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Naphthalene and Anthracene Degrading Potentials of Bacteria Isolated from Soil Samples with Evidence of Polycyclic Aromatic Hydrocarbon(PAH) Contamination

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NAPHTHALENE AND ANTHRACENE DEGRADING POTENTIALS OF BACTERIA ISOLATED FROM SOIL SAMPLES WITH EVIDENCE OF POLYCYCLIC AROMATIC HYDROCARBON(PAH) CONTAMINATION

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Polycyclic Aromatic Hydrocarbon (PAH) from crude oil and other sources is one of the most serious environmental problems. PAH has an adverse effect on human health, water bodies and agricultural products. Removal of PAH through the physical and chemical methods are expensive and not environmentally friendly. Bioremediation, which is the use of microorganisms to degrade environmental pollutants like naphthalene and anthracene and render them less toxic, provides a cheaper and safer method. The objective of this research is to study the naphthalene and anthracene degrading potentials of bacteria isolated from soil samples with evidence of cude oil contamination. Another objective of this study is to determine the effect of pH on the biodegradation potentials of good bacterial degrader that have been identified. The soil samples were enriched in a mineral nutrient that contains Phenol as the only carbon source.

The Bacteria that can grow and be isolated from the mineral nutrient are those that can degrade phenol and make use of its carbon source for growth. These isolated and identified bacteria were further inoculated into mineral nutrient that contained naphthalene and anthracene as the only source of carbon and their growth rate is determined over time. The higher the growth of a bacterium in the medium, the higher the naphthalene and anthracene degrading potential of the bacterium. This research revealed that *Pseudomonas aeruginosa* and *Bacillus thuringiensis* are good degraders of both naphthalene and anthracene. Acidic condition favored the degrading potentials of the 2 bacteria than the basic conditions. Concentration of the PAH (Naphthalene and anthracene) was found to play a significant role in the biodegradation potential of the 2 selected bacteria. The higher the concentration, the lower the degradation potential.

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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
CaCl2	Calcium Chloride
CFU	Colony Forming Units
$C_{14}H_{10}$	Butane
Et al	et alia
FAD	Flavine Adenine Dinucleotide
Fe2 +	Iron (II)
FeCl3	Iron (III) Chloride
g/cm³	Gram per Cubic Centimeter
H_2O	Water
KH2PO4	Monopotassium Phosphate
K2HPO4	Dipotassium Phosphate
Mg/L	Milligram per Liter
MgSO4	Magnesium Sulfate
mL	Milliliters
mol/L	Moles per Liter
MSM	Mineral Salt Medium
NAD^+	Nicotinamide Adenine Dinucleotide
$NADP^+$	Nicotinamide Adenine Dinucleotide phosphate
nm	Nanometers

OD	Optical Density
%	Percentage
pH	Potential of Hydrogen
Rpm	Revolutions per Minute
SOC	Synthetic Organic Compounds
sp.	Specie
USEPA	U.S. Environmental Protection Agency

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CHAPTER 1

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAH) are pollutants that are common in various habitats. PAH are parts of the fossil fuel that are formed due to incomplete combustion of fossil fuels, gas and oil, domestic waste, smoke, soot etc. (Tao et al., 2010). PAH are found in all parts of the environment including the air, water, and soil. PAH is formed when 2 or more aromatic rings are fused. The fused rings can be linear, angular, or clustered. The fused rings range from Naphthalene (2 fused benzene rings) to coronene (7 fused benzene rings) (Kim et al., 2013). PAH are less soluble in water, but they have high boiling, high melting point and low vapor pressure. The molecular weight of PAH is related to its melting point, boiling point, vapor pressure and solubility. High molecular weight is associated with an increase in melting and boiling points and decrease in vapor pressure and solubility. PAH undergoes various stages and reactions in the environment. These include volatilization, photo-oxidation, adsorption by soil particles, leaching and microbial degradation (Tay et al., 2017). PAH may enter the food chain by various methods. One of these methods is when PAH accumulates and gets deposited in soils, sediments, and particulates materials in the water bodies. PAH enters and get accumulated in Fishes because they are highly hydrophobic in fatty tissues. If these fishes are consumed by men, the accumulated PAH are passed into human body, and this leads to serious health issue (Hussein et al., 2016).

Anthracene with molecular formular $C_{14}H_{10}$, whose biodegradation is studied in this research using bacteria isolated from crude oil and petroleum contaminated soil samples, is a three-ring linear PAH. It is an isomer of Phenanthrene with the difference that phenanthrene is arranged in angular manner.

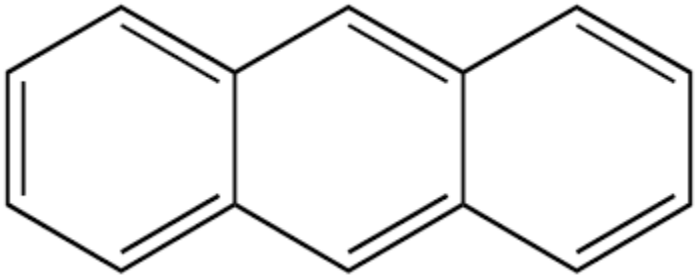

Figure 1: Anthracene

Anthracene is considered by USEPA as a priority pollutant, but it is not acutely toxic, carcinogenic, or mutagenic (Gao et al., 2015). Anthracene and its Methyl derivatives are pollutants found in Cigarette smoke, coal liquefaction products, diesel exhaust and shale oils. When anthracene enters the body system, it launches an attack on the skin, stomach, intestine, and Lymphatic tissues (Tay et al., 2017). Anthracene is found in the water (More than 95%) while 1.4% can be found in the atmosphere. Anthracene has a great affinity to soil particles (Tay et al., 2017).

