ISOLATION AND CHARACTERIZATION OF PHENANTHRENE DEGRADING *Bacillus subtilis* FROM HYDROCARBON POLLUTED SOIL IN ZARIA, KADUNA STATE NIGERIA

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A MASTER OF SCIENCE DEGREE IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY,
FACULTY OF LIFE SCIENCES,
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ZARIA, NIGERIA

July, 2021

DECLARATION

I declare that the work in this dissertation entitled ‘’Isolation and Characterization of Phenanthrene Degrading *Bacillus subtilis* from Hydrocarbon Polluted Soil in Zaria Kaduna state Nigeria’’ has been carried out by me in the Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria under the supervision of Prof I.O Abdullahi and Dr M.B Tijjani.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation has been previously presented for another degree or diploma at this or any other institution.

Usman Olayinka Odunola	……	…….
Name	Signature	Date

CERTIFICATION

This dissertation entitled ‘’Isolation and Characterization of Phenanthrene Degrading *Bacillus subtilis* from Hydrocarbon Polluted Soil in Zaria Kaduna State Nigeria ‘’by Usman Olayinka Odunola meets the regulation governing the award of degree of Master of Science of the Ahmadu Bello University Zaria, and is approved for its contribution to knowledge and literally.

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DEDICATION

I dedicate this work to God Almighty, the Sustainer of life and my Mother Mrs Maureen Usman.

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are pollutants that accumulate in the soil and sediments due to their insolubility in water and lack volatility, and thus require eco-friendly remedial strategies. A bacterial strain isolated and characterized from hydrocarbon polluted soil was identified as *Bacillus subtilis* and studied for its ability to degrade phenanthrene as sole source of carbon and energy. The colour of colonies changed to navy blue on plate with indole indicating the presence of dioxygenase enzyme. The population density of *Bacillus subtilis* increased from 1.2×10^{6} to 8.1×10^{7}cfu/ml on phenanthrene within 20 days. A significant positive relationship was observed between *Bacillus subtilis* and dilutions factors (10^{-4}-10^{-8}) in days. Gas chromatography readings were taken on days (0. 10 and 20) to analyze the degradation ability of *Bacillus subtilis* on phenanthrene at concentration 100mg/l. The percentage degradation of phenanthrene was 67.61%. The rate of degradation decreased from 5.15 mg L^{-1} day^{-1} to 4.35 mg L^{-1} day^{-1} with degradation constant rate ranging from 0.051K to 0.056K and half life 13.58 days and 12.38 for 10 and 20 days. This study reveals that the *Bacillus subtilis* isolated has the potential of degrading PAHs and can be useful in decommissioning environment with toxic compounds effectively.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Polycyclic aromatic hydrocarbons (PAHs) are chemical compounds containing only carbon and hydrogen that is composed of multiple benzene rings (Smith, 1990). They are released to the environment as a result of the incomplete combustion of fossils fuels or by accidental discharge during the transport, use and disposal of petroleum products (Smith, 1990). Phenanthrene a major pollutant is the smallest polycyclic aromatic hydrocarbon to have a 'bay and a kay' region that are highly reactive. The epoxides formed at the 'bay and kay' region are as reactive as the parent compound such as Pyrene and Anthracene which are suspected to be carcinogens (USEPA, 1986). PAHs constitute a group of priority environmental pollutants, which are ubiquitous contaminants in soils and sediments. They are of environmental concern, because of their toxic, mutagenic or carcinogenic effects (Santa *et al.,* 2002). In recent years, the biodegradation of PAHs has received considerable attention and a variety of microorganisms have been reported to play important roles including species of *Pseudomonas, Alcaligenes, Mycobacterium, Rhodococcus, Sphingomonas* (Han *et al.*, 2016, Zhao *et al.*, 2019).

Anthropogenic activities, particularly oil spills, engine oil spills, domestic combustion of wood, coal, and bush burning are significant sources of polycyclic aromatic hydrocarbons released into the environment. PAHs enter the environment through the air as the sooty part of smoke or ash, then absorbed to particulate matters and get deposited into the lithosphere and hydrosphere as a result soil concentration tended to increase particularly in urban areas (Wilson and Rose, 2007). Human exposure to PAHs is via inhalation, ingestion, or dermal contact with high risks to public health. Exposure to PAHs can also occur if the skin touches contaminated soil or product like

heavy oils, coal tar, roofing tar or creosote. Once the PAHs enter human body, they can spread and target fat tissues, organs which include kidneys, lungs and liver (Zhao *et al.*, 2009).

PAHs released into the environment could be removed by many process including volatilization, adsorption of soil particles, however the process for successful removal and elimination of PAHs from the environment is microbial transformation and degradation (Wilson and Rose, 2007). In addition, the use of microorganisms could offer advantages for several reasons because they are adapted to the contaminated environment, thus allowing the innoculum to survive (Kazuga *et al.*, 2001) and microorganisms are able to extend through the soil by propagation, because bacteria can access xenobiotics (Eniola *et al.*, 2014).

Recently, attention has been turned towards diverse PAHs metabolizing bacteria degrading mechanism (Pinyakong *et al.,* 2014). In order to achieve an efficient bioremediation process, it is interesting to find new bacteria that can degrade PAHs in a new pathway without accumulating potentially toxic metabolites (Obayori *et al.*, 2008). Therefore the present study aims to investigate the biodegradation potentials of indigenous bacteria isolated from hydrocarbon polluted soil that can degrade phenanthrene and render it less toxic to the environment.

1.2 Statement of the Research Problem

Polycyclic aromatic hydrocarbons are pollutants produced via natural and anthropogenic sources, generally created during incomplete combustion of solid and liquid fuels. Phenanthrene is known to cause hazardous effects on humans and animals, plants, ecosystem and agriculture by reducing soil fertility and low production yield as a result of contaminated soil (Obayori *et al.*, 2008). In Zaria, Kaduna State Nigeria, there are large numbers of hydrocarbon polluted soils which are contaminated by phenanthrene. This poses risks to human or animal health. Thus,

there is need for a robust, efficient, eco- friendly removal of PAHs through microbial transformation and degradation from the environment.

1.3 Justification of the Study

The chemical methods to remove hydrocarbon pollutants in general are too complex, expensive, and environmentally unfriendly and do not ensure total elimination of the pollutants and frequently result in the introduction of new toxic substances into the environment (Obayori *et al.*, 2008). There is therefore the need to develop an efficient, cost effective, sustainable and eco-friendly approach for removing polyaromatic hydrocarbon from polluted soil (Stanley *et al.*, 2015). A limited number of PAHs degrading microorganisms have been reported but due to lack of understanding about their degradative mechanism, this study seeks to isolate indigenous bacteria strain with efficient degradation potentials for phenanthrene that can be used as a tool for bioremediation.

1.4 Aim of the Study

The aim of this study was to isolate and characterize phenanthrene degrading *Bacillus subtilis* from hydrocarbon polluted soil in Zaria, Kaduna State Nigeria

1.5 Objectives of the Study

The objectives of this study were to:

1. determine the physicochemical and microbiological parameters of the soil in the study area
2. determine the total hydrocarbon content and phenanthrene peak in the soil sample used during the study

3. isolate phenanthrene degrading *Bacillus subtilis* from the polluted soil and characterize it based on cultural morphology and biochemical characteristics
4. determine the growth rate and pattern of phenanthrene degradation of *Bacillus subtilis*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Hydrocarbons

Hydrocarbons are organic compounds which contain only carbon and hydrogen. Structurally they can be grouped into three broad classes namely saturated hydrocarbons, unsaturated hydrocarbons and aromatic hydrocarbons (Kazuga *et al.*,2001). Saturated hydrocarbon contains carbon and hydrogen, unsaturated hydrocarbon carbon contains multiple carbon bonds while aromatic are compounds with special cyclic compounds (Kazuga *et al.*,2001). Hydrocarbon are the primary components of crude oil, this is because decomposed organic matters are the parent material for crude oil. Hydrocarbons differ in their susceptibility to microbial attack and, in the past, have generally been ranked in the following order of decreasing susceptibility: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes. Biodegradation rates have been shown to be highest for saturates, followed by the light aromatics, with high-molecular-weight, aromatics and polar compounds exhibiting extremely low rates of degradation (Kazuga *et al.*,2001).

2.2 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons also known as arenes, contain at least one or more aromatic ring specifically benzene rings. Aromatic hydrocarbons range from monocyclic aromatic hydrocarbon which consists of only one benzene ring to the polycyclic aromatic hydrocarbon where benzene rings are fused together in different arrangements (Margesin *et al.,* 2003). Based on the number of fused benzene rings, PAHs are classified into low molecular weight compounds and high molecular weight compound. PAHs are uncharged, non polar molecules

found in coal and tar deposits, they are majorly produced by thermal decomposition of organic matter, they are generally toxic to the environment and have accumulated in the environment due to their ubiquitous existence (Eniola *et al.*, 2014).

Different aromatic compounds co-exist as complex mixtures in petroleum refinery and distillation sites. There are three major categories: polycyclic aromatic hydrocarbons (PAHs), Heterocyclics, and Substituted aromatics. PAHs are a group of chemicals that contain two or more fused aromatic rings in linear, angular, or cluster arrangements (Keum *et al.,* 2006).

Physical and chemical properties of PAHs vary with the number of rings and hence their molecular weight. Chemical reactivity, aqueous solubility and volatility of PAHs decrease with increasing molecular weight. As a result, PAHs differ in their transport, distribution and fate in the environment and their effects on biological systems.

The USEPA has identified 16 PAHs as priority pollutants (Figure 2.1). Some of these PAHs are considered to be possible or probable human carcinogens, and hence their distributions in the environment and possible exposure to humans have been of concerns (Eniola *et al.,* 2014).

High molecular-weight PAHs are given more attention as they are recalcitrant. In general, PAHs are relatively stable and recalcitrant in soils and less easy to degrade than many other organic compounds (Margesin *et al.,* 2003). They are difficult to remove from contaminated soil using the treatments that have been used successfully to clean soils contaminated with more degradable or volatile organic compounds such as alkanes (Gratia *et al.,*2006).

Three major sources of PAHs are

I. Petrogenic
II. pyrogenic
III. biogenic

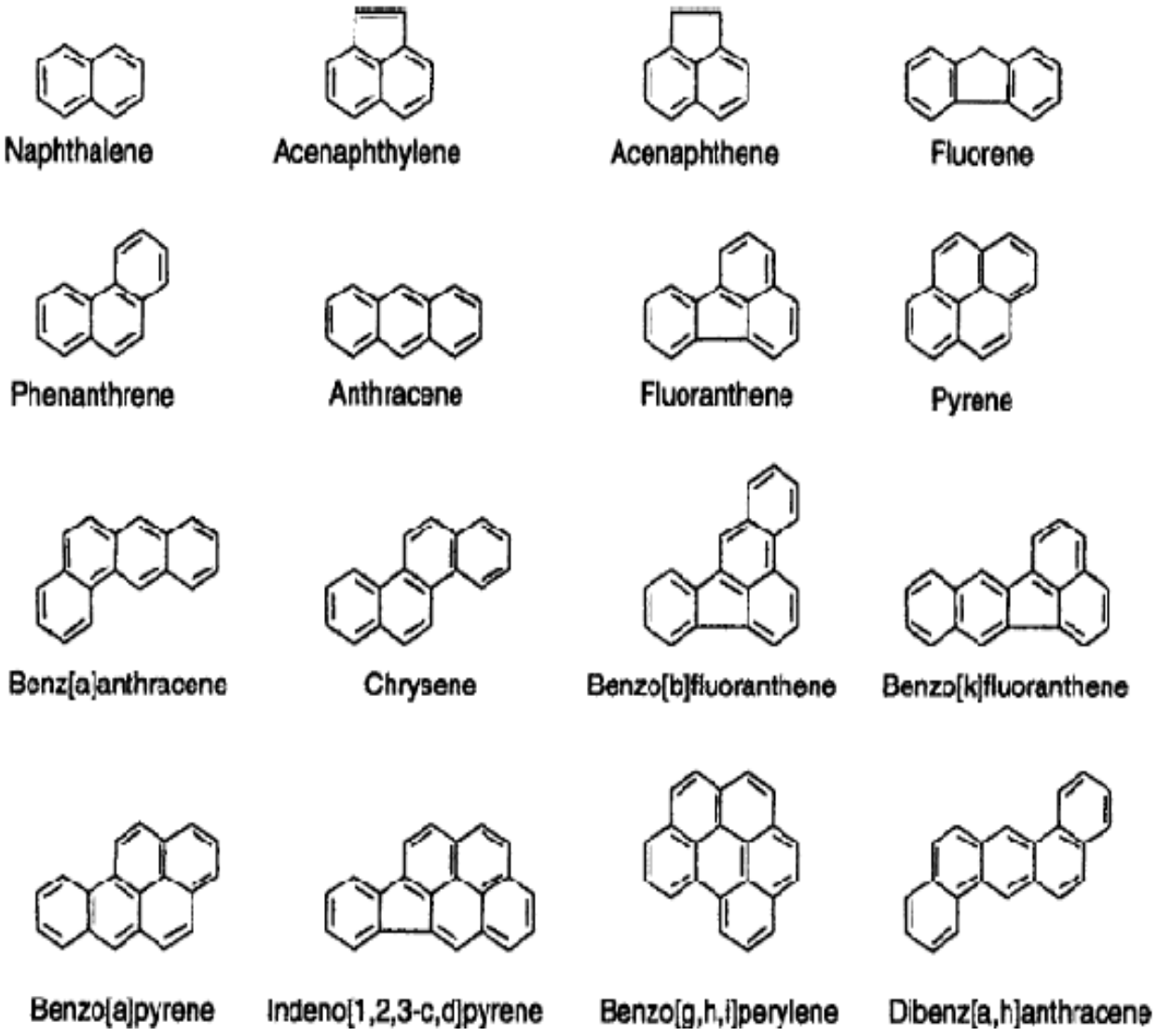


Figure 2 1: Chemical structures of some common polycyclic aromatic compounds (USEPA, 1986).

Petrogenic PAHs are from petroleum and petroleum-derived products, and are often marked as abundance of alkyl-substituted PAHs such as alkyl naphthalenes, alkyl phenanthrenes and alkyl dibenzothiophenes. Pyrogenic PAHs are produced from combustion processes and are comprised of predominantly unsubstituted PAHs. Biogenic aromatic compounds including aromatic amino acids, lignin compounds and their derivatives are of biotransformation origin (Kim *et al* ., 2003). Exposures from various occupations and high levels of pollutant mixtures containing PAHs are known to result in symptoms (Table 2.1) such as eye irritation, nausea, vomiting and diarrhea. Mixtures of PAHs are also known to cause skin irritation and inflammation.

Phenanthrene, Anthracene, benzo(a)pyrene, and naphthalene are direct skin irritants and skin sensitizers i.e. major cause of an allergic skin response in animals and humans (Cocker *et al.,* 2006).

2.3 Physical Properties of PAHs

The fused benzene rings in PAHs are in linear (anthracene), angular (phenanthrene) or clustered (dibenzopyrene) arrangements. PAHs are hydrophobic and have electrochemical stability which makes them persistence in the environment (Cerniglia and Shuttleworth 2002).

Generally PAHs solubility decreases and hydrophobicity increases with an increased number of benzene fused rings, further more volatility decreases with an increase number of fused benzene rings (Margesin *et al.,* 2003). Polycyclic aromatic compounds are also lipophilic which gives them a place in the environment, primarily in soil and sediment. PAHs have characteristics UV Absorbance spectra with many bands each unique for each ring structure thus each isomer has a different UV absorbance spectrum (200nm-400nm) which helps in the identification of PAHs (Cerniglia and Shuttleworth 2002).

Table 2.1: Hazardous effects of Polyaromatic hydrocarbons

Sector	Hazardous effects
Agriculture	Soil fertility reduces, physiological properties and have adverse effect on seed germination
Human	It causes skin cancer, sinonasal cancer, lungs cancer and adverse effects on central nervous system which leads to eye irritation, nausea, vomiting and diarrhea
Ecosystem	Imbalance of the eco system and food chain, loss of biodiversity
Plant and animal	Loss of chlorophyll and disease such as cancer and central nervous system disorders

Source: (Cocker *et al.*, 2006).

2.4 Environmental Fate of PAHs

PAHs released to the atmosphere are subject to short and long range transport and are removed by wet and dry deposition onto soil, water and vegetation through volatilization. In surface water, PAHs can photolyse, oxidize, bind to suspended particles, sediments or accumulate in aquatic organisms (Margesin *et al.,* 2003). In sediments, PAHs can biodegrade or accumulate in aquatic organism through spillage (Obayori *et al.,* 2013) whereas PAHs in soil can volatilize and undergo abiotic degradation such as photolysis and oxidation. They can also enter ground water and be transported within the aquifer (Cerniglia and Shuttleworth 2002).

PAHs may accumulate in high concentrations in terrestrial environments near coal gasification sites and tar oil distillation plants. Major sources of PAHs are incomplete combustion of organic materials, gas production, wood treatment facilities, and waste incineration. PAHs are formed naturally during thermal geologic reactions associated with fossil-fuel and mineral production, and during burning of vegetation in forest and bush fires (Margesin *et al.,* 2003). Anthropogenic sources, particularly fuel combustion, automobiles, spillage of petroleum products, and waste incinerators are significant sources of PAHs into the environment (Cerniglia and Shuttleworth 2002).Tobacco cigarette smoking is a significant source of PAH exposure to smokers and secondary smokers. PAHs generated during anthropogenic combustion activities are primarily transported via atmospheric deposition (Cerniglia and Shuttleworth 2002). Transport of PAHs in the environment is determined to a large extent by chemical properties such as water solubility, vapour pressure, Henry' law constant, octanol- water partition coefficient and organic carbon partition coefficient (Margesin *et al.,* 2003).

