 minutes, the mixture was shaken on an orbital shaker for 30 minutes at 150rpm after which it was allowed to stand for 10 minutes. The pH meter was standardized using buffer 7.0 and 4.0. Finally the pH value was read on the pH meter (Eckerts and Sim, 1995).

3.11.2 Determination of nitrogen

The Kjeldahl method or Kjeldahl digestion (Nelson and Sommers, 1996) in analytical chemistry is a method for the quantitative determination of nitrogen contained in organic substances. The method was used as follows:

3.11.2.1 Digestion

Two gram of the soil sample was weighed into a digestion flask and then digested by heating it in the presence of 40 mL sulfuric acid (an oxidizing agent which digests the soil), 10 mL of 10 M anhydrous sodium sulfate (to speed up the reaction by raising the boiling point) and the catalyst copper (to speed up the reaction). Digestion converts any nitrogen in the soil (other than that which is in the form of nitrates or nitrites) into ammonia, and other organic matter to $C0_2$ and H_20. Ammonia gas is not liberated in an acid solution because the ammonia is in the form of the ammonium ion (NH_4^+) which binds to the sulfate ion (SO_4^{2-}) and thus remains in solution:

3.11.2.2 Neutralization

After the digestion was completed the digestion flask was connected to a recieving flask by a tube. The solution in the digestion flask was then made alkaline by addition of 10 mL of 10 M of sodium hydroxide NaOH, which converts the ammonium sulfate into

ammonia gas. The ammonia gas that was formed was liberated from the solution and moves out of the digestion flask and into the receiving flask which contains an excess of 5 mL boric acid. The low pH of the solution in the receiving flask converts the ammonia gas into the ammonium ion, and simultaneously converts the boric acid to the borate ion.

3.11.2.3Titration

The nitrogen content was then estimated by titration of the ammonium borate formed with standard 0.1 M hydrochloric acid, using a methyl red indicator to determine the end-point of the reaction.

The concentration of hydrogen ions (in moles) required to reach the end-point was equivalent to the concentration of nitrogen that was in the original soil.

3.11.3 Determination of available phosphorous

Phosphorous content of the soil was determined using Bray No.1 method described by Bray and Kurtz (1945). One gram of air-dried soil sample was passed through a 2 mm sieve, and introduced into a centrifuge tube and 7 mL of 1M NH_4F and 25 mL of 0.5 M HCl was added to 460 mL distilled water. The mixture was shaken for one minute on a mechanical shaker and the suspension centrifuged at 2000 rpm for 15 minutes. 2 mL of the clear filtrate was introduced into a 20 mL test tube, 5 mL of distilled water and 2 mL of ammonium molybdate solution was added. The content was mixed properly and 1 mL of $SnCl_2$. $2H_2O$ dilute solution was added and mixed again. After 5 minutes, the percentage transmittance was measured on a spectrophotometer (Jenway 6305, UK) at 660 nm wavelength. A standard curve within the range of 0-1µg P/mL (or ppm P) was prepared. The optical density of the standard solution was plotted against the µg P/mL

and the content of extractable phosphorous in the soil was calculated using Equation 3.1 (Bray and Kurtz, 1945).

$$\text{P (ppm)} = \frac{\text{Off curve reading} \times \text{dilution factor} \times \text{volume of extract}}{\text{Original weight of soil}} \quad (3.1)$$

3.11.4 Determination of organic carbon

This method is adapted from Heanes (1984). It is a complete oxidation procedure. Ninety eight grams of reagent-grade Potassium dichromate ($K_2Cr_2O_7$) was dissolved in distilled water and diluted to 2 liters. For standard solutions, 1.00 ml of the 5 standard solutions was added into 5 digestion tubes. Five ml of $K_2Cr_2O_7$ solution was added to samples and standards followed by 10 ml of concentrated H_2SO_4. It was then capped with a rubber stopper, and allowed to swirl on a vortex mixer until the soil sample was completely dispersed. It was then placed in a digestion block and preheated to 150 ^oC for exactly 30 minutes. The tubes where allowed to cool then it was diluted to 50 ml, mixed, and allowed to stand overnight.

The standards and samples were read on a spectrophotometer at a wavelength of 600 nm using a 1 cm cell. The standards contain 0, 2.50, 5.00, 7.50, and 10.00 mg of C.

Calculations:

To determine the amount of C from a standard curve, zero % of organic carbon (OC) was calculated using equation 3.2

Organic matter content may be estimated by multiplying organic C by a factor of 1.8

$$\%\, OC = \frac{mg\ C}{mg\ of\ sample} \times \frac{100}{1} \quad (3.2)$$

3.11.5 Determination of moisture

Moisture content of the soil was determined using the gravimetric method described by Black (1965) and Agbenin (1995). The moisture can was weighed using an electronic weighing balance. The can and the soil sample were weighed and transferred to a hot spot conventional oven (Genlag, MIN0150). The sample was dried in the oven at 105 ^{o}C for 5 hours, after which it was transferred to desiccators and allowed to cool. The weight of the oven-dried sample was obtained using electronic balance and the percentage moisture content calculated using the equation 3.3

$$\% \text{ Moisture content} = \frac{B-C}{B-A} \times 100 \quad (3.3)$$

Where:

A = Weight of moisture can (grams)

B = weight of can + wet sample (grams)

C = Weight of can + oven-dried sample (grams)

3.11.6 Determination of organic matter

This method of (Nelson and Sommers, 1996) was used where 250 mg of soil sample was weighed into a 50 ml digestion tubes. For standard solutions, 1.00 ml of the 5 standard solutions was added into 5 digestion tubes. After taking 1 ml each of the standards, the pipette was rinsed with 1.00 ml of distilled water into the digestion tubes. Five ml of $K_2Cr_2O_7$ solution was added to the samples and standards. Then10 ml of concentrated H_2SO_4 was added, it was capped with a rubber stopper, and swirl on a vortex mixer until the soil sample was completely dispersed. It was then placed in a digestion block preheated to 150 ^{o}C for exactly 30 minutes, the tubes were allowed to cool and diluted to 50 ml, mixed, and allowed to stand overnight. The standards and samples were read on a spectrophotometer at a wavelength of 600 nm using a 1 cm cell. The standards contain 0, 2.50, 5.00, 7.50, and 10.00 mg of Carbon.

Calculations:

To determine the amount of carbon from a standard curve, % Organic carbon was calculated in equation 3.4

$$\%\ organic\ carbon = \frac{mg\ carbon}{mg\ of\ sample} \times \frac{100}{1} \quad (3.4)$$

Organic matter content may be estimated by multiplying organic carbon by a factor of 1.8

3.11.7 Determination of electrical conductivity

The method of Black. (1965) was used when ten grams (10 g) of soil was weighed and transferred into 100 ml beaker, 10ml distilled water was added and stirred properly using a glass rod. This was allowed to stand for 30 minutes with intermittent stirring. To the soil water suspension in the beaker the electrical conductivity meter (Thermo Orion 135A Japan) was inserted and swirled gently, after approximately 60 seconds or after the EC reading has stabilized, the digital display on the meter was read.

3.11.8 Determinaton of exchangeable acidity (titration method)

Three grams of air-dried soil was weighed (grind to pass a 2 mm sieve) into folded filter paper placed on the extraction cups. 50 ml of 1.0 *N* KCl solution was poured through the soil in the filter paper and the leachate collected. 5 drops of phenolphthalein indicator was added to the leachate. The leachate was titrated with 0.05 *N* NaOH to pink end point and the volume (ml) of NaOH used was recorded Agbenin. (1995).

For exchangeable acidity in soil

Four mililiters of 3 *N* NaF was added to the titrated extract. The mixture was titrated with 0.05 *N* HCl to pink end point. The volume (ml) of HCl used was recorded.

Calculation

Exchangeable acidity (meg/100 g)

$$= \frac{V*0.05*100}{W} = V*1.67 \quad (3.5)$$

Where;

V = Titre volume of NaOH used (ml)

W = weight of soil sample used (g)

3.11.9 Determination of particle size of the soil structure and type

The soil particle size was determined by the method described by Bouyoucos (1962) and United States Environmental Protection Agency (USEPA, 1996). Forty grams (40 g) of soil was weighed into 600 mL capacity beaker, 60 mL of dispersing solution was added and the beaker was covered with watch glass and left overnight. Quantitatively, content of the beaker was transferred to a soil stirring cup and the cup was filled with water to about three quarters after which the suspension was stirred for three minutes with stirring paddle. The suspension was transferred into one litre calibrated cylinder (hydrometer jar) and was brought to a volume with water. Blank was determined by adding 60 mL of dispersing solution. It was mixed thoroughly and the hydrometer was inserted to take its reading and recorded as (Rb).

Determination of clay was done by mixing the suspension in the hydrometer jar with paddle, the paddle was withdrawn carefully and after 4 hours, hydrometer was inserted and reading was taken.

$$\% \text{ clay in soil (w/w)} = \frac{(Rc-Rb) \times 100}{\text{Oven-dry soil (g)}} \quad (3.6)$$

$$\% \text{ silt in soil (silt + clay) (w/w)} = \frac{(\text{Rsc}-\text{Rb}) \times 100}{\text{Oven}-\text{dry soil (g)}} \quad (3.7)$$

After the values of clay and silt have been determined, the value of sand was obtained by subtracting the values of silt and clay from 100. The soil was classified using the textural triangle.

3.12 Determination of Total Petroleum Hydrocarbon

The extent of hydrocarbon utilization in the crude oil was estimated spectrophotometrically (Adesodun and Mbagwu 2008). Fifty gram of soil samples was taken from each microcosm and put into a 250 ml flask, 100 ml diethyl ether was added, the mixture was shaken vigorously on an orbital shaker for 30 minutes at 2100 rotation per minute (RPM) to allow diethyl ether extract the oil from the soil sample. The solution was then filtered using whatman filter paper containing anhydrous sodium sulphate to extract water. The absorbance of the mixture was measured spectrophotometrically at a wavelength of 620 nm using n-hexane as blank (UV-2450 12550, Japan). The total petroleum hydrocarbon was estimated with reference to a standard curve derived from fresh crude oil of different concentrations diluted with diethyl ether.

3.13 Gas Chromatography- Mass Spectrophotometry

The model of the instrument used for the GCMS analysis is agilent technologies 7890 coupled with a mass spectrometer of 5975, the carrier gas was helium and the stationary phase was a column of model agilent technologies HP5MS of length 30m internal diameter of 0.320mm and the thickness of 0.25 micro litre. The oven temperature program 80 ^{o}C held for 2 minutes at 10 ^{o}C per minute to the final temperature of 240

ºC held for 10 minutes. The volume of sample injected was 1 microlitre, the scan range was from 50 to 550Amu (Chaprao *et al*., 2015).

3.14 Molecular Identification of Microorganisms

The molecular identification of the microoganisms (Bacteria and fungi) was analysed to characterize the obtained organisms molecularly.

3.14.1 Extraction of bacterial DNA

Single colonies of bacteria grown on medium were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker for 48 h at 28 ºC. After this period, cultures were centrifuged at 4600 g for 5 min. The resulting pellets were resuspended in 520 µl of TE buffer (10mMTris-HCl, 1mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37 ºC, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and votexed. The suspension was incubated for 10 min at 65 ºC and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200 g for 20 min. The aqueous phase was then transferred to a new tube and isopropanol (1: 0.6) was added and DNA precipitated at –20 ºC for 16 hours. DNA was collected by centrifugation at 13000 g for 10 min, washed with 500 µl of 70% ethanol, air dried at room temperature for approximately three hours and finally dissolved in 50 µl of TE buffer. PCR sequencing preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25mM MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template. PCR

was carried out in a Gene Amp 9700 PCR System Thermal cycler (Applied Biosystem Inc., USA) with a Pcr profile consisting of an initial denaturation at 94°C for 5 min; followed by a 30 cycles consisting of 94°C for 30 s, 50°C for 60s and 72°C for 1 minute 30 seconds ; and a final termination at 72°C for 10 mins. And chill at 4°C.GEL (Wawrik *et al*., 2005).

