BIOREMEDIATION OF SPENT ENGINE OIL POLLUTED SOIL USING BACTERIAL ISOLATES

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SEPTEMBER, 2023

ABSTRACT

Spent Engine Oil (SEO) is a harmful environmental contaminant, affecting soil, ground water and their microbial communities. This study was carried out to remediate SEO polluted soil using indigenous oil-degrading bacteria. Soil samples were collected from spent oil contaminated sections of various automobile workshops in Minna. Bacteria were enumerated from the soil using the spread plate technique, onto Nutrient agar and oil agar plates. The bacterial isolates were characterized based on colony morphology and standard biochemical tests. Gram staining and biochemical tests were carried out using morphological and biochemical techniques using the taxonomic scheme of Bergey's manual of determinative bacteriology. The bacterial isolates were screened for their potential to utilize SEO by growing them separately on Nutrient broth at 37°C for 24 h. The utilization of spent lubricating oil as the sole carbon source and energy by the selected bacterial isolates were determined using mineral salt medium (MSM). The best potential SEO degraders were used for the bioremediation process. Changes in microbial population of the respective experimental setup were determined every 2 weeks. Total petroleum hydrocarbon (TPH) and percentage degradation was determined by measuring absorbance at 272nm. Degradation percentage of SEO contaminated soil treated with the test organisms was determined as the difference between the initial and final TPH concentrations of bioremediated soil. The physicochemical properties of the soil samples (pH, moisture, crude nitrogen, organic carbon, phosphorus, particle size) were determined before and after remediation. The rate of bioremediation of SEO contaminated soil was determined based on total petroleum hydrocarbon (TPH) present in contaminated soil and gas chromatography-mass spectrometry (GC-MS) was used to determine the hydrocarbon compound present in the SEO before and after remediation. The total bacterial count of SEO polluted soil ranged from 7×10^3 to 17×10^3 CFU/g while count for potential oil degraders ranged from 1×10^4 to 3×10^6 CFU/g, various bacteria species including *L. casei*, *S. epidermidis*, *S. aureus*, *B. larvae, B. megaterium*, *B. subtilis* and *B. azotofamans* were identified in SEO polluted soil with *B. megaterium*, *B. azotofamans* and *S. epidermidis* been the best potential SEO degraders after screening. During the bioremediation process, total bacterial count was highest in the first week ranging from 2.60×10^7 to 4.00×10^7 CFU/g. Significant reduction ($P<0.05$) in bacterial count was observed in the second week (1.50×10^6 to 1.68×10^7 CFU/g). A steady increase was observed up till week six (2.20×10^7 to 3.20×10^7 CFU/g). Significant decrease in the TPH value was observed in all treatment groups during the course of this study as shown by the TPH values at week 0 (0.7, 2.80, 2.66 and 1.42mg/L) and at end of the experiment, week 6 (0.68, 1.53, 0.65 and 0.72mg/L). *Bacillus megaterium* (75.6%) recorded the highest level of degradation followed by *S epidermidis* (48.6%) and *B. azotoformans* (44.7%). There was no significant difference ($p>0.05$) between the pH of soil samples in all the treatments, but the pH of the treatment significantly differed ($p<0.05$) from the control. There was no significant difference ($p>0.05$) in the Organic carbon, Phosphorus and particle size of soil samples in all the treatments and their controls. The crude nitrogen content of contaminated soil treated with *B. azotoformans* (0.395) significantly differ ($p<0.05$) from other treatment *B. megaterium* (0.508) and *S. epidermidis* (0.512) and the control (0.512). The analysis of the physical properties of the soil samples showed that all the soil samples fall in the sandy loamy texture class due to their high sandy percentage (75.80 – 77.84%). Gas chromatography-Mass spectrometry results of bioremediated soil after six weeks showed a significant reduction in the intensity of the hydrocarbon peak, confirming the SEO biodegradation potential of the test isolates. The result of this study showed that these isolates, notably, *B. megaterium*, isolated from SEO was able to utilise the hydrocarbons in spent engine oil and can be developed for the remediation of soil contaminated with SEO.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Spent engine oil (SEO) is a frequent and harmful environmental contaminant that is not naturally found in the environment, but substantial amounts are released when motor oil is changed and disposed of in gutters, water drains, open unoccupied plots, and farmlands, which is a typical practice among technicians (Iren and Ediene, 2017). Crude oil and refined petroleum products can harm plants and soil bacteria, as well as contaminate ground water (Adeleye *et al.*, 2021). Spent engine oil has a significant negative impact on soil and soil microorganisms. Due to inadequate aeration, immobilization of soil nutrients, and decrease of soil pH, it produces an unfavorable environment for soil life (Obi *et al.*, 2016). It has been shown that marked changes occur in soil contaminated with hydrocarbons and these changes affect the physical, chemical and microbiological properties of the soil (Iren and Ediene, 2017).

Because petroleum hydrocarbon contamination of soil is now widely recognized as a potential health risk, attempts have been undertaken in recent years to develop effective, low-cost, and environmentally friendly soil remediation processes, such as bioremediation as well as phytoremediation (Akpabio *et al.*, 2017; Ajiboye *et al.*, 2020). Microorganisms break down petroleum hydrocarbons by converting them to less toxic molecules (e.g., alcohols, acids, carbon dioxide, and water) (Ani *et al.*, 2019). Because exploration and downstream utilization are linked to economic development, petroleum hydrocarbon is a major environmental problem around the world today (Umoren *et al.*, 2019). Automobile workshops in Nigeria are poorly managed, and they can be sources of frequent flow of wasted spent oil from the crank cases of vehicles and motorbikes, which

can be unattractive and pollute the environment. Clean-up of mechanic sites is still a challenge since the operators are often unaware of the negative impact on the environment. There is also a likelihood of percolation to ground water and a pint of engine oil is capable of contaminating 100,000 liters of ground water (Soretire *et al.,* 2017). However, the ability of resident microflora in the soil to digest petroleum hydrocarbons is extremely important, and when contaminants enter natural ecosystems, they interact with these organisms, resulting in a breakdown in a process known as biodegradation. Hydrocarbons, including polycyclic aromatic hydrocarbons (PAHs), have been long recognized as substrates supporting microbial growth. A wide range of hydrocarbon utilizers (HCUs) found to be useful in the soil include the following species, *Pseudomonas, Rhodococcus, Mycobacterium, Bacillus, Acinetobacter, Providencia, Flavobacter, Carynebacterium sp.* And *Streptococcus* (Tudararao-Aherobo and Mesogboriwon, 2020). Other organisms such as fungi are also capable of degrading the hydrocarbons in engine oil to a certain extent, but they take longer periods of time to grow when compared to their bacterial counterparts (Nkereuwem *et al.,* 2020).

Bioremediation is described as any technique that uses microorganisms, their enzymes, and metabolic processes to return the environment changed by xenobiotics to a fit-for-purpose state, employs biodegradation to achieve its goal. In-situ bioremediation involves treating the contaminated material at the site, while ex-situ involves the removal of the contaminated materials to be treated elsewhere. Riskuwa and Ijah (2016) opined that utilizing naturally occurring microorganisms rather than augmenting resident flora have several advantages. They claimed that when natural populations evolved over time, they adapted to make proliferation and survival in that location easier. Secondly, according to them, the ability to consume hydrocarbons is dispersed among a varied number of microbial populations that exist in natural ecosystems and may metabolize various

hydrocarbons either individually or in combination. Adebiyi (2021) however submitted that microbial diversity in certain ecosystems even though rich in certain species might be deficient in certain proven strains of microorganism capable of degradation of petroleum hydrocarbons. They have shown that introduction of microorganism to polluted land area increased the rate of remediation by as much as 50%.

Research on the isolation of bacteria associated with engine oil contaminated soil was carried out recently by Adeleye *et al*. (2019). They selected different mechanic sites in a town within the Northern part of Nigeria. They suggested that *Bacillus subtilis* and *Aspergillus niger* are most adapted to conditions present in soils contaminated with used engine oil and hence could be exploited in bioremediation activities.

1.2 Statement of the Research Problem

When auto mechanics service vehicles, they risk polluting the environment if they do not properly dispose of old engine oil. As more and more people buy cars in Nigeria, there has been a corresponding rise in demand for and production of engine oil (EO). As a result, the maintenance and repair of these cars results in enormous quantities of wasted engine oil (SEO). Most workers at Nigeria's car factories treat SEO like it is garbage and throw it out the window (Iren and Ediene, 2017). There is lack of effective regulation in the handling of used motor oil in Nigeria, which leads to its careless disposal into soil drains and, on occasion, open water. Because of the harm SEO causes to the natural world, immediate action is necessary. Land that has been polluted by used motor oil is not only unsightly, but also detrimental to plant growth and the survival of microorganisms. After being contaminated by SEO, ground water is no longer fit for human consumption. It causes a significant drop in fish population, bioaccumulation, and biomagnification in coastal waters by negatively impacting fish development, survival, and overall health.

Therefore, the goal of this research is to evaluate the efficacy of bacterial isolates at hydrocarbon remediation in spent engine oil polluted soils in Minna, Nigeria.

1.3 Aim and Objectives of the Study

The aim of this study was to bioremediate Spent Engine Oil (SEO) polluted soil using indigenous bacterial isolates.

The objectives of the study were to:

i. enumerate and isolate bacterial species from spent engine oil (SEO) polluted soil.

ii. screen the isolates for potentials to utilize SEO.

iii. determine the physicochemical properties of the spent engine oil contaminated soil before and after bioremediation.

iv. remediate spent lubricating oil contaminated soil with isolates that demonstrates high potential to utilize SEO.

v. determine the rate of bioremediation of SEO.

1.4 Justification for the Study

Bioremediation is a technology applied to clean up polluted environment using microorganisms. Its potential contribution as a countermeasure biotechnology for decontamination of polluted systems could be enormous as these microorganisms can play a crucial role in maintaining ecosystem and biosphere to develop sustainable environment (Varjani and Srivastava, 2015). Bioremediation is a preferred method for decontaminating oil polluted environs compared to other conventional methods of remediation, mainly due to it been simple and easy to maintain, its applicability over large areas and it results in complete removal of contaminants with minimal disruption to the ecosystem (Anthony, 2006). Bioremediation allows for natural organisms to degrade the

toxic hydrocarbons into simpler compounds which poses no threat to the environment, thus eliminating the need to remove and transport such toxic compounds (Kumar *et al.,* 2011). It is an environmentally friendly technique worthy of serious consideration in the context of the sustainability of the intervention of decontamination of environmental matrices, it has cheap operation costs, and cost effective compared to other technologies like chemical and physical processes which may generate additional contaminants in the catchment area of interest after treatment.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Spent Engine Oil

The environmental damage caused by spent-engine oil (SEO) is on its way to becoming more widespread than the damage caused by crude oil. After servicing vehicle engines, generating sets, and other types of engines (Ikhajiagbe and Ogwu, 2020; Isikhuemhen *et al*., 2003), repair shops that specialize in either mechanical automotive or electrical engine repair produce spent-engine oil, also known as used engine oil. This type of oil is sometimes referred to as recycled engine oil. It can range in color from dark brown to black and is typically detrimental to the health of the soil (Ismail *et al*., 2014). It is a common practice, particularly among motor mechanics in Nigeria, to dispose of SEO by throwing it into gutters, water drains, open vacant lands, and even into farms (Obi *et al.*, 2016). Used engine oil is known to be hazardous to both the health of humans and the organisms that live in the soil because it contains a variety of chemical compounds, some of which range from low to high molecular weight (C15-C21), as well as lubricants, additives, products of decomposition, and heavy metals. Some of these compounds have been found to be carcinogenic (Riskuwa and Ijah, 2016; Nkereuwem *et al.*, 2020).

In Nigeria, it is common for auto mechanics to not have a designated and appropriate method for disposing of spent engine oil that is used in their various workshops. This results in the oil being dumped into gutters, water drains, and soil (Iren and Ediene, 2017). Used lubricating oils that have been obtained after an engine has been serviced and then drained from an automobile or generator are what are referred to as “spent” engine oils. The percentage of aromatic and aliphatic hydrocarbons, nitrogen and sulphur compounds, and metals such as manganese, calcium, zinc, and lead that are present in spent oils is

significantly higher than that of fresh oils. Because the engine is subjected to wear and tear, these metals make their way into fresh oil (Komolafe *et al.*, 2021).

According to Nkereuwem *et al*. (2020), engine oil is the type of oil that primarily serves the purposes of lubricating the moving parts of motor engines, preventing corrosion in motor engines, cleaning the motor engines, cooling the engine by transporting heat away from the moving parts and improving sealing. The modern-day engine oils are a derivation of chemical compounds that are produced either with or without the use of petroleum. Because of this, most engine oils are made by blending hydrocarbon-based oils, such as mineral oil, which are organic compounds that only contain hydrogen and carbon. Blending is the primary method used to produce engine oils (Chukwura, *et al.*, 2016).

Used motor oil does significant damage to the soil and the microflora that live there. It creates conditions in the soil that are unfavourable for the existence of life as a result of poor aeration, immobilization of soil nutrients, and a reduction in soil pH (Musa *et al.*, 2021). It has been demonstrated that significant alterations in properties take place in soil that is contaminated with hydrocarbon. These contaminations have an impact on the soil's chemical, physical, and microbiological properties (Abioye *et al.*, 2021). Some of these heavy metals are essential micronutrients for plants at low concentrations, but at higher concentrations, they can cause metabolic disorders and growth inhibition in plants. The primary constituents that are characteristic of used engine oil are aliphatic and aromatic hydrocarbons such as phenol, naphthalene, benzo (a) anthracene, benzo (a) pyrene, and fluoranthene. Other components include fluoranthene (Jain *et al.*, 2011).

Depletion in the nutrient status (nitrogen and phosphorus), inhibition of microbial activities, and degradation of soil physical properties have been reported in spent-oil

contaminated soils. These effects can be traced back to the spent oil (Lee *et al*., 2007; Tudararao-Aherobo and Solomon, 2020). It has also been reported that the formation of waxy texture in soils contaminated with used lubricating oil contributes to reduce oxygen content in soils of this type (John and Okpokwasili, 2012; Jadhav *et al*., 2013). Oil spills on agricultural land typically result in a retardation of plant growth (Obire and Anyanwu, 2009; Abioye *et al*., 2021), in addition to a reduction in the population of soil microflora and fertility (Gkorezis *et al.*, 2016). Without bioremediation of the soil, oil pollution causes heavy metals to build up in the soil and then eventually move into plant tissues (Obuotor *et al*., 2016; Musa *et al*., 2021). This happens even in the absence of bioremediation of the soil.

Following an oil spill of any kind, the Polycyclic Aromatic Hydrocarbons (PAHs), which are a component of spent oil, are an important contaminant that are retained in the environment. PAHs have the potential to affect the endocrine system of animals and cause disruption (Tudararao-Aherobo and Solomon, 2020). In Nigeria, spent oil has polluted the soils that are used for agricultural purposes and has also affected the aquatic and marine plants and animals. The ground water has also been polluted, which has the effect of polluting the crops and farm animals (Umana *et al*., 2016). Because of this, there is a demand for the bioremediation of contaminated used engine oil (hydrocarbon). The remediation of contaminated areas traditionally makes use of processes involving the physical, the chemical, and the mechanical realms. Incineration, the manufacture of bricks, and skimmers are all examples of methods used in physical remediation. More than 10–15 percent of the oil that has been spilled cannot be biodegraded using this method (Agbor *et al*., 2015). The use of chemical surfactants as a remediating agent, on the other hand, is not recommended due to the fact that these substances have toxic effects on the local flora and fauna (Ani *et al.*, 2019). On the other hand, this kind of treatment

system requires heavy machinery, and the environmental consequences of this pollutant removal could result in a significant increase in the amount of air pollution (Bhupathiraju *et al.*, 2002).

In the process of hydrocarbon biodegradation, fungi and bacteria are both utilized (Snape, *et al.*, 2001). The filamentous fungi have a number of characteristics that make them suitable candidates for the role of degrading agents. A fungus quickly attaches itself to the substratum, and then it begins to digest the substratum by secreting extracellular enzymes (Ajiboye *et al.*, 2020). Fungal growth is not inhibited by environmental stresses such as low pH, poor nutrient levels, or low water activity. In comparison to other remediation methods, such as physical and chemical methods, the use of fungal bioremediation is an appealing choice because it is uncomplicated, easy to maintain, affordable, and can be manufactured in large quantities (Adeleye *et al.*, 2021). In addition to this, it has a low energy requirement and maintains the structure of the soil. *Aspergillus, Penicillium, Fusarium, Rhizopus, Alternaria*, and *Cladosporium* species have been discovered to be efficient hydrocarbon biodegrades, according to research conducted by Adeleye *et al.* (2019).

2.1.1 Effect of spent engine oil pollution in soils

Used motor oil that finds its way into the ground may be absorbable on the surfaces of mineral and organic soil constituents, fixed within the soil's pores and fissures, found in mobile form, or it may form a continuous cover on the soil's surface (Adebiyi, 2021). Certain soil parameters, such as pH value, cation exchange capacity, and redox properties, are impacted when land is contaminated with crude oil. These include the mineral and organic matter content of the soil. As a result of the hydrophobic nature of used engine oils, contaminated soils have a lower water holding capacity, moisture content, and

hydraulic conductivity than unpolluted soils do. This is because of the hydrophobic nature of used engine oils (Ujowundu *et al*., 2011). The presence of crude oil in the soil produces an anaerobic condition, which, when combined with water logging and acidic metabolites, leads to a high accumulation of aluminium and manganese ions, both of which are detrimental to the development of plants. The carbon that is found in oil products is responsible for the significant increase in the amount of organic carbon that is found in soil that is caused by oil pollution. As a result of the effects of oil pollution, the humus in the soil becomes richer in humic acids, but the degree to which the organic matter in the soil is humified decreases (Ajiboye *et al*., 2020). These shifts in the soil properties have a significant impact on the biotic components of the ecosystem, or they cause toxicity among them.

2.1.2 Toxicity of spent engine oil

The fundamental determinant of how dangerous a spill of petroleum hydrocarbons will be is the chemical composition of the polluting petroleum product. The solubility of alkanes and aromatic hydrocarbons in water is relatively low, with benzene exhibiting the highest level of solubility at 1780 mg/L (Adebiyi, 2021). Lipophilic compounds are substances that have a log octanol/water partition coefficient (KOW) value that falls between 2 and 6, and these are the substances that are considered to be the most biologically active in terms of toxicity and bioaccumulation (Riskuwa and Ijah, 2016) This category contains a large number of hydrocarbons; for example, the log KOW values for n-hexane and n-decane are 2.91 and 5.58, respectively. Because petroleum products are typically found in the form of complex mixtures of individual substances, the concentration of those individual components is a crucial factor in determining their impact on the environment. N-Alkanes, for example, have a lower toxicity level and a shorter half-life than aromatic compounds. In particular, polycyclic aromatic

hydrocarbons, or PAHs, such as benzo(a)pyrene, have been shown to be carcinogenic and have been linked to a wide variety of issues affecting human health as well as diseases affecting aquatic organisms (Tudararao-Aherobo and Solomon, 2020).

2.1.2.1 Toxicity to plants

The pollution caused by used engine oil results in a stunting of plant growth as well as a reduction in crop production. The rate of seed germination in soil that is contaminated with petroleum or a derivative of petroleum has been reported by many authors to be lower. For instance, Soretire *et al*. (2017) tested numerous plant species, including grasses, legumes, herbs, and commercial crops, to determine whether or not they were able to germinate in diesel fuel contaminated soil at concentrations of 25 and 50 g/kg. The authors observed delayed and decreased seed germination and suggested that this may be due to a volatile component (light hydrocarbons) that may be phytotoxic and capable of entering easily through the plant cell walls. This may be due to the fact that the authors observed light hydrocarbons. Additionally, used engine oils have the potential to form a film on the seed, which would prevent oxygen and water from reaching the seed. Oyedele *et al.* (2016) investigated the effect of 25, 50, 75, and 100 g/kg of spent oil on the seed germination, shoot height, and biomass of six species of herbaceous plants. These species included one species of the Fabaceae family (*Medicago truncatular*), four species of the Gramineae family (*Bromous mermis, Secal seral, Triticum sativa, and Agropyron deserterum*), and (*Linum ussitasimum*). All of the species' responses to the contaminated soils were found to be dose-dependent, as was reported by the authors. *Linum ussitasimum*, *A. deserterum, B. mermis* showed only 16.2, 15, and 2.7 percent germination, respectively, whereas *M. truncatular* showed 63.5 percent germination.