Table 2.2: Chemical properties of Polyaromatic hydrocarbons

PAHs	Mass (Da)	Vapour pressure (Pa)	Log K_{ow}	Solubility
Naphthalene	128.18	12.0	3.58	30
Acenaphthene	154.20	4.02	3.92	3.6
Phenanthrene	178.24	0.0161	4.46-4.63	1-2
Fluorene	166.23	0.13	4.18	2.00
Anthracene	178.24	0.001	4.45	0.015
Pyrene	202.26	0.0006	5.88-6.7	0.12-0.18
Chysene	228.30	6.08×10^{-7}	5.01-7.10	0.00015
Fluoranthene	202.26	0.001	5.22	0.25
Benz(a)anthracene	228.30	2.0×10^{-5}	5.99	0.01
Acenaphthylene	155.20	3.87	3.90	3.88
Benzo(a)pyrene	252.32	7.0×10^{-7}	5.78-6.5	0.001-0.006

Keys: K_{ow} : Octanol water coefficient; Da=Dalton, Pa=pressure

Source: Cerniglia and Shuttleworth, 2002

Petroleum refining and transport activities are major contributors to localized loadings of PAHs into the environment. Such loadings may occur through discharge of industrial effluents and through accidental release of raw and refined products. Heterocyclic compounds including dibenzothiophene and carbazole are components of creosote, crude oils, and shale oils and often co-exist in the environment with PAHs and other aromatic compounds (Lee *et al.,* 2007).

Dibenzothiophene is a sulfur heterocyclic compound and is quite persistent in the environment. Carbazole, a nitrogen heterocyclic is carcinogenic and toxic. Dibenzofuran and its substituted analogues are found in several woody plants as stress chemicals, and are called phytoalexins. However, most of the environmental concerns with dibenzofuran are related to its halogenated analogues, especially its chloro/bromo derivatives (Cerniglia and Shuttleworth 2002).

2.5 Biodegradation of Polyaromatic Hydrocarbons

Biodegradation of PAHs is catalysed by multi component enzymes from microbes, a key enzymes for attacking the aromatic structure of PAHs under aerobic condition is the initial dioxygenase which is substrate specific. Most PAHs undergo biodegradation which involves the biological mediated breakdown of complex substances to simplier forms which often time yield to Carbondioxide, Water and cell biomass as products of complete minerlization of the pollutant (Gratia *et al.,*2006). Ring cleavage play an important role in the degradation of aromatic compounds, they catalyse the incorporation of two atoms of molecular oxygen into substrate (Gratia *et al.,*2006).

Biodegradation is the ultimate fate of hydrocarbons released into the environment, although it is mostly a slow process. It is the process whereby microorganisms (especially bacteria, yeasts, fungi, algae) chemically transform compounds such as petroleum hydrocarbons into simpler

products (Gratia *et al.*,2006). This can be done both aerobically and anaerobically. Aerobically, these organisms share the ability to insert one or two of the oxygen atoms of diatomic O_2 into a hydrocarbon molecule, thereby activating and making it accessible to the central metabolism of the organism (Kazuga *et al.*, 2001). The oxygenated hydrocarbon then serves as a source of reductant for the growth of the organism, and oxygen is required as the terminal oxidant (Hasanshahian *et al.*, 2008). Anaerobically, the hydrocarbon still acts as a reductant, and in the absence of oxygen something else must serve as a terminal electron acceptor such as sulfate, nitrate, and ferric ions (Yakimov *et al.*, 2003).

Microbes typically have a carbon: nitrogen: phosphorus ratio of approximately 100:10:1, but crude oil contains no significant amounts of either nitrogen or phosphorus (Matthew *et al.*, 2001). Oil-degrading microbes must therefore obtain these elements in useful forms from elsewhere in the environment, and if there is a significant amount of hydrocarbon it is likely that the growth of the degrading organisms will be limited by the supply of these nutrients (Wilkes *et al.*, 2002).

2.6 Metabolism of Phenenthrene

Phenanthrene is a low molecular weight which is a three ring angular poly aromatic hydrocarbon and a major constituents of coal derivatives and oil fuels. It is known to be a human skin photosensitizer, an inducer of sister chromatid exchanger and a potent inhibitor of gap junctional intercellular communication (Samanata *et al.*, 2002). It has mass of 178.24 Da vapour pressure of 0.0161 pa, its octanol water coeffifient ranges from 4.46-4.63 and its solubility also from 1-2 (Cerniglia and shuttleworth, 2002). The catabolism of phenanthrene by bacteria is metabolized to 1-hydroxy 2 napthoic acid (IN2HN) by initial dioxygenation which is further degraded through

two dihydroxynapthalene and is later converted to salicylic acid. Salicylic acid is further degraded via the formation of either catechol or gentisic acid. Both catechol and gentistic acid undergo ring fission to form Tri carboxylic acid (TCA) cycle intermediates. In another pathway acid, 1N2HN undergoes ring cleavage leading to formation of ophthalic acid, which is further converted to protocatechuic acid which is finally cleaved to form pyruvic acid and ultimately enters the TCA cycle (Habe and Omori, 2002). The degradation normally involves the formation of the cis-diol that spontaneously converts into catechol. This catechol is subsequently subjected to two types of aromatic ring cleavage pathways called 'ortho' and 'meta' cleavages that are catalyzed by dioxygenase enzymes. In the ortho pathway, catechol, in the presence of the enzyme 1, 2- dioxygenase is converted to cis, cis-muconic acid that is further transformed to β-ketoadipic acid. This product is cleaved to produce succinate and acetyl CoA, intermediates of the tricarboxylic acid (TCA) cycle (Margesin *et al.,* 2003).

In the meta pathway, the enzyme 2, 3-dioxygenase cleaves the aromatic ring of catechol in the 2,3 position to produce an intermediate called hydroxymuconicsemialdehyde (Habe and Omori, 2002) which proceeds to give a mixture of pyruvate and acetaldehyde as final products. The breakdown products of the ‘ortho’ and ‘meta’ pathways are then utilised by the organism for biosynthesis of cell materials or oxidized to carbon dioxide (CO_2) yielding energy (Habe and Omori, 2002). In biotransformation process, biological agents like micro organism convert the complex organic contaminants to other simpler organic compound mostly to non toxic or less toxic substance which can be finally mineralized by other organism to carbondioxide, water and inorganic compounds in co- metabolism (Medina Bellver *et al.*,2005).

Ring cleaving dioxygenase play an important role in the degradation of phenanthrene. They catalyse the incorporation of two atoms of molecular oxygen into substates. Based on the mode

of ring cleavage, they are grouped as extradiol dioxygenase which require non heam Fe II and cleave the aromatic ring proximal to one semialdehyde and the other is intradiol dioxygenase which require non heam Fe III which cleave the aromatic ring between hydroxylated carbon atoms yielding a muconic acid. Apart from dioxygenation of phenanthrene at the 3,4- position, there are reports of phenanthrene degradation involving initial dioxygenation at the 9,10-position of phenanthrene (Sutherland *et al.,* 1990; Narro *et al.,* 1992; Moody *et al.,* 2001).

In addition, there are a few reports on the assimilation of phenanthrene via initial dioxygenation at the 1,2-position of phenanthrene. Jerina *et al.*,(1976) detected cis-1,2-phenanthrenedihydrodiol as a minor pro- duct of phenanthrene degradation in the mutant strains *Beijerinckia* B-836 and *Pseudomonas putida*. Moreover, dioxygenation at the 1,2-position of phenanthrene by *Burkholderia* sp, *Sphingomonas* sp P2, *Mycobacterium vanbaalenii* PYR-1 and *Sinorhizobium* sp (Balashova *et al.,* 1999; Pinyakong *et al.,* 2003; Keum *et al.,* 2006; Kim *et al.,* 2007). (Figure 2.2)

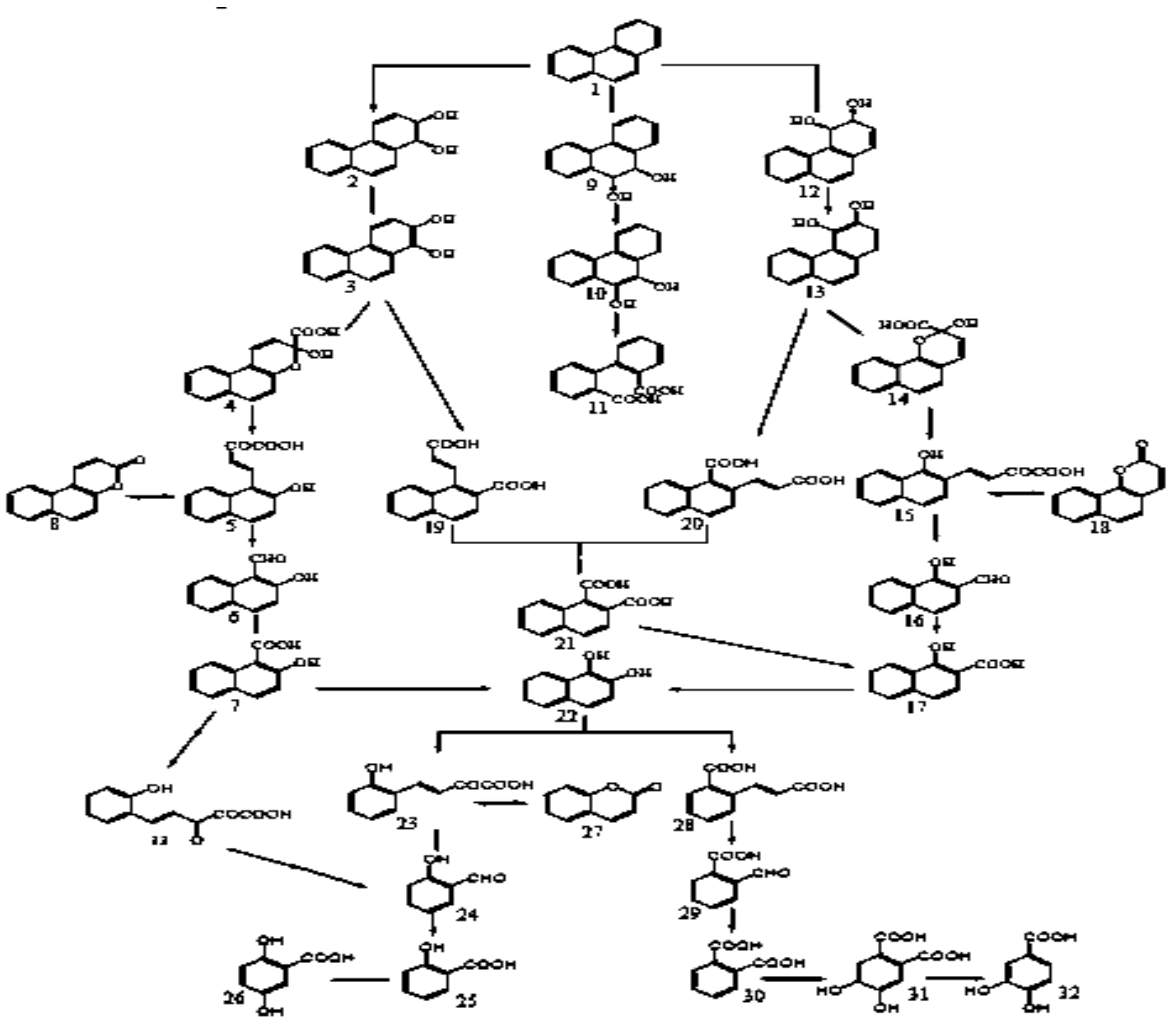


Figure 2.2: Metabolic pathways for the degradation of phenanthrene in bacteria (Samanta *et al*., 1999).

Keys: Compound designations: 1, phenanthrene; 2, cis-1,2-dihydroxy-1,2-dihydrophenanthrene; 3, 1,2-dihydroxyphenanthrene; 4, 3-hydroxy-3H-benzo[f]chromene-3-carboxylic acid; 5, 4-(2-hydroxy-naphthalen-1- yl)-2-oxo-but-3-enoic acid; 6, 2-hydroxy-naphthalene-1-carbaldehyde; 7, 2-hydroxy-1-naphthoic acid; 8, 5,6-benzocoumarin; 9, cis-9,10-dihydroxy-9,10-dihydrophenanthrene; 10, 9,10-dihydroxyphenanthrene; 11, 2,2'-diphenic acid; 12, cis-3,4-dihydroxy-3,4-dihydrophenanthrene; 13, 3,4-dihydroxyphenanthrene; 14, 2-hydroxy- 2H-benzo[h]chromene-2-carboxylic acid; 15, 4-(1-hydroxynaphthalen-2-yl)-2-oxo-but-3-enoic acid; 16, 1- hydroxy-naphthalene-2-carbaldehyde; 17, 1-hydroxy-2-naphthoic acid; 18, 7,8-benzocoumarin; 19, 1-(2- carboxy-vinyl)-naphthalene-2-carboxylic acid; 20, 2-(2-carboxy-vinyl)-naphthalene-1-carboxylic acid; 21, naphthalene-1,2-dicarboxylic acid; 22, naphthalene-1,2-diol; 23, 2-hydroxybenzalpyruvic acid; 24, salicylic aldehyde; 25, salicylic acid; 26, gentisic acid; 27, coumarin; 28, 2-carboxycinnamic acid; 29, 2-formylbenzoic acid; 30, phthalic acid; 31, 3,4-dihydroxyphthalic acid; 32, protocatechuic acid; 33, trans-2,3-dioxo-5-(2'- hydroxyphenyl)-pent-4-enoic acid.

2.7 Environmental Factors Affecting Phenanthrene Degradation

Microbial degradation of Phenanthrene in the environment is largely determined by a combination of abiotic factors, which limit the rate of degradation. Such factors include temperature, oxygen, nutrients (nitrates and phosphates), pressure, salinity and chemical composition of the pollutant (Habe and Omori, 2002). Microorganisms usually bacteria utilize the Phenanthrene as a sole source of carbon and energy in a process known as bioremediation. In bioremediation process, the micro organism undergo acclimatization which start after prolong exposure to the polluting compounds and eventually consequence is the degradation of the pollutant (Jain and Bajpai, 2005). The optimization of biodegradation process involves many factors among which are existences of microbial consortia capable of degrading the pollutant, the bioavailability of the contaminant to microbial attack and certain abiotic factors such as soil type, temperature, soil pH, oxygen level of the soil, electron acceptor agents, nutrient content of the soil which contributes to the microbial growth (Pukall *et al.,* 2001).

2.7.1 Temperature

Temperature affects the rate of Phenanthrene degradation by stimulating evaporation and weathering of lighter components which may be toxic to exposed microbial strains (Nakamuraa *et al.,* 2007). Phenanthrene degradation is faster at the mesophilic temperature of $25^{O}C$, However, some micro-organisms associated with the deep sea and ice water are known to degrade Phenanthrene under low temperature conditions and such organisms are very sensitive to high temperature conditions (Pukall *et al.,* 2001). Temperature also affects enzyme activities and thus rate in the metabolic pathway (Margesin *et al.,* 2003).

2.7.2 Oxygen

The initial steps in the degradation of Phenanthrene by bacteria involve the oxidation of the substrate by oxygenating enzymes for which molecular oxygen is needed (Margesin *et al.,* 2003). Availability of oxygen in soils, sediments is low thereby requiring artificial aeration, for the purpose of rehabilitating those areas after an oil spillage. Aerobic degradation is more rapid while anaerobic biodegradation are slow and often relying on a consortium of bacteria (Margesin *et al.,* 2003).

2.7.3 Nutrients

Crude oil supplies excess of carbon but the microorganism also requires other elements for growth, especially nitrogen and phosphorus that are limiting in the soil environment. The possibility of adding mineral nutrients in the form of oleophilic fertilizers had been considered in pilot and actual field situations for oil spill abatement (Lee *et al.,* 2007). Biodegradation is enhanced by nitrate and phosphates in sites only when the background nutrients are insufficient so addition of nutrient may have a minor effect (Margesin *et al.,* 2003).

2.7.4 Salinity

The effect of salinity on Phenanthrene degradation has been studied in polluted soil and sediment and the results showed a general decrease in the rate of Phenanthrene metabolism with increase in salinity and it also affects the osmotic pressure in bacterial cell (Malatova *et al.,* 2005).

2.7.5 Pressure

High pressure in deep sea environments reduces the biological activity of microorganisms. Hydrocarbon compounds in the deep sea environment are degraded slowly and persist for decade (Margesin *et al.,* 2003).

2.7.6 Bioavailability

Microorganism main metabolic process is via soluble PAHs which are limited by low solubility and strong sorption in micropores or organic matter. Addition of biosurfactant or homogenization can improve PAHs transport to degraders (Margesin *et al.,* 2003).

2.8 Microorganisms in Phenanthrene Degradation

A large number of bacteria that metabolize phenanthrene have been isolated such as *Alcaligenes denitrificans, Rhodococcus* sp, *Pseudomonas* sp, *Mycobacterium* sp and *Bacillus* sp (Margesin *et al.,* 2003). A variety of bacteria can degrade phenanthrene completely to CO_2 and metabolic intermediate. Phenanthrene degrading microorganisms display various physiological adaptations for survival in contaminated soils, such as high specific affinities for Poly aromatic hydrocarbon or biofilm formation on PAH contaminated soil. Various bacteria have been reported by several researchers to be involved in the degradation of phenanthrene as shown in the (Table 2.3).