3.14.2 Extraction of fungal DNA

The fungi DNA Extraction Protocol was carried out using the method adopted by Wawrick *et al*. (2005).

One hundred mg of fungal mycelia was added into sterile mortal, one ml of DNA Extraction Buffer (DEB) containing proteinase K (0.05 mg/ml) was added and macerated with sterile pestle. The extract was transferred into 1.5 ml of eppendorf tube. Fifty microliter (50 µl) of 20 Sodium Dodecyl Sulphate (SDS) was added and incubated in a water bath at 65°C for 30 minutes. The tubes were allowed to cool at room temperature. One hundred µl of 7.5 M Potassium Acetate was added and mixed briefly. It was centrifuged at 13000 rpm for 10 minutes. The supernatant was transferred into new fresh autoclaved tubes Two to three volumes of cold Isopropanol / Isopropyl alcohol was added to the supernatant, the tubes were inverted 3-5 times gently and incubated at -20 ^{0}C for 1 hour It was centrifuge at 13000 rpm for 10 minutes and the supernatant was discarded. Five hundred µl of 70 % ethanol was added and centrifuged for 5 minutes at 13000 rpm. The supernatant was carefully discarded with the DNA pellet intact. Traces of ethanol was removed and the DNA pellets were dried at 37 °C for 10-15 minutes. The DNA pellets were resuspended in 50 µl of Tris-EDTA (TE) buffer. The DNA was aliquoted and stored at -20 °C for further lab analysis.

3.14.2.1 Polymerase Chain Reaction (PCR)

To use the ITS gene for characterization of fungi, ITS universal primer set which flank the ITS1, 5.8S and ITS2 region was used;

ITS 1: 5’ TCC GTA GGT GAA CCT GCG G 3’

ITS 4: 5’ TCC TCC GCT TAT TGA TAT GC 3’

PCR conditions include a cycle of initial denaturation at 94 ^{0}C for 5 min, followed by 35 cycles of each cycle comprised of 30 secs denaturation at 94 ^{0}C, 30 secs annealing of primer at 55 ^{o}C , 1.5 min extension at 72 ^{0}C and a final extension for 7 min at 72 ^{o}C.

3.14.2.2 Integrity

The integrity of the amplified about 1.5 Mb gene fragment was checked on a 1 % Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5 % agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60 °C and stained with 3 µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter of 10 X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4 µl of each PCR product and loaded into the wells after the 100 bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120 V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100 bp molecular

weight ladder that was ran alongside experimental samples in the gel (Wawrik *et al*., 2005).

3.14.2.3 Purification of Amplified Product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3 M and 240 µl of 95 % ethanol were added to each about 40 µl PCR amplified product in a new sterile 1.5 µl tube eppendorf, mix thoroughly by vortexing and kept at -20 °C for at least 30 min. Centrifugation for 10 min at 13000 g and 4 °C followed by removal of supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µl of 70 % ethanol and mixed then centrifuged for 15 min at 7500 g and 4 °C. Again all supernatant were removed (invert tube on trash) and inverted tube on paper tissue and let it dry in the fume hood at room temperature for 10-15 min. then resuspended with 20 µl of sterile distilled water and kept in -20 ^{o}C prior to sequencing. The purified fragment was checked on a 1.5 % Agarose gel ran on a voltage of 110 V for about 1hr as previous, to confirm the presence of the purified product and quantified using a nanodrop of model 2000 from thermo scientific (Wawrik *et al*., 2005).

3.14.2.4 Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130 xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of Big Dye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis

3.15 Data Analysis

Data were subjected to analysis of variance ($p \leq 0.05$) using SPSS version 20 and the averages were compared by Duncan Multiple Range Tests (DMRT) $p \leq 0.05$. The effect of studied factors was considered significant when $p \leq 0.05$.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1: Physicochemical Properties of Uncontaminated Soil and Agro waste used for Biodegradation Studies

The physicochemical properties of the uncontaminated soil used for this study is presented in Table 4.1. The pH of the soil was slightly acidic (5.61) which was within the acceptable limit (5.5 – 8.5) for effective bioremediation as reported by Vidali (2001). The Nitrogen content 0.31 % of the soil was low, hence the need for amendment with organic stimulants (horse dung and unripe plantain peel). Other physicochemical parameters analyzed were nitrogen (0.31 %), phosphorous (10.11 mg/kg), organic carbon (4.07 %) and moisture content (8.20 %). The texture of the soil is sandy loam. The two agro-waste used for the study are horse dung and unripe plantain peel. The pH of the horse dung was acidic (4.3), nitrogen (0.98 %), phosphorous (4.0mg/kg), organic carbon (11.12%) and moisture content (14.4%) whereas the pH of the unripe plantain peel was slightly acidic (5.7), nitrogen (0.93 %), phosphorous (31.0 mg/kg), organic carbon (6.42 %) and moisture content (10.1 %).

The low level of carbon and nitrogen (C, and N) in the uncontaminated soil sample could have been caused by leaching or erosion. The presence of these limiting nutrients (C and N) in the agro-waste samples analysed in this study is in consonance with the earlier reports of (Agarry *et al*., 2010, Abioye *et al*., 2012, Akpe *et al*., 2015). They noted that the addition of these limiting nutrients obtained from the agro-waste(s) is a key factor in achieving effective biodegradation of hydrocarbons by loosening the compactness of the soil making sufficient aeration available for the indigenous bacteria present in the soil, thereby enhancing metabolic activities.

Table 4.1: Physicochemical Properties of Soil and Agro waste (Horse dung and Unripe plantain peels) used for Biodegradation studies

Parameter	Soil	Horse dung	Unripe plantain peel

pH	5.61	4.3	5.7
Nitrogen (%)	0.31	0.98	0.93
Phosphorous ($mgkg^{-1}$)	10.11	4.03	31.0
Organic carbon (%)	4.07	11.12	6.42
Moisture	8.20	14.4	10.1
Organic matter (%)	7.02	----	----
Sodium ($Cmolkg^{-1}$)	0.34	----	----
Potassium ($Cmolkg^{-1}$)	0.28	----	----
Calcium ($Cmolkg^{-1}$)	6.64	----	----
Magnesium ($Cmolkg^{-}$)	2.91	----	----
Cation exchange capacity ($Cmolkg^{-1}$)	10.40	----	---
Electrical conductivity (Us/cm)	60	----	---
Exchangeable Acidity ($Cmolkg^{-1}$)	0.27	----	--
Sand (%)	44.24	---	---
Silt (%)	30.28	---	---
Clay (%)	25.48	---	---
Texture	Sandy loam		

4.2 Counts of Aerobic Heterotrophic Bacteria and Fungi in Uncontaminated Samples (Soil, Horse dung and Unripe plantain peels).

The number of aerobic heterotrophic bacteria and fungi found in uncontaminated soil samples and agricultural waste (horse dung and unripe plantain peel) indicates that the

uncontaminated soil sample had the highest number of bacterial growth of 323×10^5cfu/g, the horse dung agro-waste recorded bacteria count of 235×10^5cfu/g whereas the unripe plantain peel had the lesser number of bacteria count of 207×10^5cfu/g. The highest number of fungal growth of 112×10^5cfu/g was recorded on the unripe plantain peel, the uncontaminated soil sample recorded 100×10^5cfu/g of fungal growth whereas the horse dung had the lowest fungal count of 99×10^5cfu/g. The uncontaminated soil had a greater heterotrophic bacterial count than the agro waste (horse dung and unripe plantain peel). This contradicts with the findings of Abioye *et al*., (2010) who stated that organic amendments have compositions that may stimulate the growth of microbiota. The increase in the number of organisms present in soil compared to the agro waste could be as a result of environmental factors such as climate, vegetation, topography and time.

Table 4.2: Counts of Aerobic Heterotrophic Bacteria 10^8 and Fungi $\times10^5$ (Cfu/g) in Uncontaminated Samples (Soil, Horse dung and Unripe plantain peels)

Uncontaminated Sample	Bacteria	Fungi
Soil	323.±10.54	100.±5.86
Horse dung	235.±4.51	99.±5.51
Unripe plantain peel	207.±6.24	112.±3.00

4.3 Bacterial Counts in Contaminated Soil during Biodegradation Studies

Tables 4.3 and 4.4 shows the heterotrophic and hydrocarbon utilizing bacterial counts of contaminated soil samples collected during the study period. Bacterial counts were found to be greater in crude oil-polluted soil samples than in control soil samples. Treatment G (1000 g of soil contaminated with 5 % (50 g) crude oil mixed with 20 % (200 g) of horse dung and unripe plantain peels) had the highest amount of

heterotrophic and hydrocarbon-utilising bacteria, with counts of 866×10^5cfu/g and 598×10^5cfu/g, respectively. Thus, higher bacterial counts were observed in the treatments with an increase in the combination of both agro waste. This showed that the soil treatments mixed with both agro waste (horse dung and unripe plantain peel) enhanced the bacterial growth rate. Overall, these findings indicate that agro waste amendment had no negative impact on aerobic bacterial activity.

An increase in the number of bacteria in agro-waste treatment could be caused by favourable soil adsorbent conditions, which resulted in an increase in a large number of microbial populations and activities, resulting in high energy requirements (carbon) for hydrocarbon micro-organisms. Agarry *et al*. (2012) and Vasilyeva *et al*. (2020) made similar observations. For soil amended with agrowaste treatment; the total hydrocarbon utilizing bacteria count (THUB) with a combination of 100g each of the agro-wastes (horse dung and unripe plantain peel) increased from 39×10^5 to 587×10^5 CFU/g; whereas the treatment with the combination of 200 g each of the agro-waste (horse dung and unripe plantain peel) increased from 49×10^5 to 598×10^5 CFU/g. In the unamended soil (natural attenuation), the total aerobic heterotrophic bacteria count (TAHB) increased from 207×10^5 to 235×10^5 CFU/g. This showed that adding agro-waste to the soil treatment speed up bacterial development, which explains the higher bacterial counts compared to the control soil treatment (natural attenuation). Meynet *et al*. (2019) presented a study with similar findings in which they reported an increase in bacterial count when waste was added. Furthermore, it was discovered that treatments altered with a greater amount of agro-waste had higher bacterial counts, ranging from 866×10^5cfu/g to 866×10^5cfu/g. Counts of heterotrophic and hydrocarbon-utilising bacteria all increased gradually throughout the study.