Every plant species experienced a decrease in seedling height as well as a decrease in biomass. In a similar manner, Umoren *et al.* (2019) investigated the effects of 1, 2, 3, 4, and 5 percent diesel contamination on four crop plants: *Arachis hypogaea, Sorghum bicolor, Zea mays,* and *Vigna unguiculata*. They found that all of the test plants were able to tolerate diesel fuel contamination at levels ranging from 1 to 3 percent, as shown by the fact that seed germination ranged from 89 to 33 percent. In the presence of 1 percent diesel, it was reported that 89 percent of the seeds of *Z. mays* and *S. bicolor* germinated, whereas only 77 and 68 percent of the seeds germinated in *A. hypogaea and V. unguiculata* respectively. The researchers found that as the levels of contamination increased in the four different test plants, there was a corresponding decrease in the number of seeds that successfully germinated. There was a complete blockade of seed germination in both S. *bicolor* and *V. unguiculata* when the contaminated diesel was at a concentration of 4 percent. Wheat (*Triticum aestivum L.),* barley (*Hordeum vulgare L.*), alfalfa (*Medicago sativa L.*), and clover were the four species that were studied by Umana *et al*. (2017) in their investigation of the effect of 2 ml/kg (v/w) of a mixture of gasoline and diesel fuel on seed germination and seedling growth characteristics (*Trifolium resupinatum L.*). The authors reported that the stress caused by the mixture of gasoline and diesel fuel caused a delay in the beginning of the germination process. After 5, 8, 11, and 14 days, they observed inhibition of germination with a decrease of 70.92 %, 53.19 %, 25.01 %, and 8.46 % in wheat; 61.58 %, 31.78 %, 8.48 %, and 4.18 % in barley; 85.97 %, 35.84 %, 7.68 %, and 2.15 % in clover; and 91.76 %, 56.68 %, 37.55 %, and 26.26 % in alfa.

The germination of perennial ryegrass was another topic of investigation for Al-Hawash *et al.* (2018), who investigated the impact of diesel concentrations of 0, 0.64, 1.6, 4.0, and 13.6 percent (w/w). The amount of diesel that was added to the soil determined how long

it took for perennial ryegrass to germinate, which ranged from 11 to 30 days. The decomposition of n-C10 and n-C11 was found to be linked to the process. Diesel with a weight-to-weight concentration of 13.6% was found to delay the germination of seeds by up to 24 weeks.

Cowpea (*Vigna unguiculata*) seedlings were used in the study by Hoang *et al*., (2022) to investigate plant-derived saponin enhances biodegradation of petroleum hydrocarbons in the rhizosphere of native wild plants. The authors reported that 0.25 %, 0.5 %, 1.0 %, and 2.0 % crude oil induced stress in the seedlings. This stress was apparent from the dose-dependent increase in free sugar, total protein, and amino acids, as well as a decrease in the chlorophyll contents of the leaves of the seedlings. They also observed an inhibition of starch phosphorylase and amylase, both of which influence the process of starch assimilation.

Diesel fuel has the potential to either acutely or chronically harm the plants. Several studies have shown that oil pollution inhibits plant growth in a variety of ways, including a lower germination rate, a shorter root length, a shorter shoot length, a lower plant weight, and so on. For instance, Romera (2017) demonstrated the effect of 0.2, 0.4, 0.6, 0.8, and 1 percent (v/w) of gasoline fuel/diesel mixture on the germination of seeds, survival of seedlings, and growth of *Vigna unguiculata* over a period of 10-38 days. They found that the mixture had no effect on the growth of *Vigna unguiculata*. When there was a higher concentration of petroleum products, there was a discernible decline in the number of seedlings that survived. At each concentration of gasoline fuel and diesel, the researchers found that a plant's percent t seed germination, dry weight, leaf area, shoot and root lengths were all shorter than normal. In a similar vein, Chaudhary and Kim (2019) investigated the effects of diesel on the viability of plant seeds from four different

species: *Atriplex halimus, Cochorus olitorius, Hordeum spontaneum*, and *Triticum aestivum*. After ten days, the researchers found a decrease in the germination rate of *C. olitorius* seeds of 57.7 and 76.9 percent respectively when the diesel concentration was 100 and 5000 mg/kg. While the seeds of *H. spontaneum, T. aestivum*, and *A. halimus* showed a decline of less than 30 percent when exposed to 100 mg/kg of diesel, these same seeds showed a decline of 11.1, 20, and 29.4 percent when exposed to 5000 mg/kg of diesel, respectively. The authors also observed a significant decrease (> 50 percent) in both the length and weight of *C. olitorius* sprouts when exposed to 5000 mg/kg diesel. This is in contrast to the most resistant *T. aestivum* sprouts, which showed a decrease of less than 15 percent in both the length and fresh weight of the sprouts.

Additionally, Ehiosun *et al.* (2022) investigated the impact of diesel pollution levels of 2, 4, 6, 8, and 10 percent, as well as nutrient amendments, on the growth characteristics of eggplant plants (*Solanum melongena*). Ehiosun *et al.*, (2022) found that the negative effects of diesel pollution on plant growth parameters varied depending on the concentration of the pollution. It was possible to reverse the damaging effects of diesel oil pollution by applying nutrient amendments to the soil, such as cow dung, poultry and pig waste, as well as inorganic fertilizer. *Zea mays* and *Arachis hypogaea* were used in the experiment by Daccò *et al.* (2020) to investigate the effect of 1, 2, 3, 4, and 5 percent of spent and unspent diesel. They found that spent diesel had a seed germination rate of 40 percent for *Z. mays* and 22 percent for *A. hypogaea*, while unspent diesel had a seed germination rate of only 10 percent for Z. mays and 0.5 percent for *A. hypogaea* at a level of contamination of 5 percent. In addition, the authors found that the growth of radicles and plumules was inhibited by 75 percent in *Z. mays* and 80-83.4 percent in *A. hypogaea*, respectively, when diesel was present at a concentration of 5 percent.

2.1.2.2 Toxicity to microorganisms

According to research by Ekhause and Nkwell (2011), n-alkanes are known to be toxic to microorganisms. It was demonstrated by Dong *et al.* (2019) that crude oil and refined oil are both toxic to natural bacterial populations isolated from pristine sediments, with refined oil being the more toxic of the two. It was discovered that the volatile aromatic compounds found in crude oils, such as a-pinene, limonene, camphene, and isobornyl acetate, are toxic to the microorganisms (Emilio *et al.*, 2021). Even for bacteria that were able to survive in the presence of dissolved aromatic hydrocarbons like naphthalene, researchers observed an increase in the lag phase and a decrease in the growth rate (Zhou *et al*., 2019). In *Saccharomyces cerevisiae*, the toxic effects of cyclohexane that were reported by Chaudhary and Kim (2019) included inhibition of oxygen uptake in intact cells and isolated mitochondria, impairment of ATP synthesis and potassium uptake, and dissipation of the mitochondrial membrane potential. All of these effects were caused by cyclohexane. The mechanism of cyclic hydrocarbons' toxic effect on membranes was analysed in detail by Zhang *et al.* (2016). The build-up of these compounds in the membranes of microorganisms has a significant impact on the structural and functional properties of the membranes. The insertion of hydrocarbons changes the structure of membranes by causing fluidity and protein conformations to shift. This causes a disruption in the functions of the barrier and the energy transduction channels, while also affecting the activity of membrane-bound and embedded enzymes (Sharuddin *et al.*, 2021).

2.3 Treatment of Contaminated Soil

Due to the toxic effects that petroleum Hydrocarbon (PHC) has on a variety of biotic components, the contaminated soils need to be cleaned up as soon as possible. For the remediation of PHC-tainted soils, there is a wide variety of treatment options available,

including chemical, biological, and physical approaches. These techniques can be utilized to restore a level of microbial diversity that is very close to the original (Akpabio *et al.*, 2017).

2.3.1 Physical methods

Landfilling, capping and containing, and incineration are the three disposal methods that are used most frequently. A landfill is a carefully engineered pit that is dug in the ground where contaminated soil from the actual site is excavated and placed, followed by being covered with soil and spread evenly in layers. This pit is known as a landfill. By using this method, it is expected to segregate and contain the hazardous waste, as well as prevent the contamination of ground water and surface water. In the method known as "cap and contain," the contaminated soil is treated right there on the site. The contaminated site is covered with a cap, much like a landfill, and it is monitored on a regular basis to ensure that the contamination is decreasing. Incineration is a method of waste disposal that involves the burning of potentially harmful materials. These materials are burned in incinerators, which produces ash, gas, heat, and steam (Kumar *et al.,* 2011).

2.3.2 Chemical methods

Ozonation and surfactant washing are the two chemical processes that are utilized most frequently for the removal of PHC. For instance, Daccò *et al.* (2020) demonstrated in situ flushing for the remediation of soil that was contaminated with 55,156 g/kg SEO by using alternate periods of water and water/surfactant. At the end of the period of six weeks, the authors reported that the removal efficiency of SEO was 98 percent. Molecular ozone or the products of its decomposition, such as hydroxyl radicals, can react with organic compounds and change them into oxidized products. These oxidized products are more water-soluble, less toxic, and/or more bioavailable than the parental compounds. For

instance, Tian *et al.* (2022) investigated the microbial mechanisms of refractory organics degradation in old landfill leachate by a combined process of UASB-A/O-USSB. After 900 minutes of ozonation, the authors reported that 50 percent of the SEO had been removed. They also noticed that the native microbes that were treated were very sensitive to the ozone, and as a result, they saw a decline in the population of microbes as the ozonation time increased. The conventional physical and chemical remediation approaches will result in enormously high costs for the clean-up of contaminated sites (Rahmati *et al.*, 2022). These methods have not been successfully implemented, in part because there is a lack of public acceptance and in part because of the complexity of the technology (Daccò, 2020; Rahmati *et al.*, 2022). In addition, for these methods, the potential dangers to the environment and the impact they could have on the variety of soil are major sources of concern. Therefore, alternative methods such as biological methods are required in order to restore polluted sites in a manner that is risk-free, friendly to the environment, less expensive, and labour intensive than the current approach.

2.3.3 Biological methods

2.3.3.1 Phytoremediation/Rhizoremediation

The use of plants for the in-situ remediation of contaminated soil, sludge, sediment, and groundwater is referred to as phytoremediation. This can be accomplished through any one of the following mechanisms: extraction, filtration, stabilization, degradation, or evapotranspiration. The rhizoremediation potential of a variety of plants with extensive fibrous root systems, including common grasses, corn, wheat, soybeans, peas, and beans, was investigated by studying a number of these plants (Zand *et al*., 2016). Bioremediating soil polluted with 20,000 mg/kg gasoline and diesel compounds to a depth of 3 meters, several trees belonging to the family Salicaceae, including poplar and willow, were planted. These trees have the ability to grow quickly and have deep rooting capabilities

(Nwankwegu *et al.*, 2022). However, the authors found that willows could not be used for phytoremediation at high concentrations, such as greater than 5000 mg of hydrocarbons per kilogram, because of the toxic effects that would result. Therefore, the use of willows for bioremediation in and of itself was restricted, localized, and reserved for areas with low levels of hydrocarbon contamination.

The technique of phytoremediation was utilized by Nooraldeen *et al.* (2018) in order to treat soil that was contaminated with PAHs. Within a period of six months, it was discovered that three different plant species, namely alfalfa (*Medicago sativa*), switch grass (*Panicum virgatum*), and little bluestem grass (*Schizachyrium scoparium*), could remediate 72 % of the total PAH. Phytoremediation of two to four ring alkylated PAHs in crude oil-contaminated soil was investigated by Umana *et al.* (2016). The researchers used treatment systems that involved a combination of fescue (*Lolium arundinaceum*), ryegrass (*Lolium multiflorum L.*), or bermudagrass (*Cynodon dactylon L.*). The authors reported a pattern of degradation that went from 2-ring to 3-ring to 4-ring, with the rate of degradation decreasing with increasing alkylation of larger ringed structures. In addition to this, they found that the addition of plants and fertilizer sped up the degradation process. Al-Hawash *et al*. (2018) observed that *Vigna unguiculata* had the ability to remediate soil that had been polluted with 5 % (v/w) crude oil by reducing the total hydrocarbon contents by 54 % within two months. This was accompanied by an improvement in the growth and yield of the cassava crop that was grown in the phyto-remediated soil.

Following the completion of phytoremediation, the plants will be able to be harvested, processed, and eventually discarded. In most cases, the plants have an effect on the microbial community of the rhizosphere, which is important for rhizoremediation

(Sharuddin *et al.*, 2021). On the other hand, numerous plant species are said to be capable of producing enzymes like cytochrome P450 and peroxidase, which are both essential for the metabolism of n-alkanes. Mikolasch *et al.* (2019) demonstrated that the cell cultures of *Cinchona robusta and Dioscorea composita* had the ability to take up and metabolize n-hexadecane, which resulted in the formation of hexadecanol and cis-13-Octadecenoic acid. In addition to this, they found that the levels of cytochrome P450 and peroxidase were increased, which lends credence to the notion that these enzymes play a role in the biotransformation of n-hexadecane. Broad bean (*Vicia faba*), maize (*Zea mays*), and wheat (*Triticum aestivum*) plants were studied by Adebiyi (2021) to determine whether or not they had the potential to stimulate the microbial degradation of soil pollutants in desert soil that was contaminated with 2.2-2.3 % crude petroleum oil. In comparison, the rhizosphere soil of *Z. mays* and *T. aestivum* showed a reduction of 16.8 and 13.7 % of SEO, respectively, while the rhizosphere soil of *V. faba* showed a reduction of 30 % of SEO. This indicates that *V. faba* is more effective at degrading PHCs than either *Z. mays* or *T. aestivum*. Mikolasch *et al.* (2019) also reported that the rhizosphere of *V. faba* was able to reduce 5.3 % of the hardly degradable resins fraction along with 47.0 % of the saturated and 26.2 % of the aromatics fractions as compared to 37.4 % and 8.2 % for Z. mays and 33.2 % and 3.9 % for *T. aestivum* rhizospheres respectively. These % ages compare favourably to those of *Z. mays* and *T. aesti.*

The surface area and the depth that the roots occupy are the only areas that can be cleaned up by phytoremediation. Second, there is an increase in the amount of time needed because the plants involved have a low biomass and grow slowly. In plant-based systems for the remediation of pollution, it is impossible to prevent the seepage of contaminants into the groundwater (Jadhav *et al.*, 2013).

2.3.3.2 Bioremediation using microorganisms

The term "microbial bioremediation" refers to treatment processes that use microorganisms such as bacteria, fungi, yeast, or their enzymes to break down potentially harmful substances into substances that are less toxic or nontoxic, thereby restoring the site that was contaminated (Zhang *et al*., 2022). Bioremediation can be accomplished through the use of microorganisms in a variety of ways, including land farming, composting, biopiling, slurry bioreactors, natural attenuation, bioventing, biosparging, biostimulation, and bioaugmentation to name a few (Zhou *et al.*, 2019). In the following sections, various aspects of the use of microorganisms in the cleaning up of PHC-contaminated soils will be discussed.

2.4 Different Microbial Bioremediation Techniques

The subsequent treatment, which may consist of land farming, composting, biopiling, or slurry bioreactors, comes after the removal of contaminated soils from excavation sites. This step comes immediately after the removal of the contaminated soils. Land farming entails the spreading of excavated contaminated soils in a thin layer on the ground surface of a treatment site and stimulating aerobic microbial activity within the soils by means of aeration and/or the addition of nutrients, minerals, and water/moisture. This is done so that the soils can be used as a growing medium for vegetation. This is done in order to prepare the soils for agricultural use, which is why it is done. Land farming was reported by Emilio *et al.* (2021) to have been effective in cleaning up diesel-contaminated soils in the Canadian Arctic. The authors demonstrated that the addition of fertilizer led to a 90 % increase in the amount of bioremediation achieved with 2800 mg/kg SEO. They also demonstrated an important reduction in hydrocarbon content of 80 % due to aeration brought on by rototilling over the course of three years, with rototilling taking place once every four days. Zhang *et al.* (2022) reported in their study on the bioremediation of

petroleum waste sludge in landfarming sites an enhanced degradation of petroleum hydrocarbons (PHC), in particular n-alkanes, with a removal efficiency of between 75 and 100 percent after 14 months. Their research focused on the bioremediation of petroleum waste sludge.

2.5 Microorganisms Involved in SEO Bioremediation

In their metabolic processes, a wide variety of microorganisms, such as bacteria, fungi, and microalgae, are able to use petroleum hydrocarbons as their one and only source of energy. This is the case because petroleum hydrocarbons are readily available to them. According to Vysotskaya *et al*. (2019), research on bacteria that were responsible for the dissipation of oil slicks on water that had been conducted and published in 2013 by Söhngen had opened up a new field in the fight against oil pollution. Sohngen's research (2013) had been on bacteria that were responsible for the dissipation of oil slicks on water. It is interesting to note that the work was initially started with the intention of determining the potential pathogenicity of bacteria. At some point in time, the application of these organisms in the process of bioremediation became the primary focus of attention. There is a wide variety of bacterial genera, each of which is recognized for its own unique capacity to degrade hydrocarbons in an *efficient manner. The overwhelming majority of them are members of bacterial species such as Aeromonas, Alcaligenes, Acinetobacter, Arthobacter, Bacillus, Brevibacterium, Flavobacterium, Geobacillus, Micrococcus, Mycobacterium, Ochrobactrum, Pseudomonas, Rhodococcus, Sphingomonas, Thermus*, and *Xanthomonas*, among others (Statsenko and Blinokhvatov, 2019; Vysotskaya *et al*., 2019; Yerima *et al.*, 2020).

Rahman *et al.* (2002) utilized both individual strains of *Micrococcus* sp. GS2-22, *Corynebacterium* sp. GS5-66, *Flavobacterium* sp. DS6-86, and *Pseudomaonas* sp. DS10-

129 in their degradation studies, in addition to their consortium. They found that the bacterial consortium was able to degrade 78% of 1% crude oil, which was a higher %age than the individual cultures were able to achieve individually. This indicates that the consortium is more effective than the individual cultures. Zand *et al*. (2016) discovered and described two new species of bacteria: *Microbacterium oleivorans* sp. Nov. and *Microbacterium hydrocarbonoxydans* sp. These two types of bacteria are capable of degrading crude oil. *Bacillus* sp. SV9, *Acinetobacter* sp. SV4, and *Pseudomonas* sp., SV17 were isolated from contaminated soil in Ankleshwar, India (21°60' N 73°00' E), and they were tested for their ability to degrade a complex mixture of petroleum hydrocarbons (including alkanes, aromatics, resins, and asphaltenes), sediments, heavy metals, and oil.