Table 2.3: Selected Phenanthrene Degrading Microorganisms

Phenanthrene degrading microorganisms	References
Proteus mirabilis strain 10c	Obayori *et al.*, 2017
Sphingomonas sp HS362	Hwa *et al.*, 2005
Pseudomonas sp ARP26	Coral *et al.*, 2005
Staphylococcus sp PN/Y	Malick *et al.*, 2007
Bacillus subtilis MI6K and M19F	Oyetibo *et al.*, 2017
Arthrobacter sulphureus RKJ4	Samanta *et al.*, 1999
Mycobacterium sp PYR1	Kelly *et al.*, 2007
Burkholderia cepacia BU3	Kim *et al .*, 2003
Pseudomonas putida P16	Seo *et al.*, 2007
Sphingomonas paucimobilis EPA 505	Story *et al.*, 2001
Pseudomonas aeruginosa	Weissentels *et al.*, 1990
Acidovoras delafiedii P41	Samanta *et al.*, 1999
Brevibacterium sp HL4	Samanta *et al.*, 1999
Mycobacterium vanbaalenii PY9	Kim *et al.*, 2007

2.9 Laboratory Techniques in Bioremediation of Phenanthrene

These techniques involve treating contaminated soil at the site of pollution. It does not require any excavation. It is accompanied by little or no disturbance to soil structure. Some in situ bioremediation techniques might be enhanced such as bioventing and biosparging while others are intrinsic bioremediation or natural attenuation (Eniola *et al.,* 2014).

2.9.1 Bioventing

This technique involves controlled stimulation of air flow by delivering oxygen to unsaturated zone in the contaminated soil in order to increase bioremediation, by increasing activities of indigenous microbes. In bioventing, amendments are made by adding nutrients and moisture to enhance bioremediation with the ultimate goal to achieve microbial transformation of pollutants to harmless state.

Frutos *et al.,* (2010) reported the effectiveness of bioventing treatment in remediation of phenanthrene contaminated soil and recorded 93 % contaminant removal after seven months.

2.9.2 Bioslurping

This technique combines vacuum enhanced pumping, soil vapour extraction and bioventing to achieve bioremediation of contaminated soil. It can also be used to remediate soils contaminated with volatile and semi-volatile organic compounds (Eniola *et al.,* 2014).

2.9.3 Biosparging

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

unlike bioventing air is injected at the saturated zone, which can cause upward movement of volatile organic compounds to the unsaturated zone to promote biodegradation. The effectiveness of biosparging depends on two major factors which are soil permeability, which determines pollutant bioavailability to microorganisms and pollutant biodegradability (Eniola *et al.*, 2014).

2.9.4 Bioaugmentation

It involves the application of autochthonous or allochthonous wild type or genetically modified microorganisms to polluted hazardous waste sites in order to accelerate the removal of undesired compounds. This approach is to enhance the degree or rate of degradation of the complex pollutants by the addition of pollutant-degrading microorganisms. Enhancing the microorganisms of the contaminated site will not only improve the elimination of the pollutants from the particular site but also at the same time increases the genetic capacity of the desired site (Eniola *et al.*, 2014).

2.9.5 Biostimulation

It is a remediation technique that is highly efficient, cost effective and eco-friendly in nature. Biostimulation refers to the addition of limiting nutrients like phosphorus, nitrogen, oxygen, electron donors to severely polluted sites to stimulate the existing bacteria to degrade the hazardous and toxic contaminants. The addition of limiting nutrients improves the degradation potential of the inhabitant microorganisms (Eniola *et al.*, 2014).

2.10 Analytical Techniques of Bioremediation of Phenanthrene

Analytical techniques for bioremediation studies of phenanthrene contaminated soil includes

2.10.1 Gas Chromatography

Gas chromatography is at technique which uses gas as the mobile phase while the stationary phase can either be solid or non volatile liquid. It is a technique for separating or identifying

components in a mixture. Gas chromatography is divided into three parts, the injector, the column, and the detector. All this three components work together to give the GC its analytical efficiency (Chavez *et al.*, 2004).

2.10.2 High-Performance Liquid Chromatography

It is a specific form of column chromatography generally used to separate, identify, and quantify the active compounds. HPLC mainly utilizes a column that holds packing material (stationary phase), a pump that moves the mobile phase through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvents used. The sample to be analyzed is introduced in small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase. The amount of retardation depends on the nature of the analyte and composition of both stationary and mobile phase. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time (Chavez *et al.*, 2004).

2.10.3 Molecular Analytical Methods

Proteomics and metabolomics have been recently employed in studies of environmental microbiology and have shown their high impact on the field of biodegradation and bioremediation (Chavez *et al.*, 2004). Proteomics is an effective technique to identify proteins and their functions involved in the biodegradation of aromatics while metabolomics can be used to profile degradation products of PAHs and primary metabolites in response to PAH exposures.

A good number of genomic sequences or expressed sequence tags (ESTs) of bacteria are used for effective bioremediation. These include several PAH degrading bacteria in the genus of *Mycobacterium, Acinetobacter, Arthrobacter, and Burkholderia* (Gratia *et al.*, 2006). This

revealed that large changes at genomic and proteomic levels occurred during the PAH catabolism, compared with the catabolism of natural carbon sources. This include up-regulation of the genes related to PAH catabolism, removal of reactive oxygen species, primary carbon metabolism, including one-carbon metabolism, energy production, e.g., Adenosine tri phosphate (ATP) synthesis, various transporters, and synthesis of energy reservoir, e.g., polyphosphate. Many of these phenomena have been demonstrated by specific studies. For example, polyphosphate accumulation during PAHs catabolism was confirmed by electron microscopy (Hanson *et al.*, 1996). The accumulation of polyphosphate and polyphosphate kinase (PPK) under stressed conditions are common in all biota. Up-regulation of superoxide dismutase or catalase/peroxidase is also common phenomena during PAHs or toxic chemical catabolism (Gratia *et al.*, 2006). The activation of one-carbon metabolism is another common phenomenon in bacteria during xenobiotic metabolism. The up- regulation of one-carbon metabolism may be induced by several factors, including limitation of primary metabolite pools, introduction of one carbon unit, e.g., formate in PAH metabolism, and the loss of concerted regulation of general metabolism. Another common phenomenon is the changes of fatty acid content or composition in bacteria (Sokolovska *et al.*, 2003).

As proteomics is advancing rapidly in environmental microbiology, metabolomics will also become a powerful tool. In conjunction with functional genomics, proteomics and metabolomics are becoming indispensable in elucidation of mechanisms in biodegradation and biotransformation of organic pollutants in the environment, particularly for the situations of multiple chemicals and microbial consortia (Sokolovska *et al.*, 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sampling site

The sampling site was polluted mechanic workshop in Samaru Zaria, Kaduna State Nigeria. The coordinates of the site were longititude $5^{o}\,27^{'}\,4^{''}$, N and latitude $2^{o}\,10^{'}\,5^{''}$ E.

3.2 Sample Collection

Soil sample was collected at 5cm at different sampling points using a sterile spoon after clearing debris from the soil surface (Alam *et al.,* 2011). Samples for both physicochemical and microbiological analyses were collected in clean polyethylene bags, labeled and transported to the Environmental Microbiology Laboratory, Department of Microbiology, Ahmadu Bello University, Zaria for subsequent analysis and stored at 4℃.

3.2.1 Sample Preparation

The soil from different sampling points were mixed together to make a composite sample, air dried and debris was removed. The sample was sieved through a 2mm sieve and stored in a cool dry place for biodegradation studies (Prenafeta-Boldu *et al.,* 2004).

3.3 Determination of Physicochemical Parameters of the soil sample

The physicochemical parameters of the soil sample analyzed were pH, Moisture content, Organic matter content, Total nitrogen, Available phosphorus and Potassium, Sodium, Magnesium and Calcium content will be determined according to standard methods.

3.3.1 pH

The pH of the soil was measured using pH meter (Jenway 3510, Jenway, Staffordshire, England) This was done at the site and then confirmed in the laboratory. Twenty grams (20g) of air dried composite soil sample was weighed into a 50ml beaker. To this, 50 ml of distilled water was added, stirred by a glass rod and left to stand for 30 minutes. After 30 minutes, the soil solution was stirred and the pH was measured using an electrode (Olsen *et al.,* 2006).

3.3.2 Moisture Content

Hundred grams (100g) of the soil sample was transferred to tare moisture dry container and covered with the lid. The lid was removed and the container was placed in the drying oven at 75°C for sixteen (16) hours. After the material has dried to a constant weight, the container was removed from the oven and the lid was replaced firmly. The moisture content in mass (w/g) was obtained using this formula below:

$$\text{Mass of water:} \quad (W_w) = W_1 - W_2$$

$$\text{Mass of solid particle:} \quad (W_S) = W_2 - W_C$$

$$\text{Water content (\%):} \quad WC = 100\ (W_w / W_s)$$

Where:

Wc = mass of container and lid, (g)

W_1=mass of container, lid and moist specimen, (g)

W_2= mass of container, lid and oven-dried specimen in (g) (Okalebo *et al.,*2002).

3.3.3 Organic Matter Content

One (1) gram of the soil sample was weighed into 250 ml Erlenmeyer flask, 10 ml of potassium dichromate solution was added using a pipette then 20 ml of concentrated sulphuric acid was added using a measuring cylinder. The flask was swirled and left to stand for 30 minutes. Two hundred (200) ml of distilled water was added slowly followed by the addition of phosphoric acid. To the flasks, one (1) ml of diphenylamine indicator was added and titrated against 0.5N ferrous ammonium sulphate to a green colour end point. A blank was prepared following the same procedure with the exception of soil sample.

$$\% = M \times \frac{V1-V2}{s} \times 0.39 \times mcf$$

Where M = Molarity of ferrous sulphate solution (from blank titration)

V_1 =ml of ferrous sulphate solution required for blank

V_2 = ml of ferrous sulphate solution required for sample

$0.39 = 3\times10^{-3}100\%\times1.3$ (3 = equivalent weight of carbon)

S = weight of air-dry sample in gram

mcf = Moisture correction factor

% Organic matter content was obtained by multiplying the % C by 2 (i.e.$\%C \times 2$) (Okalebo *et al.*, 2002).

3.3.4 Total Nitrogen Content

Twenty (20) g of composite dry soil samples was placed in a distillation flask containing twenty (20) ml of distilled water and 100 ml of 0.32% potassium permanganate solution was added. In a 250 ml of conical flask, few drops of mixed indicator 20 ml of 2% boric acid solution was added

(3 parts of Bromocresol green and 1 part of methyl red indicator). The end of the delivery unit was dipped into the conical flask and 100 ml of 2.5% solution of sodium hydroxide was poured into the distillation flask and corked immediately.

Distillation of ammonia was collected approximately with 50 ml of the distillate into the boric acid solution. The distillate was titrated against 0.02N sulphuric acid till the green colour disappears (Subbiah and Asiza, 2004).

The total nitrogen was obtained using the formular below:

$$\%N = \frac{a-b}{s} \times M \times 1.4 \times mcf$$

Where:

a = ml of HCl required for titration of sample

b = ml of HCl required for titration of blank

s = air-dry sample weight in gram

m= mass of air-dry sample

mcf = Moisture correction factor

$1.4 = 14 \times 10^{-3} \times 100\%$ (14 = atomic weight of Nitrogen) (Subbiah and Asiza,2004).

3.3.5 Available Phosphorus

The soil samples was prepared by weighing 2.5 g of soil in 250 ml of Erlenmeyer flask followed by the addition of 25ml of Brays P1 Solution (0.03M NH_4F + 0.025N HCl) and shaken for 5 minutes using a mechanical shaker. After 5 minutes, the solution was filtered and 2ml was pipetted into 25 ml of volumetric flask. The pH was adjusted with the aid of paranitrophenol indicator, 2% ammonia and 5N sulphuric acid. To these solutions 2 mL of reagent B (1.056 g of ascorbic acid was dissolved in 200 mL of the molybdate solution) was added and made up to

volume with distilled water. A blank was prepared following the same procedure without soil sample. The absorbance of the solution was measured at 660 nm using a UV Spectrophotometer (Okalebo *et al.,* 2002).

The available phosphorus was obtained using the formular below

$$\% P = \frac{a - b}{s} \times mcf$$

a= mg/L in sample extract

b= mg/L in blank

s= sample weight in grams

mcf= moisture correction factor

Conversion factor for reporting;

$P_2O_5 = 2.31 \times P$

3.3.6 Potassium, Sodium, Calcium and Magnesium Content

A flame photometer was used for the purpose of determining the potassium and sodium contents of the soil sample. Potassium and sodium was determined quantitatively when they are atomized from solution and placed near a bunsen burner. Dried potassium chloride (kCl) (1.9117g) was dissolved in distilled water and made up to a volume of 1L. Dried sodium chloride (NaCl) (2.5422g) was also dissolved in distilled water and made up to 1L volume as a standard curve solution was produced with 1N ammonium acetate (pH 7). An aliquot of the solution to be analyzed was transferred into a 50ml volumetric flask using a pipette and completed with 1ml

ammonium-acetate at pH 7.0. Concentration of K or Na in samples was calculated by slope calculation for every concentration of the standard solution (10-100ppm) the concentration is divided by the reading of the apparatus. Magnesium and calcium were determined by atomic adsorption (Okalebo *et al.*, 2002).

3.4 Analytical Techniques of the Soil

3.4.1 Total Hydrocarbon content of the Soil Sample

The total hydrocarbon content of the soil sample (10g) was extracted with (10ml) dichloromethane solvent and determined by Gas chromatography (HP 6890, Agilent Technologies, Santa Clara, (U.S.A)

3.4.2 Level of Phenanthrene in the Soil Sample

The phenanthrene content of the soil sample (10g) was extracted with (10ml) dichloromethane solvent and determined by Gas chromatography (HP 6890, Agilent Technologies, Santa Clara, (U.S.A)

3.5 Microbiological Analysis of the Soil

3.5.1 Total Heterotrophic Bacterial Counts

Total heterotrophic bacterial was enumerated by plating aliquots (0.1ml) of appropriate dilution factors ($10^{-6,}$ 10^{-7} and 10^{-8}) of soil sample on nutrient agar (Oxiod Limited, Basingstoke, Hampshire, England). The colonies were counted after 5 days of incubation at room temperature ($27^{o}C\pm2$) with a Stuart Scientific Colony Counter (Bibby Sterilin Stone Staffordshire United Kingdom) (Chavez *et al.*, 2004).

3.5.2 Total Hydrocarbon Utilizers Counts

The population of hydrocarbon utilizers was estimated on mineral salts medium (MSM) (Kastner *et al.,* 1994) that contained (1000ml) $NaHPO_4$ (2.2g) KH_2PO_4 (0.8g) NH_4NO_3 (3.0g) $CaCL_2 2H_2O$ (10g) which was supplemented with yeast extract (0.005g) as a source of growth factor and pH was adjusted using base to 7.2. The mineral salts medium was fortified with sterilized nystatin (0.5g) to knockoff the growth of fungi. Sterile crude oil (Escravos light) served as a source of carbon and energy (Raymond *et al.,* 2006). The colonies were counted after 7 days of incubation at room temperature ($25^oC \pm 2$) with a Stuart Scientific Colony Counter (Bibby Sterilin Stone Staffordshire United Kingdom)

3.6 Preparation of Phenanthrene Solution

The phenanthrene chemical was purchased from Sigma Aldrich (USA) through Cardinal Scientific Supply Lagos, Nigeria. The solution was prepared by weighing 0.1 g of phenanthrene into a clean beaker, twenty milliliter (20ml) of acetone was also added to it in the beaker and was allowed to dissolve (Cerniglia, 1992). One millimeter (1ml) of the prepared phenanthrene and acetone solution was pipetted into dry Erlenmeyer flasks. Fifty (50ml) of mineral salts medium broth was added into the flask, covered with cotton wool and foil and autoclaved at 121°C for 15 minute (Obayori *et al.,* 2017).

3.6.1 Enrichment and Isolation of Phenanthrene Degrading Bacterium

Phenanthrene degrading bacteria isolated using mineral salts medium (broth) supplemented with 0.025% phenanthrene as a source of carbon and energy. It was fortified with (0.5g per 1000ml) of nystatin to knockoff fungal growth. Air dried contaminated soil sample (5g) was added to 50ml of mineral salts medium (broth) and the suspension was incubated using rotary shaker at 150rpm at room temperature (27°C ± 2°C) in the dark until there was turbidity in 7 days

(Obayori *et al.,* 2017). One (1) millimetre of the enriched media was transferred into fresh mineral salts medium (broth) containing the phenanthrene solution and incubation was done in the same manner. After four (4) consecutive transfers suspected phenanthrene degrader was isolated by plating out dilutions from the final flask on Luria-Bertani (LB) agar. The colonies that appear were further sub-cultured once onto LB agar for growth. Ability to grow on phenanthrene was checked by plating out on phenanthrene mineral salts medium (agar). The most promising phenanthrene degrading microorganism was used for further study and was maintained in glycerol: Nutrient broth (1:1) at 4℃ (Obayori *et al.,* 2017).