Statistically, during crude oil breakdown, the total heterotrophic bacterial numbers and the hydrocarbon-using bacterial counts of the three treatments differed substantially at $p \leq 0.05$. (Tables 4.3. and 4.4)

Table 4.3: Total Aerobic Heterotrophic Bacterial Counts (x10^5 (Cfu/g) of Contaminated Soil during Crude Oil Biodegradation

Treatment	Time (days)				
	0	14	28	42	56
A	34.±1.53^{e}	103.±4.04^{d}	184.±1.73^{d}	203.±4.73^{e}	505.±2.00^{d}
B	66.±2.56^{d}	198.±7.77^{c}	376.±3.51^{c}	567.±3.06^{d}	842.±2.52^{c}

C	68.±2.08^{cd}	206.±5.57^{c}	377.±5.29^{c}	571.±5.86^{cd}	841.±3.06^{c}
D	73.±1.53^{bc}	221.33.±3.51^{b}	387.±4.04^{c}	581.±2.52^{bc}	853.±3.61^{b}
E	76.±1.16^{ab}	230.±3.06^{ab}	402.±8.74^{b}	582.±7.51^{bc}	853.±4.04^{b}
F	73.±1.51^{bc}	229.±4.16^{ab}	403.±6.35^{b}	590.±2.08^{ab}	851.±1.53^{b}
G	80.±1.00^{a}	241.±3.61^{a}	429.±3.61^{a}	601.±3.61^{a}	866.±2.00^{a}
H	8.±1.73^{f}	88.±1.53^{e}	170.±2.31^{d}	195.±3.00^{e}	488.±4.36^{e}

Values are means ± Standard deviation of duplicate values. Values with the same superscript in columns are not significantly different ($P \leq 0.05$)

KEY

A= 1000 g of Soil + 5 % (50 g) (w/w) crude oil (Control), B=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100g) of horse dung, C=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of unripe plantain peels, D=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of horse dung + 10 % (100 g) of unripe plantain peels, E=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung, F= 1000 g of Soil + (50 g) (w/w) crude oil + 20 % (200 g) of unripe plantain peels, G=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung + 20 % (200 g) of unripe plantain peels, H=1000 g of Autoclaved Soil +5 % (50 g) crude oil +2 % (20 g) of sodium azide (Negative control).

Table 4.4: Hydrocarbon Utilizing Bacterial Counts (x10^5 (Cfu/g) in Soil during Crude Oil Biodegradation.

Treatment	Time (days)				
	0	14	28	42	56
A	18.±0.58^{e}	57.±1.53^{e}	91.0±1.00^{d}	152.±3.06^{c}	358.±2.08^{d}
B	31.±0.58^{d}	95.00±2.00^{d}	236.±4.58^{c}	378.±2.52^{b}	570.±1.53^{c}

C	30.±0.58[d]	92.±2.08[d]	230.±3.00[c]	378.±5.57[b]	567.±3.06[c]
D	39.±0.58[c]	119.±1.53[c]	254.±3.61[b]	382.±3.51[b]	587.±2.65[b]
E	43.±0.58[b]	129.±3.06[b]	250.±2.52[b]	382.±2.08[b]	580.±1.16[b]
F	44.±1.00[b]	132.±3.06[b]	250.±1.00[b]	383.±3.61[b]	581.±1.00[b]
G	49.±2.65[a]	148.±7.64[a]	266.±4.16[a]	395.±1.73[a]	598.±2.00[a]
H	2.±0.58[f]	39.00±1.00[f]	84.±3.61[d]	143.±3.51[c]	350.±3.51[e]

Values are means ± Standard deviation of duplicate values. Values with the same superscript in columns are not significantly different ($P \leq 0.05$)

KEY

A= 1000 g of Soil + 5 % (50 g) (w/w) crude oil (Control), B=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100g) of horse dung, C=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of unripe plantain peels, D=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of horse dung + 10 % (100 g) of unripe plantain peels, E=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung, F= 1000 g of Soil + (50 g) (w/w) crude oil + 20 % (200 g) of unripe plantain peels, G=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung + 20 % (200 g) of unripe plantain peels, H=1000 g of Autoclaved Soil +5 % (50 g) crude oil +2 % (20 g) of sodium azide (Negative control).

4.4: Fungal Counts in Soil during Biodegradation Studies

The total and hydrocarbon utilizing fungal counts of soil samples during the study period are presented in Tables 4.5 and 4.6. Fungal counts were found to be greater in crude oil-polluted soil samples than in control soil samples. The highest total and hydrocarbon-utilizing fungal counts were found to be 84×105cfu/g and 54×105cfu/g,

respectively Hydrocarbon utilising fungal (HUF) populations were substantially greater in organic waste-treated soils than in unmodified and poisoned control soils. The presence of significant amounts of nitrogen and phosphorus in organic waste, particularly high levels of nitrogen in agro waste, which are important nutrients for fungal biodegradation activities, could explain enhanced fungal counts in supplemented soils (Abioye *et al*., 2012).

The isolates' capacity to exploit Used Engine Oil (UEO) as a sole source of carbon could explain why fungal growth increased in the media containing used crude oil. It's also likely that the fungus thrived due to favorable cultural settings. This is in accord with the findings of Mbachu *et al*. (2018) who found that fungal isolates such as *Aspergillus niger* grew well on mineral salt medium (MSM). Total and hydrocarbon-utilizing fungal counts all increased gradually during the course of the experiment. During crude oil breakdown, the total heterotrophic fungal counts and the hydrocarbon-utilizing fungal counts of the three treatments differed significantly at $p \leq 0.05$.

Table 4.5: Total fungal Counts $\times 10^5$ (Cfu/g) of Contaminated Soil during Crude Oil

Biodegradation

Treatment	Time (days)				
	0	14	28	42	56

A	2.±0.58^{d}	9.±0.58^{c}	15.±1.53^{d}	29.±0.58^{e}	53.±3.51^{c}
B	4.±0.58^{cd}	12.00±2.00^{c}	24.±2.52^{c}	51.±1.53^{cd}	74.0±1.00^{b}
C	4.±0.58^{cd}	12.±1.53^{c}	23.±4.16^{c}	49.00±1.00^{d}	71.±2.89^{b}
D	6.±0.58^{bc}	18.±1.53^{b}	34.±2.52^{b}	58.±1.53^{b}	77.±1.53^{b}
E	7.±1.00^{b}	19.00±3.61^{b}	38.±1.00^{b}	57.±1.53^{b}	76.±2.52^{b}
F	9.±1.00^{b}	19.00±1.00^{b}	40.±2.08^{b}	55.±1.53^{bc}	77.±2.08^{b}
G	12.±1.52^{a}	27.±3.06^{a}	51.00±4.0^{a}	65.00±2.00^{a}	84.±2.52^{a}
H	2.±0.58^{d}	7.±0.58^{c}	12.±1.00^{d}	20.00±1.00^{f}	50.±2.08^{c}

Values are means ± Standard deviation of duplicate values. Values with the same superscript in columns are not significantly different ($P \leq 0.05$)

KEY

A= 1000 g of Soil + 5 % (50 g) (w/w) crude oil (Control), B=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100g) of horse dung, C=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of unripe plantain peels, D=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of horse dung + 10 % (100 g) of unripe plantain peels, E=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung, F= 1000 g of Soil + (50 g) (w/w) crude oil + 20 % (200 g) of unripe plantain peels, G=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung + 20 % (200 g) of unripe plantain peels, H=1000 g of Autoclaved Soil +5 % (50 g) crude oil +2 % (20 g) of sodium azide (Negative control).

Table 4.6: Hydrocarbon Utilizing Fungal Counts (x10^{5} (Cfu/g) in Soil during Crude Oil Biodegradation.

Treatment	Time (days)				
	0	14	28	42	56
A	1.±0.58^{de}	3.±0.58^{d}	6.±1.16^{cd}	11.±1.53^{d}	18.±1.16^{e}

B	3.±1.00^{cd}	8.±1.00^{c}	9..±2.08^{c}	25.±1.00^{c}	40.±1.53^{cd}
C	2.±0.58^{cd}	7..±0.58^{c}	16.±1.53^{b}	22.±2.08^{c}	38.±1.53^{d}
D	4.±0.58^{bc}	9..±1.00^{bc}	17.±2.00^{b}	28.±1.56^{bc}	48.±1.00^{b}
E	5.±0.58^{ab}	11.±1.14^{ab}	20.±1.00^{b}	35.±4.36^{b}	47.±0.58^{b}
F	5.±0.58^{ab}	10.±1.53^{bc}	20.±1.53^{b}	35.±3.00^{b}	44.±1.53^{bc}
G	0.6±0.58^{a}	13.±1.53^{a}	29.±3.61^{a}	44.±2.52^{a}	54.±1.16^{a}
H	0.3±0.58^{e}	1..±0.57^{d}	3.±0.58^{d}	7.±1.16^{d}	16.±2.65^{e}

Values are means ± Standard deviation of duplicate values. Values with the same superscript in columns are not significantly different ($P \leq 0.05$)

KEY

A= 1000 g of Soil + 5 % (50 g) (w/w) crude oil (Control), B=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100g) of horse dung, C=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of unripe plantain peels, D=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of horse dung + 10 % (100 g) of unripe plantain peels, E=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung, F= 1000 g of Soil + (50 g) (w/w) crude oil + 20 % (200 g) of unripe plantain peels, G=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung + 20 % (200 g) of unripe plantain peels, H=1000 g of Autoclaved Soil +5 % (50 g) crude oil +2 % (20 g) of sodium azide (Negative control).

4.5: Morphological and Biochemical Characterization of Bacterial Isolates from crude oil contaminated soil

Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Micrococcus luteus, Pseudomonas aeruginosa and *Escherichia coli* were identified from crude oil polluted

soil based on morphological and biochemical characteristics (Table 4.7). *Bacillus*, *Staphylococcus*, and *Pseudomonas* species have been isolated from oil-polluted soil and linked to crude oil degradation, confirming previous research (Vinothini *et al.*, 2015; Chen *et al.*, 2017; Kiamars *et al*., 2019; Hajieghrari and Hejazi, 2020; He *et al*., 2020). In all treatments, *Bacillus* sp exhibited the highest frequency of isolation, which corresponds to Adeyemo *et al*. (2013). *Bacillus* sp. was also shown to have a high frequency of incidence in their research. This could be due to the fact that they produce spores, which aid microorganisms in surviving in severe environments. The number of microbial counts in soil samples contaminated with crude oil and agro-waste dropped from day zero to day fifteen as compared to the number of microbial counts in uncontaminated soil (Obiakalaije *et al*., 2015). *Bacillus cereus, Pseudomonas aeruginosa,* and *Staphylococcus aureus* may utilize crude oil as a carbon source, according to Mariano *et al*. (2008). *Bacillus* species capacity to mineralize crude oil may explain its ability to survive high crude oil concentrations. According to Chikere and Ekwuabu (2014), the microbial group that can survive in this environment has developed enzymatic and physiological responses that allow the hydrocarbons that are available as substrates to be utilised. When hydrocarbon is used as a growth substrate, the organisms release additional cellular enzymes and acids that can break down the hydrocarbon molecule by shortening the long hydrogen and carbon chains and converting hydrocarbon into simpler forms or products that the organisms can absorb for nourishment and growth. Organisms capable of thriving on crude oil-polluted soil.

Table 4.7: Morphological and Biochemical Characteristics of Bacterial Isolates

Tests													
Shape	Gram reaction	Catalase	Citrate	Oxidase	Urease	Starch hydrolysis	Methyl red	Voges proskaur	Motility	Lactose	Indole	Glucose	Suspected organisms
Rod	+	+	+	-	+	+	-	+	+	-	-	+	*Bacillus cereus*
Rod	+	+	+	-	-	+	-	+	+	-	-	+	*Bacillus subtilis*
Cocci	+	+	-	-	-	-	-	-	+	-	-	+	*Staphylococcus aureus*
Cocci	-	+	+	-	-	-	-	-	+	+	-	+	*Escherichia coli*
Cocci	+	+	-	-	+	-	-	-	+	-	-	+	*Micrococcus luteus*
Rod	-	+	+	+	-	-	-	-	+	-	-	-	*Pseudomonas aeruginosa*

Key: +Positive, -Negative

4.6: Morphological and Biochemical Characteristics of Fungal Isolates

The following fungal isolates were found in crude oil-contaminated soil: *Aspergillus niger, Aspergillus flavus, Mucor, Penicillum*, and *Rhizophus* Table 4.8 shows the results.