Zand *et al*. (2016) isolated a psychrotrophic *Rhodococcus* species from oil-contaminated ground water. Within just 20 days, this particular species of *Rhodococcus* was able to degrade a wide variety of petroleum hydrocarbons by almost 90 %, including crude oil, diesel oil, and gasoline. In addition, this *Rhodococcus* species was able to thrive in a medium containing 7 % NaCl and maintained a temperature of 4 degrees Celsius. Researchers Polyak *et al*. (2020) discovered that two strains of *R. wratislaviensis* and *R. aetherivorans* were capable of breaking down more than 15 petroleum compounds from a mixture of benzene, toluene, ethylbenzene, m-xylene, p-xylene, o-xylene, octane, hexadecane, 2,2,4-trimethylo. The co-culture was responsible for the complete degradation of the compounds. An intriguing discovery was made regarding the compound's MTBE, ETBE, isooctane, cyclohexane, and 2-EHN, which were resistant to the capabilities of these strains to degrade them. Sambuu *et al*. (2019) conducted an investigation into the hydrocarbon degrading potential of a *Proteus vulgaris* strain that was isolated from fish samples. Research on the utilization of kerosene, diesel oil, jet fuel,

and engine oil as carbon sources by autotrophic nitrifying bacteria, *Nitrosomonas* and *Nitrobacter* species, was carried out by Nuhu and Salisu (2022). They discovered that a mixed culture of the isolates was able to degrade 52 % of the crude oil, followed by *Nitrosomonas sp*., which degraded 40 %, and *Nitrobacter sp*., which degraded 20 %. It was also discovered by Chukwura *et al*. (2016) that white-rot fungi such as *Pleurotus tuberregium, Pleurotus ostreatus, Bjerkandera adusta, Irpex lacteus,* and *Lentinus tigrinus* degrading polyaromatic hydrocarbons. These fungi are known as white-rot. These fungi are responsible for the breakdown of polyaromatic hydrocarbons (Isikhuemhen *et al.*, 2003; Schwartz *et al.*, 2012). Additional research on the capability of *P. tuberregium* to bioremediate soils that had been contaminated by crude oil was carried out by Isikhuemhen *et al.* (2003). Additionally, they made use of it to facilitate the germination of *Vigna unguiculata* seeds as well as the growth of seedlings. Research was carried out by Obire and Anyanwu (2009) to determine the effect that varying concentrations of crude oil had on the microbial communities that are found in soil. Some of the fungi that were found included *Alternaria, Aspergillus, Candida, Cephalosporium, Cladosporium, Fusarium, Geotrichum, Mucor, Penicillium, Rhizopus, Rhodotolura, Saccharomyces, Torulopsis*, and *Trichoderma* species. The authors reported that as the concentration of crude oil increased, there was a decrease in the order of occurrence of a wide variety of fungal genera within both total fungi and petroleum-utilizing fungi. This phenomenon is referred to as fungal diversity. They demonstrated that higher concentrations of crude oil have a negative impact on fungal diversity while simultaneously increasing the population of a smaller number of fungi. More specifically, they increased the population of *Aspergillus* sp., *Cladosporium* sp., *Mucor* sp., *Penicillium* sp., *Trichoderma* sp., and *Candida* sp.

Particularly well represented among the yeast species that have been described in the scientific literature as having the ability to use hydrocarbons as a source of energy are the genera *Candida, Clavispora, Debaryomyces, Leucosporidium, Lodderomyces, Metschnikowia, Pichia, Rhodosporidium, Rhotorula, Sporidiobolus, Sporobolomyces, Stephanoascus,* and *Trichosporon*, (Obuotor *et al.*, 2016). *Candida digboiensis* was isolated by Hoang (2022) from soil samples that were contaminated with acidic oily sludge (pH 1–3). This *Candida* strain had the ability to degrade 73% of the total petroleum hydrocarbons present in the medium in just one week when the pH of the medium was set to 3.

The diesel-degrading yeasts *Rhodotorula aurantiaca* and *Candida ernobii* were investigated by Ehiosun *et al.* (2022) to determine whether or not they had the potential to be utilized in the production of biofuel. After a period of twenty days, they reported that *C. ernobii* had completely degraded the octadecane, tetradecane, and 5-methyl-octane. They were able to achieve a degradation rate of 93% for decane, 38.4% for nonane, and 22.9% for dodecane from diesel with a concentration of 12% using *R. aurantiaca*.

2.5.1 Appraisal of literatures on bioremediation of spent engine oil using bacteria isolate

The research conducted by Adeleye *et al.* (2021) investigated the bioenhancement of bacteria that were isolated from polluted spent engine oil (SEO) in order to facilitate the removal of total petroleum hydrocarbons (TPH) and polyaromatic hydrocarbons (PAHs) from spent engine oil (SEO) contaminated soil. Before the addition of sterilized biostimulants such as powdered cow dung (CD), powdered cocoa pod husk (CPH), and compost, sterilized soil was contaminated with three different levels of SEO. This was done before the soil was given sterilized biostimulants (made from fresh CPH and CD).

Polyethylene bags containing the mixture had a bacterial inoculum of *Staphylococcus aureus* and *Bacillus areus* coculture added to them. The volume of the addition was 150 milliliters. This was a factorial experiment that had a completely randomized design laid out for it (CRD). The levels of TPH and PAHs were measured on the first day, the fifth week, and the tenth week of the room's incubation process, respectively. The influence of biostimulants on the TPH and PAHs degradation potential of the bacterial co-culture produced the results that showed an increase in the degradation of the hydrocarbon contents that was statistically significant (p 0.05). When compared to the other biostimulants, compost resulted in the greatest enhancement of TPH reductions (315 and 380 mg kg–1) at the tenth week. The contamination levels tested were 5 % and 15 % SEO, respectively. When compared to other biostimulants, compost led to significantly greater PAHs reductions (48.8, 39.6, and 94.6 mg kg–1) on a 5 %, 10 %, and 15 % SEO contamination level respectively. When compared to the soil samples that had been bioaugmented and biostimulated, the quantity of SEO contents that had been degraded was noticeably higher in the bioaugmented and biostimulated soil samples. The technology that was used in this investigation has the potential to be put to use in an efficient manner for the bioremediation of pollution caused by petroleum hydrocarbons (Umana *et al*., 2017).

Ajiboye *et al.* (2020) also carried out research on the bioremediation of spent engine oil on selected contaminated soils in the Ilorin Metropolis area for their study. The purpose of this study was to investigate the bioremediation of used engine oil on a few different contaminated soils located within the metropolitan area of Ilorin. In order to accomplish this goal, soil samples were gathered from three different mechanic shops located along the Taiwo axis in the metropolitan area. After that, the soil samples were put through a bioremediation process that utilized the land-farming method. Standard procedures were

used to conduct an analysis on the physicochemical parameters of soil samples taken both before and after the bioremediation process. The bacteria were isolated by following the appropriate protocols and then identified by applying both biochemical and genetic techniques. Particle size (all sandy in nature), pH (6.00 0.14 – 6.20 0.14), organic carbon (14.65 3.20 – 17.54 1.87), organic matter (33.50 0.85 – 43.45 9.12), and heavy metals (ND – 11.74 0.07) are some of the results for the physicochemical parameters of the soil samples before bioremediation. Following the application of bioremediation, the pH, organic carbon, organic matter, and heavy metal concentrations reached the following values: 8.25 0.07 – 8.90 0.14; 13.07 0.05 – 13.25 0.84; 37.25 1.06 – 44.80 1.13; ND – 9.40 0.04. The values for the bacterial count were as follows: 8.00 1.41 – 67.50 2.12 x 105 CFU/g before bioremediation of the soil samples, and 6.50 2.12 – 164.00 11.31 x 105 CFU/g after bioremediation of the soil samples, respectively. Bacterial isolates were identified as *Pseudomonas* sp., *Enterobacter* sp., *Acinetobacter* sp., and *Bacillus* sp., while the bacteria that utilize hydrocarbons were identified as *Thalassospira 26esophile* strain JCM 18969; *Pseudomonas fluorescens* F113; *Siccibacter turicensis* LMG 23730; *Pseudomonas.* Thus, the spent engine oil contaminated soils were effectively bioremediated by the bacteria isolates, resulting in a reduction of the amount of hydrocarbon pollutants.

In addition to this study, Obuotor *et al.* (2016) carried out research on the topic of enhancing the biodegradation of spent engine oil contaminated soil by using organic wastes. In comparison to the biodegradation method, the physical and chemical methods of cleaning up contaminated soil are significantly less friendly to the environment. Within the scope of this study, Oyedele *et al*. (2016) investigated the potential of various organic wastes to speed up the biodegradation of soil contaminated with spent engine oil (SEO). A total of seven treatments, each with two replicates, were conducted using one kilogram

of uncontaminated soil that was thoroughly mixed with ten % (w/v) of SEO. Spent Fruit Residues (SFR), Cassava Peel (CP), and a combination of Bean Husk and *Chromolaena odorata* (BHC) were added at 10 % and 20 % (w/w), and an untreated control was included in the study as well. Standard microbiological methods were used to determine the Total Heterotrophic Bacterial Count (THBC), Total Fungal Count (TFC), Total Hydrocarbon Degrading Bacterial Count (THDBC), and Total Hydrocarbon Degrading Fungal Count (THDFC) of the contaminated and uncontaminated soils. Using a 2,6-dichlorophenol indophenol indicator, the potential for SEO utilization in the isolated organisms was investigated. Gas chromatography-mass spectrometry was utilized in order to ascertain the levels of hydrocarbons present in the soils (GC-MS). A statistical analysis was performed on the information that was gathered. The THBC contained anywhere from 1.3x106 to 2.9x106 CFU g-1, the TFC contained anywhere from 5.4x104 to 2.0x105 CFU g-1, the THDBC contained anywhere from 0.5x103 to 1.9x104 CFU g-1, and the THDFC contained anywhere from 2.0x103 to 1.0x104 CFU g-1. The bacteria that were isolated included *Pseudomonas* spp., *Bacillus* spp., *Klebsiella* spp., *Proteus mirabilis, Burkholderia cepacia, Micrococcus luteus, Providencia rettgeri, Enterococcus faecalis, Streptococcus bovis*, and *Enterobacter cloacae*. The fungi that were isolated included Candida with an absorbance of 0.26 and 0.49 at 600 nm, respectively, *Pseudomonas aeruginosa* and *Aspergillus niger* utilized the oil more effectively than the other isolates. According to the results of the GC-MS analysis, the SFR 20 % (w/w) treatment caused the greatest amount of %age degradation (70.5 %). This study demonstrates that the residues of spent fruit can speed up the biodegradation of soil that is contaminated with spent engine oil (Chaillan *et al*., 2004).

Ismail *et al.* (2014) conducted research on the biodegradation of spent engine oil (SEO) by bacteria isolated from the rhizospheres of *Cajan cajan* and *Lablab purpureus* plants.

In phytoremediation studies, this was done with the intention of identifying the species of bacteria that were the most effective at breaking down SEO. Enrichment culture was used to isolate and identify bacteria capable of degrading hydrocarbons. The medium used was oil agar that had been supplemented with 0.1 % by volume of SEO. The total heterotrophic and oil-utilizing bacterial count revealed the presence of a significant number of bacteria, the majority of which were found in the rhizosphere soil. These counts varied between 54 108 and 144 108 CFU/g and between 4 108 and 96 108 CFU/g, respectively. The %age of oil-utilizing bacteria ranged from 0% (in uncontaminated soil without rhizosphere) to 76% (in oil-contaminated soil with rhizosphere) (contaminated rhizosphere). Using turbidimetry, it was determined that five different bacterial species grew to their full potential and degraded the oil at rates of 68 %, 62 %, 59 %, 58 %, and 45 %, respectively. These species are *Pseudomonas putrefacience* CR33, *Klebsiella pneumonia* CR23, *Pseudomonas alcaligenes* LR14, *Klebsiella aerogenes* CR21 Chromatographic analysis carried out with GC-MS revealed the presence of hydrocarbons with a lower molecular weight in the residual oil after 21 days, indicating that the oil had degraded. In contrast, the undegraded oil (the control) had hydrocarbons with a higher molecular weight after the same amount of time. Because it was determined that the isolated species had a high capacity for SEO biodegradation, these organisms have the potential to be useful tools in the process of remediating SEO-contaminated soil (Saeed *et al*., 2022).

2.6 Factors Affecting Bioremediation

The process of bioremediation is influenced by a wide variety of factors, each of which needs to be managed, controlled, and improved. The presence of a microbial population that is capable of degrading a contaminant is one of the most important factors. Another is the accessibility of the contaminant to the microorganisms. Environmental factors such

as the type of soil, temperature, pH, moisture, nutrients, and oxygen are also important (Nooraldeen, 2018).

However, in the case of oil pollution, the composition of the petroleum hydrocarbon pollutant and its inherent biodegradability is the first and most important parameter. In general, the susceptibility drops down the list as follows: n-alkanes > branched alkanes > low-MW aromatics > cyclic alkanes. It has been demonstrated that saturates have the highest rates of biodegradation, followed by light aromatics, while high-molecular-weight aromatics and polar compounds have extremely low rates of biodegradation (Hazaimeha *et al.*, 2019). Chemical structure, degree of substitution, solubility, viscosity, and concentration of various components were the properties of petroleum hydrocarbon products that had an effect on their biodegradation (Jain *et al.*, 2011). For instance, Grigoriadi *et al*. (2019) found that the simple aromatic hydrocarbons (naphthalene and 2-methylnaphthalene) degraded more quickly than the n-alkanes did.

Temperature is one of the physical factors that plays a significant part because it has an effect on the solubility of PHC and the physiology of the microbial flora. For instance, Gkorezis *et al*. (2016) observed that at low temperatures, the viscosity of the oil increased, while the volatility of the toxic low molecular weight hydrocarbons decreased. This resulted in a delay in the beginning of the process of biodegradation. In order for the biodegradation of hydrocarbon pollutants to be successful, certain nutrients, like nitrogen, phosphorus, and in some cases iron, are essential ingredients. As a result, the addition of nutrients is going to be required in many different situations in order to boost the biodegradation of oil pollutants. On the other hand, excessive nutrient concentrations can also inhibit the activity of biodegradation. Oxygenases are responsible for the first steps in the breakdown of PHC by bacteria and fungi (Tian *et al.*, 2022). These steps involve

the oxidation of the substrate, which necessitates the presence of molecular oxygen. Hence, aerobic conditions are essential for PHC degradation (Garcia *et al*., 2017).

In order to achieve a higher level of PHC degradation, one of the most important steps is for the local microbial community to produce and release biosurfactants. These biosurfactants contribute to the formation of emulsions, which in turn increases the bioavailability of the substance for the microorganisms to consume. As a result, the vast majority of bacteria that are capable of efficiently degrading crude oil also possess significant emulsifying activity. The moisture content of the soils has a direct impact on the growth and metabolism of living things, and the optimal rates of PHC biodegradation can be seen in soils with moisture contents ranging from 30 to 90 % water saturation (Garcia *et al*., 2017).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study area was automobile workshops Mechanic garage opposite House of Assembly Quarters (OPP), Keteran Gwari mechanic garage (KG), and Unguwan Daji (UD), within Minna metropolis, Niger State, Nigeria. Minna is situated on latitude of $9^o37^'$ North and longitude of $6^o33^'$ East. Minna is a city in the Middle Belt of Nigeria. It is the capital of Niger State, one of Nigeria's 36 Federal States (Figure 3.1). It consists of two major ethnic groups: the Gbagyi and the Nupe. Minna has a typical Middle Belt tropical savanna climate with two seasons: an arid, dusty, harmattan-dominated dry season from November to April and a humid, oppressive wet season dominated by monsoonal air masses from May to October. Temperatures are hot to sweltering year-round, except at the height of the wet season when air temperatures are merely very warm but the high humidity makes it equally or more uncomfortable than the hotter dry season (Yerima *et al*., 2020).

3.2 Collection of Samples

Soil samples were collected from spent oil contaminated sections of various automobile workshops (Opposite House of Assembly Quarters, Keteran Gwari mechanic garage and Unguwan Daji) in Minna. The samples were collected with a soil auger at a depth of 0 – 15 cm. Sample were collected in sterile polythene bags, properly packaged and transported to the Microbiology laboratory at the Department of Microbiology, Federal University of Technology, Minna for analysis.

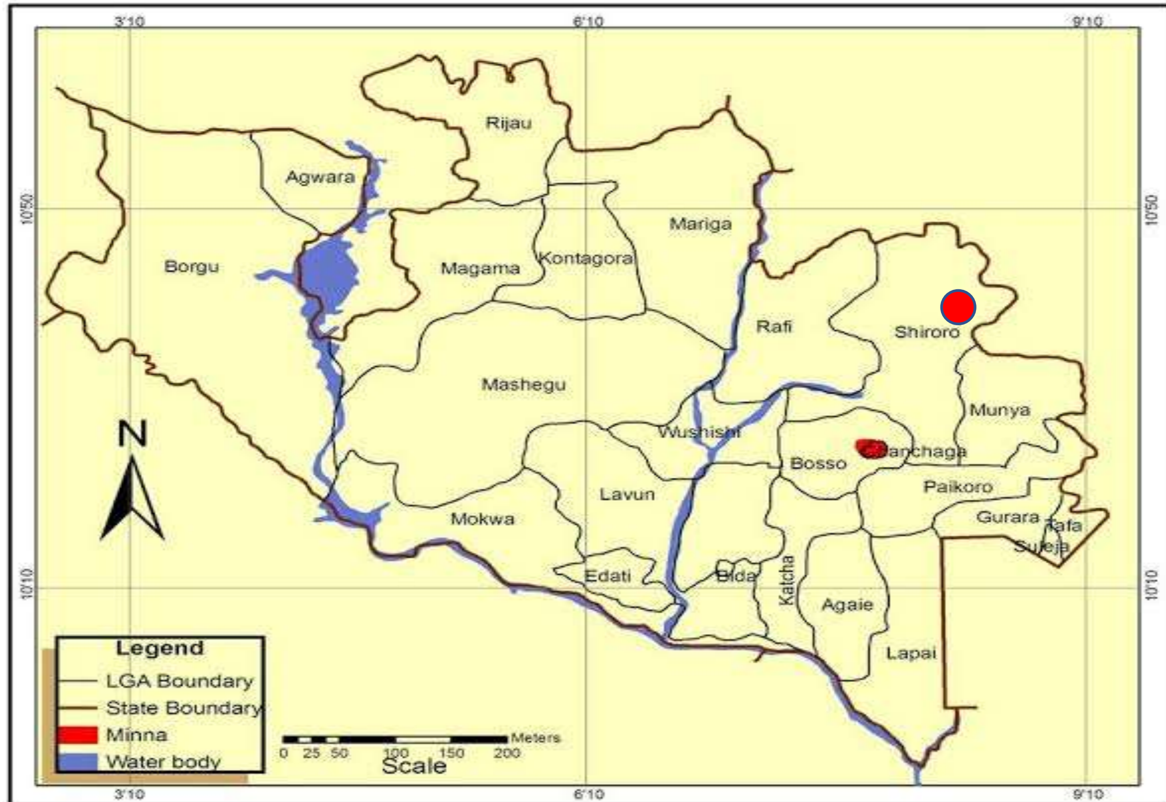

Figure 3.1: Map of Niger State showing Minna as the Study Area.

Source: Department of Urban and Regional Planning, Federal University of Technology, Minna.

3.3 Enumeration of Bacteria

Bacteria were enumerated using the spread plate technique, 0.1ml of serially diluted samples was aseptically inoculated onto Nutrient Agar (NA) and Oil Agar (OA) plates. Oil Agar was composed of 1.2g KH_2PO_4, 1.8g K_2HPO_4 4.0g NH_4Cl, 0.2g $MgSO_4.7H_2O$, 0.1g NaCl, 0.01g $FeSO_4.7H_2O$ and 20g agar per litre and supplemented with 0.1% SEO. The pH was adjusted to 7.4. The Nutrient agar plates were incubated at $35\pm2^{\circ}C$ for 24-48 hours while the OA plates were incubated at $30\pm2^{0}C$ for 5-7 days, as described by Ijah *et al.* (2008). After incubation

bacterial colonies were enumerated and the result was expressed in colony forming units per gram of soil (CFU/g). The bacterial isolates were repeatedly sub-cultured onto fresh agar plates to obtain pure cultures which were maintained on agar slants for further identification and characterization (Cheesbrough, 2014).

3.4 Characterization and Identification of Bacteria

The bacterial isolates were characterized based on colony morphology and standard biochemical tests. Gram staining and biochemical tests were carried out using morphological and biochemical techniques using the taxonomic scheme of Bergey's manual of determinative bacteriology, Holt *et al.* (1994) and Cheesbrough (2014).