3.7 Morphological and Biochemical Characterization of the Isolate

The colonial morphology of the selected bacterial isolate was examined by light microscope includes shape of the cell, size, colour, form, margin, elevation. Presence of Endospore and Gram-staining were tested according to Barrow and Feltham, (1995). Conventional biochemical test includes Catalase, Oxidase, Carbohydrate fermentation, Motility, and Citrate utilization test. Furthermore, organisms were tested for ring hydroxylating dioxygenase enzyme and Analytical profile index

3.7.1 Gram Staining

A thin smear of fresh culture (24 hours old) of isolate was made by placing a full loop of sterile water on a clean oil free glass slide. The smear was heat-fixed and stained with crystal violet as primary dye for one minute and rinsed with running water (Habe *et al.,* 2002). Lugol´s iodine was added as mordant and allowed to stay for one minute and rinsed with water. The smear was decolourized by adding alcohol (95%). The smear was counter stained with safranin for 60 seconds, rinsed with water, air dried and examined under the light microscope using ×100 oil immersion objective lens (Barrow and Feltham, 1995).

3.7.2 Endospore Staining

A smear of 24- hour old of isolate was heat fixed on different glass slides and was flooded with malachite green with blotting paper and heated over a bunsen flame for 10 minutes. Stains was added to the slide to avoid drying and subsequently washed under running tap and counter stained with safranin for twenty seconds. The slide was washed, blot dried and examined under oil immersion. Endospores will stain green while vegetative cell will stain pink (Barrow and Feltham, 1995).

3.7.3 Catalase

A pure culture of 24- hours old of the test organism was prepared with the use of sterile distilled water on a clean glass slide. Few drops of hydrogen peroxide (3%) was added using a dropping pipette

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$ (Barrow and Feltham, 1995).

3.7.4 Oxidase

One drop of oxidase reagent (1% tetra methyl phenyllenediamine dihydrochloride) was added to isolated organism growing on the agar. The development of an intense purple colour by the bacterial cell within ten seconds indicates test positive (Barrow and Feltham, 1995).

3.7.5 Motility

The isolated organism was inoculated in a motility medium by making a fine stab with an inoculating needle to a depth of 2cm of the bottom of the test tube and incubated at room temperature ($27℃ \pm 2$) for 48 hours. The motile organism will grow and spread out from the line of inoculation and the medium will be cloudy (Barrow and Feltham, 1995).

3.7.6 Citrate Utilization

Simmons citrate agar slant was used with strict adherence to manufacturer's specification during preparation. The isolated organism was transferred to a sterile agar slant and incubated at 35°C for 24h. Growth on the slant and change in colour from green to blue indicated positive result (Barrow and Feltham, 1995).

3.7.7 Carbohydrate Fermentation

One (1) % sugars (Lactose, Maltose, Surcose, Glucose and Fructose) solution was added to 2ml of the basal medium containing the 2 drops of indicator (phenol red) which was sterilized and autoclaved for 121^{o}C for 15 minutes and durham tubes were inserted inversely. The media was inoculated with the isolated organism and incubated at 35℃ for 5 days watching daily for colour change. Acid production was indicated by appearance of a yellow colour in the medium and gas production by the presence of an air space in the inserted durham tubes (Barrow and Feltham, 1995).

3.7.8 Test for Ring Hydroxylating Dioxygenase

The aromatic ring dioxygenase activity of phenanthrene was examined using indole. The formation of indigo from indole which is a presumptive test for the presence of aromatic ring dioxygenase (Ensley *et al.,* 1983). Dioxygenase cleaves indole with the production of indigo (navy blue colour). Bacteria possessing dioxygenase reduce indole to indigo while colonies of such isolate turn navy blue. The isolate was streaked on Luria - bertani agar and incubated at room temperature for 48h. Then indole crystals (5g) was placed on the lid of the petri dish containing the isolate and incubated. After 24-48h incubation at 27^{o}C, colonies produced navy

blue colour and that indicated positive for dioxygenase enzyme activity (Chikere and Omadudu 2015)

3.7.9 Analytical Profile Index (Microbact)

The Microbact 12E (MB12E) (Microbact systems, disposable products Adelaide, South Australia) was used for the identification of the isolated bacteria. It consists of dehydrated substrates for 12 different biochemical tests placed in the wells of a microtitre tray (lysine decarboxylase, ornithine, hydrogen sulphide production, glucose, mannitol, xylose, indole, citrate, tryptophan deaminase, voges proskauer, methyl red and orthonitrophenylgalactose pyranoside) The isolate was tested in each well tray. A saline suspension of the test organism was added to each of the wells and was overlaid with sterile paraffin oil and incubated overnight at 37°C. Suitable reagents were added and colour changes of the tests was recorded. The result was transcribed into a code of organisms which was identified by the use of a computer based profile register postman as followed by manufacturer specification.

3.8 Biodegradation Experiment

3.8.1 Inoculum Preparation

Bacterial cell (2g) was washed in ten ml of sterile mineral salts medium and centrifuged at 3 revolution per minutes (rpm) for 15 minutes consecutively three times and the supernatant was decanted at each time. This serves as a source of innoculum for the experiment (Obayori *et al.*, 2017).

3.8.2 Experimental Setup

Ten Erlenmeyer flask was setup containing 100 parts per million (ppm) of phenanthrene solution. 50ml of mineral salts medium was added to each flask and autoclaved at 121°C for 15

minutes. Each flask was inoculated with the innoculum (1 ml) and incubated at room temperature 27℃ for 24h (Obayori *et al.,* 2017).

3.8.3 Determination of Growth Profile of the Isolate

The Erlenmeyer flask was shaken at 150 rpm in the dark for 15 minutes in which 1ml of the suspension was taken and dissolved into 9ml of physiological saline and serial dilution was further done up to 10^{10} in sterile test tubes to reduce microbial concentration. Aliquot of 0.1ml was taken at appropriate dilutions ($10^{-6,}$ 10^{-7} and 10^{-8}) and was spread out with a sterile bent glass rod aseptically into nutrient agar and this was done in duplicates and incubated at room temperature 27℃ for 24hours. The colonies were counted in colony forming unit per ml (cfu/ml) (Obayori *et al.,* 2017) with a Stuart Scientific Colony Counter (Bibby Sterilin Stone Staffordshire, United Kingdom)

3.8.4 Extraction of Residual Phenanthrene

Residual phenanthrene was extracted by liquid liquid extraction method according to the method described by Sarma *et al.,* (2004). Twenty milliliter (20ml) was extracted using a glass separating funnel with a stopper and a cork. Twenty milliliter (20ml) of hexane was added to the mixture and shaken vigorously for 30 minutes. The hydrocarbon layer was collected into a glass beaker and allowed to evaporate. The concentrated 1ml of extract was transferred to a sample vial and stored in a refrigerator to await gas chromatography analysis (Sarma *et al.,* 2004).

3.8.5 Gas Chromatography

Gas chromatography is used in analytical chemistry for separating and analyzing chemical components of a sample mixture and detects how much is present. These chemical components are usually organic molecules or gases (Chavez *et al.,* 2004).

3.8.6 Analytical Method

The standard chromatogram (Chlorobenzene) was used to standardize the gas chromatography in preparation of the sample analysis. One microlitre (1µl) of the extract was injected into the GC and a corresponding chromatogram was generated. The chromatogram of both the standard and the test sample was compared in respect to the concentration of the standard. Hexane extracts (1.0 µl) of residual phenanthrene was analyzed with Hewlett Packard 5890 Series II gas chromatography equipped with flame ionization detector (FID). The column was OV-3 with length of 60m. The carrier gas was nitrogen, the injector and the detector temperature was maintained at 220℃ and 270℃ respectively. The column temperature was programmed at an initial temperature of 50℃, this was held for 2 minutes and then ramped at 5℃ /min to 250℃ and held for 5 minutes. Air flow rate was 450ml/min, hydrogen 45ml/min and nitrogen 22ml/min. The GC runs was carried out on the sample on day 0, 10 and day 20. The PAH data was fitted to the first order kinetics model

$Y= ae^{-kt}$

Where Y was the residual PAH in culture

a is the initial PAH in culture

K is the degradation time and

t is the time in days

As calculated as

Half –life = Ln (2)/k (Yeung *et al*., 1997).

3.9 Chromatographic Analysis of Phenanthrene Degradation

The sum peak area of the standard chromatogram and substrate chromatogram were compared in relative to concentration of the standard to give the concentration of the of the test substrate (phenanthrene)

$$\text{Concentration of phenanthrene} = \frac{\text{sum peak area of sample} \times \text{concentration of standard}}{\text{sum peak area of the standard}}$$

The percentage residual of the substrate (phenanthrene) and % degradation activity on phenanthrene was calculated at different days using the following formula

$$\text{Residual of phenanthrene \%} = \frac{\text{concentration of phenanthrene} \times 100}{\text{Initial concentration of sample at day 0}}$$

Degradation activity of the organism % = 100- % residual phenanthrene

$$\text{Rate of degradation per day} = \frac{\text{Initial concentration of phenanthrene Co} \times \text{final concentration}}{\text{day}}$$

$$\text{Degradation constant K} = \frac{\text{Ln Co} - \text{Ln C}}{\text{T(days)}}$$

$$\text{Halflife } t_{1/2} = \frac{0.693}{\text{K}}$$

(Yeung *et al*., 1997)

2.9.1 Statistical Analysis

The data collected was presented as mean ± standard deviation. The results were analysed using analysis of variance (One way Anova) and the differences between the mean were further analysed using Duncan's new multiple range test, T independent (Paired samples) and the *p* value < 0.05 was significant.

CHAPTER FOUR

4.0 RESULTS

4.1: Physicochemical Properties of the Polluted Soil Sample

The result of physicochemical properties of the polluted soil sample used in this study is presented in Table 4.1. The results revealed that the pH of the soil sample was acidic 6.85, total organic carbon was 9.5 %. The results also showed that among the nutrient composition in the soil, calcium recorded the highest 0.046 Cmol/Kg followed by magnessium 0.35 Cmol/Kg with potassium being the least 0.013Cmol/Kg. The soil sample was equally high in water content 22.0%.

4.2: Microbiological Properties of the Soil

Total hydrocarbon degrading and heterotrophic bacteria in the soil were 4×10^6 cfu/g and 3×10^6 cfu/g respectively (Table 4.2).

4.3: Analytical Properties of the Soil

The total hydrocarbon content was 6764mg/kg and phenanthrene peak of the polluted soil sample was 622.410 m^2 as shown in Figure 4.1. Hydrocarbons ranging from mono aromatic, saturated, arenes, aliphatic and poly aromatic hydrocarbons were present in the soil sample. The lowest peak was phytane and ethane which amounted to 150.mg/kg and 178. mg/kg respectively while the highest peak was decane with a value of 2164.mg/kg.

Table 4.1: Physicochemical properties of the polluted soil sample in the study

Physicochemical parameters	Quantity
Ph	6.85±0.106
Moisture Content (%)	22.0±0.707
Organic Carbon Content (%)	9.51±0.707
Available Phosphorous(%)	0.3770±0.036
Total Nitrogen (%)	0.140±0.007
Calcium (Cmol/Kg)	0.0466±0.283
Magnesium (Cmol/Kg)	0.35±0.021
Potassium (Cmol/Kg)	0.013±0.001
Sodium (Cmol/Kg)	0.063±0.001

Keys: Cmol/kg-Centimole per kilogram. %- percentage

Table 4.2: Microbiological properties of the polluted soil sample in the study

Microbiological parameters	Amount **(**($\times 10^6$cfu/g)
THB	3.00±0.212
THUB	4.00±0.141

Keys: All values are expressed as Mean±SD THB- total heterotrophic bacteria, THUB- total hydrocarbon utilizing bacteria SD-standard deviation, Cfu/g-colony forming unit per gram.

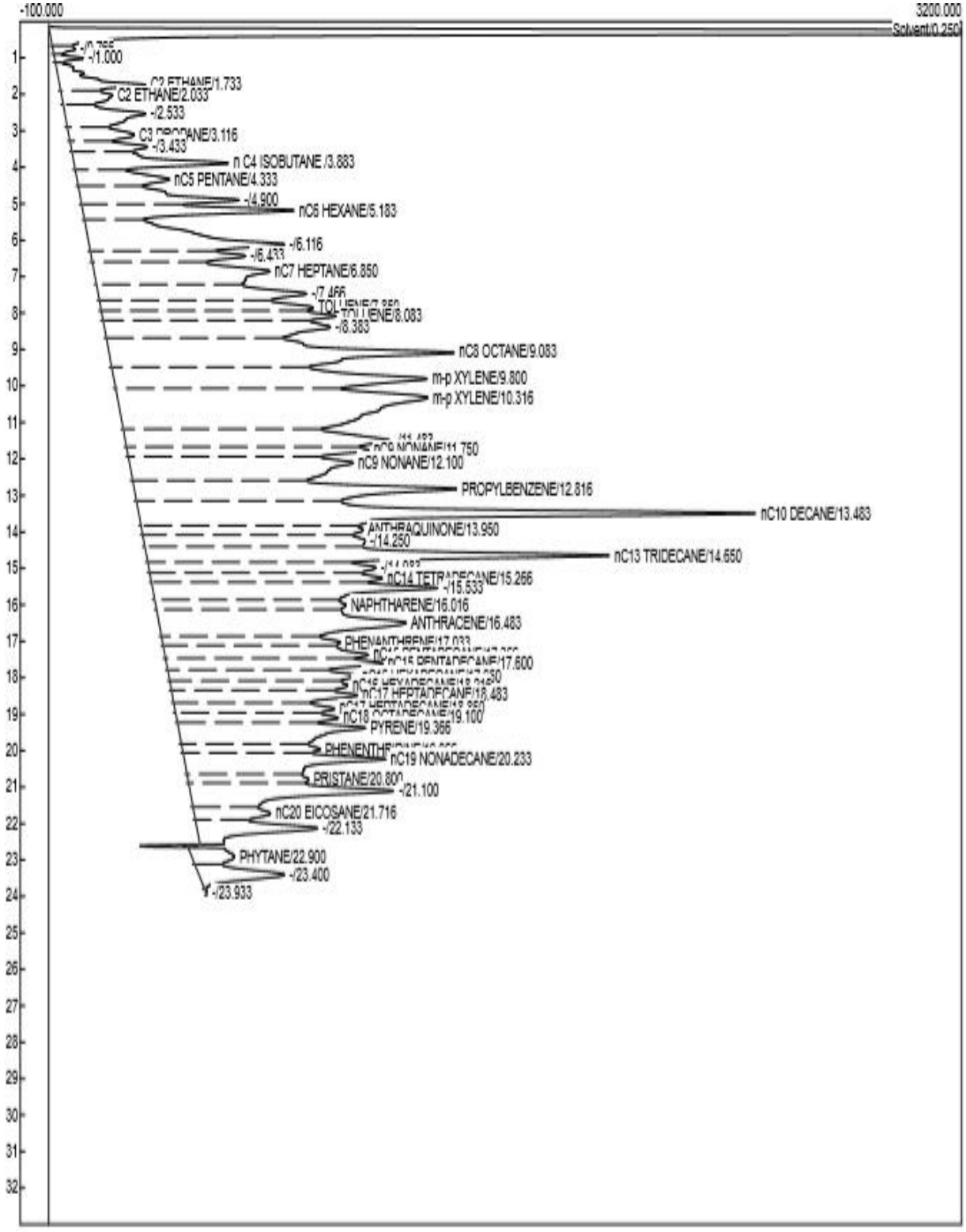


Figure 4.1: Chromatogram of Total hydrocarbon content and phenanthrene peak of the polluted soil sample.

4.4 Cultural Morphological and Biochemical Characteristics of Phenanthrene Degrader

The turbidity of the Erlenmeyer flask containing the isolate increased as the number of days increases, phenanthrene crystals disappeared in the flask between 5^{th} and 6^{th} day of every transfer which is an indication that the organism utilizes phenanthrene as a sole source of carbon and energy. The optimal condition for growth in mineral salts medium was pH 7.0 and temperature 28℃ with 100ppm of phenanthrene in shaken culture. These colonies were grown on phenanthrene coated Luria bertani agar plate, clear zones were visualized indicating the phenanthrene degrading ability, despite this, these organisms were also tested for their phenanthrene degrading ability in broth containing Mineral salts medium only strain p 10 was able to degrade phenanthrene with no accumulation of degradative products.

Morphologically, isolate p10 is a Gram positive bacterium, rod shape with shiny pigmentation. The isolate was positive for motility and endospore formation which was located at the terminal position. Isolate p10 was positive to catalase and negative to oxidase. It utilized citrate as a sole source of carbon. The isolate was able to produce navy blue colouration when grown in the presence of indole crystals indicating production of ring hydroxylating dioxygenase and was unable to utilize most sugars such as glucose, sucrose, maltose when tested, but lactose and fructose were utilized by the isolate p10.

The analytical profile index of the organism was *Bacillus subtilis* (90%) with code number (645220030) *Bacillus subtilis* was positive for lysine and hydrogen sulphide production and negative for tryptophan deaminase. (Table 4.3)

Table 4.3: Cultural Morphological and Biochemical Characteristics of *Bacillus subtilis* isolated from the study

Characteristics	Results
Margin of the colony	Coiled
Surface of the colony	Smooth
Elevation of the colony	Raised
Pigmentation of the colony	Shiny
Cell arrangement	Cluster
Shape	Short rods
Dioxygenase activity	Navy blue (+)
Gram staining	+
Spore staining	+
Motility	+
Citrate utilization	+
Catalase	+
Oxidase	-
Lactose	+
Glucose	+
Sucrose	-
Maltose	-
Fructose	-
Lysine	+
Ornithine	+
Mannitol	+
Hydrogen sulphide	+
Indole	+
Tryptophan deaminase	-
Urease	-
Voges proskauer	-
Methyl red	+
Profile code	645220030
probability	90%

Keys: Identification threshold was set at 90% as the cut-off point; Lys = Lysine decarboxylase; Orn = Ornithine decarboxylase; H_2S = hydrogen sulphide production; Glu = Glucose; Man = mannitol; Xyl = Xylose; ONPG =Orthonitrophenylgalactosepyranoside; Ind = Indole; Cit = Citrate; TDA = Tryptophan deaminase; VP = Voges-Proskauer; MR = Methyl red; + = Positive; - Negative.