These isolates were identified using the morphological and microscopic characteristics. These species of fungi have been implicated in crude oil degradation which is similar observation with the findings of Adekunle and Adeniyi, (2015), Egbo *et al*. (2018), Al-Dossary *et al.* (2019) and Bessong *et al* 2019. Adeyemo *et al*. (2013) also isolated and observed a significant frequency of *Aspergillus* sp in crude oil polluted soil during their research.

When cultured in single cultures, Adams *et al.* (2014) discovered that *Penicillium* sp. and *Aspergillus* sp. could break down hydrocarbons. In the Nigerian Niger Delta, Obire (1988) discovered various species of oil-degrading fungi in the genera *Aspergillus* sp. and *Penicillium* sp., which were also discovered in this study. Ugboma and Ibietela (2020) discovered that fungi are superior degraders than other bioremediation techniques, including most bacteria. Fungi's mycelia can permeate oil, increasing the amount of surface area accessible for bacteria to decompose. The findings of this investigation revealed that there were a reasonable number of active indigenous hydrocarbon-using fungi in the oil-contaminated soil. According to Adekunle and Adebambo (2007), the energy generated is used to synthesise cell components and release carbon (iv) oxide, water, and biomass power

Table 4.8: Morphological Characteristics and Identification of Hydrocarbon-Utilizing Fungi During Bioremediation Study

Colour of aerial/hypae	Colour of substrate hypae	Nature of hyphae	Shape and kind of spore	Appearance of sporangiophore (Conidiophore)	Probable Organisms
White	Brown	Long wolly, non-septate or long slender	Spherical black sporangia	Long, erect, single and non-septate	*Mucor mucedo*
White	Dark grey	Non-septate	Round conidia	Simple erect conidiophores	*Rhizopus microsporus*
Yellow	Brown	Septate hyphae	Circular conidia	Long, erect aseptate	*Aspergillus flavus*
Yellow	Brown	Septate hyphae	Circular conidia	Long, erect aseptate	*Aspergillus niger*
Pale green	Brown	Septate branched hyphae	Circular conidia	Long, erect sponrangiophore	*Penicillium chrysogenum*

4.7 Molecular Identification of Microorganisms

The molecular result revealed that the isolate has a size of 1500bp (plate I and plate II) and the sequence of the 16S rRNA revealed the identity to be *Bacillus subtilis* strain MT152288 for the bacterial isolate and *Aspergillus flavus* strain MT152296 for the fungal isolate with 99 % homology.

The documented electrophosesis gel image illustrated in plates I and plate II shows the lane which is labelled M (molecular marker), and 1 to 2 representing DNA extracted from the oil agar bacteria and fungi plates. The direction of the band migration from negative charge carried by their sugar phosphate backbone (Wawrick, 2005). Lane 1 is more prominent than lane 2 for the fungi gel image. The both patterns (Bacteria and Fungi) shows that the two bands were 1500 bp in size with reference to the 1 kb DNA Ladder.

Plate I: Agarose Gel Electrophoresis Indicating the Positive Amplification of the Bacteria 16S Region. Mk- indicates Molecular Marker and 1 Sample VBB (*Bacillus subtilis*)

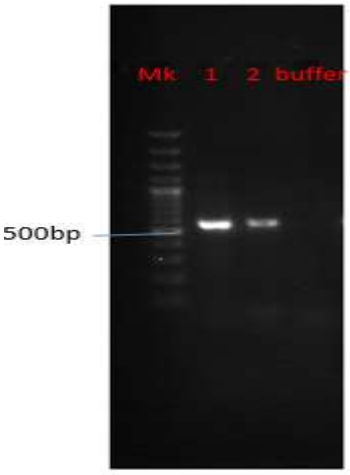

Plate II: Agarose Gel Electophloresis Indicating the Positive Amplification of the ITS Region of the Fungi Isolate. Mk-buffer indicates Molecular Marker, Fungal Isolate, Fungi Positive Control Buffer Negative Control.

4.8 Physicochemical Parameters of Contaminated Soil During Biodegradation

The physicochemical parameters were analysed for uncontaminated soil sample used for the bio remediation study. It consists of the following: pH, Nitrogen, Carbon and Phosphorus.

4.8.1 pH profile of crude oil contaminated soil during biodegradation

Figure 4.3 depicts the pH profile of a polluted soil sample that is being biodegraded. The pH of soil samples decreased steadily from day 0 to day 56 of biodegradation. After 56 days, the pH of all treatments dropped drastically from 6.02 to 4.10. The decrease in pH suggests that as the biodegradation time lengthens, the rate of crude oil biodegradation in polluted soil accelerates.

The pH decline seen in this study could be due to acidic metabolites produced by crude oil biodegradation. Because the pH of the crude oil plus agro-waste polluted soil was lower than that of the control soil sample, this result was not unexpected (just crude oil contaminated soil). The pH of crude oil contaminated soil incubated with *Pseudomonas aeruginosa* UL07 reduced from 7.15 to 6.70 after 10 days, according to Riskuwa-Shehu *et al*. (2016). Previous research has discovered a similar drop in pH levels (Abiodun *et al*., 2016; Sarkar *et al*., 2017). Acidic pH values in biodegraded soil may be the optimal microorganism pH range for digesting hydrocarbons, but alkaline pH hinders hydrocarbon breakdown (Agnello *et al*., 2015). The maximal crude oil breakdown rate of *Bacillus cereus* was observed by Olama *et al*. (2013) at pH 7. The biodegradability of microbial populations, as well as the solubility and absorption/desorption of ions and pollutants, are all affected by pH changes (San Martn, 2011; Wang *et al*., 2018).

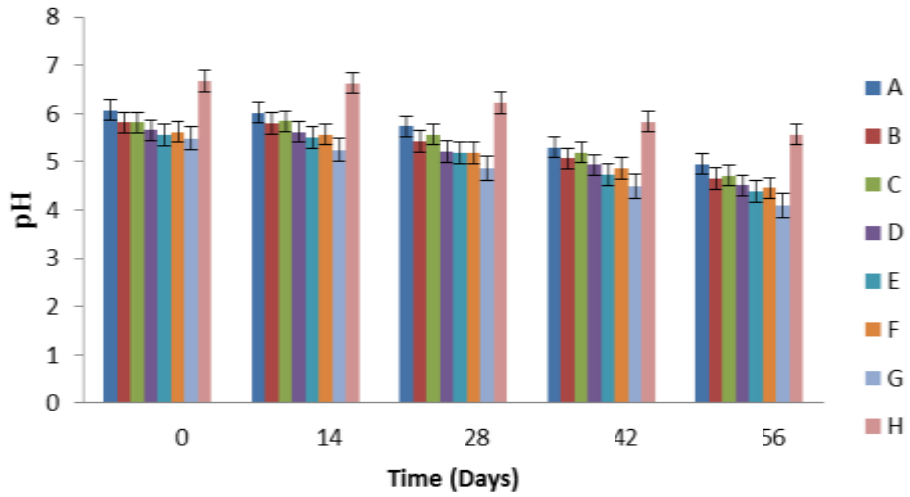

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Figure 4.3: pH Profile of Crude Oil Polluted Soil During Biodegradation

KEY

A= 1000 g of Soil + 5 % (50 g) (w/w) crude oil (Control), B=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100g) of horse dung, C=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of unripe plantain peels, D=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of horse dung + 10 % (100 g) of unripe plantain peels, E=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung, F= 1000 g of Soil + (50 g) (w/w) crude oil + 20 % (200 g) of unripe plantain peels, G=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung + 20 % (200 g) of unripe plantain peels, H=1000 g of Autoclaved Soil +5 % (50 g) crude oil +2 % (20 g) of sodium azide (Negative control).

4.8.2 Total nitrogen content of soil during crude oil biodegradation

The total nitrogen concentration of crude oil-contaminated soil increased from 0.34 % to 4.72 %, as shown in Figure 4.4 in which the treatment with the combination of 200 g each of horse dung and plantain peel had the highest increase all through the biodegradation period followed by the design with 100 g each of horse dung and unripe plantain peel. Throughout the experiment, the total nitrogen content of the control soil sample contaminated exclusively with crude oil changed marginally.

The increase in nitrogen content observed in this study could be due to the nitrogenous compound released from crude oil degradation. Increased nitrogen levels could also be attributed to microbial activities in the soil (nitrogen-fixing bacteria and algae) and phosphate-solubilizing bacteria, which fix nitrogen and build up phosphate compounds. (Adams *et al.,* 2015). Nitrogen is an essential nutrient that boosts the rate of microbial cell proliferation, shortens the lag phase of microorganisms, and supports a large microbial population, all of which contribute to an increase in the rate of hydrocarbon breakdown (Walworth *et al*., 2007). However, as documented by Maddela *et al*. (2015) and Onuoha *et al*. (2011), excessive nitrogen can hinder the soil microbial community. However, the maximal total nitrogen content found in this study falls within stimulating quantity for hydrocarbon biodegradation (Posada-Baquero *et al*., 2019).

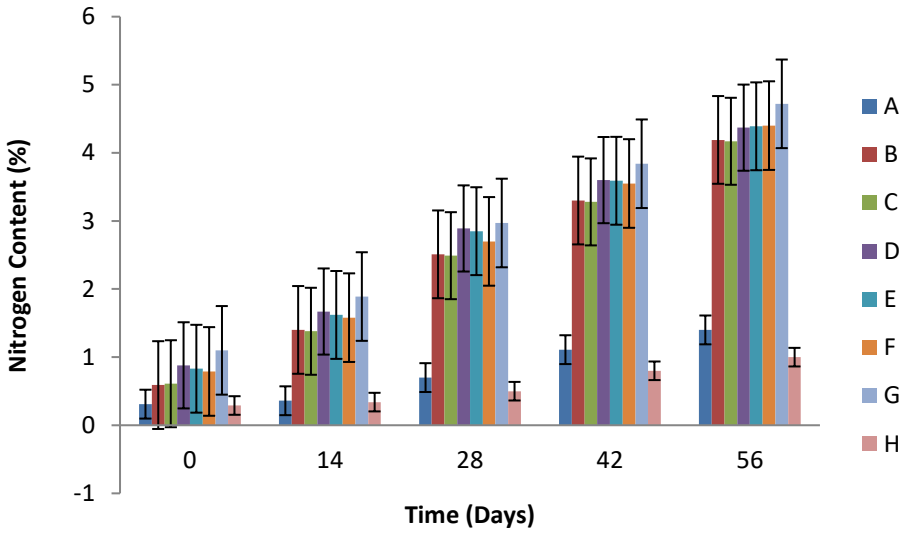

Figure 4.4: Total Nitrogen Content of Soil During Crude Oil Biodegradation

KEY

A= 1000 g of Soil + 5 % (50 g) (w/w) crude oil (Control), B=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100g) of horse dung, C=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of unripe plantain peels, D=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of horse dung + 10 % (100 g) of unripe plantain peels, E=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung, F= 1000 g of Soil + (50 g) (w/w) crude oil + 20 % (200 g) of unripe plantain peels, G=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung + 20 % (200 g) of unripe plantain peels, H=1000 g of Autoclaved Soil +5 % (50 g) crude oil +2 % (20 g) of sodium azide (Negative control).