3.4.1 Gram staining

A thin smear of each of the pure 24-hour old culture were prepared on clean grease-free slides, fixed by passing over gentle flame. Each heat-fixed smear was stained by addition of 2 drops of crystal violet solution for 60 seconds and rinsed with water. The smear was flooded with Lugol's iodine for 60 seconds and rinsed with water, decolourized rapidly with 70% alcohol and rinsed with distilled water. It was counter stained with 2 drops of Safranin for 30 seconds and finally rinsed with water, then allowed to air dry. The smears were mounted on a microscope and observed under oil immersion objective lens. Gram-positive bacteria appeared blue or purple under gram staining while gram-negative bacteria would appear red or pink under gram staining (Cheesbrough, 2014).

3.4.2 Motility test

A sterile needle was used to pick a loop of the organism and stabbed onto Nutrient Agar in glass vials. The vials were incubated at 37ºC for 24 h. Non-motile bacteria showed growth

confined to the stab line with definite margins without spreading to surrounding area while motile bacteria will show diffused growth extending from the surface (Cheesbrough, 2014).

3.4.3 Catalase test

A small quantity of culture was transferred into a drop of 3% Hydrogen peroxide solution on a clean slide with the aid of sterile inoculating loop. Production of gas bubbles indicates the presence of catalase enzyme (Cheesbrough, 2014).

3.4.4 Methyl red test

Five millimetres of glucose phosphate broth (1g glucose, 0.5% KH_2PO_4, 0.5% peptone and 100 mL distilled water) were dispensed in clean test tubes and sterilized by autoclaving at 121°C for 15 minutes. The tubes were inoculated with the test organisms and incubated at 37°C for 48h At the end of incubation, few drops of methyl red solution were added to each test tube and colour change were observed, for positive test were indicated by bright red colour while negative test were indicted by yellow colour (Iren and Ediene, 2017).

3.4.5 Voges-proskaeur test

Five millimetre of glucose phosphate broth (1g glucose, 0.5% KH_2PO_4, 0.5% peptone and 100ml distilled water) were dispensed in clean test tubes and sterilized by autoclaving at 121°C for 15 minutes. The tubes were inoculated with the test organisms and incubated at 37°C for 48 hours after incubation, 6% "α-napthtol and 6% Sodium hydroxide were added to about 1ml of the broth culture. A strong red colouration forming within 30 minutes indicates positive reaction (Musa *et al.,* 2021).

3.4.6 Indole test

Tryptone broth (5 ml) were placed into different test tubes after which a loopful of the bacterial isolates were inoculated into the test tubes, leaving one of the test tubes uninoculated to serve as control. The test tubes were incubated at 37°C for 48 h. After incubation, 0.5 ml of Kovac's reagent were added and shaken gently; it was allowed to stand for 20 minutes to permit the reagent to rise. A red or red-violet colour at the top surface of the tube indicates a positive result while yellow colouration indicates a negative result (Cheesbrough, 2014).

3.4.7 Citrate utilization test

The test organism was lightly inoculated on the Simmon's citrate agar slant and was incubated at 35^{0}C for 18 hours. (Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium). The development of blue colour, denoting alkalization were observed. For positive result, growth was visible on the slant surface and the medium were an intense Prussian blue. Trace or no growth were visible and no colour change occurred for negative result (Cheesbrough, 2014).

3.4.8 Starch hydrolysis

Nutrient agar was prepared with 1% soluble starch and sterilized by autoclaving at 121°C for 15 minutes. The medium was poured into sterile plates and allowed to solidify at room temperature (37°C) the plates were inoculated by streaking the organisms once across the medium. The plates were incubated at 37°C for 24 hours after which they were flooded with Gram's iodine. Hydrolysed starch appears as a clear zone due to alpha amylase activity while reddish brown zones around the colony indicates partial hydrolysis of starch to dextrans (Cheesbrough, 2014).

3.4.9 Oxidase test

This test was used to differentiate *Pseudomonas sp*. from other Gram-negative enteric bacteria. Few drops of the enzyme indo-phenol Oxidase were added onto the colonies of the test organisms, Oxidase positive colonies quickly became dark – purple within 10 seconds (Cheesbrough, 2014).

3.4.10 Coagulase test

The tube coagulase test was carried out. Nutrient broth weighed 0.5mL were dispensed into test tubes and the test organisms inoculated into the broth. The cultures were incubated at 37°C for 30 minutes. Then 0.5 mL of blood plasma were added and incubated at 37°C for 4 hours and observed for signs of coagulation at 1 and 4 hours interval. For a positive result, the blood plasma was converted into a shift gel or soft gel, best observed by tilting the tube to the horizontal position, while negative result showed no coagulation (Cheesbrough, 2014).

3.4.11 Urease test

This was done to determine the ability of the isolated organism to produce the enzyme, urease for the decomposition of urea. The colonies were inoculated heavily over the entire surface of the urea medium and incubated at 37^{0}C. Observation were made at interval of 24 hours, 48 hours and after overnight incubation. In urease positive cultures, the colour of the medium changed from dark brown to red or purple, while white colour was observed in urease negative cultures (Holt *et al.,* 1994; Cheesbrough, 2014).

3.5 Screening of bacterial isolates for potentials to utilize spent engine oil

Selected bacterial isolates were grown separately in nutrient broth at 37^{0}C for 24 hours. The utilization of spent lubricating oil as the sole carbon source and energy by the selected

bacterial isolates were determined using the mineral salt medium (MSM) of Zajic and Supplisson (1972). Five millilitres (5ml) of mineral salts medium were dispensed in each test tubes containing 0.05 ml of spent lubricating oil. After sterilization at 121^oC for 15 minutes, the test tubes were allowed to cool before being inoculated with 0.1 ml of Nutrient broth grown bacterial isolates. The test tubes were incubated at room temperature (28±2^0C) for 10 days according to the methods of Musa *et al*. (2021). The growth of the organism in the oil medium at the end of incubation were determined by the use of a spectrophotometer (MODEL 752) to examine the degree of turbidity of the oil medium. The tube with the highest turbidity was regarded to have the highest potential to bioremediate Spent Engine Oil (SEO).

3.6 Remediation of Spent Engine Oil Polluted Soil

3.6.1 Experimental setup

Two kilograms (2 kg) of moistened soil were poured in clay pots in duplicates. Treatment one: Only unpolluted soil (for control), Treatment two: Soil + 5.0 % (w/w) SEO, Treatment three: Soil + 5.0 % (w/v) SEO + Bacterial isolates. Each treatment was carried out in triplicate and was observed for six (6) weeks. The experiment was conducted at the Biological garden of the Federal University of Technology, Minna. This setup was a completely randomized design (CRD). The bioremediation setup is as shown in Table 3.1.

Table 3.1: Bioremediation Setup

Design	Treatment
1	Sterilized soil + SEO + Sodium azide
2	2Kg soil + 5.0% (w/w) SEO
3	2Kg soil + 5.0% (w/w) SEO + Bacterial isolates

Key: SEO = Spent Engine Oil

3.6.2 Microbial counts and identification

Changes in microbial population of the respective experimental setup were determined by spread inoculating 0.1 mL of serially diluted sample onto Nutrient agar plates (NA) for the enumeration of total heterotrophic bacteria, and incubated at 30°C for 24 hours. Spent Engine oil degrading bacteria (SEO) were enumerated on spent engine oil agar (SEOA). The spent engine oil plates were incubated at 30°C for 5 days. The colonies that developed after incubation were counted and expressed as colony forming units per gram (CFU/g) of soil sample.

3.6.3 Determination of total petroleum hydrocarbon (TPH) and percentage degradation

Total petroleum hydrocarbon (TPH) were determined spectrophotometrically based on the method of Ibrahim (2016). Ten (10) grams of each soil sample was weighed into a 250 mL capacity conical flask and 50 mL of diethyl ether was added, shaken vigorously for 30

minutes in an orbital shaker. The diethyl ether extracts were filtered with Whatman No. 1 filter paper, the liquid phase of the extract was collected, and the TPH contents were determined by measuring the absorbance at 272 nm using a spectrophotometer (Model, 752). A standard curve was prepared using known concentrations of SEO (0.5–5 mg/L). Accordingly, the relationship between the SEO concentration and the absorbance using Equation 3.1:

$$Y = 1.0641X \quad (3.1)$$

Where Y is the measured absorbance of sample and X is the concentration of SEO in the sample.

Degradation percentage was determined as the difference between the initial and final TPH concentrations using Equation 3.2:

$$Degradation\ (\%) = \left[\frac{O_i - O_r}{O_i}\right] X\ 100 \quad (3.2)$$

where O_i is the initial SEO concentration (mg/L) and O_r is the residual SEO concentration.

3.7 Physicochemical Analysis of the Soil Samples

Physicochemical analysis refers to things that involve the principles of both physics and chemistry, meaning that they are dependent on, or produced by, the combined actions of physical and chemical attributes (Vysotskya *et al.*, 2019). The parameters that were analysed we re: pH, moisture content, organic carbon content, Nitrogen, particle size and textural class, phosphorus, potassium.

3.7.1 Determination of pH

The pH of the soil samples was determined by the potentiometric method. Ten grams of each sample were introduced into a 50 mL capacity beaker after which 20 millilitres of 0.01 M calcium chloride solution was added and stirred. The sample was allowed to stand for 30 minutes with occasional stirring so that the sediments could settle. The glass electrode of Testronic digital pH meter (Model 511) were immersed into the suspension to determine the pH reading of the sample. The test was repeated for each sample and the average of the duplicate samples were recorded (Abioye *et al.*, 2021).

3.7.2 Determination of moisture content

Moisture content of the soil samples were determined using the gravimetric methods described by Black (1965) and Agbenin (1995). The moisture-cans were weighed using an electronic weighing balance. The cans and the samples were weighed and transferred to a hot spot conventional oven Genlab - MIN0150. The samples were dried in the oven at 105°C for 5 hours. The samples were transferred to a desiccator and allowed to cool. The weights of the oven-dried samples were obtained using the electronic balance and the percentage moisture content calculated using Equation 3.3.

$$\%Moisture = \frac{B-C}{B-A} \, x \, 100 \qquad (3.3)$$

Where:

A= Weight of moisture can (g)

B=Weight of can + Wet sample (g)

C=Weight of can + Oven-dried sample (g)

3.7.3 Determination of organic carbon content

This was carried out using the method of Agbenin (1995) in which 1 g of each (0.5 mm) sieved soil sample were weighed in duplicates and transferred to a 250 ml capacity Erlenmeyer flask. Ten (10) millilitres of 1M $K_2Cr_2O_7$ solution were introduced into each flask and swirled gently. Twenty (20) milliliters of concentrated H_2SO_4 were added rapidly using an automatic pipette, directing the stream into the suspension. The flask was immediately swirled gently until the sample and reagents were mixed, then swirled more vigorously for one minute. The flask was rotated again and allowed to stand on a sheet of asbestos for 30 minutes after which 100 ml of distilled water were added. Three to four drops of the indicator (Barium-diphenylamine-Sulphonate) were added and then titrated with 0.5 M ferrous sulphate solution. As the end point were approached, the solution was taken on a greenish cast and then change to dark green. At this point, the ferrous sulphate was added drop by drop until the colour changed sharply from blue to red (maroon colour) in reflected light against a white background. The blank was prepared in the same manner but without the sample to standardize the dichromate. The percentage carbon was calculated using Equation 3.4

$$\% \text{ Organic carbon in soil } = \frac{(me\ K2Cr2O7 - me\ FeSO4) \times 0.003 \times\ 100 \times (f)\ (air-dry\ basis)}{1g\ of\ air\ dry\ soil} \quad (3.4)$$

Where:

f (Correction factor) = 1.33

me = Molarity of solution x cm^3 of solution used

% Organic matter in soil = % Organic Carbon × 1.729 (International Institute for Tropical Agriculture (IITA), 1979).

3.7.4 Determination of total nitrogen

In carrying out this experiment, the Macro-Kjeldahl method described by Black (1965) and Agbenin (1995) were used. Five (5) grams of each soil sample were weighed and moistened with a small amount of water into a Kjeldahl flask. Forty (40) millilitres of concentrated H_2SO_4 and three Kjeldahl tablets were added and the mixture were heated at 150°C for 2 hours and at 390°C for 4 hours. After the digestion, the mixture was cooled, filtered and made up to 100 cm^3 with distilled water. A 10ml aliquot of the filtrate were introduced into the reaction flask and 10 millilitres of 10 M NaOH solution were added. The solution inlet of the apparatus was corked and steam distilled. The distillate was collected in a 50 millilitres capacity conical flask containing 5 millilitres of boric acid (4%) with two drops of mixed indicator (0.02g methyl red mixed with 0.1g bromocresol green and 43.8 millilitres of ethanol and 16.2 millilitres of distilled water). Moistened red litmus paper were used to determine the presence or absence of NH_3 coming directly from the condenser. The distillate was titrated with standardized 0.1M HCl. The total nitrogen was calculated using Equation 3.5

$$\%\text{Nitrogen} = \frac{(Volume\ of\ HCl\ cm3sample - Volume\ of\ HClcm3\ blank) \times 0.14\ x\ df}{Volume\ of\ aliquot \times Weight\ of\ sample} \quad (3.5)$$

Where:

HCl = Volume of Hydrochloric acid in cm^3

df = Dilution factor.

3.7.5 Determination of particle size and textural class of soil

In carrying out this experiment, the hydrometer method described by IITA (1979) were used. Fifty (50) grams of oven dried soil sample, which were passed through a 2 mm sieve were introduced into a milkshake mix cup and 50 cm^3 of 5% sodium hexa-metaphosphate were added along with 100 cm^3 of distilled water. Stirring rod were accustomed to mix the sample, this were allowed for 30 minutes. The contents of the cylinder were thoroughly mixed by inversion several times until all soil were in suspension. The cylinder was positioned on a flat surface area and time observed. Immediately, the hydrometer was introduced into the soil suspension slowly till the hydrometer were floating. The first reading on the hydrometer were taken 40 seconds after the cylinder were set down. The hydrometer was removed and temperature of the suspension recorded.

After the first hydrometer reading, the suspension was allowed to stand for 3 hours before a second reading was considered. In addition, the temperature from the suspension were considered. According to IITA (1979), the first reading measures the percentage of silt and clay in suspension. The second reading indicates the percentage of total clay in the suspension. Results were corrected to a temperature of 20°C. In addition, 2.0 were subtracted from every hydrometer reading to compensate for added dispensing agent. The percentage weight of sand, silt and clay for the soils were calculated using equation 5 (IITA, 1979). After the values for silt and clay have been determined, the value for sand were obtained by subtracting the values of silt and clay from 100. The soil was classified using the textural triangle using Equation 3.6

$$C = R - R_L + 0.36T \times \frac{100}{50} \tag{3.6}$$

Where:

C = Corrected hydrometer reading

R = Reading of the sample

R_L = Reading of the blank

T = Change in temperature (^oC)

3.7.6 Determination of available phosphorus

For this determination, the Bray No. 1 method of Bray and Kurtz (1945) were used. One gram of each air-dried soil sample that was passed through a 2 mm sieve were weighed into a 15 milliliters centrifuge tube and 7 cm^3 of 1 M NH_4F and 25 milliliters of 0.5 M HCl to 460 milliliters distilled water (extracting solution). The mixture was shaken for 1 minute on a mechanical shaker and the suspension centrifuged at 2000 rpm for 15 minutes. Two milliliters of the clear filtrate was introduced into a 20 cm^3 test tube, 5 cm^3 of distilled water and 2 cm^3 of ammonium molybdate solution was added. The content was mixed properly and 1 cm^3 of $SnCl_2 .2H_20$ dilute solution was added and mixed again. After 5 minutes, the percentage transmittance was measured on the spectrophotometer at 660 nm wavelength. A standard curve within the range of 0-1μg P/ml (or ppm P) was prepared. The optical density (OD) of the standard solution was plotted against the μg P/ml (or ppm P) and the content of extractable phosphorus in the soil was calculated using equation 6 (Bray and Kurtz, 1945) using Equation 3.7

$$\text{ppm } P = \frac{Off\ curve\ reading \times Dilution\ factor \times Volume\ of\ extract}{Initial\ weight\ of\ soil} \tag{3.7}$$

3.7.7 Determination of potassium

Potassium was determined according to the methods of IITA (1979) and Agbenin (1995). To five grams of each sample, 30 cm^3 of 1 M NH_4OAc was added and shaken on a mechanical shaker for 2 hours. It was centrifuged at 9000 rpm for 10 minutes and the clear supernatant was carefully decanted into a 100 cm^3 volumetric flask. Another 30 cm^3 of NH_4OAc solution was added and shaken for 30 minutes. It was centrifuged at 9000 rpm for 10 minutes and the supernatant was transferred into the same volumetric flask. This was repeated and the supernatant were transferred into the same volumetric flask. It was made up to the 1 litre mark with the NH_4OAc solution. The potassium and sodium were determined on a flame photometer (Jenway PFP-7) after calibration with sodium and potassium standards.

3.8 Gas Chromatography- Mass Spectroscopy (GC-MS)

A microliter of the leftover oil sample was analysed using the gas chromatography mass spectrometry (GC/MS) QP-2010 in scan mode. The GC was equipped with a cross-linked 5% phenyl methyl siloxane capillary column with the designation QP-5MS and used helium as the carrier gas. The temperature programme started at 60 °C and climbed by 10 °C every minute until it reached 200 °C, where it remained for 8 minutes.

3.9 Data Analysis

The samples were assayed and analysed in triplicates and data generated from SPSS (Version 20) were reported as Mean± Standard Error. One-way analysis of variance (ANOVA) and Fisher's Least Square Difference (LSD) were used to determine significant difference within and between groups, considering a level of significance of less than 5% ($P<0.05$)

CHAPTER FOUR

4.0 RESULTS AND DISCUSION

4.1 Results

4.1.1 Enumeration and identification of bacteria from SEO polluted soil

The bacterial count of soil samples is shown in Tables 4.1 and 4.2. The highest total bacterial count was observed in soil samples collected from mechanic workshop in Site C (17×10^3 CFU/g) followed by Site A (7×10^3) and Site B (1×10^3). Similarly, the count of bacterial isolates plated on oil agar showed that soil samples collected from Site C (3×10^6 CFU/g) had the highest count followed by Site A (1×10^4) while no potential hydrocarbon degraders were observed in site B. Various bacterial species were identified from the SEO polluted soil. Table 4.3 shows the biochemical characteristics of isolated bacterial species. Species identified were *Lactobacillus casei*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *B. larvae, Bacillus megaterium*, *Bacillus subtilis* and *Bacillus azotofamans*.