4.5 Growth Rate of *Bacillus subtilis*

The growth rate and pattern kinetics of the *Bacillus subtilis* supplemented with phenanthrene as a sole source of carbon and energy obtained from the biodegradation experiment showed initial slow growth between day 0 to day 4, but grew exponentially from day 8 to day 20. (Table 4.4) The bacterial population density increased from $1.2.\times 10^{6}$ to 8.1×10^{7} in 20 day from 10^{-7}. At day 8, with dilution factor (10^{-4}) bacterial population were too numerous to count and day 12 at dilution factor (10^{-8}) there was absence on growth (Table 4.4).

Degradation kinetics of phenanthrene by *Bacillus subtilis* was monitored for 20 days. The chromatogram of residual phenanthrene readings were taken on day 0, 10 and 20. The retention time for day 0 chromatogram was 16 minutes, the area was 11705 m^2 and the height was 594 m^2 while the retention time for day 10 was 16 minutes, the phenanthrene area was 7027m^2 and the height was 569 m^2 and the retention time for day 20 was 16 minutes, the area was 3792 m^2 and the phenanthrene height was 277m^2. The chromatogram shows decrease in residual phenanthrene in the soil sample from day 0 (128.7 mg/l) to day 20 (41.7 mg/l) (Table 4.5). Degradation kinetics of *Bacillus subtilis* showed that 67.61% in 20 days at the rate of $4.35mg1^{-1}d^{-1}$ (Table 4.6). The biodegradation constant (k) and half life ($t_{1/2}$) were 0.056 d^{-1} and 12.38 days respectively (Table 4.6). *Bacillus subtilis* showed a linear retrogressive curve indicating exponential increase (Figure4.2).

Table 4.4: Total viable counts of *Bacillus subtilis* in different dilutions factor

	Viable counts of *Bacillus subtilis*(Cfu/g)					
Dilutions factor	day 0	day 4	day 8	day 12	day 16	day 20
10^{-4}	$1.17\times10^{5} \pm 0.06^{a}$	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-5}	$1.1\times10^{4} \pm 0.03^{b}$	$4.81\times10^{4}\pm 0.10^{c}$	$5.21\times10^{4}\pm 0.15^{d}$	TNTC	TNTC	TNTC
10^{-6}	$1.0\times10^{5}\pm 0.15^{c}$	$4.21\times10^{5}\pm 0.10^{b}$	$5.01\times10^{5}\pm 0.10^{c}$	$5.91\times10^{5}\pm 0.15^{b}$	TNTC	TNTC
10^{-7}	$1.2\times10^{6}\pm 0.20^{c}$	$4.1\times10^{6}\pm 0.20^{8b}$	$4.9\times10^{6}\pm 0.10^{b}$	$5.8\times10^{6}\pm 0.10^{b}$	$7.3\times10^{6}\pm 0.10^{b}$	$8.1\times10^{7}\pm 0.05^{b}$
10^{-8}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}	$1.1\times10^{8}\pm 0.10^{a}$	$1.2\times10^{8}\pm 0.10^{c}$

Keys : (TNTC**)** To numerous to count**,** CFU- colony forming unit per gram Result are values of three independent test (n = 3)

Mean values with the identical superscript letter are significantly different at $p > 0.05$

Duncan new multiple range test was used to analyse data

Table 4.5: Mean residual Percentage of Phenanthrene Degraded by *Bacillus subtilis* during the study

Days	Peak area of phenanthrene (m^2)	Concentration of phenanthrene mg/L	Residual of phennanthrene (%)
0	11705.53±0.106	128.7±0.111	
10	7027.78±0.108	77.21±0.121	60.03±0.100
20	3792.81±0.102	41.71±0.113	32.39±0.101

Keys: Values are means of duplicate readings obtained from difference between amounts of residual phenanthrene on Day 0 of incubation of bacteria to Day 20.Standard Peak Area = 9091.2088,Standard Concentration = 99.99 mg/L,Initial concentration of phenanthrene= 128.74 mg/L mg/L milligram per litre

$t = 1.940 \times 10^3$

Confidence interval = 0.05

T test (paired samples) was used to analyse data

Table 4.6: Percentage kinetics of degradation of phenanthrene by *Bacillus subtilis* during the study

Days	Phenanthrene degraded (%)	Rate of degradation (mg L^{-1} day^{-1})	Degradation rate constant (K)	Half-life Days ($t^{1/2}$)
10	39.970±0.100	5.15±0.101	0.051±0.100	13.58±0.101
20	67.61±0.101	4.35±0.111	0.056±0.103	12.38±0.111

Keys:Values are means of duplicate readings obtained from difference between amounts of residual phenanthrene on Day 0 of incubation of bacteria to Day 20, Half-life ($t^{1/2}$) Constant = 0.693, mg/L day-miligram per litre in day.

t=1.940 ×10^3

Confidence interval = 0.05

T test (paired samples) was used to analyse data

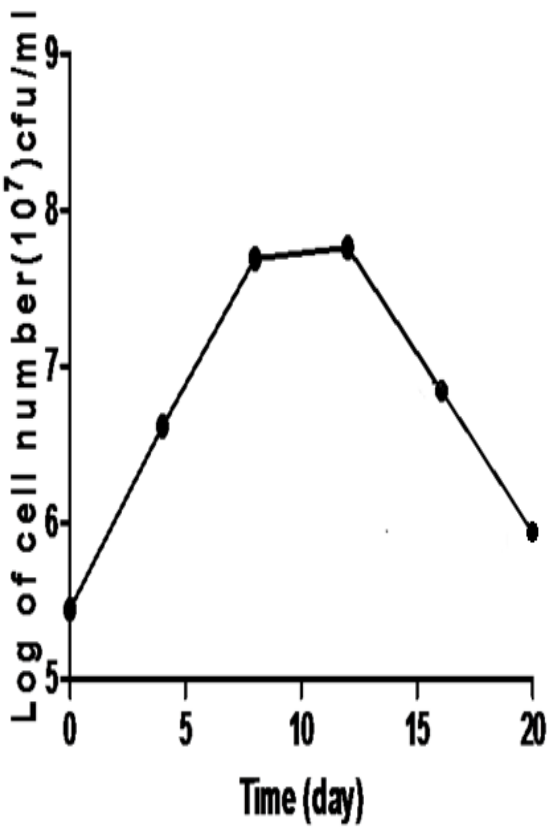


Figure 4.2: Growth profile of *Bacillus subtilis* on phenanthrene as a sole source or carbon and energy on mineral salts medium.

CHAPTER FIVE

5.0 DISCUSSION

Polyaromatic Hydrocarbons are a diverse group of priority pollutants containing two or more fused benzene rings that are arranged in linear, angular and cluster conformations. Cleanup of environmental priority pollutants is important due to their acute toxicity, low bioavailability, hydrophobicity, mutagenicity and carcinogenicity (Nkansah *et al.,* 2011). The biodegradation of PAHs is mainly through the activities of microorganisms.

Physicochemical of the polluted soil sample in this study indicated low concentrations of macro minerals such as nitrogen, phosphorous, sodium, potassium and calcium. This may be attributed to the high demand of these inorganic nutrients by microorganism for sugar phosphorylation, synthesis of amino acids, nucleic acids, increase in cell division, growth, biomass and other cellular processes (Salam *et al.,* 2014).

In biodegradation, presence of these macronutrients in low concentration in soil sample may be an impediment for pollutant degradation (Vidali 2001). The pH of the polluted sample was slightly acidic pH6.85 which indicate that phenanthrene degradation is accompanied with lowering of the pH (Obayori *et al.,* 2011). This may be connected with the fact that phenanthrene degradation usually leads to the production of organic acids such as pyruvic acid and salicylic acid as their metabolites which invariably leads to lowering the pH (Obayori *et al.,* 2011).

The total hydrocarbon content 6764 mg/kg and phenanthrene content 622mg/kg of the polluted soil sample were higher when compared to the baseline limit set for the total petroleum hydrocarbon by the petroleum regulatory body in Nigeria as 50 mg/kg (Department of Petroleum

Resources 1991). The value recorded is the average value recently recorded in the oil spill site in the oil producing Niger Delta region in Nigeria (Allinor *et al.*,2017).

The phenanthrene degrading *Bacillus subtilis* isolated in the polluted soil collected from a mechanic garage in Samaru, Zaria Kaduna State. During the growth of *Bacillus subtilis* on phenanthrene in batch culture, the water soluble intermediates causing a change in colour of the medium from clear to yellow which was detected due to the accumulation of compounds and their meta cleavage products such as pyruvic acid and salicylic acid may be attributed to the colour changes. Isolate primed as seed for bioremediation must be able to compete favourably with autochthonous community been in a nutrient limiting environment, highly compacted and diverse contaminated soil environment hence, some microorganisms have been involved in the degradation of phenanthrene mostly autochthonous organisms found in the polluted region as a result of selective pressure and acquisition of degradative abilities such as gene encoding different enzymes, resistance and degradative plasmids has been involved in biodegradation (Salam *et al.,* 2011).

Bacillus subtilis was found to posses dioxygenase enzyme activity which was observed from the Meta cleavage pathway of indole to indigo with the production of navy blue colouration. The detection of dioxygenase enzyme activity in some of the hydrocarbon utilizing bacteria is important because the enzyme converts polycyclic aromatic hydrocarbon into metabolic intermediates that are channeled through the meta cleavage pathway to the tricarboxylic acid cycle and finally yielding carbon dioxide, energy, biomass and water (Iwai *et al.,* 2011). Different authors like Obayori *et al., (*2011), Thavamani *et al.,* (2012) and Chikere and Omadudu, (2015) reported some members of the genus *Pseudomonas*, *Bacillus*, that posses the

dioxygenase enzyme which has contributed to the ability method of removing PAHs from environment due to the presence of dioxygynase gene that code for this enzyme.

The ability of PAH degraders to resist some antibiotics revealed their bioremediation potentials for PAHs impacted soil as reported by Obayori *et al.,* (2017). Arbabi *et al.,*(2009) reported some species of *Bacillus* that were among the group of PAHs degraders which degraded 50% of the initial concentration of phenanthrene in silty contaminated soil and 60% in clayey contaminated soil within three weeks is lower than *Bacillus subtilis* isolated during the study to degrade initial phenanthrene concentration 67.61% within 20 days in loamy contaminated soil which indicates that the type and properties (structure and texture) of contaminated soil greatly affect the rate at which pollutant such as hydrocarbons are removed from the environment.

Two strains of *Bacillus subtilis* M16K and M19F were also reported by Oyetibo *et al.,* (2017) to degrade significant amount of phenanthrene with 88.6% and 86.97% respectively within 20 days. The frequent involvement of several strains of *Bacillus* in removal of PAHs suggests its effectiveness in bioremediation of hydrocarbon polluted soil.

The Degradation rate utilization of *Bacillus subtilis* in the study on phenanthrene is 0.0156 h^{-1} which is lower compared to those reported in previous findings Boldrin *et al* .,(1993) who reported 0.111 and Obayori *et al.,* (2008) who reported 0.082 and 0.067 h^{-1} . Earlier reports have indicated that growth and utilization rate of isolates on substrate are not only predicted on the intrinsic properties of the isolates but also the culture conditions such as crystal size of substrate and variations in physic chemical parameters (Boldrin *et al.,* 1993). It is not unlikely that the organisms has special mechanism for the uptake of the hydrocarbon as substrate as microorganisms are generally known to take up the hydrocarbons dissolved in the aqueous phase

and such mode may become limiting at low aqueous solubility (Maier, 2009). Several mechanisms have been adduced for utilization of substrate at such low solubility namely production of biosurfactant, uptake system with high substrate affinity, reduction of distance between cells and substrate by means of cell surface structures which promote adhesion to hydrophobic surfaces (Bastiaens *et al.,* 2000).

In this study, *Bacillus subtilis* degraded 67.61% of the initial concentration of phenanthrene (100 mgI^{-1}) in 20 days. This degradation rate is lower than 87.92% (100 mg l^{-1}) in 20 days reported by Obayori *et al* .(2017) who discovered *Proteus mirabilis* 10c strain for the first time to degrade phenanthrene effectively and 99% (100 mg l^{-1}) in 16 days reported for *Diaphorobacter*. It is however higher than 61.5%, 65.8% and 33.7% reported for *Leclercia adecarboxylata* (Sarma *et al.,* 2004), *Bacillus cereus* py5 and *Bacillus megaterium* py6 within 20 days respectively. The degradation rate is however slower than 72% (500 mg I^{-1}) reported by Sokolovska *et al.,* (2003) for *Rhodoccocuss* sp UWI within two weeks. However, *Rhodoccocuss* sp UWI has always been effective for biodegradation purposes and has degraded various PAHs such as Pyrene, Naphthalene and Flourathrene in high concentration as a result of the dioxgenase gene which help in the breakdown and meta cleavage into the Tricarboxylic pathway intermediates(Klankeo and Pinyakong, 2006).

In this study, population density of *Bacillus subtilis* increased from 1.2×10^{6} to 8.1×10^{7}cfu/ml on phenanthrene within 20 days, this is quite higher than the study reported by Adeyemo *et al.,* (2018) where population density increased from 1.0×10^{7} to 2.8×10^{8} 10^{8}cfu/ml within 20 days. The rate of degradation decreased from 5.15 mg L^{-1} day^{-1} to 4.35 mgL^{-1} day^{-1} within 20 days at constant degradation rate from 0.051 K to 0.056 K within the same period and the half life were

13.58 days to 12.38 days respectively. The increase and decrease could be as result of long exposure of degradation day, concentration of phenanthrene and degradative abilities of the microorganisms.

Polluted soil environment such as mechanic work shop used in this study is predominantly inundated with complex hydrocarbon such in the workshop as spent engine oil, diesel with their aliphatic, alicyclic and aromatic components. As such there is need to assay the specificity pattern of PAHs degraders on complex hydrocarbons such as crude oil. The degradation of aliphatic fraction of crude oil to <10% of their initial concentration give credence to the assertions that aliphatic component are the most readily degraded fraction of crude oil (Jain *et al.*, 2005). While organisms with great specificity for PAHs and aliphatic hydrocarbons are not readily isolated from the environment, a number of remarkable isolates with such capability have been reported (Kanaly and Harayama 2010).

This study describes biodegradation potentials of phenanthrene by *Bacillus subtilis* isolated from polluted soil. It also shows the degradation ability of the isolate of poly aromatic and complex hydrocarbons mixtures. The ability of the isolate to posses dioxygenase enzyme revealed its bioremediation potentials for PAHs impacted soil and can be used as a tool for the recovery of polluted soil.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

The physicochemical parameters of the polluted soil sample indicated low amount of minerals such as magnesium (0.35Cmol/kg), potassium (0.013 Cmol/kg), calcium (0.046Cmol/kg) and high water content (22.0%) with slight acidic pH of 6.85.

The total hydrocarbon content of 6764mg/kg and phenanthrene content of 622mg/kg found in polluted soil sample were very high when compared to the base line limit set by petroleum regulatory body is 50mg/kg

Bacillus subtilis was enriched in mineral salts medium for 30 days and the medium was fortified with yeast extract for optimal growth and was identified on the basis of cultural morphological, biochemical test and analytical profile index.

Bacillus subtilis showed broad versatility in its action to degrade phenanthrene and utilized it as a sole source of carbon and energy. Its ability to degrade phenanthrene at 67.61% within 20 days was demonstrated which led to a corresponding increase in bacterial cell growth, rapid multiplication of cell biomass and cell components from 1.2×10^6 to 8.1×10^7cfu/ml.

6.2 RECOMMENDATIONS

1. There is need for more studies on primary metabolites produced during biodegradation process that can probably be used for industrial processes.
2. *Bacillus subtilis* isolated from the study can be used for bioremediation of phenanthrene contaminated soils.

REFERENCES

Adeyemo, C,A and Obayori , O.S (2018). Biodegradation of phenanthrene by Bacillus sp from mechanic work shop , Lagos Nigeria. *Journal of Nigeria Society of Microbiology*.**8**:14-19

Alam J. and Rizvi S.H.A. (2011) .Isolation and characterization of microorganism from oil contaminated sites. *journal of a Advanced Applied Science.* **2**: 455-460.

Alexander, M. *Biodegradation and Bioremediation.* (1999). 2nd Edition.; *Academic Press*: San Diego, USA. **12:**325-327.

Arbabi, M; Sedeghi, M and Anyakora, CH (2009). Phenanthrene Contaminated Soil Biotreatment Using Slurry Phase Bioreactor. *American Journal of Environmental Sciences* **5** (3):223-229.

Balashova, N.V.; Kosheleva, I.A.; Golovchenko, N.P.; Boronin, A.M. (1999).Phenanthrene metabolism by *Pseudomonas* and *Burkholderia* strains. *Journal of Biochemistry*, **35**, 291-296.

Bastiaens , L Springeal, D. Wattain , R. (2000).Isolation of adherent polycylic aromatic hydrocarbon (PAH) degrading bacteria using PAH sorbing carriers. *Journal of Applied Environmental Microbiology*. **66**:1834-1843.