4.8.3 Total Phosphorous content of soil during crude oil biodegradation

Phosphorous is also one of the nutrients needed for the breakdown of hydrocarbons (Sarkar *et al*., 2018). As a result, the phosphorus content of soil samples was evaluated during biodegradation, and it increased from 10.14 ppm to 15.3 ppm. Figure 4.5 shows the final results. The phosphorus content of samples increased from day 0 to day 56 on the control soil sample, with a maximum value (15.3 ppm) obtained for a soil sample of 200 g of horse dung and unripe plantain skin.

There was an increase in the content of phosphorous, this might be correlated with the higher Phosphorous content in the unripe plantain peel agro waste. On the other hand, as shown by Brune *et al.*, (2012), plantain peel agro waste may boost cation exchange capacity and reduce aluminium (Al) concentration in acid soil, releasing more accessible Phosphorous into soils and boosting bioremediation.

Wlodarczyk *et al*. (2021) also found that applying animal dung to sandy soil not only enhances crop development but also considerably improves the polluted soil's Phosphorus availability, resulting in increased bioremediation. They also observed that agro waste amendment raised total and accessible phosphorous concentrations considerably, implying that agro waste could be a viable source of high phosphorous when applied in larger quantities. According to Brown e*t al.* (2017), the nutrient removal rate of agro waste for total Phosphorous increased as the amount of agro waste amendment increased, implying that agro waste has a high adsorption potential for total Phosphorous.

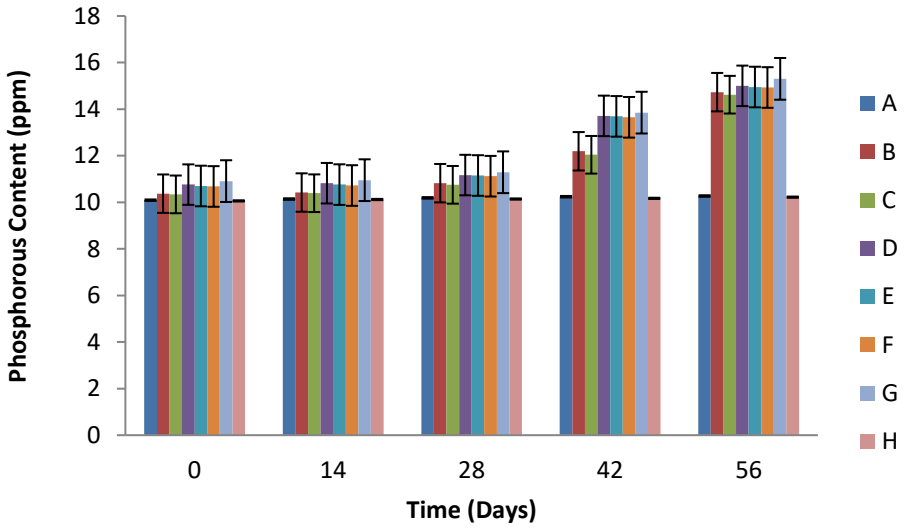

Figure 4.5: Total Phosphorous Contents of Soil during Crude Oil Biodegradation

KEY

A= 1000 g of Soil + 5 % (50 g) (w/w) crude oil (Control), B=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100g) of horse dung, C=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of unripe plantain peels, D=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of horse dung + 10 % (100 g) of unripe plantain peels, E=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung, F= 1000 g of Soil + (50 g) (w/w) crude oil + 20 % (200 g) of unripe plantain peels, G=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung + 20 % (200 g) of unripe plantain peels, H=1000 g of Autoclaved Soil +5 % (50 g) crude oil +2 % (20 g) of sodium azide (Negative control).

4.8.4 Organic carbon content of soil samples during crude oil biodegradation

The results for organic carbon in soil samples during biodegradation are shown in Figure 4.6 The organic carbon content of soil samples polluted by crude oil, horse faeces, and unripe plantain peel was 14.02 % to 9.15 % lower than that of the control soil sample (A).

According to various studies crude oil is an organic substance. Therefore the drop in organic carbon content could be attributable to the degradation of crude oil by soil microbial flora. Orji *et al*., (2021) discovered that the composition of organic carbon in soil contaminated by crude oil had changed. According to Ijah *et al*. (2008), a rise in organic carbon in crude oil polluted soil has resulted in a decrease in nitrogen, phosphorus, and pH. As a result, the decrease in organic carbon correlates with an increase in nitrogen and phosphorus content, as well as a drop in pH value, as revealed in this study.

Liu and Hsu (2013) investigated the impacts of soil organic matter and bacterial populations on the bioremediation of crude oil polluted soil and discovered that the total petroleum hydrocarbon degradation decreased as organic carbon increased. Due to increased microbial activity, the decrease in organic carbon reported in the soil tests compared to the soil control sample leads in effective hydrocarbon decomposition. Hydrocarbon degraders use hydrocarbon as their sole carbon source to efficiently purify the oil component. Ekperusi *et al*. (2015) discovered a considerable decline in organic soil carbon towards the end of their experiment causing a reduction in the last phase compared to the initial phase.

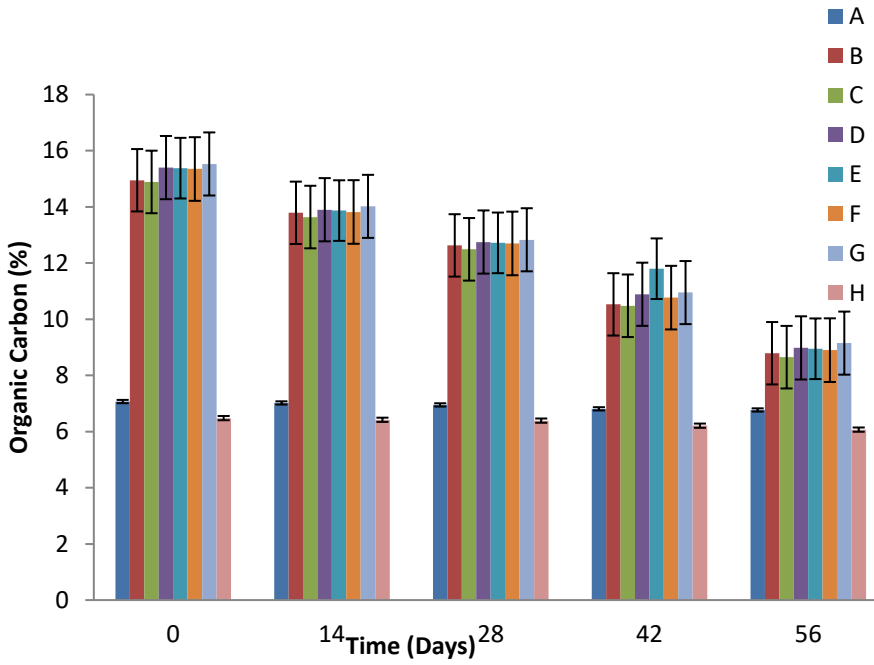

Figure 4.6: Organic Carbon Content of Soil Samples During Crude Oil Biodegradation

KEY

A= 1000 g of Soil + 5 % (50 g) (w/w) crude oil (Control), B=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100g) of horse dung, C=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of unripe plantain peels, D=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of horse dung + 10 % (100 g) of unripe plantain peels, E=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung, F= 1000 g of Soil + (50 g) (w/w) crude oil + 20 % (200 g) of unripe plantain peels, G=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung + 20 % (200 g) of unripe plantain peels, H=1000 g of Autoclaved Soil +5 % (50 g) crude oil +2 % (20 g) of sodium azide (Negative control).

4.9 Percentage (%) Biodegradation of Crude Oil

The petroleum hydrocarbon degradation is the ability of the organisms to utilize crude oil and grow on oil agar thereby breaking down complex harzardous hydrocarbons into simpler nontoxic forms. In all of the treatments, biodegradation began within the first week of remediation and progressed until the 56th day. Table 4.9 below shows the percentage biodegradation for the different treatments calculated at days 14, 42 and 56, respectively.

The highest percentage biodegradation of crude oil was recorded at day 56 from the treatment G (1000 gof soil contaminated with 5 % (50 g) crude oil mixed with 20 % (200 g) of horse dung and unripe plantain peel) from 44.94 % at day 14 to 89.05 % at day 56. This observation indicates there was an increase in the degradation of petroleum hydrocarbon in crude oil contaminated soil when a higher quantity of the two agro-wastes (horse dung and unripe plantain peel) were mixed, the breakdown of petroleum hydrocarbon in crude oil contaminated soil increased. This could be because the mixture of animal and plant waste acted as an adsorbent in the soil, allowing the pollutant to become strongly bonded to it and, therefore, fostering the growth of degrading bacteria (Yelebe *et al.*, 2015). The presence of organic wastes in soil may result in increased oil biodegradation in modified soil, which may contribute to loosening the compactness of the soil and providing adequate aeration to the indigenous organisms present on the soil, thereby improving their metabolism activity in contaminated soils. It could possibly be because these organic wastes (horse dung and unripe plantain peel) have the potential to swiftly restore the soil's physicochemical properties, neutralizing the oil's damaging effects on the microbial population (Abioye *et al*., 2012). On the other hand, the soil polluted with 5 % (50 g) crude oil combined with 2 % (20 g) sodium azide had the lowest percentage biodegradation (13.34 %) of crude oil at day 56.

Table 4.9 Percentage Biodegradation of Crude Oil in Soil Remediated with Agrowaste

Percentage Crude Oil Degradation (%)

Treatment	Time (days)		
	14	42	56
A	7.21	27.77	34.32
B	30.51	76.20	83.91
C	27.7	75.87	81.84
D	33.91	78.10	86.73
E	39.22	81.92	86.89
F	35.32	78.27	85.32
G	44.94	84.32	89.05
H	2.57	10.28	13.34

KEY

A= 1000 g of Soil + 5 % (50 g) (w/w) crude oil (Control), B=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100g) of horse dung, C=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of unripe plantain peels, D=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 10 % (100 g) of horse dung + 10 % (100 g) of unripe plantain peels, E=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung, F= 1000 g of Soil + (50 g) (w/w) crude oil + 20 % (200 g) of unripe plantain peels, G=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung + 20 % (200 g) of unripe plantain peels, H=1000 g of Autoclaved Soil +5 % (50 g) crude oil +2 % (20 g) of sodium azide (Negative control).

This could be due to sodium azide's toxicity, resulting in a decrease in the survival of the organisms present. The trend in hydrocarbons reported in the study utilizing bacterial counts accords with the increase in percentage biodegradation of crude oil attained in this research. The high biodegradation rates at this level of oil pollution could be due to an increase in the activity of hydrocarbon-utilizing bacteria (Abioye *et al*., 2012).

The natural breakdown of petroleum components into smaller molecular units appears to be a natural process in which crude oil was used as source of carbon leading to the biodegradation of crude oil by microorganisms (Zhang *et al*., 2015). Other studies (Abioye *et al*., 2012 ; Wang *et al*., 2019) discovered that increasing incubation time in waste oil-contaminated soil could dramatically reduce total hydrocarbon petroleum concentrations (Abioye *et al*., 2012 ;Wang *et al*., 2019;). Similar biodegraded findings have been found when polycyclic aromatic hydrocarbons (Chen *et al*., 2015), volatile petroleum hydrocarbons (Qin *et al*., 2013), total petroleum hydrocarbons (Qin *et al*., 2013), phenanthren (Biswas *et al*., 2019), and 2.6 dichlorophenol have been used (Agarry *et al*., 2013). This increased biodegradation of petroleum hydrocarbons can be attributed to agro-wastes as a soil adsorbent, which has a stimulating effect by increasing nutrient supply (nitrogen, phosphorous, and micronutrients) and establishing a habitat for increased microbial activity (Park *et al.*, 2011 ; Agary *et al*., 2015).