Table 4.1 Total Bacterial Count of Soil Samples on Nutrient Agar

S/N	Locations	Bacterial colony count (CFU/g)		
		Plate 1	**Plate 2**	**Average**
1	A	4×10^3	10×10^3	7×10^3
2	B	1×10^3	1×10^3	1×10^3
3	C	20×10^3	13×10^3	17×10^3

Keys: A: Ungwan Daji; B: Ketaren Gwari: C: Opposite Assembly Quarters

Table 4.2 Bacterial Count of Soil Samples on Oil Agar

		Bacterial Colony Count (CFU/g)		
S/N	**Locations**	**Plate 1**	**Plate 2**	**Average**
1	A	-	1×10^4	1×10^4
2	B	-	-	-
3	C	3×10^6	2×10^6	3×10^6

Keys: A: Ungwan Daji; B: Ketaren Gwari; C: Opposite Assembly Quarters; -: No growth

Table 4.3 Biochemical Characteristics of Bacterial Isolates

Isolates code	Gram Reaction	Starch	Catalase	Citrates	Sucrose	coagulate	Glucose fermentation	Growth on MSA	Suspected organisms
UD_3(NA)	Gram positive rod	+	+	+	+	-	-	Grow and appears yellow change MSA colour	*Lactobacillus casei*
UD_2(NA)	Gram positive cocci	+	+	+	+	+	-		*S. auerus*
UD_1(NA)	Gram positive long rod	+	-	+	-	-	-	Grow yellow and change colour of MSA	*Lactobacillus casei*
OPP_2(NA)	Gram positive rod in chains	+	+	-	+	-	+		*B. larvae*
KG_2 (NA)	Gram positive cocci in clusters	-	+	+	+	-	-	Grow but does not change media	*Staphylococcus epidermidis*
KG_3 (NA)	Gram positive rod in chains	+	-	-	+	-	+		*B. megaterium*
OPP_5(NA)	Gram positive short rod chains	+	+	+	+	-	+	Grow yellow and change colour of MSA	*B. larvae*
OPP_3(NA)	Gram positive rod in chains	+	+	+	+	-	+		*Bacillus megaterium*
OPP_1(NA)	Gram positive rod in chains	+	+	+	-	-	+	Grow and appears yellow and change colour of MSA	*B. azotoformans*

KG_1 (NA)	Gram positive short rods	-	+	-	+	-	+	Grow yellow and change colour of MSA	*B. subtilis*
OPP_6(NA)	Gram positive long rods in chains	+	+	+	+	-	+	Grow yellow and change colour of MSA	*L. casei*
OPP_4(NA)	Gram positive rod in chains	+	+	+	+	-	+	Grow yellow and change colour of MSA	*L. casei*

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4.1.2 Total bacterial count during bioremediation of SEO contaminated soil

The bacterial count of SEO contaminated soil from week 0 to week 6 is shown in Figure 4.1. The count was at its highest in the initial week of contamination 3.00×10^7, 4.00×10^7, 2.60×10^7 and 3.20×10^7 CFU/g for the Control, *B. azotoformans*, *B. megaterium* and *S. epidermidis* treated soil respectively. Significant reduction in bacterial count was observed in the second week (1.50×10^6, 1.28×10^7, 1.68×10^7 and 1.40×10^7 CFU/g). A steady increase in bacterial count was observed up till week six (3.20×10^7, 2.20×10^7, 3.10×10^7 and 2.40×10^7 CFU/g).

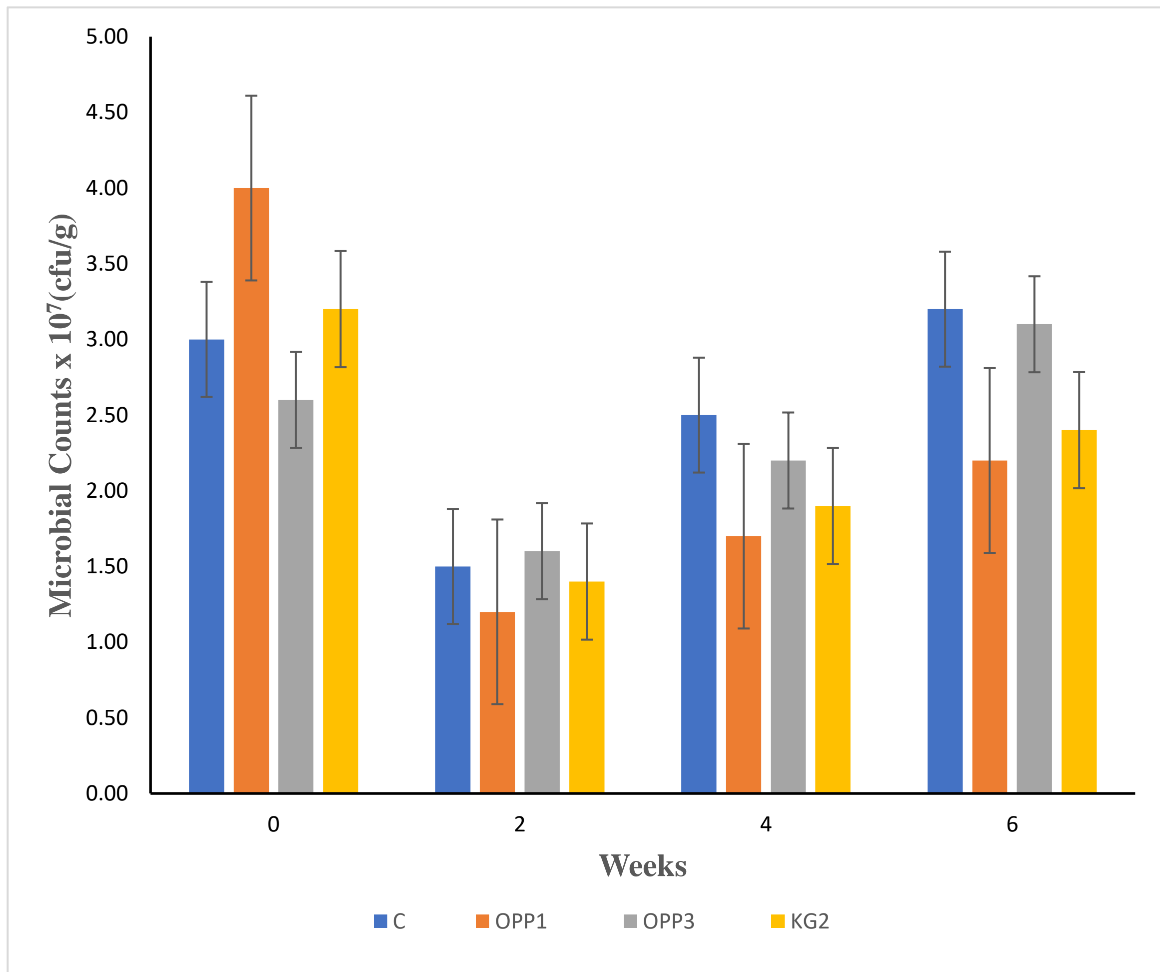

Figure 4.1 Microbial Count of Contaminated Soil

4.1.3 Total Petroleum Hydrocarbon (TPH) and percentage degradation of SEO contaminated soil

The rate of bioremediation of SEO contaminated soil was determined based on total petroleum hydrocarbon (TPH) present in the contaminated soil. Significant decrease in the TPH value of the soil was observed during the course of this experiment. The Total Petroleum Hydrocarbon of the soil from week 0 to week six is shown in Figure 4.2. Significant reduction in the TPH value was observed in all treatment groups during the course of this study. The initial TPH values (week 0) were 0.7, 2.80, 2.66 and 1.42mg/L for the Control, *B. azotoformans*, *B. megaterium* and *S. epidermidis* treated soil respectively and at end of the experiment (week 6) the recorded TPH values were 0.68, 1.53, 0.65 and 0.72mg/L. Degradation percentage of SEO contaminated soil treated with various test organisms was determined as the difference between the initial and final TPH concentrations of bioremediated soil. The percentage degradation of bioremediated SEO contaminated soil after six weeks is shown in Table 4.4. SEO contaminated soil treated with *B. megaterium* (75.6%) recorded the highest level of degradation followed by *S epidermidis* (48.6%) and *B. azotoformans* (44.7%).

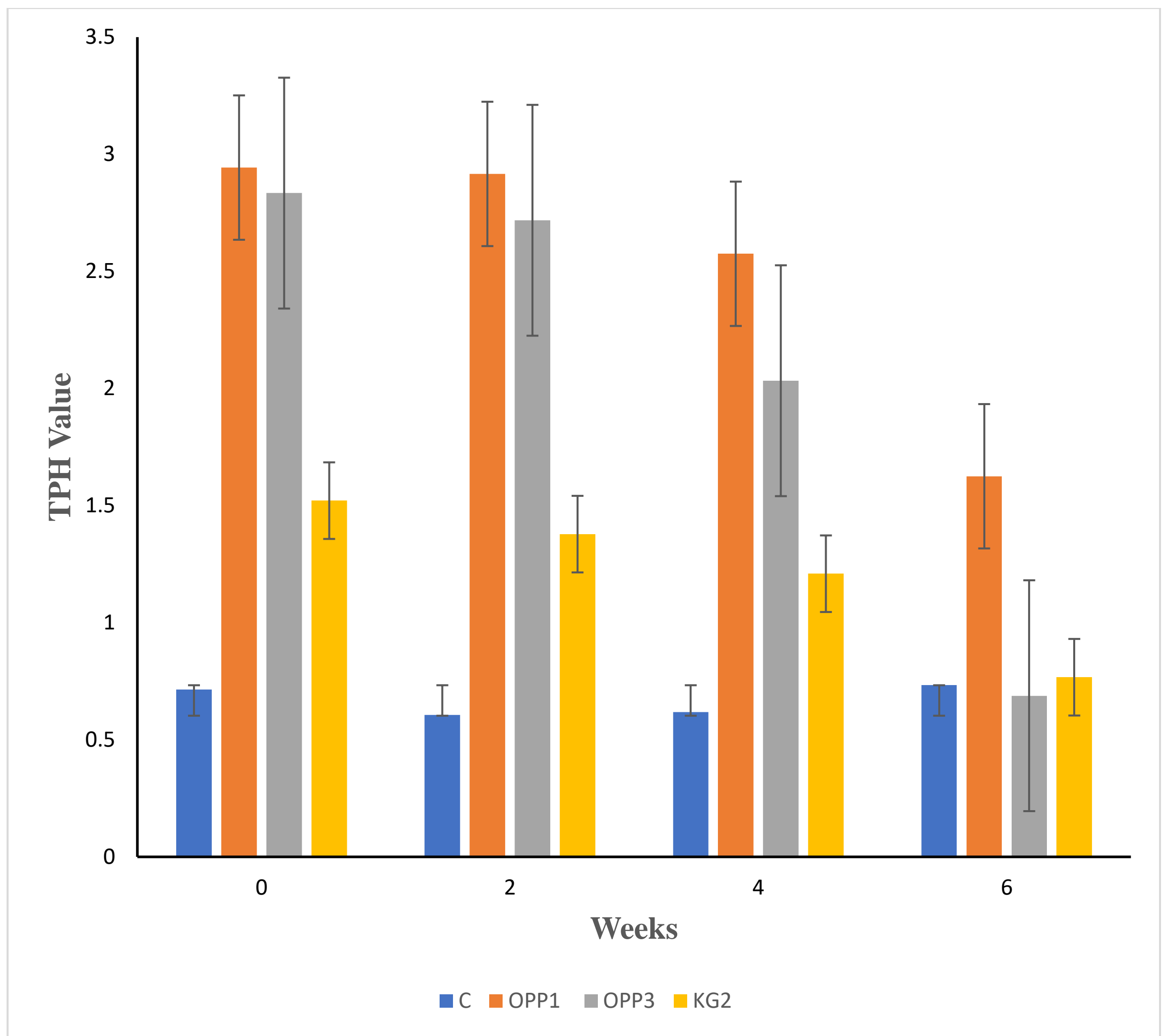

Figure 4.2 Total Petroleum Hydrocarbon in Soil

Table 4.4: Percentage Degradation of SEO Contaminated Soil by Test Organisms After Six Weeks Bioremediation

Sample	Degradation (%)
Control	2.86
Opp1 *(B. azotoformans)*	44.7
Opp3 *(B. megaterium)*	75.6
KG2 *(S epidermidis)*	48.6

4.1.4 Physical and chemical Properties of the spent engine oil contaminated soil

The chemical properties of the spent engine oil contaminated soils are shown in Table 4.5. Spent engine oil (SEO) contaminated soil treated with *B. azotoformans* had a pH of 7.548, while the pH of soil treated with *B. megaterium* and *S. epidermidis* were 7.860 and 7.075 respectively. The untreated soil (control) had a pH of 9.915. There was no significant difference ($p>0.05$) between the pH of soil samples in all the treatments, but the pH of the treatment significantly differed ($p<0.05$) from the control. The contaminated soil treated with *B. azotoformans, B. megaterium* and *S. epidermidis* had organic carbon content of 0.655, 0.625 and 0.638 respectively while the control had of 0.683. There was no significant difference ($p>0.05$) between the Organic carbon of soil samples in all the treatments, also there was no significant difference ($p>0.05$) between the Organic carbon of the treatments

and the control. Similarly, there was no significant difference ($p>0.05$) between the Phosphorus and particle size of soil samples in all the treatments and their controls. Contaminated soil treated with *B. azotoformans, B. megaterium* and *S. epidermidis* had Phosphorus content of 0.555, 0.513 and 0.528 respectively while the control had of 0.555. The particle size of all the treatments 0.074 while the control was 0.075. The crude nitrogen content of contaminated soil treated with *B. azotoformans* (0.395) significantly differ ($p<0.05$) from other treatment *B. megaterium* (0.508) and *S. epidermidis* (0.512) and the control (0.512). The analysis of the physical properties of soil samples used in this study showed that all the soil samples fall in the Sandy loamy texture class due to their high Sandy % as seen in Table 4.6

Table 4.5 Chemical Properties of the SEO Contaminated Soil Treated Test Isolates

Physicochemical	*B. azotoformans*	*B. megaterium*	*S. epidermidis*	Control
pH	7.548±0.717[a]	7.860±0.274[a]	7.075±0.807[a]	9.915±0.670[b]
Moisture (%)	1.108±0.227[a]	0.875±0.217[a]	0.543±0.274[b]	0.585±0.247[b]
Crude Nitrogen (%)	0.395±0.054[b]	0.508±0.110[a]	0.512±0.133[a]	0.512±0.203[a]
Organic carbon (%)	0.655±0.011[a]	0.625±0.018[a]	0.638±0.019[a]	0.683±0.020[a]
Phosphorus (%)	0.555±0.009[a]	0.513±0.041[a]	0.528±0.028[a]	0.555±0.011[a]
Particle size (mm)	0.074±0.000[a]	0.074± 0.000[a]	0.074±0.000[a]	0.075±0.001[a]

Note: (a, and b, are subscript), Values with same superscript in the same column are not significantly different, but values with different superscript in the same row are significantly different at ($p<0.05$).

Table 4.6 Physical Properties of Spent Engine Oil Contaminated Soil

Sample	Clay %	Silt %	Sandy%	Texture class
Normal soil	19.02	5.18	75.80	Sandy Loamy
Control	17.42	5.38	77.20	Sandy Loamy
B. azotoformans	16.78	5.58	77.64	Sandy Loamy
B. megaterium	16.40	5.44	78.6	Sandy Loamy
S. epidermidis	16.56	5.60	77.84	Sandy Loamy

4.1.5 GC-MS chromatogram of the spent engine oil

The GC-MS Chromatogram of SEO before it was subjected to bioremediation (week 0) and after bioremediation (week six) is shown in Tables 4.7 – 4.12 and Figure 4.3 – 4.8. The GC-MS Chromatogram of the SEO after six weeks bioremediation with *B. azotofamans, B. megaterium* and *S. epidermidis* showed a reduction in the intensity of the hydrocarbon peak when compared to the results from week 0.

Table 4.7 Individual Hydrocarbon Identified in SEO Sample Treated with the Bacterial Isolate *B. azotofamans* at week zero

PK	RT	Area%	**Hydrocarbon**
1	6.9626	0.1002	Eugenol
2	10.5793	0.1828	Phenol, 2-methoxy-4-(2-propenyl)-, acetate
3	10.9103	0.0599	Heptacosane
4	11.9222	0.0563	Dodecane, 2-methyl-
5	13.1958	0.1296	Hexadecane
6	14.1594	0.1023	9H-Fluorene, 2-methyl-
7	14.3373	0.181	2,2,9-Trimethyldec-5-ene-3,8-dione
8	14.6095	0.1884	1,1'-Biphenyl, 3,3',4,4'-tetramethyl-
9	14.776	0.0929	1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl-
10	15.4016	0.2283	Heptadecane
11	15.6001	0.2036	Decane, 2-methyl-
12	15.6899	0.102	Hexacosane
13	16.6143	0.1101	Dotriacontane, 1-iodo-
14	17.5182	0.1008	Octadecane
15	19.5491	0.0804	Nonadecane
16	20.636	0.1901	n-Hexadecanoic acid
17	21.4928	0.0628	Methoxyacetic acid, 2-tetradecyl ester
18	23.3547	0.078	Tetratetracontane
19	23.6424	0.3499	9,12-Octadecadienoic acid (Z,Z)-
20	23.8107	0.6387	cis-Vaccenic acid
21	24.077	0.4734	5-Eicosene, (E)-
22	24.4857	0.6291	Hexadecane, 1-(ethenyloxy)-
23	24.6466	0.3835	Octadecane, 1-chloro-
24	24.8316	0.3611	1-Docosene
25	25.142	1.1004	Octadecane, 1-bromo-

26	25.3054	0.6036	Octadecanal
27	25.8114	3.7672	2-Piperidinone, N-[4-bromo-n-butyl]-
28	25.9471	1.6906	1-Nonadecene
29	26.1318	0.743	Octatriacontyl pentafluoropropionate
30	26.2307	1.3862	1-Docosene
31	29.0914	0.2448	Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-
32	29.2555	0.263	1-Docosene
33	29.6742	0.7089	Cyclodocosane, ethyl-
34	30.0853	0.9139	Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-
35	30.2461	0.6038	Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)- 2,6,10,14-Tetramethyl-7-(3-methylpent-4-enylidene)
36	30.3431	0.4188	pentadecane
37	30.655	1.2182	Tetradecane, 1-bromo-
38	31.4224	24.6564	Octatriacontyl pentafluoropropionate
39	32.309	2.3299	Heneicosane, 11-cyclopentyl-
40	32.8261	14.3409	Triacontyl acetate
41	34.5377	0.9084	Squalene
42	34.6708	0.4348	Tetratriacontyl pentafluoropropionate
43	35.5241	23.0054	Octadecane, 1-iodo-
44	38.133	15.5345	Cyclodocosane, ethyl-
45	38.5408	0.0422	Octatriacontyl pentafluoropropionate

PK: Peak; RT: Retention time

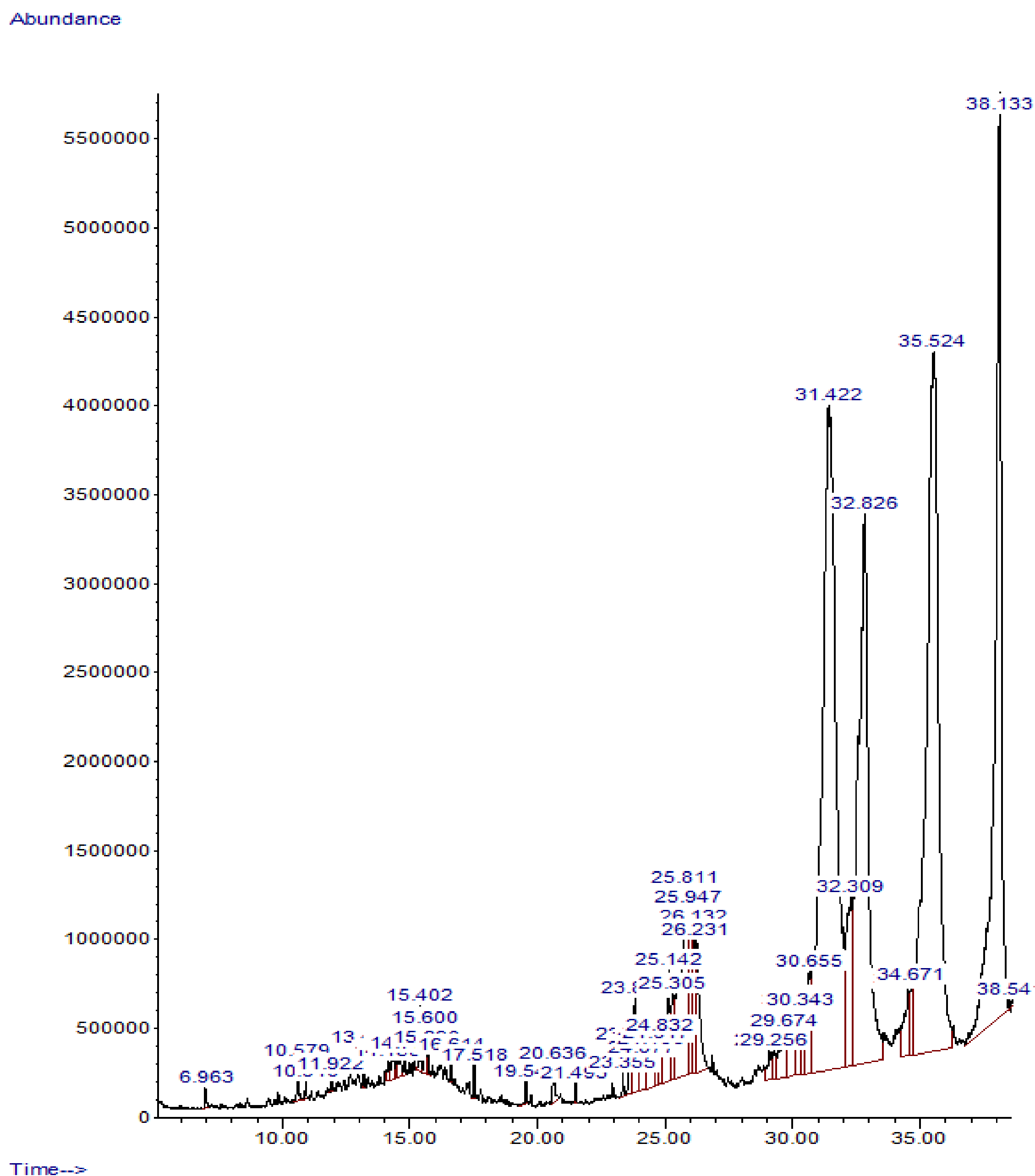

Figure 4.3 Gas Chromatography-Mass Spectroscopy for SEO sample treated with *B. azotofamans* at week zero