Barrow G.I and Feltham R.K (1995).Cowan and Steel. *Manual of idenitification of Medical bacteria. 3rd edition.* Cambridge University.Cambridge.**12**:234-345

Bauchop, T and Elsden, S.R (2008) The growth of Microorganism in relation to their energy. *Journal of General Microbiology* **23**: 457-459

Boldrin, B. Thiem, A. Fritzsche , C (1993) .Degradation of phenanthrene, flourene , fluoranthene and pyrene by *Mycobacterium* sp. *Applied Environmental Microbiology*. **59**: 1927-1930

Brar S. K.,Verma M., Surampalli R.Y., Miscra K., Tyagi R.D., Meunier N. and Blais J. F. (2006). Bioremediation of hazardous wastes: A Review, *Practical Periodical Management*. **10**:59-72.

Chávez, F.P.; Lünsdorf, H.; Jerez, C.A.(2004). Growth of polychlorinated-biphenyl-degrading bacteria in the presence of biphenyl and chlorobiphenyls generates oxidative stress and massive accumulation of inorganic polyphosphate. *Applied Environmental. Microbiology*.**70**:3064-3072.

Cerniglia, C.E.(1992). Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation*. **3**:351-368.

Cerniglia C.E. and Shuttleworth K.I. (2002).Methods for Isolation of PAH-degrading Microorganism and Procedure for Determination of Biodegradtion Intermediates and Enviromental Monitoring of PAHs. In: Hurst,C.J., Crawford R.C., Kaudsen G.R.,

Mclnerny M.J. and Stetzenbach, I.D. (Eds). *Manual of Enviromental Microbiology 2nd Edition.* ASM Press, Washington, D.C pp.**28**:972-986.

Chikere, C.B and Omadudu, K (2015).Dioxygenase screening in imdigenous hydrocarbon utilizing bacteria from polluted sites in Niger Delta Nigeria. *Nigerian journal of microbiology*, **28**:2883-2891

Cocker, W.F.; Jones, G.E. (2006).Mineralization of phenanthrene by a *Mycobacterium* sp. *Applied Environmental Microbiology* **54**: 937-944.

Coral, S.; Karagol, M.L. and Stanley, G.A.(2005).Surfactant-enhanced biodegradation of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia. Biotechnology Bioengineering* **59**: 482-494.

Chun Zhang, S.A.; Harper, J.P.; Churchill, P.F. (2014). Isolation and characterization of a *Mycobacterium* species capable of degrading three- and four-ring aromatic and aliphatic hydrocarbons. *Applied. Environmental. Microbiology*. **65**:549-552.

Department of Petroleum Resources (DPR), (1991). Guidelines and standard for the petroleum industry in Nigeria. Department of Petroleum Resources, Lagos Nigeria.

Eniola K.I.T., Adegbola G.M. and Opasola O.A. (2014). Biodegradation of Used Engine Oil by Bacteria Isolated from Soil Contaminated by Used Engine Oil in Ogbomoso, Nigeria. *Journal of Environmental Science, Toxicology and Food Technology*, **8**(5):66-70.

Ensley, B.D, Ratzkin, B.J Osslund, T.D, Simon, M.JWackett,L.P and Gibson, D.T(1983) Expression of naphthalene oxidation genes in *Escherichia coli* results in the biosyenthesis of indigo. Science **222**:167-169

Frutos, Liu X, Yang W, Xu F, Wang W, Feng L, Bartlam M, Wang L, Rao Z (2010).Crystal structure of long-chain alkane monooxygenase (LadA) in complex with coenzyme FMN: unveiling the longchain alkane hydroxylase. *Journal of Molecular Biology* **376**:453–465

Gratia, C.A.; Potochi, B.B.; Santodonato, J. (2006) Exposure to carcinogenic PAHs in the environment.*Journal of Environmental. Science. Technology.***26**:1278-1284.

Habe, H., Ashikawa Y., Saiki Y., Yoshida T.,Nojiri H. and Omori, T (2002). *Sphingomonas* sp. Strain KAI, carrying a Carbazole Dioxygenase Gene Homologue Degrades Chlorinated dibenzo-p-dioxins in Soil federation of European Microbiological Society (FEMS). Microbiology, **211**:43-49.

Han, K., Jung Y.T and Son, S.Y.(2003). Phylogenetic anaylsis of phenanthrene-degrading bacteria *Sphingomonas. Journal of Microbiology and Biotechnology*, **13**:942-948.

Hanson, R.S.; Hanson, T.E. (1996) Methanotrophic bacteria. *Microbiology Review*. **60**: 439-471.

Hasanshahian, Y.; Phale, P.S. (2008).Biodegradation of phenanthrene by *Pseudomonas* sp. strain PP2: novel metabolic pathway, role of biosurfactant and cell surface hydrophobicity in hydrocarbon assimilation. *Applied. Microbiology. Biotechnology*. **61**:342-351.

Hwa, C.; Frache, G.; Poupin, P.; Maunit, B.; Muller, J.F.; Ferard, J.F. (2005). Isolation and characterization of a gene cluster in PAH degradation in *Mycobacterium* sp. strain SNP11: Expression in *Mycobacterium smegmatis* mc2155. *Journal of Microbiology* **158**: 175-186.

Iwai, S; Johnson, T.A.; Chai, B.; Hashham, S.A and Tiedje, J.M (2011). Compararison of the specificities and efficacies of primer for aromatic dioxgynase gene analysis of environmental samples. *Apllied Environmental Microbiology.***77**:3551-3557.

Jain, P.K. and Bajpai, V. (2005): Biotechnology of Bioremediation: A Review. *International Journal of Environmental Sciences.* **3**(1): 535-549

Jerina, Y.; Gardner, D.R.; Miller, C.D.; Chen, D.; Anderson, A.J.; Weimer, B.C.; Sims, R.C. (1976).Study of biochemical pathways and enzymes involved in phenanthrene degradation by *Mycobacterium* sp. strain KMS. *Applied Environmental. Microbiology* **72**: 7821-7828.

Kanaly, R.A. and Harayama, S. (2010). Biodegradation of High- Molecular- Weight Polycyclic Aromatic Hydrocarbons by Bacteria. *Journal of Bacteriology* .**182**:2059-2067

Kastner M, Breuer J,and Mahro B (1994) enumeration and characterization of soil microflora from hydrocarbon contaminated soil sites able to mineralize polycyclic aromatic compounds. *Journal of Applied Microbiology and Biotechnology* **41**: 267-274

Kazunga C, Aitken M.D., Gold A. and Sangaiah R. (2001). Flouranthene -2,3- and -1,5- diones are novel products from the bacterial transformation of fluoranthene, *Journal of Enviromental ,Sceince and Technology,* **35**:917-922

Kelley, I.; Freeman, J.P.; Evans, F.E.; Cerniglia, C.E. (2007).Identification of metabolites from the degradation of fluoranthene by *Mycobacterium* sp. Strain PYR-1. *Applied Environmental. Microbiology* **59**: 800-806.

Keum, Y.-S.; Seo, J.-S.; Li, Q.X.; Kim, J.H. (2006).Comparative metabolomic analysis of Sinorhizobium sp. C4 during the degradation of phenanthrene. *Applied Microbiology Biotechnology*. **80**:863-872.

Kim, S.J.; Kweon, O.; Jones, R.C.; Freeman, J.P.; Edmondson, R.D.; Cerniglia, C.E. (2003) Complete and integrated pyrene degradation pathway in *Mycobacterium vanbaalenii* PYR-1 based on systems biology. J*ournal of Bacteriology.* **189**:464-472.

Kim, A.M.; Widdel, F. (2004). Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. *Biodegradation* **11**: 85-105.

Kim, M.; Yano, H.; Ono, A.; Miyazaki, R.; Ishii, H.; Genka, H.; Top, E.M.; Tsuda, M. (2005) Genomic and functional analysis of the IncP-9 naphthalene-catabolic plasmid NAH7 and its transposon Tn4655 suggests catabolic gene spread by a tyrosine recombinase. *Journal of Bacteriology* **188**: 4057-4067.

Kim, Y.H.; Freeman, J.P. (2007) Effects of pH on the degradation of phenanthrene and pyrene by *Mycobacterium vanbaalenii* PYR-1. *Applied Microbiology Biotechnology*.**67**: 275-285.

Klankeo, P and Pingakong (2006) Two novel phenanthrene degrading *Diaphorobacter* sp and *Pseudoxanthomonas* sp isolated from soil. *Journal of Biological Science.* **108**:488-495

Lee, S.E.; Seo, J.S.; Keum, Y.S.; Lee, K.J.; Li, Q.X. (2007).Fluoranthene metabolism and associated proteins in *Mycobacterium* sp. JS14. *Proteomics* **7**: 2059-2069.

Maier, R.M (2009). Microoorganism and organic pollutants. *Journal of Environmental Microbiology*. **12**-387-420

Matatova, H.; Hwang, S.Y.; Kim, Y.M.; Kim, E.; Kim, Y.S.; Kim, S.K. (2005)Degradation of phenanthrene and naphthalene by a *Burkholderia* species strain.. *Journal of Microbiology* **49:** 139-144.

Mallick, S.; Chatterjee, S.; Dutta, T.K. (2007)A novel degradation pathway in the assimilation of phenanthrene by *Staphylococcus* sp. strain PN/Y via meta-cleavage of 2-hydroxy-1-naphthoic acid: formation of trans-2,3-dioxo-5-(2'-hydroxyphenyl)-pent-4-enoic acid. *Microbiology* **153**:2104-2115.

Mathew, Y.H.; Sanseverino, J.; Sayler, G.S.(2001)Analyses of polycyclic aromatic hydrocarbon-degrading bacteria isolated from contaminated soils. *Biodegradation*. **10**:149-157.

Medina-Bellver, F.H. (2005) Characterization of wood-preserving coal-tar creosote by gas-liquid chromatography. *Anaytical Chemistry*. **46**: 46-53.

Mergesin R, Labbe D, Schinner F, Greer CW, Whyte LG (2003).Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. *Applied Environmental Microbiology* **69**:3085–3092

Moody, J.D.; Freeman, J.P.; Doerge, D.R.; Cerniglia, C.E. (2001).Degradation of phenanthrene and anthracene by cell suspensions of *Mycobacterium* sp. Strain PYR-1. *Applied Environmental Microbiology*. **67**:1476-1483.

Nakamuraa, M.; Chang, H.C.; Lee, M.L. (2007).Structural characteristics of polycyclic aromatic hydrocarbons in coal tars and combustion products. *Environmental Science. Technology* **20**:1023-1027.

Narro, D.; Siddiqi, M.A.; Maccubbin, A.E.; Kumar, S.; Sikka, H.C. (1992) Degradation of polynuclear aromatic hydrocarbons by *Sphingomonas paucimobilis*. *Environmental Science Technology* **30**:136- 142.

Nkansah, M.A , Christy, A.A, Barth, T. (2011). The use of anthracene as a model compound in a comparative study of hydrous pyrolysis methods for industrial waste remediation. *Chemosphere* **84**:403-408

Obayori O.S., Ilori M.O., Adebusoye S.A., Oyetibo G.O,and Amund O.O. (2008). Pyrene degradation potentials of *pseudomonas* species isolated from polluted tropical soils.*World Joiurnal of Microbiology and Biotechnology*,**24**:2639-2646.

Obayori O.S., Ilori M.O, and Amund O.O (2011). Bacterial metabolism of pyrene, Applications and toxic effect.*Nova Science Publishers* , Newyork pp **79**-108

Obayori, O.S, Salam, L.B, Oyetibo, G.O, Idowu, M and Amund, O.O(2013) Biodegradation potential of polycyclic aromatic compounds (pyrene and phenanthrene) by proteus mirabilis isolated from animal charcoal polluyrf sites. *Journal of biocatalysis and agricultural biotechnology*, **12**: 78-84

Obayori, O.S; Ilori, M.O.; Adebusoye, S.a.; Oyetibo, G.O and Amund, O.O (2017). Degradation of spiked Pyrene and Non Pyrene Hydrocarbons in Soil Microcosm by *Pseudomonas* sp. Isolated from Petroleum Polluted Soils. *Petroleum Science and Technology* **31**: 1674-1680

Okalebo J.R; Gethuck .W and Woomer , P (2002) Laboratory methods of soil and plant analysis. A working manual, Nairobi Kenya **24**:123-156

Omadudu, K and Chikere, C.B (2015): Dioxygenase Screening in Indigenous Hydrocarbon Utilizing Bacteria from Polluted Sites in Niger Delta Nigeria. *Nigerian Journal of Microbiology*, **28**:2883-2891

Olsen, S.R., Cole, C.V., Watanabe, F.S. and Dean, L.A. (2006). Estimation of available phosphorus and pH in contaminated soils by extraction with sodium bicarbonate and electrode. USDA Circular, **939**: 1 19. Gov. Printing Office Washington, D.C.

Oyetibo, G.O.; Chein, M.F, Ikeda-Ohtsubo W.; Suzuli, H.; Obayori O.S.; Adebusoye, S.A.; Ilori, M.O.; Amund, O.Oand Endo, G (2017) Biodegradation of crude oil Phenanthrene by heaby metals resistant *Bacilus subtilis* isolated from a multi- polluted industrial waste water creek. *International Biodegradation and Bioremediation*, **120**:143-151

Pinyakong O., Habe H., Yoshida T., Nojiri H, and Omori T.(2014). Identification of three isofunctional novel salicylate 1-hydroxylases involved in phenanthrene degradation of *Spingobium* sp. Strain P2.*Journal of Biochemistry and Biophysics Resources,* **30***(***1**) 350-357.

Prenafeta-Boldu F. X., Vervoort J., Grotenhuis J. T. C. and van Groenestijn J. W. (2004).Substrate interactions during the biodegradation of benzene, toluene, ethylbenzene, and xylene (BTEX) hydrocarbons by the fungus *Cladophialophora*sp. Strain T1.*Applied and Environmental Microbiology*, **68**:26-60.

Pukall, N.; Mercadal, J.P.R.; Guerrero, L.D.; Marzio, W.D.D.; Ferrero, M.A.; Dionisi, H.M (2001). Novel aromatic ring-hydroxylating dioxygenase genes from coastal marine sediments of Patagonia. BMC *Microbiology*.**8**: 50-56

Raymond R.L., Audson J.O., Jamison V.W (2006) oil degradation in soil. *Applied Environmental Microbiology* **31**: 522-535.

Salam, L.B.; Obayori, O.S.; Akashoro, O.S and Okogie, G.O (2011) Biodegradation of bonny light crude oil by bacteria isolated from contaminated soil. *International Journal of Agriculture and Biology*, **13**:245-250

Salam, L.B.; Obayori, O.S.; Akashoro, O.S and Okogie, G.O (2014) Biodegradation of bonny light crude oil by bacteria isolated from contaminated soil. *International Journal of Agriculture and Biology*, **13**:245-250

Samanta, S.K.; Chakraborti, A.K.; Jain, R.K. (1999).Degradation of phenanthrene by different bacteria: evidence for novel transformation sequences involving the formation of 1-naphthol. *Applied Microbiology Biotechnology* **53**: 98-107.

Samanta, A.K.; Zylstra, G.J. (2002) Molecular cloning of novel genes for polycyclic aromatic hydrocarbon degradation from Comamonas testosteroni GZ39. *Applied Environmental Microbiology* **62**:230-236.

Santa S.K., Singh O.V. and Jain R.K (2002) Polycyclic aromatic hydrocarbons: Enviromental pollution and bioremediation. *Journal of Trends in Biotechnology*, **20**:243-248.

Sarma, P.M, Bhatttcharya D and Kashnan S(2004) degradation of polycyclic araomatic compounds by a newly discovered enteric bacteria. *Journal of environmental microbiology* **70**: 3161- 3166

Seo, J.S.; Keum, Y.S.; Cho, I.K.; Li, Q.X. (2007).Degradation of dibenzothiophene and carbazole by *Arthrobacter* sp. P1-1. *International. Biodeteriortation. Biodegradation.* **58**:36-43.

Singh K. and Chandra S. (2014). Treatment of hydrocarbon polluted environment through bioremediation: A review.*Pakistan Journal of Biological Science*, **17**(**1**): 1-8.

Smith M.R (1990). The biodegradation of aromatic hydrocarbons by bacteria. *Biodegradation* **1**:191-206.

Sokolovska, I.; Rozenberg, R.; Riez, C.; Rouxhet, P.G.; Agathos, S.N.; Wattiau, P. (2003).Carbon- source induced modifications in the mycolic acid content and cell wall permeability of *Rhodococcus erythropolis* E1. *Applied Environmental. Microbiology*. **69**:7019-7027.

Stanley, H.O., Amakin, M.A. and Okerentugba, P.O. (2015). Characterization of Hydrocarbon Utilizing Bacteria in Soil Samples Collected from Various Sites in Port Harcourt (Niger Delta, Nigeria). *Global Journal of Bioscience and Biotechnology*, **4**(1):6-11.

Story, S.P.; Parker, S.H.; Hayasaka, S.S.; Riley, M.B.; Kline, E.L. (2001)Convergent and divergent points in catabolic pathways involved in utilization of fluoranthene, naphthalene, anthracene, and phenanthrene by *Sphingomonas paucimobilis* var. EPA505. *Journal of Industrial Microbiology and Biotechnology*.**26**: 369-382.

Subbiah, B.V and Asija, G.L. (2004). A rapid procedure for estimation of available nitrogen in soils. *Current. Science.***25**: 259 260.

Sutherland, M.; Breur-Jammali, M.; Mahro, B. (1990) Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil. *Applied Environmental Microbiology* **64**: 359-362.