4.10 Gas Chromatography and Mass Spectroscopy (GC-MS) of Un-degraded and Residual Crude Oil Extracted from Soil

In the initial un-degraded crude oil used for the investigation, gas chromatography and mass spectroscopy (GCMS) analysis identified a total of 48 distinct hydrocarbon compounds (Figure 4.7) and 41 individual hydrocarbon compounds in soil contaminated with crude oil mixed with 20 % (200 g) of horse dung and unripe plantain peel (Treatment G). The GCMS results after 56 days of treatment G=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung + 20 % (200 g) of unripe plantain peel is shown in Table 4.9. After 56 days, the alkane contents of the biodegraded oil samples had drastically decreased. After 56 days of biodegradation, the long chain alkanes were almost entirely destroyed in all samples. The amount of ester constituents detected increased as the amount of alkane constituents dropped. Treatment G (G=1000 g of Soil + 5 % (50 g) (w/w) crude oil + 20 % (200 g) of horse dung + 20 % (200 g) of unripe plantain peel) showed the greatest reduction in hydrocarbons, in which the n-alkanes were almost completely degraded with the exception of eicosane ($C_{20}H_{42}$), naphthalene, alkyl groups of napthalenes, some aromatic and polycyclic hydrocarbon.

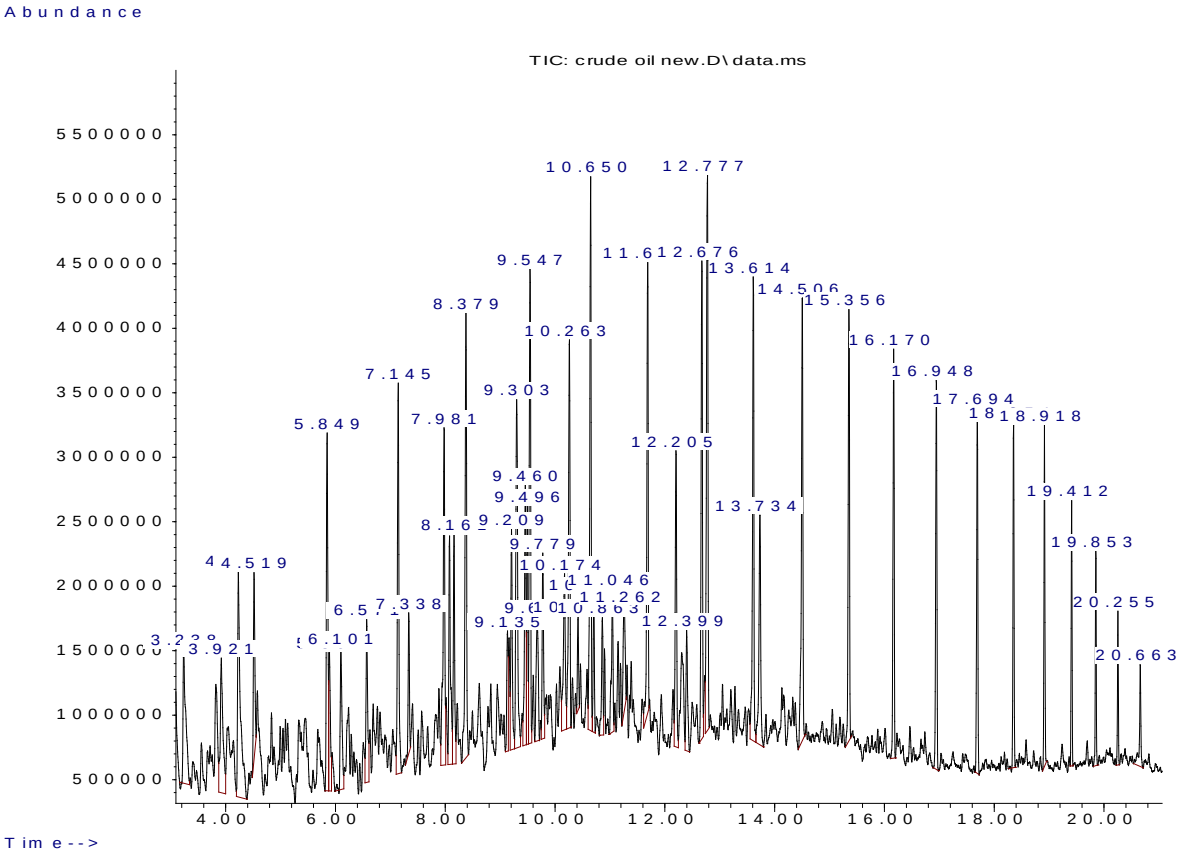

Figure 4.7: Chromatogram of Crude Oil used for Biodegradation.

Table 4.10: Individual Hydrocarbon Identified in Undegraded Crude Oil

Peak	Formula	Compound	RT	Area%
1	C_9H_20	Nonane	3.239	1.89
	C_9H_20	Nonane		
	$C_{13}H_{28}$	Decane, 2,5,6-trimethyl-		
2	C_9H_{12}	Mesitylene	3.920	1.89
	C_9H_{12}	Mesitylene		
	$C_6H_3(CH_3)_3$	Benzen,1,2,4-trimrthyl-		
3	C_9H_{12}	Mesitylene	4.235	3.26
	$C_6H_3(CH_3)_3$	Benzene,1,2,3-trimethyl-		
	C_9H_{12}	Mesitylene		
4	$CH_3(CH_2)8CH_3$	Decane	4.520	1.36
	$CH_3(CH_2)8CH_3$	Decane		
	C_9H_20	Nonane		
5	$C_{11}H_{24}$	Undecane	5.849	2.83
	$C_{11}H_{24}$	Undecane		
	$C_{11}H_{24}$	Undecane		
6	$C_{10}H_{14}$	Benzene,1,2,4,5-tetramethyl-	5.892	1.15
	$C_{10}H_{14}$	Benzene, 1-ethyl-2,3-dimethyl-		
	$C_{10}H_{14}$	Benzene,1,2,3,5-tetramethyl-		
7	$C_{11}H_2O$	1-Methyldecahydronaphthalene	6.101	1.36
	$C_{11}H_2O$	Naphthalene,decahydro-2methyl-		
	$C_{11}H_2O$	Naphthalene,decahydro-2methyl-		
8	$C_{10}H_8$	1-H-indene, 1-methylene-	6.573	1.42
	$C_{10}H_8$	Naphthalene		
	$C_{10}H_8$	Naphthalene		
9	$CH_3(CH_2)_{10}CH_3$	Dodecane	7.144	3.00
	$CH_3(CH_2)_{11}CH_3$	Tridecane		
	$CH_3(CH_2)_{12}CH_3$	Tetradecane		
10	$C_{13}H_{28}$	Undecane,2,6-dimethyl-	7.339	0.89
	$C_{13}H_{28}$	Undecane,3,6-dimethyl-		
	$C_{13}H_{28}$	Dodecane,6-dimethyl-		
11	$C_{11}H_{10}$	Naphthalene, 1-methyl-	7.982	3.01
	$C_{11}H_{10}$	Naphthalene, 2-methyl-		
	$C_{11}H_{10}$	Naphthalene, 2-methyl-		
12	$C_{10}H_{22}$	Nonane, 3-methyl-	8.077	2.00
	$C_{20}H_{42}$	Hexadecane, 2,6,11,15-tetramethyl-		
	$C_{20}H_{42}O_3S$	Sulfurous acid, dodecyl 2-ethylhexyl ester		
13	$C_{11}H_{10}$	Naphthalene, 2-methyl-	8.163	1.70
	$C_{11}H_{10}$	Naphthalene, 1-methyl		
	$C_{11}H_{10}$	Naphthalene, 2-methyl- -		
14	$CH3(CH_2)_{11}$	Tridecane	8.377	3.71
	CH_3	Tridecane		

15	$C_{14}H_3O$ $C_{13}H_{28}$ $C_{27}H_{55}Cl$	Tridecane, 2-methyl- Dodecane, 2-methyl- Heptacosane, 1-chloro-	9.135	0.81
16	$C_{15}H_{28}$	Decahydro-1,1,4a,5,6-pentamethylna Phthalene Naphthalene, decahydro-1,8a-dimeth yl-7-(1-methylethyl)-, [1R-(1.alph a.,4a.beta.,7.beta.,8a.alpha.)]- 10.alpha.-Eremophilane	9.211	1.72
17	$C_{12}H_{12}$	Naphthalene, 2,7-dimethyl- Naphthalene, 2,7-dimethyl- Naphthalene, 1,6-dimethyl	9.301	3.37
18	$C_{12}H_{12}$	Naphthalene, 2,3-dimethyl- Naphthalene, 2,6-dimethyl- Naphthalene, 1,6-dimethyl-	9.458	1.98
19	$C_{12}H_{12}$	Naphthalene, 2,6-dimethyl- Naphthalene, 2,7-dimethyl- Naphthalene, 1,6-dimethyl-	9.497	1.47
20	$CH_3(CH_2)_{12}CH_3$	Tetradecane Tetradecane Tetradecane	9.549	3.50
21	$C_{12}H_{12}$	Naphthalene, 1,3-dimethyl- Naphthalene, 2,3-dimethyl- Naphthalene, 1,3-dimethyl-	9.677	1.34
22	$C_{15}H_{28}$	Decahydro-1,1,4a,5,6-pentamethylna phthalene Naphthalene, decahydro-1,4a-dimeth yl-7-(1-methylethyl)-, [1S-(1.alph a.,4a.alpha.,7.alpha.,8a.beta.)]- 1-(p-Fluorophenyl)-4-piperidone	9.777	1.34
23	$C_{15}H_{28}$ $C_{14}H_{11}N$ $C_{14}H_{11}N$	Decahydro-1,1,4a,5,6-pentamethylna phthalene 2-Anthracenamine 2-Anthracenamine	10.173 10.263	1.68 3.06
24	$C_{16}H_{34}$ $C_{15}H_{32}$ $CH_3(CH_2)_{11}CH_3$	2,6,10-Trimethyltridecane Dodecane, 2,6,11-trimethyl- Tridecane		