Table 4.8 Individual Hydrocarbon Identified in SEO Sample Treated with Bacteria Isolate *B. azotofamans* at Week Six

PK	RT	Area %	Hydrocarbon
1	6.9487	0.5529	Eugenol
2	9.8188	0.0725	2,5-cyclohexadien-1-one, 2,6-bis(1,1-dimethylethyl)-4-hydroxy-4-methyl-
3	10.5713	0.2552	Phenol, 2-methoxy-4-(2-propenyl)-, acetate
4	20.6406	0.7482	n-Hexadecanoic acid
5	20.8759	0.1724	Tetrapentacontane, 1,54-dibromo-
6	22.9392	0.375	17-Pentatriacontene
7	23.3539	0.2718	Octadecane, 1-chloro-
8	23.6507	1.5078	9,12-Octadecadienoic acid (Z,Z)-
9	23.8187	2.4982	9-Octadecenoic acid
10	23.9848	1.2455	12-Methyl-E,E-2,13-octadecadien-1-ol
11	24.1734	0.5397	12-Methyl-E,E-2,13-octadecadien-1-ol
12	24.3207	2.3051	2-Methyl-Z,Z-3,13-octadecadienol
13	24.6387	0.8624	12-Methyl-E,E-2,13-octadecadien-1-ol
14	24.8241	1.9045	1-Docosene
15	25.527	6.875	17-Pentatriacontene
16	25.8046	4.9831	1-Docosene
17	25.9241	7.5564	Heptadecanal
18	28.6328	0.567	Octatriacontyl pentafluoropropionate
19	28.8727	0.4477	1-Dodecanol, 2-octyl-
20	29.0743	0.2914	1-Docosene

21	29.2372	0.3882	Triacontyl trifluoroacetate
22	29.6559	1.1242	17-Pentatriacontene
23	30.1545	2.6728	Tetrapentacontane, 1,54-dibromo-
24	30.4883	0.428	Octatriacontyl pentafluoropropionate
25	30.6727	0.868	Octatriacontyl pentafluoropropionate
26	30.794	0.8581	Tricosyl trifluoroacetate
27	31.0152	1.7005	Tetrapentacontane, 1,54-dibromo-
28	31.2718	1.0321	Octacosyl trifluoroacetate
29	31.6925	3.8949	Silane, trichlorooctadecyl-
30	32.5761	22.8531	Silane, trichlorooctadecyl-
31	32.6714	8.8706	Octatriacontyl pentafluoropropionate
32	32.7967	21.2799	Octacosyl trifluoroacetate
33	34.5257	0.3135	1,5,9-Undecatriene, 2,6,10-trimethyl-, (Z)-
34	34.904	0.3619	Octatriacontyl pentafluoropropionate
35	38.0359	-0.6776	Silane, trichlorooctadecyl-

PK: Peak, RT: Retention time

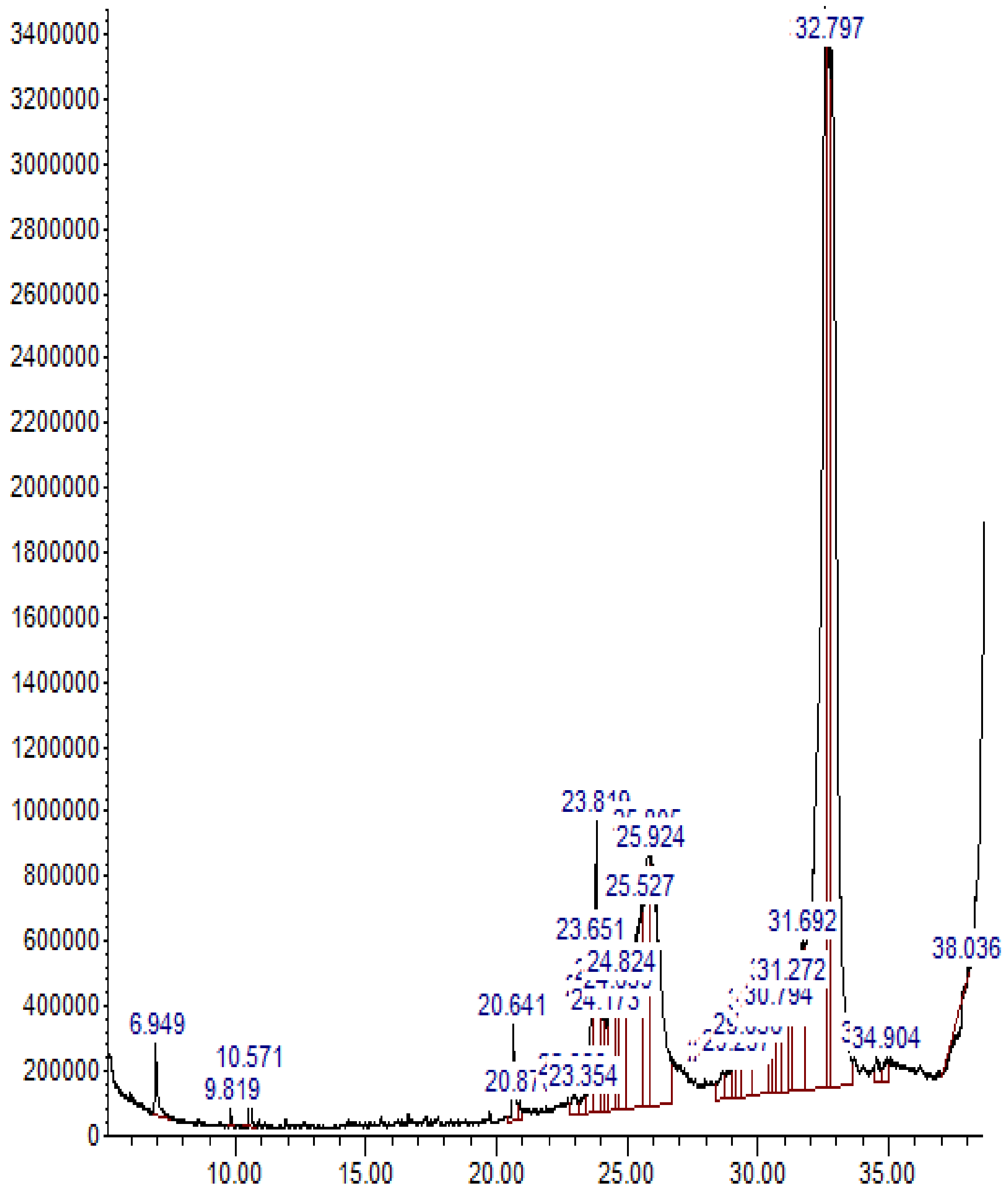

Figure 4.4 Gas Chromatography-Mass Spectroscopy for SEO sample treated with *B. azotofamans* at week six

Table 4.9 Individual Hydrocarbon Identified in SEO Sample Treated with Bacteria Isolate *Bacillus megaterium* at Week Zero

PK	RT	Area %	Hydrocarbon
1	17.5243	0.0378	Octadecane
2	19.5559	0.0547	Nonadecane
3	20.6424	0.1432	n-Hexadecanoic acid
4	21.4981	0.0921	Eicosane
5	22.9594	0.0779	Tetrapentacontane, 1,54-dibromo-
6	23.3612	0.1372	Heneicosane
7	23.6466	0.3142	9,12-Octadecadienoic acid (Z,Z)-
8	23.8132	0.4799	cis-Vaccenic acid
9	24.0827	0.4705	Oxirane, tridecyl-
10	24.3333	0.1938	1-Docosene
11	24.497	0.4956	Undecane, 5-cyclohexyl-
12	24.6542	0.4208	Octadecane, 1-chloro-
13	25.1508	1.7352	Octadecane, 1-chloro-
14	25.8248	7.8502	Tricosane
15	25.9494	3.5557	1-Docosene
16	26.2467	1.3986	Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-
17	26.3816	0.7869	Oxirane, tetradecyl-
18	26.7587	0.5744	1,19-Eicosadiene
19	26.8683	0.7547	2-Piperidinone, N-[4-bromo-n-butyl]-
20	27.056	1.3987	Tetracosyl heptafluorobutyrate
21	27.3193	2.8255	Octacosyl trifluoroacetate

22	27.5481	6.9374	Cyclohexane, (1-hexyltetradecyl)-
23	28.6768	0.5792	1-Docosene
24	28.7362	0.2446	Tricosane
25	28.9147	0.3822	Tricosane
26	29.1164	0.9053	1-Docosene
27	29.8512	20.5112	Octatriacontyl pentafluoropropionate
28	30.6624	0.2854	2-Piperidinone, N-[4-bromo-n-butyl]-
29	30.8308	0.2716	Octatriacontyl pentafluoropropionate
30	31.0246	0.4688	Dodecane, 2,6,11-trimethyl-
31	31.3025	0.3995	Propyl triacontyl ether
32	31.517	0.338	Hexacosanal
33	31.7443	0.7662	Isobutyl tetratriacontyl ether
34	31.9425	0.3911	Octatriacontyl pentafluoropropionate
35	32.3302	1.1097	1-Docosene
36	32.5989	1.4676	Hexadecane, 2,6,10,14-tetramethyl-
37	33.1033	13.4047	Silane, trichlorooctadecyl-
38	33.7381	0.5536	1-Docosene
39	34.9657	15.3225	Silane, trichlorooctadecyl-
40	36.3375	0.1239	Eicosane, 9-cyclohexyl-
41	36.9259	0.014	6-Nitroundec-5-ene
42	38.2162	11.7263	Cyclodocosane, ethyl-

PK: Peak, RT: Retention time

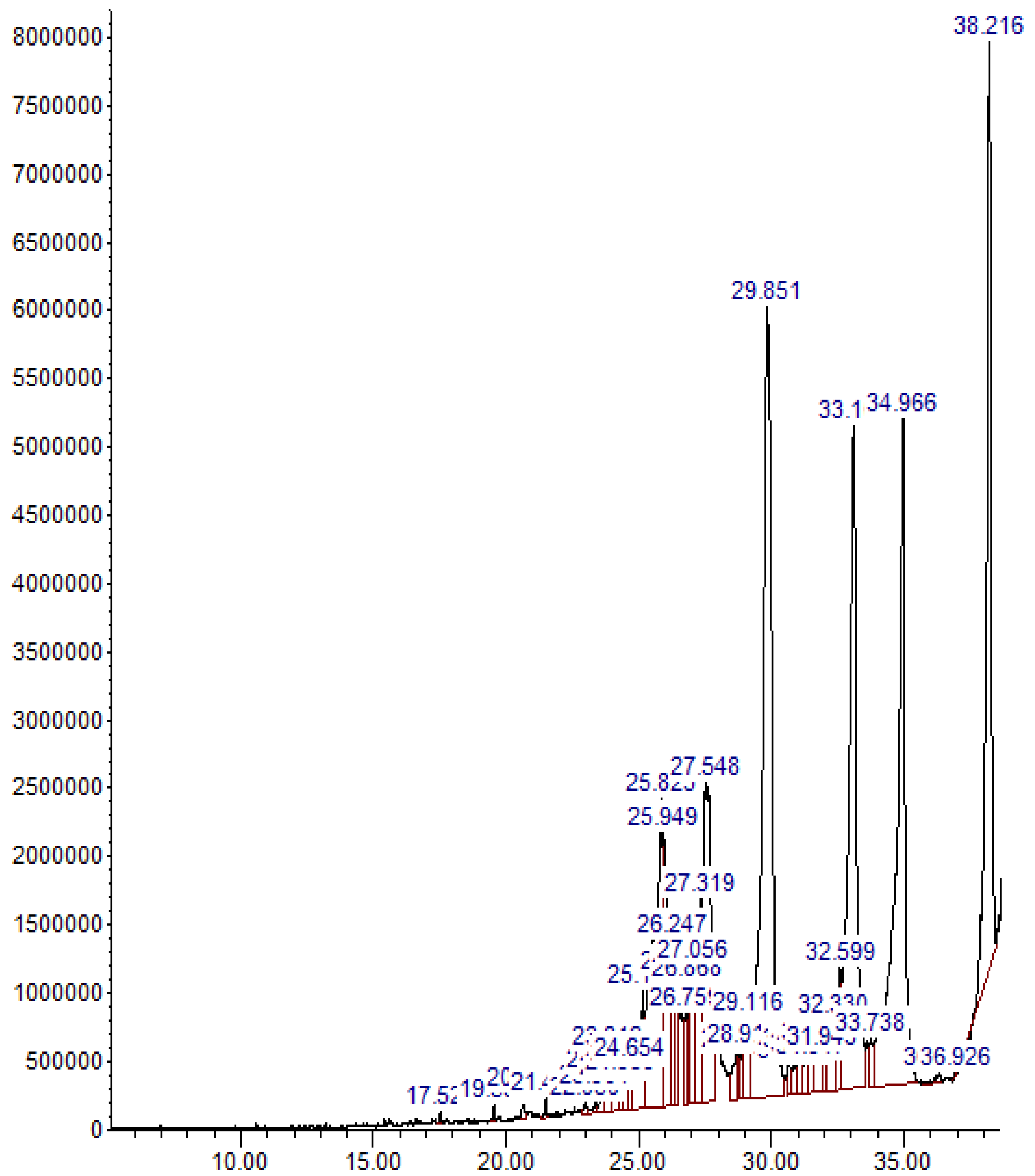

Figure 4.5 Gas Chromatography-Mass Spectroscopy for SEO sample treated with *Bacillus megaterium* at zero

Table 4.10 Individual Hydrocarbon Identified in SEO Sample Treated with Bacterial Isolate *Bacillus megaterium* at Week Six

PK	RT	Area %	Library/ID
1	6.9646	0.1323	Phenol, 2-methoxy-4-(1-propenyl)-, (Z)-
2	9.822	0.1086	9H-Fluorene, 2-methyl-
3	10.5776	0.2971	Phenol, 2-methoxy-4-(2-propenyl)-, acetate
4	10.8979	0.06	Methoxyacetic acid, 2-tetradecyl ester
5	11.9206	0.1068	Dodecane, 2-methyl-
6	12.4101	0.0638	Ethanone, 2-bromo-1,2-diphenyl-
7	12.6591	0.088	Benzene, 2,6-dimethyl-1-(phenylmethyl)-
8	14.0456	0.2422	Acetic acid, oxo(phenylamino)-
9	14.3288	0.4705	1-Octadecanesulphonyl chloride
10	14.597	0.4166	1,1'-Biphenyl, 2,2',5,5'-tetramethyl-
11	14.7704	0.3184	9-(Methylaminomethyl)anthracene
12	15.1504	0.4529	1,1'-biphenyl, 2,4,6-trimethyl-
13	15.5847	0.3999	Decane, 2-methyl-
14	16.6123	0.1518	alpha-Methylstilbene
15	20.6336	0.1518	n-Hexadecanoic acid
16	23.6471	0.3659	9,12-Octadecadienoic acid (Z,Z)-
17	23.8101	1.0697	cis-13-Octadecenoic acid
18	24.3293	0.3217	17-Pentatriacontene
19	24.8336	0.6346	Z-2-Octadecen-1-ol
20	27.0226	21.2403	Octacosyl heptafluorobutyrate
21	29.2605	1.1321	1-Hexadecanol, 2-methyl-

PK	RT	Area %	Compound
22	30.23	4.5948	Tetrapentacontane, 1,54-dibromo-
23	31.0783	26.996	Behenyl chloride
24	32.2873	1.0241	Octatriacontyl pentafluoropropionate
25	32.5567	1.5291	Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-
26	33.8215	23.9893	1-Hexadecanol, 2-methyl-
27	34.9178	0.1429	Octatriacontyl pentafluoropropionate
28	35.1257	0.1348	1-Hexacosene
29	35.6074	0.3971	Octatriacontyl pentafluoropropionate
30	36.2395	0.4819	Tetrapentacontane, 1,54-dibromo-
31	38.1738	12.485	Octatriacontyl pentafluoropropionate

PK: Peak, RT: Retention time

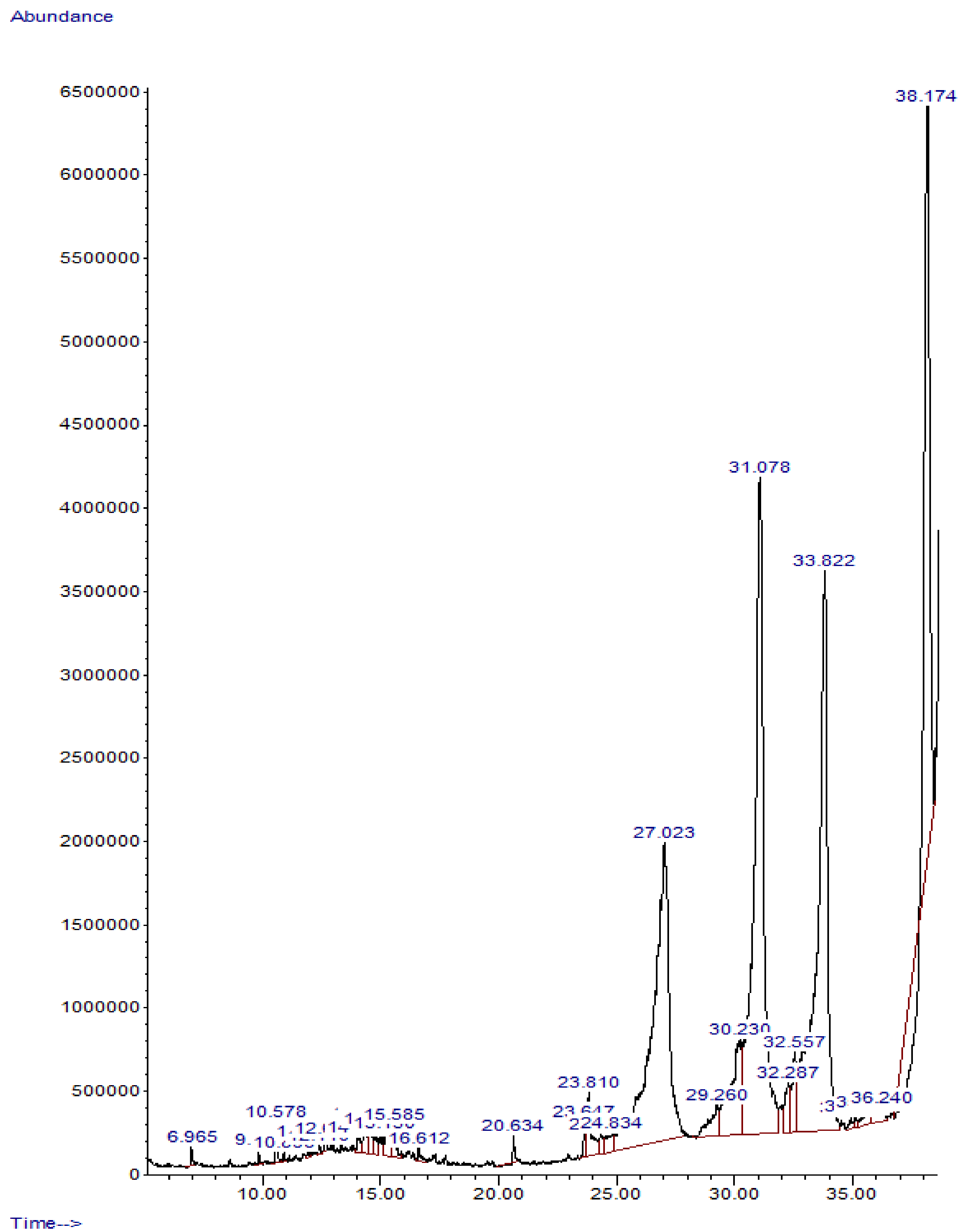

Figure 4.6 Gas Chromatography-Mass Spectroscopy for SEO Sample Treated with *Bacillus megaterium* at Week Six