Thavamani, P.; Megharaj, M and Naidu, R (2012) Bioremedaition of high molecular weight poly aromatic hydrocarbons co- contaminated with metals in liquid and soil slurries by metal tolerant PAHs degrading bacterial consortium *Global Journal of Bioscience and Biotechnology*. **23**:823-835

U.S. Environmental Protection Agency (USEPA), (1986) Test method for evaluating solid waste SW-846, Third Edition., Volume. 1A. USEPA, Washington, DC.

Vidali, M (2001) Bioremediation, an overview. Journal of Pure Applied Chemistry **73**:1163-1172

Weissenfels, W.D.; Beyer, M.; Klein, J.(1990) Degradation of phenanthrene, fluorene, and fluoranthene by pure bacterial cultures. *Applied Microbiology Biotechnology* (1990) **32**:479-484.

Wikes, S.C.; Jones, K.C. (2002).Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): A review. *Environmental Pollution,* **81**:229-249.

Wilson, S.C. and Rose, M.C (2007). Remediation of soil contaminated with poly aromatic hydrocarbons (PAHs): A review. *Enviromental Pollution*, **35**:229-249.

Yakimov, J.W.C.; Lai, K.M.; Wan, C.K.; Ma, K.K.; Fang, M. (2003).Isolation and optimization of PAH- degradative bacteria from contaminated for PAHs bioremediation. Water Air Soil Pollution **139**:1-13.

Yeung, P.Y., Johnson, R.L and XU, J.G. (1997). Biodegradation of petroleum in soil as affected by heating and forced aeration. *Journal of Enviromental Biology*, **26**:1511-1576

Zhao, H.P., Wu, Q.S., Wang, L.,Zhao, X.T., and Gao, H.W.(2009). Degradation of phenanthrene by bacterial strain isolated from soil in oil refinery fields in Shanghai, China *Journal of Hazard*. **164**:863-869.

APPENDICES

Appendix I Media preparation

The media used for this study was Nutrient agar, Luria-Bertani agar and Mineral salts medium (MSM) (broth and agar). The media was purchased from Sigma Aldrich (USA) through Cardinal Scientific Supply, Lagos, Nigeria. There was strict adherence to manufactures specification in media preparation and the media was autoclaved at 121℃ for 15 minutes.

Appendix II Mineral salts medium

$NaHPO_4$ (2.2g)

KH_2PO_4 (0.8g)

NH_4NO_3 (3.0g)

$CaCL_2 2H_2O$ (10g)

Distilled water 1L

pH 7.0

Appendix III Luria- Bertani medium

peptone (10g)

yeast extract (5.0g)

NaCl (10g)

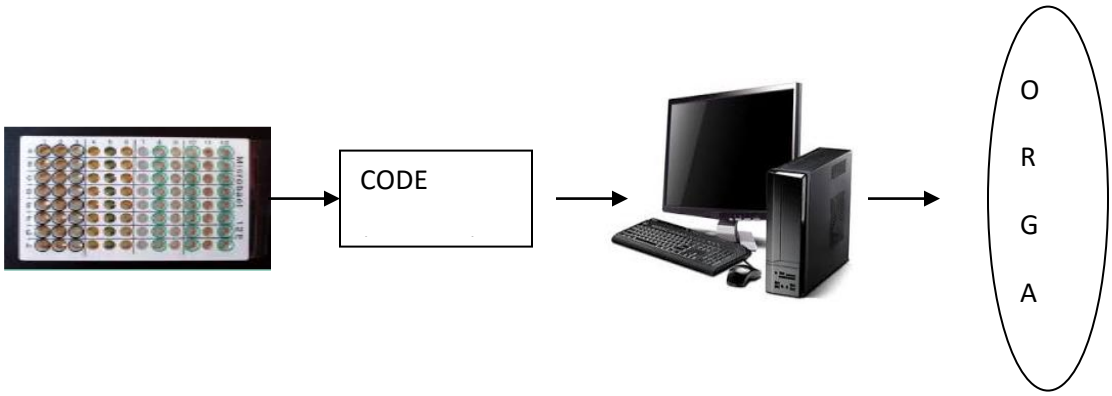
Bacteriological agar (20g)

Distilled water 1L

pH 7.0

Appendix IV Identification of bacteria using the Microbact 12E system

The Microbact 12E (MB12E) (Microbact Systems, Disposable Products, Adelaide, South Australia) is a commercial identification system for enterobacteria and other Gram negative bacteria. It consists of dehydrated substrates for 12 different biochemical tests placed in the wells of a microtitre tray. Eight organisms can be tested in each 96 well tray. Here, a saline suspension of the test organism was added to each of the 12 wells and appropriate wells were overlaid with sterile paraffin oil. After overnight incubation at 37°C suitable reagents were added and a colour change of the different tests was recorded. The results were transcribed into a code and organisms was identified by use of a computer based profile register (Mugg and Hill, 1981).



Appendix V Preparation of bacterial suspension

This was prepared by two well isolated colonies from each 24 h overnight bacterial culture and emulsifying in 2.5mL of normal saline (0.85M NaCI) and mixed to ensure a homogenous suspension. One hundred microlitre (100 uL) of bacterial suspension was added into each reaction well of the Microbact 12E system to initiate biochemical reaction.

Appendix VI Biochemical tests: The biochemical tests carried out using the Microbact 12E system were indicated. The reaction in well (1) tested for the presence of lysine decarboxylase in an organism and was characterized by the conversion of lysine to cadaverine, giving a blue or

green positive colored reaction. Well No. (8) reaction tested for the presence of tryptophanase in an organism, giving the ability to break down tryptophan into indole and pyruvate. After inoculation of the bacterial suspension into all the wells, wells 1, 2 and 3 were layered with 50 uL of mineral oil according to the Manufacturer's directives. Thereafter, the inoculated rows were resealed with an adhesive seal. This was followed by aerobic incubation at 37^0C for 24 h. A freshly prepared Kovac's reagent (25 uL) was then added to well 8 after 24 h of incubation to give a pink-red color for a positive indole reaction. 1 25-uL each of Voges Proskauer reagents 1 & II (VPI & VPII) were added to well 10 for a positive pink-red acetoin reaction, tryptophan deaminase (RDA) reagent was added to well 12 for a cherry red indole pyruvate positive. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and NCTC 10662, and *Salmonella typhimurium* 42R500 were used for quality control.

Appendix VII Computer aided identification Package

The Microbact™ Computer Aided Identification Package was consulted for the identification choice. The percentage figure shown against the organism name is the percentage share of the probability for that organism as a part of the total probabilities for all choices. Note: Miscellaneous Gram-negative bacilli - Weakly positive reactions are recorded as negative results. The results of tests for oxidase, nitrate reduction and motility are included as part of the reaction pattern. An octal coding system has been adopted for Microbact™ . Each group of three reactions produces a single digit of the code. Using the results obtained, the indices of the positive reactions are circled. The sum of these indices in each group of three reactions forms the code number. This code is entered into the computer package. The system was observed for colour change with or without the addition of suitable reagents according to the manufacturers' instructions. Each biochemical test is given a numerical value, awarded only to a positive

reaction. These values are converted into a code number which identifies the organism by the appropriate profile register. Using the results obtained, from each group of three reactions a 9 (nine) digit code number is produced.

Appendix VIII Substrate and reaction table for Microbact 12E system

Well No.	Designation	Reaction Principle	Reaction colours		Comments
			Negative	Positive	
1	**Lysine**	Lysine decarboxylase	Yellow	Blue-green	Green or blue is positive reaction. Bromothymol blue indicates formation of the specific amine cadaverine.
2	**Ornithine**	Ornithine decarboxylase	Yellow-green	Blue	Green should be regarded as a negative reaction. The pH shift indicated by bromothymol blue caused by formation of the specific amine putrescine is greater than that caused by lysine decarboxylation.
3	**H_2S**	H_2S production	Straw colour	Black	H_2S is produced from thiosulphate. H_2S reacts with ferric salts in the medium to form a black precipitate.
4	**Glucose**	Glucose fermentation	Blue-green	Yellow	Bromothymol blue indicator changes from blue to yellow when the carbohydrate is utilized to form acid.
5	**Mannitol**	Mannitol fermentation	Blue-green	Yellow	
6	**Xylose**	Xylose fermentation	Blue-green	Yellow	
7	**ONPG**	Hydrolysis of o-nitrophenyl-ß-d-	Colourless	Yellow	ß-galactosidase hydrolysis of the colourless ONPG

		galactopyranoside (ONPG)by action of ß-galactosidase			releases yellow orth-onitrophenol.
8	**Indole**	Indole production from tryptophan	Colourless	Pink-red	Indole is formed from metabolism of tryptophan.. Indole Kovacs reagent forms a pink-red complex with indole.
9	**Urease**	Urea hydrolysis	Straw colour	Pink-red	Ammonium released from splitting of urea causes the pH to rise - indicated by phenol red changing from yellow to pink-red
10	**VP**	Acetoin production (Voges-Proskaüer reaction)	Straw colour	Pink-red	Acetoin is produced from glucose indicated by the formation of a pink-red complex after the addition of alpha-naphthol and creatine.
11	**Citrate**	Citrate utilization (citrate is the only source of carbon)	Green	Blue	Citrate is the sole carbon source, which if utilized results in a pH rise, indicated by bromothymol blue, with a colour change from green to blue.
12	**TDA**	Production of indolepyruvate by deamination of tryptophan	Straw colour	Cherry red	Tryptophan deaminase forms indolepyruvic acid from tryptophan which produces a brown colour in the presence of ferric ions. Indole positive organisms may produce a brown colour. This is a negative reaction.

Appendix IX

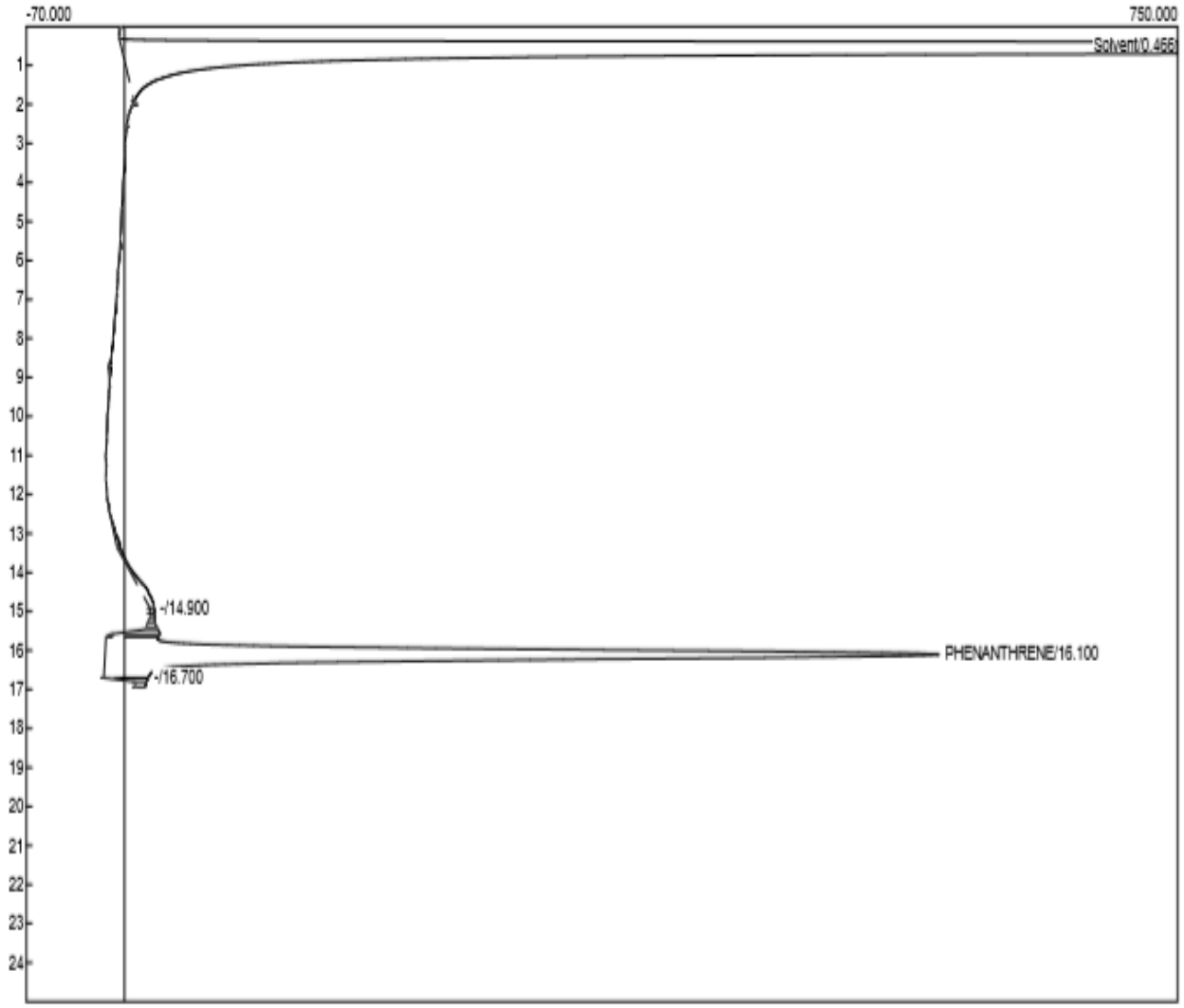


Figure 1: Chromatogram of residual phenanthrene extracted with n-Hexane from mineral salts medium culture after inoculation with phenanthrene degrading *Bacillus subtilis* and incubation at room temperature (27℃) on day 0

The retention was 16.100 minutes, phenanthrene area (11705.5356 m^2) and the height (594.150 m^2).

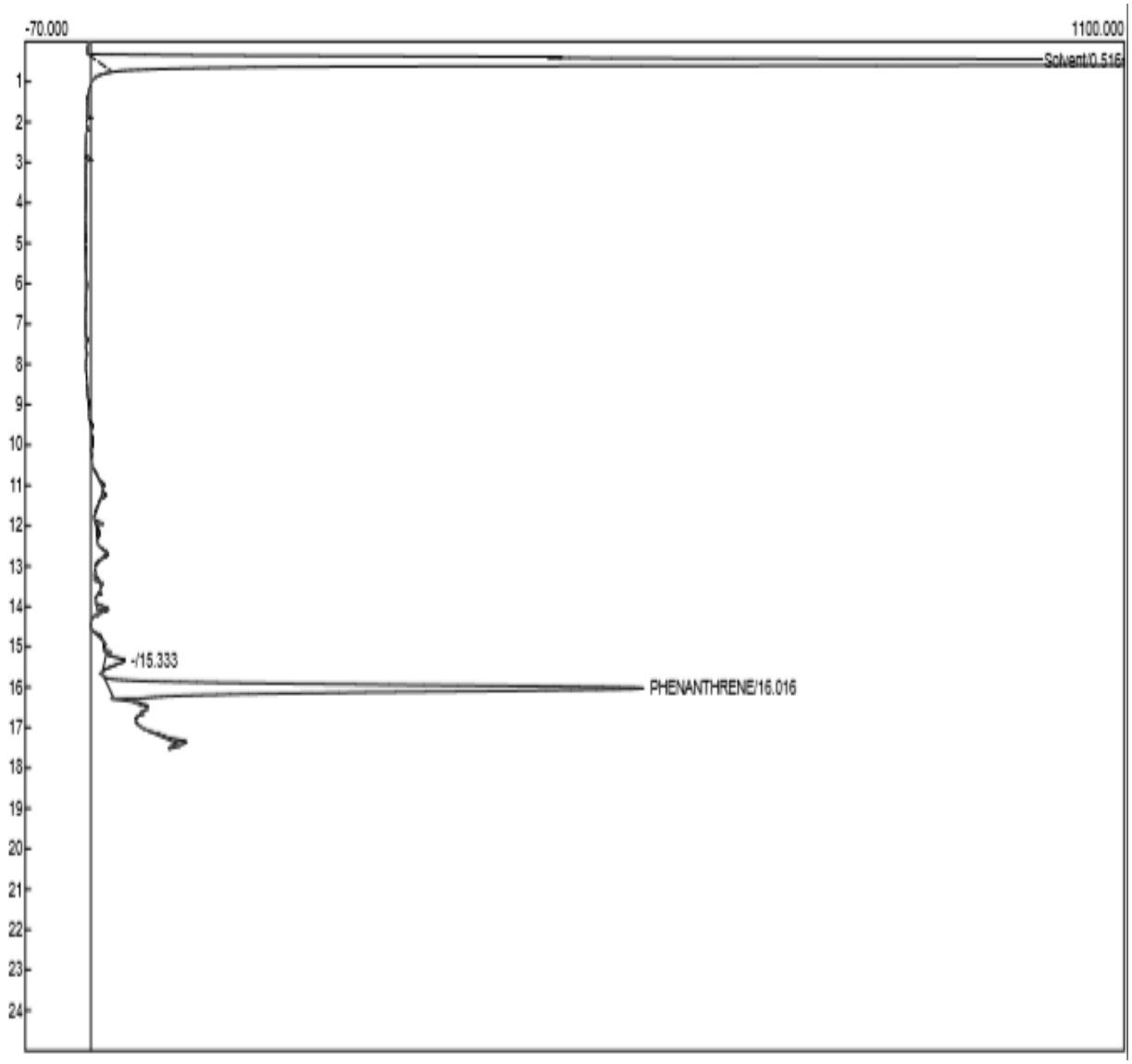


Figure 2: Chromatogram of residual phenanthrene extracted with n-Hexane from mineral salts medium culture after inoculation with phenanthrene degrading *Bacillus subtilis* and incubation at room temperature (27℃) on day 10

The retention was 16.016, the phenanthrene area (7027.7810 m^2) and the height (569.821 m^2).