25	$C_{13}H_{14}$	Naphthalene, 1,6,7-trimethyl	10.420	0.56
	$C_{13}H_{14}$	Naphthalene, 2,3,6-trimethyl		
	$C_{13}H_{14}$	Naphthalene, 1,6,7-trimethyl		
26	$C_{15}H_{32}$	Pentadecane	10.649	4.08
	$C_{15}H_{32}$	Pentadecane		
	$C_{15}H_{32}$	Pentadecane		
27	$C_{13}H_{14}$	Naphthalene, 1,6,7-trimethyl-	10.706	0.88
	$C_{13}H_{14}$	Naphthalene, 1,6,7-trimethyl-		
	$C_{13}H_{14}$	Naphthalene, 1,4,6-trimethyl-		
28	$C_{13}H_{14}$	Naphthalene, 1,6,7-trimethyl-	10.863	0.88
	$C_{13}H_{14}$	Naphthalene, 1,4,6-trimethyl-		
	$C_{13}H_{14}$	Naphthalene, 1,6,7-trimethyl-		
29	$C_{13}H_{14}$	Naphthalene, 1,6,7-trimethyl-	11.044	1.21
	$C_{13}H_{14}$	Naphthalene, 2,3,6-trimethyl-		
	$C_{13}H_{14}$	Naphthalene, 2,3,6-trimethyl-		
30	$C_{13}H_{14}$	Naphthalene, 2,3,6-trimethyl-	11.263	1.11
	$C_{13}H_{14}$	3-(2-Methyl-propenyl)-1H-indene		
	$C_{13}H_{14}$	Naphthalene, 2,3,6-trimethyl-		
31	$C_{16}H_{34}$	Hexadecane	11.687	3.31
	$C_{16}H_{34}$	Hexadecane		
	$C_{16}H_{34}$	Hexadecane		
32	$C_{19}H_{40}$	Pentadecane, 2,6,10-trimethyl-	12.206	2.26
	$CH_3(CH_2)_{12}CH_3$	Tetradecane		
	$C_{16}H_{34}$	Dodecane, 2-methyl-8-propyl-		
33	$C_{14}H_{11}NO_2$	9H-Fluorene, 2-methyl-	12.401	1.01
	$C_{14}H_{11+}$	9H-Fluorene, 9-methyl-		
	$C_{14}H_{12}$	9H-Fluorene, 1-methyl-		
34	$CH_3(CH_2)_{15}CH_3$	Heptadecane	12.678	3.40
	$CH_3(CH_2)_{12}CH_3$	Tetradecane		
	$CH_3(CH_2)_{15}CH_3$	Heptadecane		
35	$C_{19}H_{40}$	Pentadecane, 2,6,10,14-tetramethyl	12.778	4.57
	$C_{19}H_{40}$	Pentadecane, 2,6,10,14-tetramethyl		
	$C_{19}H_{40}$	Pentadecane, 2,6,10,14-tetramethyl		
36	$CH_3(CH_2)_{16}CH_3$	Octadecane	13.616	3.44
	$C_{16}H_{34}$	Hexadecane		
	$C_{16}H_{34}$	Hexadecane		

37	$C_{20}H_{42}$ $C_{20}H_{42}$ $CH_3(CH_2)_{10}CH_3$	Hexadecane, 2,6,10,14-tetramethyl- Eicosane Dodecane	13.735	2.19
38	$CH_3(CH_2)_{17}CH_3$ $CH_{16}H_{34}$ $CH_3(CH_2)_{17}CH_3$	Nonadecane Hexadecane Nonadecane	14.506	3.55
39	$C_{20}H_{42}$ $C_{16}H_{32}$ $CH_3(CH_2)_{17}CH_3$	Eicosane Hexadecane Nonadecane	15.354	3.06
40	$C_{21}H_{44}$ $C_{16}H_{34}$ $C_{16}H_{34}$	Heptadecane, 2,6,10,15-tetramethyl Hexadecane Hexadecane	16.168	2.62
41	$C_{16}H_{34}$ $C_{21}H_{44}$ $CH_3(CH_2)_{17}CH_3$	Hexadecane Pentadecane, 8-hexyl- Nonadecane	16.949	2.55
42	$C_{23}H_{48}$ $C_{21}H_{44}$ $CH_3(CH_2)_{24}CH_3$	Tricosane Eicosane, 10-methyl- Hexacosane	17.692	2.25
43	$H(CH_2)_{24}H$ $H(CH_2)_{24}H$ $C_{21}H_{44}$	Tetracosane Tetracosane Pentadecane, 8-hexyl-	18.354	1.89
44	$H(CH_2)_{24}H$ $C_{21}H_{44}$ $C_{21}H_{44}$	Tetracosane Heneicosane Pentadecane, 8-hexyl-	18.916	1.69
45	$CH_3(CH_2)_{16}CH_3$ $C_{25}CH_{52}$ $CH_3(CH_2)_{24}CH_3$	Octadecane Heptadecane, 9-octyl- Hexacosane	19.411	1.22
46	$C_{27}H_{56}$ $C_{25}H_{52}$ $C_{23}H_{48}$	Heptacosane Heptadecane, 9-octyl- Heptadecane, 9-hexyl-	19.854	1.05
47	$C_{27}H_{56}$ $CH_3(CH_2)_{24}CH_3$ $CH_3(CH_2)_{15}CH_3$	Heptacosane Hexacosane Heptadecane	20.254	0.70

48	$C_{27}H_{56}$ $C_{20}H_{42}$ $C_{23}H_{48}$	Heptacosane Eicosane Tricosane	20.663	0.77

Key:PK= Peak number, RT= Retention time.

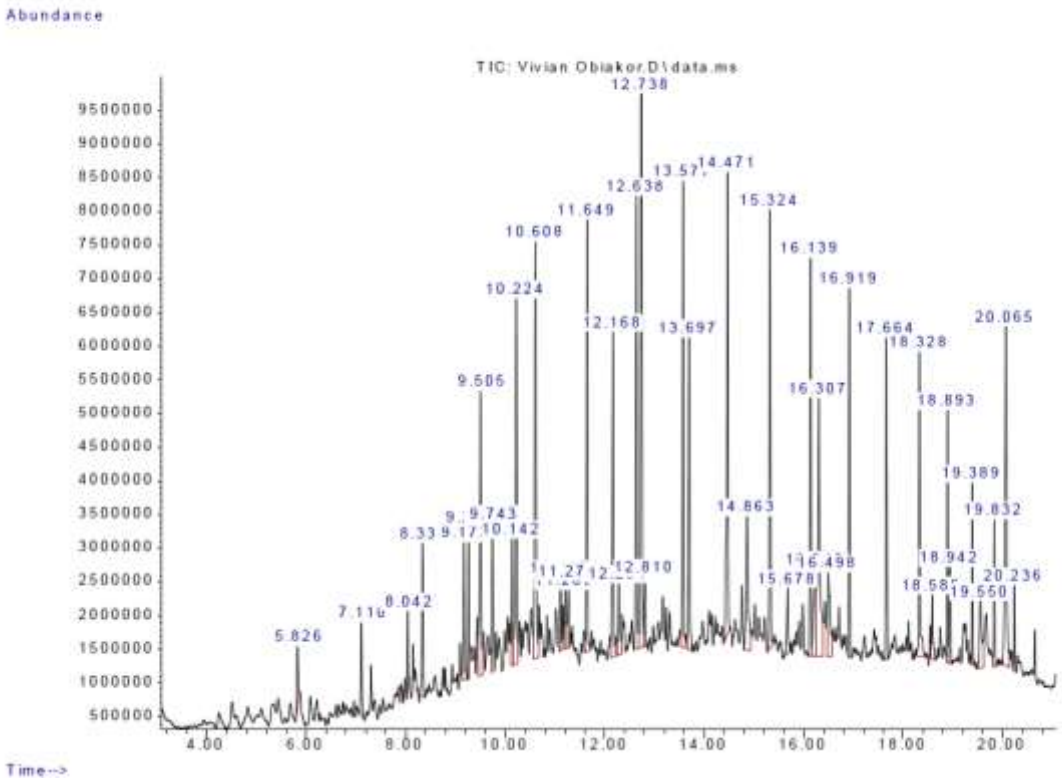

Figure 4.8: Chromatogram of degraded Crude Oil after 56 days.

The GCMS data in table 4.10 gave critical information on microorganisms' biotransformation of hydrocarbons. These studies also showed that different components of crude oil have various degrees of degradability; for example, while aromatics and aliphatic hydrocarbons were quickly degraded, resins and asphaltenes are inherently resistant (Sharma *et al*., 2018). Long chain alkanes may be degraded the most by the activities/effects of hydrocarbon degrading bacteria. Sharma *et al*. (2018) reported that bacteria with alkane hydroxylase can assimilate alkanes with a molecular weight greater than C_{20}.

It was discovered that only the asphaltene proportion (esters) of hydrocarbons remained after 56 days; the aliphatic alkanes such as Nonane, Decane and Octadecane which are highly cytotoxic thereby posing harm to humans and animals were degraded to lesser cytotoxic aliphatic hydrocarbons such as Tetradecane, Hexadecane and Pentadecane. and also a good number of aromatic fractions of hydrocarbon had been completely degraded such as Trimethyl benzene and Cyclohexyl benzene which were degraded to Benzene, Dimethyl naphthalene which was further degraded to Napthalene. Furthermore, Aliphatic hydrocarbons such as Dodecane, Undecane were degraded to Tetradecane, Pentadecane, Hexadecane etc whereas Aromatic hydrocarbons such as Dimethylnapthalene, Cyclohexylbenzene, Ethylbenzene were degraded to lesser Aromatic hydrocarbons such as Toulene and Benzene after the 56 days degradation process. Asphaltene is frequently thought to be resistant to microbial attack (Koshlaf *et al,* 2017). However, because this hydrocarbon component is significantly more polar, it can easily be adsorbed to the bottom of the contaminated soil after biodegradation. The peak and retention of soil samples were likewise linked to alkane degradation. Several enzymes work together to break down hydrocarbon components in the microbial environment. In the case of polyaromatic hydrocarbons, the process begins with oxidation, in which the enzyme groups oxygenases aid in the separation of the aromatic ring (Xenia and Refugio, 2016). Microbes metabolize hydrocarbons to create fatty acids and ester, which they subsequently incorporate into their cells, according to Fuentes *et al*., (2014).

Table 4.11: Individual Hydrocarbon Identified in Degraded Crude Oil after day 56

Peak	Formula	Compound	RT	Area %
1	$CH_3(CH_2)_9CH_3$	Undecane	5.825	0.73
		Carbonic acid, prop-1-		
	$C_{17}H_{32}O_3$	en-2-yl tridecyl ester		
	$C_{18}H_{37}Cl_3Si$	Silane, trichlorooctadecyl-		
2	$CH3(CH_2)_{10}CH_3$	Dodecane	7.111	0.97
	$C_{21}H_{44}$	Heptadecane, 2,6,10,14-tetramethyl		
	$C_{17}H_{32}O_3$	Carbonic acid, prop-1-		
		en-2-yl tri ecyl ester		
3	$C_{21}H_{44}$	Hexadecane, 2,6,11,15-tetramethyl	8.044	0.60
	$C_{20}H_{42}$	Octane, 2,6-dimethyl-		
	$C_{17}H_{36}$	Heptadecane, 2,6-dimethyl-		
			8.339	1.47
4	$CH_3(CH_2)_{10}CH_3$	Tridecane		
	$CH_3(CH_2)_{10}CH_3$	Tridecane		
	$CH_3(CH_2)_{17}CH_3$	Nonadecane		
5	$C_{15}H_{28}$	Decahydro1,1,4a,5,6pent	9.173	1.48
		amethylnaphthalene		
	$C_9H_{14}O$	2-Cyclopenten-1-one,		
		2,3,4,5-tetramethyl		
	$C_9H_{12}O$	4-Isopropylphenol,		