Table 4.11 Individual Hydrocarbon Identified in SEO Sample Treated with Bacterial Isolate *Staphylococcus epidermidis* at Week Zero

PK	RT	Area %	Hdrocarbon
1	6.9758	0.136	Dodecane
2	9.4958	0.1321	2-Indanylacetic acid
3	9.6893	0.076	1,4:5,8-Dimethanobiphenylene, 1,4,4a,4b, 5,8,8a, 8b-octahydro-
4	9.826	0.1968	Ethanone, 1-(3,4-dimethoxyphenyl)-
5	10.0964	0.0759	Naphthalene, 1,6,7-trimethyl-
6	10.5824	0.3662	Butylated Hydroxytoluene
7	10.9119	0.1794	Tetradecane
8	11.3686	0.1024	Naphthalene, 2,3,6-trimethyl-
9	11.9194	0.078	Dodecane
10	13.1963	0.0567	Hexadecane
11	15.4035	0.0606	Heptadecane
12	15.5993	0.0742	Diphenylacetylene
13	17.5238	0.0746	Octadecane
14	19.5536	0.1389	Nonadecane
15	20.423	0.1976	di-p-Tolylacetylene
16	20.6492	0.384	n-Hexadecanoic acid
17	20.9088	0.5056	Hexadecane, 1-chloro-
18	21.0759	0.2353	1-Octadecene
19	21.2175	0.1739	Octadecane, 1-chloro-
20	21.5004	1.0506	Eicosane

21	21.7059	0.6137	Octadecane, 1-chloro-
22	21.9061	1.0419	E-15-Heptadecenal
23	22.2737	2.9601	1,2-Benzisothiazole, 3-(hexahydro-1H-azepin-1-yl)-, 1,1-dioxide
24	22.3805	0.988	Octadecane, 1-chloro-
25	22.4672	0.7909	Octadecane, 1-chloro-
26	22.586	1.6469	Octadecane, 1-chloro-
27	22.6908	1.4838	Oxirane, tetradecyl-
28	22.8315	0.9939	Octadecane, 1-chloro-
29	22.9478	2.5313	Octadecane, 1-chloro-
30	23.3576	0.6653	Heneicosane
31	23.6453	0.439	Linoelaidic acid
32	23.8186	0.3745	9-Octadecenoic acid
33	25.1491	0.1159	Docosane
34	25.309	0.0474	1-Docosene
35	25.8223	0.2703	Carbonic acid, eicosyl vinyl ester
36	25.9557	0.101	1-Docosene
37	26.0441	0.0904	Octatriacontyl pentafluoropropionate
38	26.1454	0.1342	1-Docosene
39	26.2829	0.3292	17-Pentatriacontene
40	26.3914	0.2507	Tetrapentacontane, 1,54-dibromo-
41	26.5093	0.2877	Octacosyl trifluoroacetate
42	26.8738	1.5668	Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-

43	27.232	5.1654	1-Docosene
44	27.3107	3.9548	Eicosane, 9-cyclohexyl-
45	28.5333	0.103	Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-
46	28.6785	0.2972	Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-
47	28.9086	0.2437	1-Docosene
48	29.1114	0.4971	Tetradecane, 1-bromo-
49	29.2772	0.5265	1-Docosene
50	29.6956	1.4435	Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-
51	30.4293	19.0538	1-Hexadecanol, 2-methyl-
52	31.3021	14.072	1-Docosanol, acetate
53	32.3372	1.9913	1-Docosene
54	32.8333	19.5324	Octacosyl trifluoroacetate
55	34.5549	0.0618	Neopentylidenecyclohexane
56	36.3459	1.5752	Silane, trichlorooctadecyl-
57	37.0041	9.433	Silane, trichlorooctadecyl-
58	37.8772	0.0319	1,1,3-Tricyclohexylpropane

PK: Peak, RT: Retention time

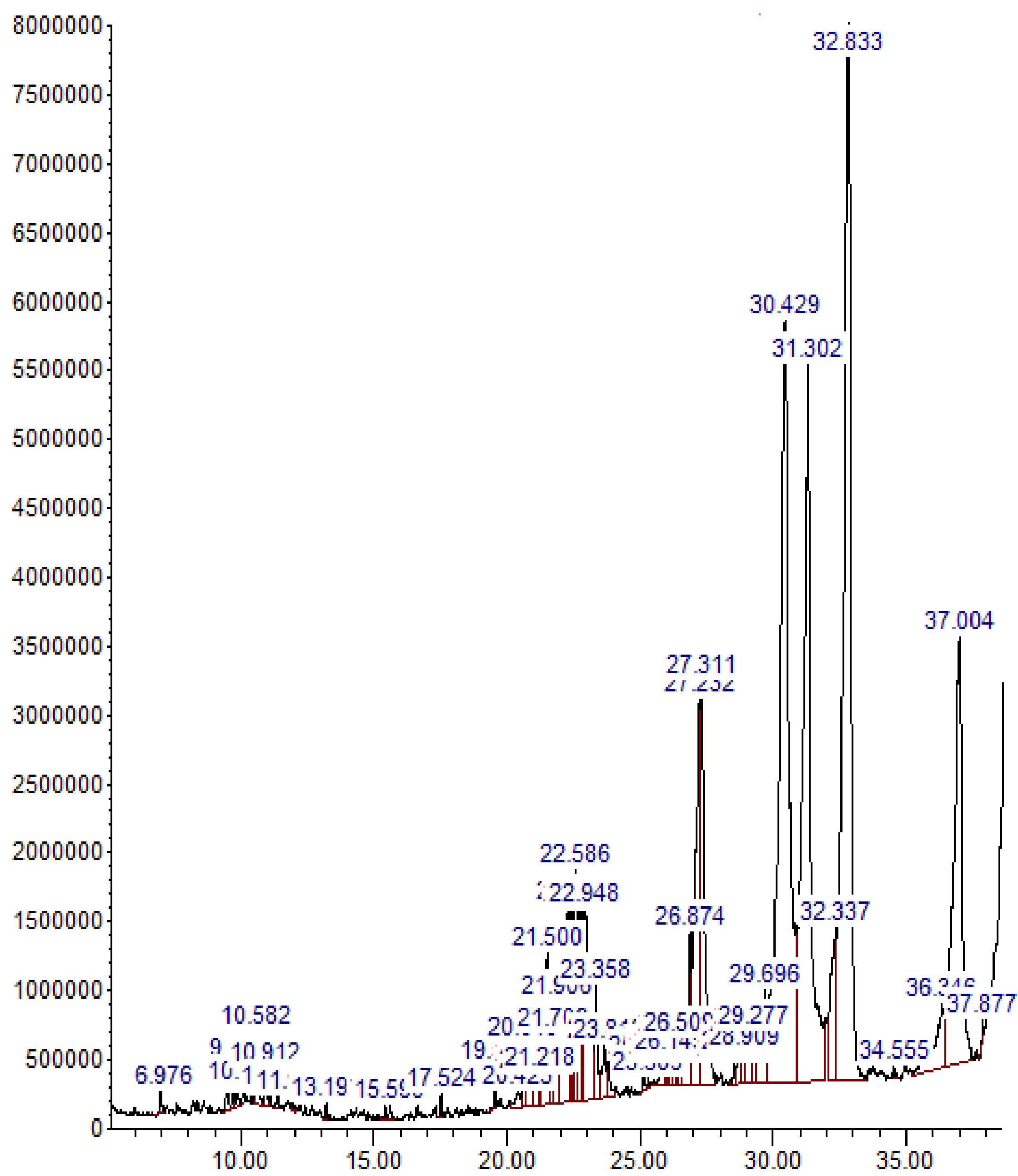

Figure 4.7 Gas Chromatography-Mass Spectroscopy for SEO Sample Treated with *Staphylococcus epidermidis* at Week Zero

Table 4.12 Individual Hydrocarbon identified in SEO Sample Treated with Bacterial Isolate *Staphylococcus epidermidis* at Week Six

PK	RT	Area %	Library/ID
1	20.6878	0.668	n-Hexadecanoic acid
2	23.6827	0.7903	Linoelaidic acid
3	23.8526	0.781	cis-13-Octadecenoic acid
4	27.2015	0.1356	Tetrapentacontane, 1,54-dibromo-
5	27.6146	0.1967	1-Docosene
6	27.8305	0.2666	1,19-Eicosadiene
7	28.2919	5.3958	Tetratriacontyl pentafluoropropionate
8	28.4394	3.6145	Tetratriacontyl heptafluorobutyrate
9	28.6424	21.3214	1-Docosene
10	30.0617	0.2128	2-Piperidinone, N-[4-bromo-n-butyl]-
11	31.0214	0.7569	2-Piperidinone, N-[4-bromo-n-butyl]-
12	31.6646	0.792	Octatriacontyl pentafluoropropionate
13	32.2024	2.1268	Silane, trichlorooctadecyl-
14	32.2909	1.9419	Octatriacontyl pentafluoropropionate
15	32.3911	1.1835	Octatriacontyl pentafluoropropionate
16	32.565	5.9195	2-Piperidinone, N-[4-bromo-n-butyl]-
17	32.904	28.5746	Silane, trichlorooctadecyl-
18	32.9536	41.5494	Silane, trichlorooctadecyl-
19	37.5889	0.0119	Hexatriacontyl pentafluoropropionate
20	37.8433	-0.258	Aspidospermidin-17-ol, 1-acetyl-19,21-epoxy-15,16-dimethoxy-
21	38.0226	0.1915	Silane, trichlorooctadecyl-
22	38.5534	-16.8844	Octadecanal
23	38.6169	0.7117	Octadecanal

PK: Peak, RT: Retention time

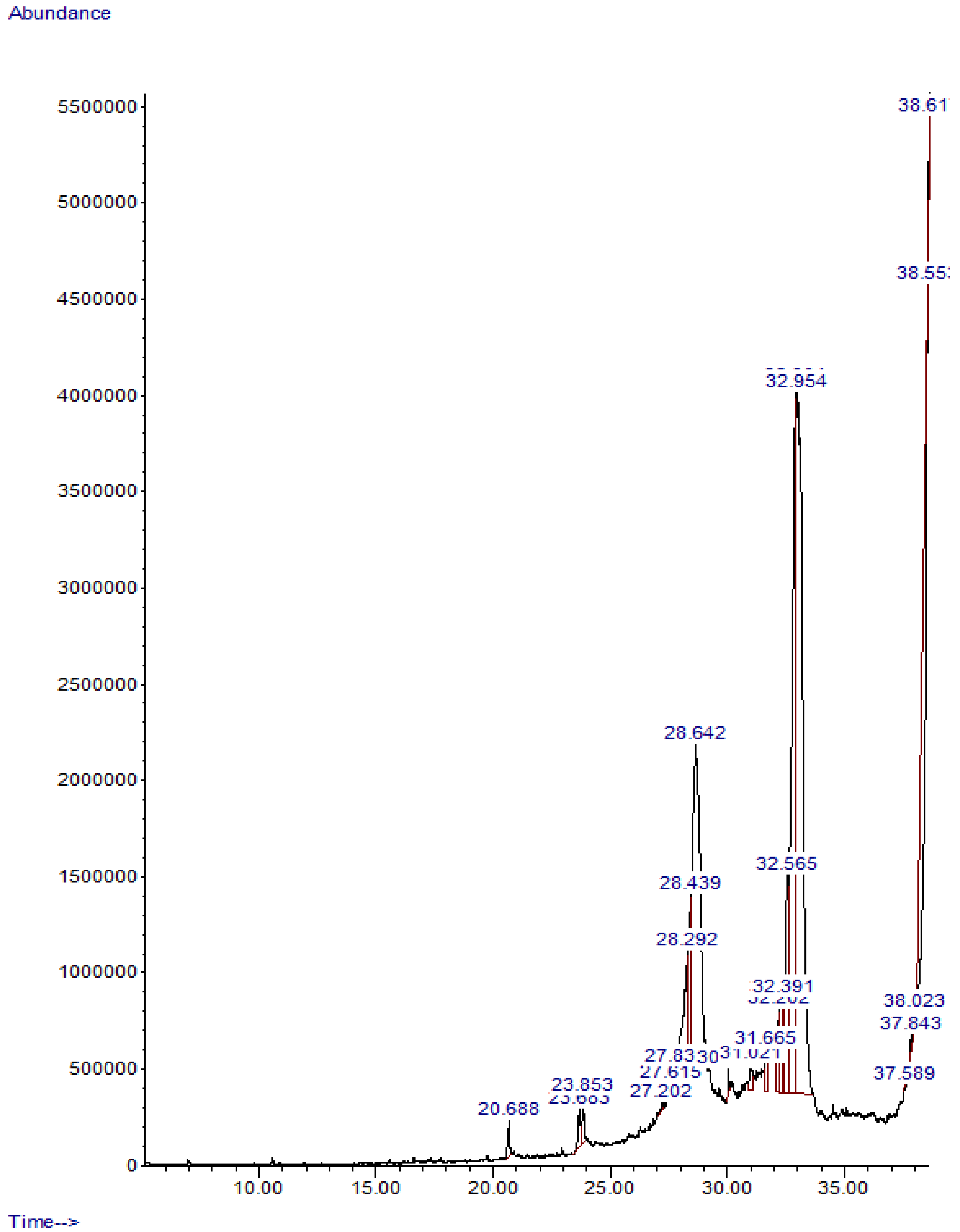

Figure 4.8 Gas Chromatography-Mass Spectroscopy for SEO Sample Treated with *Staphylococcus epidermidis* at six

4.2 Discussion of Results

4.2.1 Enumeration and identification of bacteria from SEO polluted soil

Spent engine oil causes great damage to soil and soil micro-flora. Microorganisms are important for soil quality and fertility. They play a major role in decomposition of organic matter, degradation of chemical pollutants and mineralization in the soil. Amongst the different microorganisms inhabiting the soil, bacteria are the most abundant and predominant organisms, these microorganisms serve as chemical processors, tiny biotechnologists, capable of catalysing thousands of chemical reactions that higher organisms are incapable of mediating. It has been reported that bacteria constitute the principal agents of hydrocarbon biodegradation (Tanimu *et al*., 2019). It has been shown that marked changes in properties occur in soil contaminated with hydrocarbon; affecting its microbiological properties (Okonokhua *et al*., 2007). From the current study the observed total bacteria count of SEO polluted soil obtained from automobile workshops ranged from 1 x 10^3 to 17 x 10^3 CFU/g. This is contrary to the findings of Ugoh and Moneke (2011), Tanimu *et al*. (2019) who observed higher mean total bacterial countanits in used oil contaminated soil. Tanimu *et al*. (2019) reported bacterial count from their study to range from 5.6 x 10^6 to 3.53 x 10^6 CFU/ g of soil. Ugoh and Moneke (2011) observed a bacterial count that ranged from 1.5 x 10^4 to 7.6 x 10^4. The relatively low total bacterial counts observed in oil contaminated soils in this study can be attributed to the toxic or unfavourable effect of oil contamination (Akoachere *et al*., 2008). It has been reported that oil contamination creates unsatisfactory condition for life in the soil due to poor aeration, immobilization of soil nutrients and lowering of soil pH (Ugoh and Moneke, 2011). The count of potential hydrocarbon degraders observed in this study ranged from 1 x 10^4 to 30 x 10^5 CFU/g. Similar count of hydrocarbon degraders (5.7 x 10^5 CFU/g) was observed by Muhammad *et al*. (2022). A slightly higher count of potential

hydrocarbon degraders was reported by Ayandele (2018). They reported the count of oil degrading bacteria to range from 18 x 10^6 to 47 x 10^6 CFU/g. According to Rahman *et al*. (2002) the population level of hydrocarbon utilizers and their populations within the microbial community is a sensitive index of environmental exposure to hydrocarbons. Also, the presence of hydrocarbon degraders observed in these studies can be linked to the high availability of nutrients (Ayandele, 2018). Studies have also shown that the hydrocarbon degrading microorganisms are always abundant in petroleum hydrocarbon contaminated soils and such soils serve as the good sources for the isolation of hydrocarbon-degrading microorganisms (Ekhause and Nkwell, 2011; Maddela *et al*., 2016; Maddela *et al*., 2017). Soils contain very large numbers of microorganisms which can include a number of hydrocarbons utilizing bacteria (Abdulsalam *et al*., 2012), these Microorganisms are capable of breaking down many complex molecules by adaptation of their degradative enzyme system (Ugoh and Moneke, 2011). Various bacteria species were identified from the SEO polluted soil. These species include *L. casei*, *S. epidermidis*, *S. aureus*, *B. larvae, B. megaterium*, *B. subtilis* and *B. azotofamans*. Similar and various other species of bacteria were reported in numerous studies. From the study of Musa *et al*. (2021) the following bacteria species *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Bacillus megaterium* and *Pseudomonas aeruginosa* were identified in oil contaminated soil. Ayandele (2018) identified *Pseudomonas aeruginosa, Micrococcus* sp., and *Flavobacterium* sp. from SEO contaminated soil, Ugoh and Moneke (2011) reported *Pseudomonas spp*, *Bacillus spp*., *Micrococcus* sp. and *Serratia* species. Yerima *et al*. (2020) reported *Pseudomonas*, *Streptococcus* and *Bacillus* sp. Muhammad *et al*. (2022) reported species from the genera *Acinetobacter, Bacillus, Micrococcus, Flavobacterium* and *Pseudomonas.* Ajiboye *et al*. (2020) reported *Pseudomonas spp*., *Enterobacter spp*., *Acinetobacter* sp., and

Bacillus sp. Agu (2015) isolated the Bacteria species belonging to the genera *Bacillus*, *Micrococcus*, *Corynebacterium* and *Pseudomonas* from oil contaminated soil and Ghazali *et al*. (2004) reported the various species from the genera *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Enterobacter*, *Escherichia*, *Flavobacterium*, *Norcadia*, *Pseudomonas*, *Staphylococcus* and *Vibrio* in hydrocarbon contaminated soil. The presence of these organisms in hydrocarbon polluted soil could mostly be attributed to the availability of necessary and required nutrients to support their growth (Ayandele, 2018).