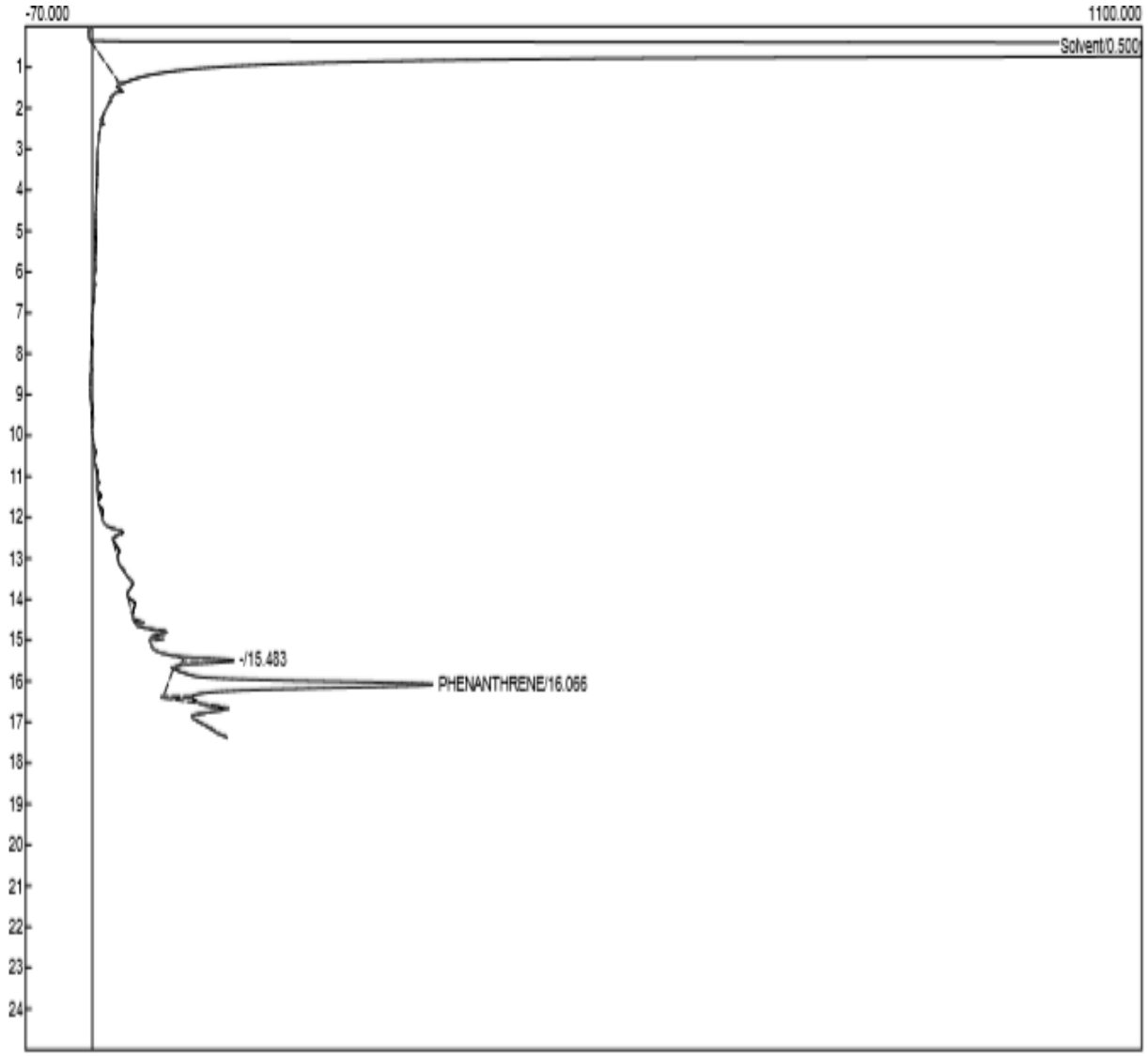


Figure 3: Chromatogram of residual phenanthrene extracted with n-Hexane from mineral salts medium culture after inoculation with phenanthrene degrading *Bacillus subtilis* and incubation at room temperature (27℃) on day 20

The retention was 16.066, the phenanthrene area (3792.8130m^2) and the height (277.484m^2)

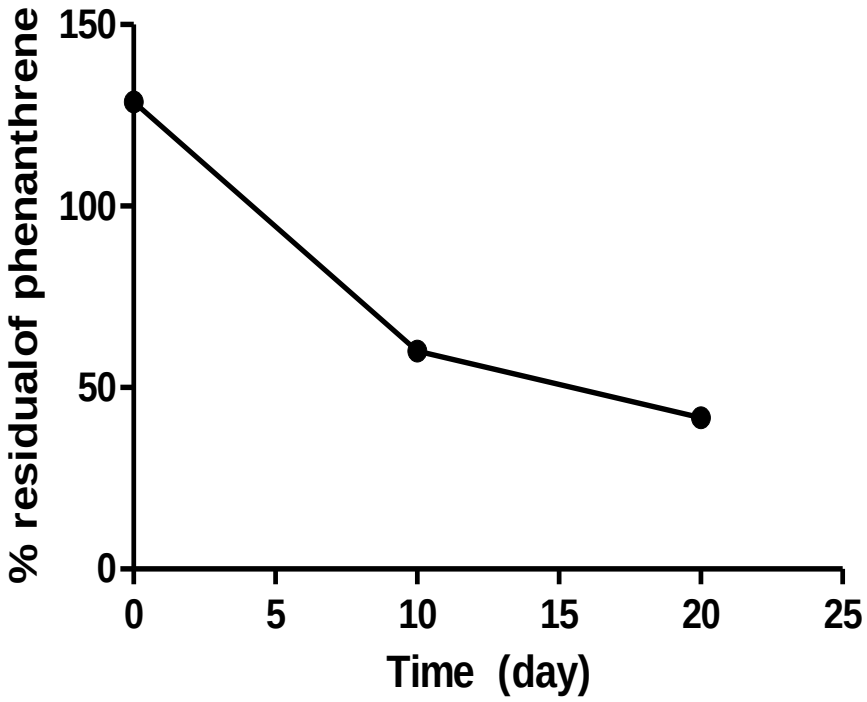


Figure 4: Residual Phenanthrene reduction dynamics within 20 days.

Appendix X

Plate I: Enrichment culture of the polluted soil sample supplemented with phenanthrene (100 parts per million) as a source of carbon and energy in mineral salts medium.

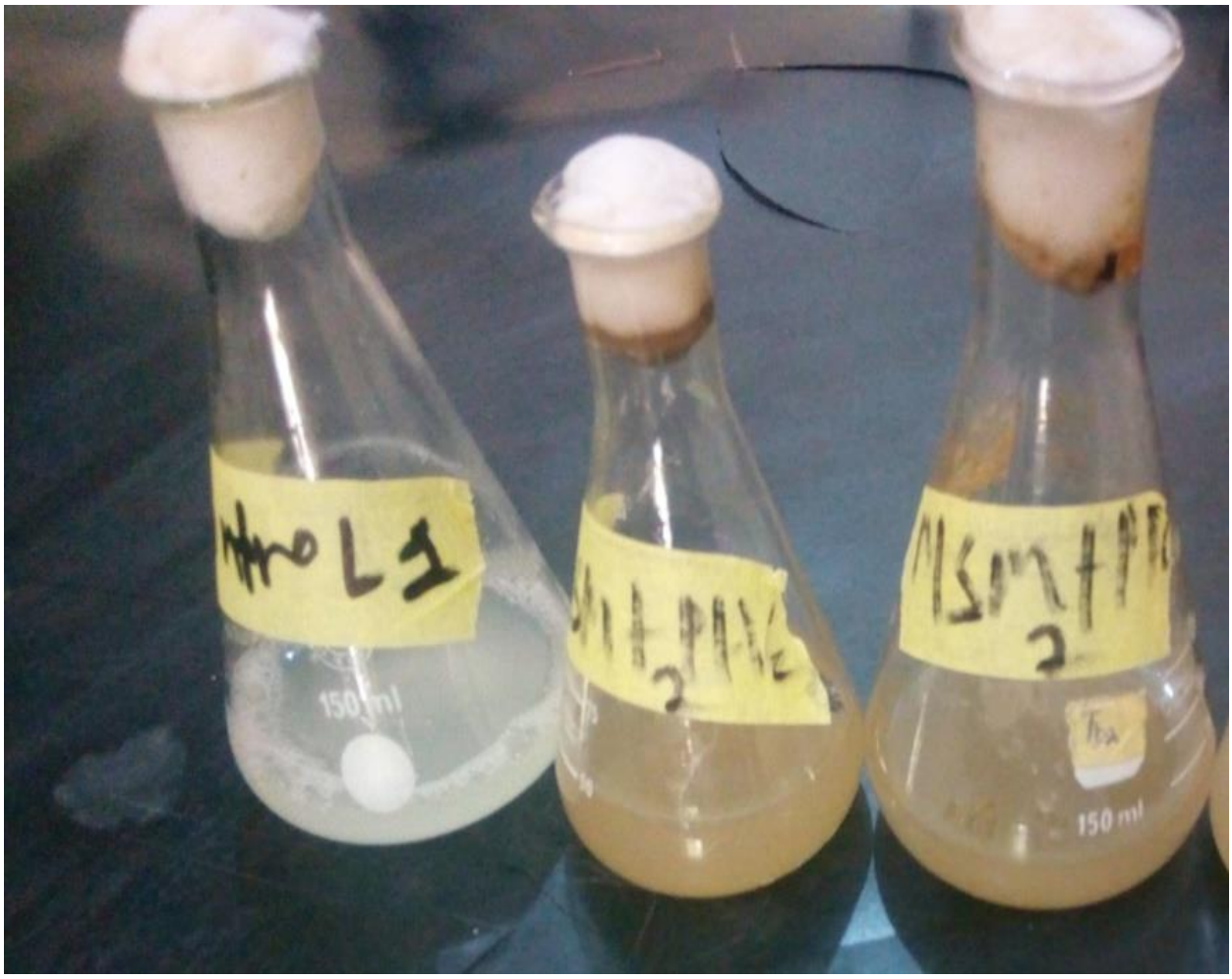


Plate II: Transferred enrichment culture of the polluted soil sample after eight days supplemented with phenanthrene as a source of carbon and energy (100parts per million) in mineral salts medium

Plate III: A 9 day old culture (Biodegradation flask) in mineral salts medium supplemented with phenanthrene (100 parts per million) as a sole source of carbon and energy showing high turbidity compared to the control flask without carbon and energy source.

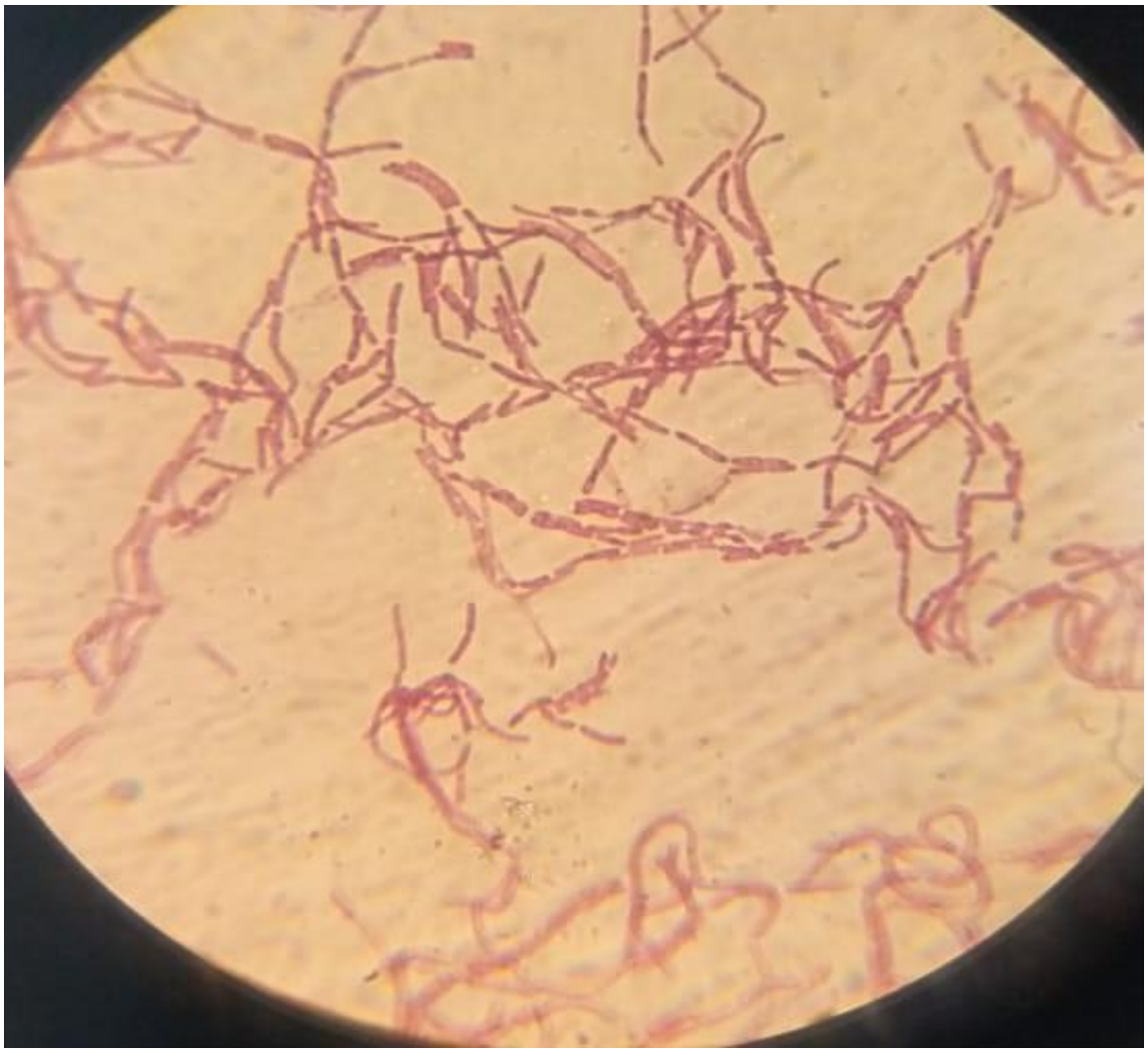


Plate IV: Microscopic picture of *Bacillus subtilis* (×100 magnification)

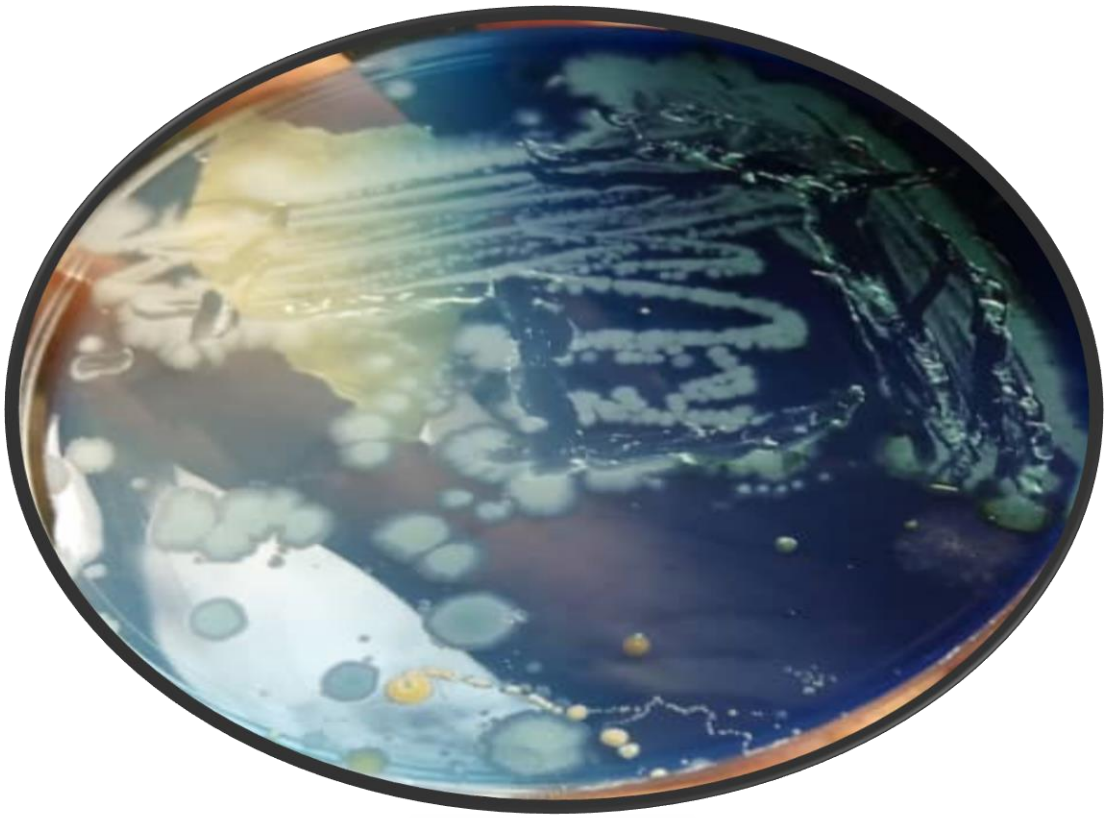


Plate V: Navy blue colouration of *Bacillus subtilis* on luria bertani agar with indole crystals which indicated it being positive for production of dioxygenase enzyme.