6	$C_{15}H_{32}$	Dodecane, 2,6,11-trimethyl-	9.263	1.39
	$C_{15}H_{32}$	Dodecane, 2,6,11-trimethyl-		
	$C_{12}H_{24}$	1-Undecene, 4-methyl-		
7	$CH_3(CH_2)_{12}CH_3$	Tetradecane	9.506	3.31
	$CH_3(CH_2)_9CH_3$	Undecane		
	$CH_3(CH_2)_{12}CH_3$	Tetradecane		
8	$C_{15}H_{28}$	Decahydro-	9.744	1.60
		1,1,4a,5,6pentamethylna		
		phthalene		
	$C_{14}H_{24}N_2O_2$	2-propenamide, N-(2,6-		
		dihydroxyphenyl)-2-		
	$C_{10}H_{16}O$	methyl-		
9.	$C_9H_{14}O$	2,6-Octadien-1-ol, 3,7-dimethyl	10.139	1.36
	$C_{11}H_{26}O_2Si$	2,6-Heptadienal, 2,4-dimethyl- Silane, chlorodiethyl(2-methylpent-3-yloxy)-		
	C_9H_{16}	Cyclopropane,1,1dimethyl2(2methyl2-propenyl)-		
10	$C_{16}H_{34}$	Hexadecane	10.225	4.02
	$C_{16}H_{34}$	Hexadecane		
	$CH_3(CH_2)_9CH_3$	Undecane		
11	$CH_3(CH_2)_{12}CH_3$	Tetradecane	10.606	4.98
	$CH_3(CH_2)_{17}CH_3$	Nonadecane		
	$CH_3(CH_2)_{12}CH_3$	Tetradecane		
12	$C_{17}H_{34}$	Undecane, 3-cyclohexyl-	11.111	0.83
	$C_{14}H_{28}O$	Tetradecanal		
	$C_{16}H_{32}O$	Hexadecanal		
13	$CH_3(CH_2)_9CH_3$	Undecane	11.211	0.64

	$CH_3(CH_2)_{11}CH_3$	Tridecane		
	$CH_3(CH_2)_{11}CH_3$	Tridecane		
14	$CH_3(CH_2)_{10}CH_3$	Dodecane	11.273	0.81
	$CH_3(CH_2)_{10}CH_3$	Dodecane		
	$C_{18}H_{37}Br$	Octadecane, 1-bromo-		
15	$C_{16}H_{34}$	Hexadecane	11.649	4.19
	$C_{16}H_{34}$	Hexadecane		
	$CH_3(CH_2)_{17}CH_3$	Nonadecane		
16	$C_{18}H_{34}$	Pentadecane, 2,6,10-trimethyl-	12.168	3.63
	$C_{16}H_{34}$	Tridecane, 5-propyl-		
	$C_{27}H_{56}$	Heptacosane		
			12.283	0.91
17	$CH_3(CH_2)_{15}CH_3$	Tetradecane		
	$C_9H_{16}BrNO$	2-Piperidinone, N-[4-bromo-n-butyl]-		
	$C_{16}H_{34}$	Pentadecane, 3-methyl-		
			12.639	4.58
18	$CH_3(CH_2)_{15}CH_3$	Tetradecane		
	$CH_3(CH_2)_{15}CH_3$	Dodecane, 2-methyl-6-propyl-		
	$C_{16}H_{34}$	Dodecane, 2-methyl-6-propyl-		
19	$C_{22}H_{46}$	Pentadecane, 2,6,10,14-tetramethyl	12.739	8.18
	$C_{20}H_{42}$	Hexadecane, 2,6,10,14-tetramethyl-		
	$C_{22}H_{46}$	Pentadecane, 2,6,10,14-tetramethyl		
20	$C_{43}H_{88}$	Tritetracontane	12.811	0.78
	$CH_{17}H_{36}O_3S$	Sulfurous acid, 2-propyltetradecyl ester		
	$C_{44}H_9O$	Tetratetracontanane		
21	$C_{16}H_{34}$	Hexadecane	13.578	4.39
	$CH_3(CH_2)_{15}CH_3$	Tetradecane		
	$C_{15}H_{32}$	Pentadecane		
22	$C_{16}H_{34}$	Hexadecane	13.697	3.43
	$C_{21}H_{44}$	Heptadecane, 2,6,10,15-tetramethyl		
	$C_{15}H_{32}$	Dodecane, 2,6,11-trimethyl-		
23	$C_{26}H_{54}$	Hexacosane	14.473	5.02
	$C_{20}H_{42}$	Nonadecane		
	$CH_3(CH_2)_{17}CH_3$	10-Methylnonadecane		
24	$CH_3(CH_2)_{14}COOH$	n-Hexadecanoic acid	14.863	2.41

	$CH_3(CH_2)_{14}COOH$	n-Hexadecanoic acid		
	$CH_3(CH_2)_{14}COOH$	n-Hexadecanoic acid		
25	$C_{20}H_{42}$	Eicosane	15.325	4.09
	$C_{21}H_{44}$	Heneicosane		
	$CH_3(CH_2)_{17}CH_3$	Nonadecane		
26	$C_{26}H_{54}$	2-Methyl-Z,Z-3,13-octadecadienol	15.678	0.79
	$C_{20}H_{42}$	8-Hexadecyne		
	$CH_3(CH_2)_{17}CH_3$	Cyclohexane,1-(1,5-dimethylhexyl		
27	$C_{26}H_{54}$	Hexacosane	16.139	3.65
	$C_{20}H_{42}$	10-Methylnonadecane		
	$C_{21}H_{44}$	Heneiocosane		
28	$C_{18}H_{32}O_2$	9,12-Octadecadienoic acid (Z,Z)	16.239	1.83
	$C_{18}H_{32}O_2$	9,12-Octadecadienoicacid (Z,Z)		
	$C_{20}H_{38}$			
29	$C_{18}H_{34}O_2$	9-Eicosyne	16.306	4.55
	$C_{18}H_{34}O_2$	9-Octadecenoic acid,		
	$C_{18}H_{36}$	9-Octadecenoic acid,		
30	C_2H_4O	1-Octadecene	16.497	1.80
	$CH_3(CH_2)_{17}CH_3$	Oxirane, tridecyl-		
	$C_{20}H_{42}O_2$	Oxirane, tridecyl- Ethanol, 2-(octadecyloxy)-		
31	$C_{21}H_{44}$	Eicosane, 10-methyl-	16.920	3.34
	$C_{13}H_{27}I$	Tridecane, 1-iodo-		
	$C_{20}H_{42}$	10-Methylnonadecane		
32	$C_{21}H_{44}$	Eicosane, 10-methyl-	17.663	2.87
	$C_{26}H_{54}$	Hexacosane		
	$C_{20}H_{42}$	10-Methylnonadecane		
	$C_{24}H_{50}$	Tetracosane		

	$C_{24}H_{50}$	Tetracosane		
33	$C_{26}H_{54}$	Hexacosane	18.330	2.88
	$C_{18}H_{34}O$	13-Octadecenal, (Z)-		
	$C_{18}H_{38}O_3$	Oxirane, tridecyl-		
34	$C_{22}H_{42}O_2$	Butyl 9-octadecenoate	18.587	0.70
	$C_{26}H_{54}$	Hexacosane		
	$C_{20}H_{42}$	10-Methylnonadecane		
35	$C_{16}H_{34}$	Dodecane,2-methyl-6-propyl-	18.892	1.90
	$C_{24}H_{38}O_4$	Bis(2-ethylhexyl) phthalate		
	$C_{18}H_{28}O_3$	8-[3-Oxo-2-(pent-2-en-		
36	$C_{16}H_{18}O_4$	1yl)cyclopent-1-enyl]octanoic acid	18.940	0.78
		Di(E)-but-2-enyl phthalate		
	$CH_3(CH_2)_{15}CH_3$	Tetradecane		
	$C_{44}H_{90}$	Tetratetracontane	19.387	1.19
	$C_{26}H_{54}$	Hexacosane		
37	$C_{18}H_{28}O_3$	Oxirane, tridecyl-		
	$C_{14}H_{28}O$	Tetradecanal	19.549	1.36
	$C_{18}H_{38}O_3$	Oxirane, tetradecyl-		
38	$CH_3(CH_2)_{16}CH_3$	Octadecane	19.835	1.05
	$C_{27}H_{56}$	Heptacosane		
	$C_{20}H_{42}$	Nonadecane, 9-methyl-		
39	$C_{29}H_{50}O$	.gamma.-Sitosterol		
	$C_{29}H_{50}O$		20.063	4.91
	$C_{27}H_{46}O$	.beta.-Sitosterol		
		Lathosterol		
40				
	$C_6H_{13}Br$	Hexadecane, 1-bromo-		
	$C_{18}H_{34}O_2$	Palmitic acid vinyl ester	20.235	0.59
	$C_{28}H_{58}$	Octacosane		
41				

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The bacterial population (aerobic heterotrophic bacteria and hydrocarbon-utilizing bacteria), fungal population and physicochemical paramaters (pH, nitrogen, phosphorous, electrical conductivity, exchangeable acidity and cation exchange capacity) of uncontaminated soil were suitable for bioremediation and greatly improved during crude oil biodegradation.

Bacillus subtilis, Micrococcus luteus, Bacillus cereus, Escherichia coli, Staphylococcus aureus and *Pseudomonas aeruginosa* were among the bacteria isolated from crude oil-contaminated soil, while *Aspergillus niger, Aspergillus flavus, Mucor mucedo, Penicillium chrysogenum*, and *Rhizopus microsporus* were among the fungi isolated from crude oil-contaminated soil, these organisms were able to breakdown hydrocarbons. Biostimulation of crude oil polluted soil with horse dung and unripe plantain peels 100 g (10 %) and 200 g (20 %) greatly influenced heterotrophic and hydrocarbon-utilizing bacterial and fungal population during biodegradation positively by enhancing the remediation process; particularly treatment with 200 g each of horse dung and unripe plantain peel.

Crude oil contaminated soil containing 20 % mixture of each of the agro-waste (horse dung and unripe plantain peel) recorded highest crude oil degradation of 89.05 % at the end of 56 days as compared to other treatments which ranged from 2.57 % to 86.89 %. The current research confirms that using agricultural waste (horse dung and unripe plantain peel) enhanced the rate of biodegradation in crude oil-contaminated soil or land habitats. The maximum total petroleum hydrocarbon (TPH) removal was 89.05 % for crude oil polluted

soil amended with various agro-wastes at the end of the 56-day remediation period. From the percentage biodegradation results, it was observed that the treatments with horse dung waste had biodegradation relatively higher (86.89 %) than the treatment with unripe plantain waste (85.32 %) and unamended contaminated soil (34.32 %) as well as soil treated with sodium azide (13.34 %).

The use of agro-waste to transform soils can be a low-cost, novel method to reduce organic compound pollution and soil exposure. Because of the low cost and limited environmental danger associated with hydrocarbon volatile losses, the bioremediation technique provided here for crude oil damaged soils may be appropriate on the ground.

5.2 Recommendations

It is recommended that:

i. Bioremediation of crude oil contaminated soil using mixture of horse dung and unripe plantain peels at a concentration of 20 % or more should be encouraged.
ii. Microorganisms such as *Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas aeruginosa Aspergillus niger, Aspergillus flavus, Mucor mucedo*. *Penicillum* chrysogenum and *Rhizopus microspores* isolated from crude oil contaminated soil should be employed in integrated management goal spills in the tropics. These organisms are effective crude oil degraders.

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APPENDIX I

***Bacillus subtilis* strain**

CCGGGGTGCTATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGT

TAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAA

CTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACA

TAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTT

GGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGAT

CGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGG

AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG

GTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGG

GCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAG

CCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCT

CGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTC

ATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAG

CGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGG

TCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACC

CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCT

TAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACT

GAAACTCAAAGGAATTGACGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT

TCGAAGCAACGCGAAGACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGA

TAGGACGTCCCCTTTCGGGGGCAGAC

APPENDIX II

Aspergillus flavus strain

ACGGGAAGTAAAAAATCGCAACAAGGTTTCCGTAGGTAAACCTGCGGAAGGATC

ATTACCGAGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTAC

CTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGG

GCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTT

GATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCG

ATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAAT

CATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGT

CCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTC

TCCGGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGC

GTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAA

TCAATCTTTTCCAGGTGACCTCGATCACAGAGGTCCCCCCCCCCA