4.2.2 Total bacterial count during bioremediation of SEO contaminated soil

The bacterial count of SEO contaminated soil during the bioremediation process was highest in the initial week of contamination (3.00×10^7, 4.00×10^7, 2.60×10^7 and 3.20×10^7 CFU/g for Control, *B. azotoformans*, *B. megaterium* and *S. epidermidis* treated soil respectively). Significant reduction in bacterial count was observed in the second week (1.50×10^6, 1.28×10^7, 1.68×10^7 and 1.40×10^7 CFU/g). Similar finding was reported by Stephen *et al*. (2013) and Tanimu *et al*. (2019). According to their study the reduction in bacterial count in SEO contaminated soil could be attributed to the death of the bacteria due to poor nutrient availability and/or the toxic nature of the used oil in the soil. After week two a steady increase in bacterial count was observed up till week six (3.20×10^7, 2.20×10^7, 3.10×10^7 and 2.40×10^7 CFU/g). Milic *et al*. (2009) and Stephen *et al*. (2013) reported similar findings. The observed increase in bacterial population after week two could indicate that the presence of spent oil either attracted hydrocarbon degrading organisms or served as substrate for the multiplication of some indigenous hydrocarbon degrading microbes already present in the soil (Tanimu *et al*., 2019).

AF4.2.3 Total Petroleum Hydrocarbon (TPH) and percentage degradation of SEO contaminated soil

The rate of bioremediation of SEO contaminated soil was determined based on total petroleum hydrocarbon (TPH) present in the contaminated soil before and after bioremediation. According to Kwaspisz *et al*. (2008) and Adeleye *et al*. (2019), biodegradation can be measured or monitored in terms of various parameter including total petroleum hydrocarbon (TPH). TPH is the mixtures of organic compounds found in or derived from crude oil (Udoetok and Osuji, 2007; Adipah, 2019). They include a broad family of several hundred hydrocarbon compounds that originally come from crude oil (Kuppusamy *et al*., 2020). Total Petroleum Hydrocarbon (TPH) is a commonly used gross parameter for quantifying environmental contamination originated by various petroleum hydrocarbon products such as fuels, oils, lubricants, waxes, and others (Schwartz *et al*., 2012). In the current study, significant decrease in the TPH value of contaminated soil was observed during the course of experiment. The initial TPH values at week 0 were 0.7, 2.80, 2.66 and 1.42mg/L for the Control, *B. azotoformans*, *B. megaterium* and *S. epidermidis* treated soil respectively and at week 6 the recorded TPH values decreased to 0.68, 1.53, 0.65 and 0.72mg/L respectively. Comparable finding was observed in several other studies including Milic *et al*. (2009), Abdulsalam *et al.* (2012), Abioye *et al*. (2012), Adams *et al.* (2014), Ayandele, (2018).

In a study on the biotreatment of soil contaminated with spent engine oil by locally isolated microorganisms by Ayandele (2018) reported significant decrease in TPH values from 20.29 PPM to 2.19, 3.18 and 4.1 PPM in soil treated with *Pseudomonas aeruginosa*, *Flavobacterium* sp. and *Micrococcus* sp. respectively. Abdulsalam *et al*. (2012) reported in

their study on the bioremediation of soil contaminated with used motor oil in a closed system that TPH values of contaminated soil was reduced from an initial value of 215, 215, 192, 326, 169 and 192 mg/kg dry weight to 111, 94, 54, 129, 66 and 54 mg/kg dry weight after 70 days of bioremediation, which are equivalent to 49, 56, 72, 60, 61 and 72% removals. Milic *et al*. (2009) in their study on Bioremediation of soil heavily contaminated with crude oil observed a decrease in the TPH value from 29.80 to 3.29 g/kg (89%) after 5.5 months. The observed reductions in TPH values could be attributed to microbial activity the SEO contaminated soil, this include production of enzymes responsible for the degradation of hydrocarbons (Duan *et al*. 2015). According to Ibrahim *et al*. (2016) and Okpashi *et al*. (2020) some bacteria species are capable of producing biosurfactants that break the interface between water in oil or oil in water due to enzymatic reactions. Degradation percentage of SEO contaminated soil treated with various test organisms was determined as the difference between the initial and final TPH concentrations of bioremediated soil. Spent engine oil contaminated soil treated with *B. megaterium* (75.6%) recorded the highest level of degradation followed by *S epidermidis* (48.6%) and *B. azotoformans* (44.7%).

4.2.4 Physical and chemical properties of the spent engine oil contaminated soil

Spent engine oil causes great damage to various soil properties, marked changes in properties occur in soil contaminated with hydrocarbon; affecting the physical, chemical and microbiological properties of such soil (Okonokhua *et al*., 2007). Various physicochemical parameters like texture, pH, Crude Nitrogen, Organic carbon, Phosphorus and Particle size were observed in this study. From the study no significant difference ($p<0.05$) was observed in the value of Crude Nitrogen, Organic carbon, Phosphorus and Particle size of the control and SEO contaminated soil, indicating that SEO contamination had no effect on these

parameters. The pH value of SEO contaminated soil significantly differed from that of the control, the pH value of SEO contaminated soil decreased from been Alkaline in the control (9.915±0.670) to a near neutral level (7.548±0.717, 7.860±0.274 and 7.075±0.807). pH values akin to those observed in this study was reported by Ilemobayo and Kolade (2008), Abioye *et al*. (2012) and Ajiboye *et al.* (2020). Ilemobayo and Kolade (2008) observed a pH value of 5.96-8.88, Abioye *et al*. (2012) reported a pH value of 6.12 and Ajiboye *et al.* (2020) reported 6.00 – 6.20. The drop in pH value of SEO contaminated soil from an Alkaline condition (9.915±0.670) to a near neutral level as observed in this study could be attributed to the fact that hydrocarbons contain many free cations causing them to have properties of a weak acid (Onojake and Osuji, 2012). pH is a critical factor for microbial growth and survival. According to Ajiboye *et al.* (2020) a near neutral pH is suitable for the growth of diverse bacterial populations. The pH values observed in this study (7.548±0.717, 7.860±0.274 and 7.075±0.807) all fall within the optimal soil pH range recommended to support bacterial growth which is from 6 to 8 (Ilemobayo and Kolade, 2008; Haritash, and Kaushik, 2009) as well as the optimum soil pH for efficient bioremediation which is 5.5 to 8.8 (Antizar-Ladislao *et al*., 2004). Soil pH is of importance as it can affect availability of nutrients (Agamuthu *et al*., 2013), it also influences the solubility and accessibility of soil components which indirectly influence biological activity in the soil (Onojake and Osuji 2012).

The analysis of the physical properties of SEO contaminated soil samples showed that all the soil samples fall in the Sandy loamy texture class. The Physical properties of soil have been considered to be very important for bioremediation because several factors affecting the degradation process like soil aeration, movement of nutrients through soil pores, water

holding capacity etc. are under the direct and indirect influence of soil physical properties (Ajiboye *et al.,* 2020). Increased ventilation has a direct impact on microbial growth, which can enhance the biodegradation of petroleum compounds (Luepromchai *et al*., 2007).

4.2.5 GC-MS chromatogram of the spent engine oil

Using the gas chromatography-mass spectrometry (GC-MS), numerous hydrocarbon were identified in the untreated SEO samples, while most of this hydrocarbon were missing after bioremediation with *B. azotoformans*, *B. megaterium* and *S. epidermidis*, in the SEO sample treated with *B. azotoformans*, 35 hydrocarbon out of an initial 45 were identified after six weeks of bioremediation. While SEO samples treated with *B. megaterium* and *S. epidermidis* had 31 and 23 hydrocarbons from an initial 42 and 58 respectively. The reductions in hydrocarbon compound observed in bioremediated SEO samples indicates their degradation to more volatile compounds that might have escaped. Similar findings were reported by Milic *et al*. (2009) and Ibrahim (2016). Ibrahim (2016) observed a reduction in the percentage of total peak area of used engine oil after treatment with *Ochrobactrum anthropi* HM-1 and *Citrobacter freundii* HM-2 isolated from oil-contaminated soil. Degradation of hydrocarbon compounds observed could be attributed to the enzymes and active compound produced by oil degrading microorganisms. According to Umana *et al*. (2017), Ani *et al*. (2019) and Umoren *et al*. (2019) reported that microorganisms have enzyme systems that enable them degrade and utilize hydrocarbons as a carbon and energy source. The GC-MS results observed in this study shows clear evidence of the capability of *B. azotoformans*, *B. megaterium* and *S. epidermidis* to degrade some the chemical components found in SEO thus confirming the SEO biodegradation potential of these isolated bacteria species.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Bacterial species were isolated from SEO polluted soil, the total bacterial count ranged from 7 x 10^3 to 17 x 10^3 CFU/g while count for potential oil degraders ranged from 1 x 10^4 to 3 x 10^6 CFU/g. Various bacteria species: *Lactobacillus casei*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *B. larvae, Bacillus megaterium*, *Bacillus subtilis* and *Bacillus azotofamans* were identified in SEO polluted soil.

In this study, the isolated bacterial species *Bacillus megaterium*, *Bacillus azotoformans,* and *Staphylococcus epidermidis* showed the highest potential for SEO degradation after screening.

The physical and chemical properties of the SEO contaminated soils did not differ significantly ($p>0.05$) from the uncontaminated soil (control). There was no significant difference ($p>0.05$) in the Crude Nitrogen, Organic carbon, Phosphorus and Particle size of SEO contaminated soil, while a slight reduction in pH was observed in the polluted soil. The pH value of SEO contaminated soil decreased from been Alkaline in the control (9.915±0.670) to a more neutral level (7.548±0.717, 7.860±0.274 and 7.075±0.807). The analysis of the physical properties of soil samples used in this study showed that all the soil samples fall in the Sandy loamy texture class.

The Gas Chromatography – Mass Spectrometry (GC-MS) of SEO before it was subjected to bioremediation (week 0) and after bioremediation (week 6) was determined. The GC-MS Chromatogram of the SEO after six weeks bioremediation with *B. azotofamans, B.*

megaterium and *S. epidermidis* showed a reduction in the intensity of the hydrocarbon peak when compared to the results from week 0.

Degradation percentage of SEO contaminated soil treated with various test organisms was determined as the difference between the initial and final TPH concentrations of bioremediated soil. Spent engine oil contaminated soil treated with *B. megaterium* (75.6%) recorded the highest level of degradation followed by *S epidermidis* (48.6%) and *B. azotoformans* (44.7%). Also, GC-MS results of SEO after six weeks bioremediation with *B. azotofamans, B. megaterium* and *S. epidermidis* showed a reduction in the intensity of the hydrocarbon peak. Therefore, the bioremediation can be employed in the clean-up of spent engine oil.

The rate of bioremediation of SEO contaminated soil was determined based on total petroleum hydrocarbon (TPH) present in contaminated soil. Significant decrease in the TPH value was observed in all treatment groups during the course of this study. The initial TPH values (week 0) were 0.7, 2.80, 2.66 and 1.42mg/L for the Control, *B. azotoformans*, *B. megaterium* and *S. epidermidis* treated soil respectively and at the end of the experiment (week 6) the recorded TPH values were 0.68, 1.53, 0.65 and 0.72mg/L.

5.2 Recommendations

Based on the findings and conclusion of the study, the following recommendations were made:

1. *Staphylococcus epidermidis, Bacillus megaterium,* and *Bacillus azotoformans* should be kept in high profile for their significance on bioremediation of spent engine oil (SEO) polluted soil.

2. Government should provide grant to further extend the research on identification and screening of more bacteria isolates for bioremediation of spent engine oil (SEO) polluted soil.
3. Encouragement should be given to the use of bacteria isolates as bioremediating agents for the purpose of degrading the hydrocarbons that are present in used engine oil.
4. The application of microorganisms by industries in order to improve bioremediation on soil in the areas where there has been an oil spill.
5. Identification of mechanisms/genes used by these isolates for bioremediation should be encouraged.

5.3 Contribution to Knowledge

The research makes several significant contributions to knowledge in the field of environmental science and bioremediation. Some of the key contributions are as follows:

1. Identification of Indigenous Oil-Degrading Bacteria: The research contributes to the knowledge of microbial diversity and potential oil-degrading bacteria in SEO-polluted soil. By isolating and identifying various bacterial species capable of degrading spent engine oil, the study enhances our understanding of the microbial community's role in remediating oil-contaminated environments.
2. Evaluation of Bioremediation Potential: The study provides valuable insights into the efficacy of indigenous oil-degrading bacteria in remediating SEO-polluted soil. By screening and comparing the biodegradation rates of different bacterial isolates, the research highlights specific strains, such as Bacillus megaterium, Bacillus

azotofamans, and Staphylococcus epidermidis, as promising candidates for effective bioremediation strategies.

3. Successful Bioremediation of Spent Engine Oil Contamination: The research demonstrates the feasibility of using selected indigenous bacterial isolates for the bioremediation of SEO-polluted soil. By tracking the reduction in Total Petroleum Hydrocarbon (TPH) values and confirming the degradation of hydrocarbon compounds through GC-MS analysis, the study showcases the successful application of microbial remediation techniques.
4. Holistic Assessment of Soil and Environmental Parameters: The research takes a comprehensive approach by analyzing both microbial and physicochemical properties of SEO-polluted soil. By evaluating changes in soil pH, moisture content, crude nitrogen, organic carbon, phosphorus levels, and particle size distribution before and after bioremediation, the study provides a complete understanding of the impact of the biodegradation process on the soil environment.
5. Contribution to Sustainable Environmental Management: By addressing the remediation of SEO-polluted soil, the research contributes to the field of sustainable environmental management. The successful bioremediation approach presented in the study offers a practical and eco-friendly solution for restoring contaminated soils and mitigating the environmental impacts of spent engine oil pollution.

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APPENDICES

Appendix I: Physicochemical Properties

sample	pH	Moisture (%)	Crude Nitrogen (%)	Organic carbon (%)	Phosphorus (%)	Particle size (mm)	Week
N	7.70	2.00	0.644	0.68	0.58	0.075	
C	8.98	1.00	0.784	0.70	0.57	0.075	
OPP_1	8.51	1.50	0.484	0.67	0.57	0.074	
OPP_3	8.29	0.50	0.663	0.65	0.55	0.074	
KG_2	8.30	1.00	0.548	0.66	0.56	0.075	**Week 0**
C	9.64	0.05	0.616	0.70	0.56	0.075	
OPP_1	7.88	1.00	0.392	0.66	0.55	0.074	
OPP_3	8.00	1.00	0.560	0.63	0.55	0.074	
KG_2	7.20	0.50	0.434	0.65	0.55	0.074	**Week 2**
C	10.20	0.49	0.392	0.68	0.55	0.074	
OPP_1	7.20	0.98	0.364	0.65	0.55	0.074	
OPP_3	7.80	1.00	0.420	0.62	0.50	0.074	**Week 4**

KG_2	6.70	0.38	0.784	0.63	0.50	0.074	
C	10.80	0.35	0.255	0.65	0.54	0.074	
OPP_1	6.60	0.95	0.341	0.64	0.55	0.074	
OPP_3	7.45	1.00	0.390	0.60	0.45	0.074	
KG_2	6.10	0.29	0.684	0.61	0.50	0.073	**Week 6**

Appendix II: One Way ANOVA on Physicochemical Properties

```
DATASET ACTIVATE DataSet4.

MEANS TABLES=phValue MoistureValue CrudeNValue OrganicValue PhosphorusValue ParticleSizeValue BY

    Isolate

  /CELLS=MEAN COUNT STDDEV

  /STATISTICS ANOVA.
```

Means

Notes

Output Created		02-May-2022 04:16:25
Comments		
Input	Active Dataset	DataSet4
	Filter	<none>
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	N of Rows in Working Data File	17

Missing Value Handling	Definition of Missing	For each dependent variable in a table, user-defined missing values for the dependent and all grouping variables are treated as missing.
	Cases Used	Cases used for each table have no missing values in any independent variable, and not all dependent variables have missing values.
Syntax		MEANS TABLES=phValue MoistureValue CrudeNValue OrganicValue PhosphorusValue ParticleSizeValue BY Isolate /CELLS=MEAN COUNT STDDEV /STATISTICS ANOVA.
Resources	Processor Time	00:00:00.02
	Elapsed Time	00:00:00.02

[DataSet4]

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
phValue * Isolate	17	100.0%	0	0.0%	17	100.0%
MoistureValue * Isolate	17	100.0%	0	0.0%	17	100.0%
CrudeNValue * Isolate	17	100.0%	0	0.0%	17	100.0%
OrganicValue * Isolate	17	100.0%	0	0.0%	17	100.0%
PhosphorusValue * Isolate	17	100.0%	0	0.0%	17	100.0%
ParticleSizeValue * Isolate	17	100.0%	0	0.0%	17	100.0%

Report

Isolate		phValue	MoistureValue	CrudeNValue	OrganicValue	PhosphorusValue	
C	Mean	9.9050	.4725	.5117	.6825	.5550	
	N	4	4	4	4	4	

	Std. Deviation	.77758	.39668	.23470	.02363	.01291	
KG2	Mean	7.0750	.5425	.6125	.6375	.5275	
	N	4	4	4	4	4	
	Std. Deviation	.93229	.31690	.15335	.02217	.03202	
N	Mean	7.7000	2.0000	.6440	.6800	.5800	
	N	1	1	1	1	1	
	Std. Deviation	.	.	.	.	.	
OPP1	Mean	7.5475	1.1075	.3953	.6550	.5550	
	N	4	4	4	4	4	
	Std. Deviation	.82774	.26247	.06273	.01291	.01000	
OPP3	Mean	7.8850	.8750	.5083	.6250	.5125	
	N	4	4	4	4	4	
	Std. Deviation	.35294	.25000	.12701	.02082	.04787	
Total	Mean	8.0794	.8229	.5150	.6518	.5400	
	N	17	17	17	17	17	

Std. Deviation	1.26600	.48047	.15974	.02877	.03335	

Report

Isolate		ParticleSizeValue
C	Mean	.0745
	N	4
	Std. Deviation	.00058
KG2	Mean	.0740
	N	4
	Std. Deviation	.00082
N	Mean	.0750
	N	1
	Std. Deviation	.
OPP1	Mean	.0740
	N	4
	Std. Deviation	.00000
OPP3	Mean	.0740
	N	4
	Std. Deviation	.00000
Total	Mean	.0742
	N	17
	Std. Deviation	.00053

ANOVA Table

		Sum of Squares	df	Mean Square		
phValue * Isolate	Between Groups (Combined)	18.793	4	4.698		
	Within Groups	6.851	12	.571		
	Total	25.644	16			
MoistureValue * Isolate	Between Groups (Combined)	2.526	4	.632		
	Within Groups	1.168	12	.097		
	Total	3.694	16			
CrudeNValue * Isolate	Between Groups (Combined)	.112	4	.028		
	Within Groups	.296	12	.025		
	Total	.408	16			
OrganicValue * Isolate	Between Groups (Combined)	.008	4	.002		
	Within Groups	.005	12	.000		
	Total	.013	16			

PhosphorusValue * Isolate	Between Groups	(Combined)	.007	4	.002		
	Within Groups		.011	12	.001		
	Total		.018	16			
ParticleSizeValue * Isolate	Between Groups	(Combined)	.000	4	.000		
	Within Groups		.000	12	.000		
	Total		.000	16			

ANOVA Table

			F	Sig.
phValue * Isolate	Between Groups	(Combined)	8.230	.002
	Within Groups			
	Total			
MoistureValue * Isolate	Between Groups	(Combined)	6.491	.005
	Within Groups			
	Total			
CrudeNValue * Isolate	Between Groups	(Combined)	1.138	.385

	Within Groups			
	Total			
OrganicValue * Isolate	Between Groups	(Combined)	5.029	.013
	Within Groups			
	Total			
PhosphorusValue * Isolate	Between Groups	(Combined)	1.967	.164
	Within Groups			
	Total			
ParticleSizeValue * Isolate	Between Groups	(Combined)	1.471	.272
	Within Groups			
	Total			

Measures of Association

	Eta	Eta Squared
phValue * Isolate	.856	.733
MoistureValue * Isolate	.827	.684
CrudeNValue * Isolate	.524	.275
OrganicValue * Isolate	.791	.626
PhosphorusValue * Isolate	.629	.396
ParticleSizeValue * Isolate	.574	.329