Naphthalene whose biodegradation is studied in this research is a white solid that exists in various forms with characteristics odor. Its molecular formular is $C_{10}H_{8}$. It has a molar mass of 128.17, a boiling point of 218°C, melting point of 80.26°C and density of 1. 1.14 g/cm³. It is a low molecular weight PAH with highest solubility among the PAHs (Polycyclic Aromatic Hydrocarbon) in the environment.

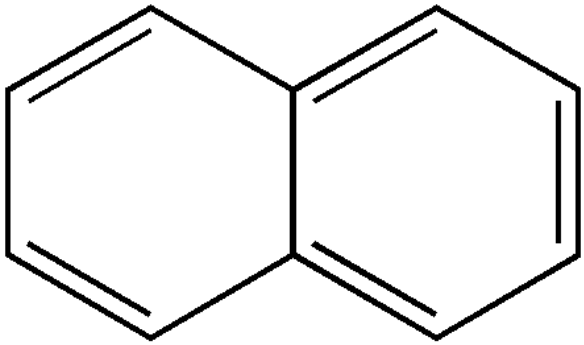

Figure 2: Structure of Naphthalene

The production of naphthalene is through crystallization and distillation of coal tar fractions. Naphthalene has a wide range of uses among which are production of insecticides, dispersant in synthetic and natural rubbers, production of naphthalene sulfonate superplasticizers for concrete and tanning agents in the leather industry. Humans are exposed to Naphthalene through inhalation in oil refineries, vehicle exhaust, gas stations etc. It has been reported that exposure to Naphthalene mothballs can lead to hemolytic anemia in glucose-6-phosphate deficiency patients.

Some enzymes like oxygenase and peroxidases in bacteria are responsible for the degradation of PAH (Tay et al., 2017). Examples of these enzymes are AlkB from *Pseudomonas*, Alkm from *Acinetobacter* sp. Strain, ADP-1, AlkB1 and AlkB2 from Rhodococcus spp; XylE, catechol 2,3 dioxygenases from *Pseudomonas* putida; NdoB as well as different dehydrogenases and protocatechuate dioxygenases in *Stenotrophomonas* spp (Das and Chandran, 2011). The enzymes produced by aerobic microorganisms oxidize low molecular weight PAH. The enzymes are responsible for various reactions like dehydrogenation, aromatic ring cleavage and aromatic-ring hydroxylation {mono-or-di-).

These reactions lead to products with higher oxidation which enhances central carbon pathway metabolism (Phale et al., 2020).

Spilled crude oil hydrocarbons are serious contaminants of soil in oil producing countries. Plants, animals, and human life are affected by contamination of soil due to petroleum hydrocarbons (Pinedo et al.,2014). From the oil produced from different nations, it has been estimated that 0.08%-0.4% spilled into the marine and ecosystem. An example of this recent spill occurred in Southern California in October 2021.The oil spill was reported to be from underwater pipe and the amount is estimated to be approximately 25,000 gallons.

Bioremediation is the use of normal microbial flora or introduced microorganisms or other forms of life to degrade environmental pollutants and render them less toxic. Bioremediation can be divided into bioaugmentation and biostimulation. Bioaugmentation is a process whereby exogenous microorganisms are inoculated into polluted sites (Szulc 2014, Van et al., 1998) . In this method, more gene pool is added to the contaminated site (Domde et al., 2007). Bio stimulation is the enhancement of the activities of the already existing microorganisms. The enhancement can be achieved by fertilization with Nitrogen and Phosphorus compounds (Varjani et al., 2017).

The 3 forms of mixtures in petroleum are saturated hydrocarbon, aromatic hydrocarbon, and non-hydrocarbon compounds. The saturated hydrocarbon's structure is simple and made up of carbon-carbon bond and carbon-hydrogen bond that can be degraded easily. It can easily disappear from the soil through photosynthesis and volatilization because it has low boiling points. Aromatic hydrocarbon structure is complex, and it also has high boiling points which makes its removal from the soil difficult.

Polycyclic aromatic hydrocarbons exist in many environmental systems (Guo et al., 2016). In the list of priority control pollutants by Environmental Protection Agency of the United States of America and European Community, 16 PAH have been included and the examples include naphthalene, acenaphthene, fluoranthene, pyrene etc. (Wang, 2014).

Polycyclic aromatic hydrocarbon (PAH) has detrimental effect on Human life and the environment. Petroleum has an abundance of carbon and low Nitrogen compounds, and it can alter the composition and structure of soil organic matter and impact the C/N, C/P, salinity, PH, and conductivity of soil (Li et al., 2009). Secondly, in unpolluted soil there is an abundance of microorganisms due to presence of healthy and clean soil in which there is no need for microorganisms that can degrade crude oil contaminants. This is not the case in contaminated soil because microorganisms that can produce enzymes that can degrade the pollutants can only survive and they form the dominant microorganisms, thereby leading to alteration and reduction in the microbial population because of hydrocarbon pollution (Deng, 2014). PAH can prevent the growth of crops and lead to reduction in the ability of crops to resist pest and diseases {Xu and Lu, 2010). The oil compound can also combine with inorganic nitrogen and phosphorus. This results in reduced nitrogen and phosphorus that is present in the soil which in turn, leads to a reduction in their availability to crops for absorption (Liao et al., 2015). PAH in petroleum chemicals have carcinogenic, mutagenic, teratogenic, and other toxic effects. It poses a serious health threat when it enters humans through breathing, skin and diet which leads to malfunctioning of liver and the kidney. In addition to the pedosphere, the oil pollutants in the soil affect the atmosphere and the water bodies as these pollutants escaped into the atmosphere and water bodies from the soil (Zhang, 2006)

Human as attempted to use physical and chemical approach to remove crude oil and its polyaromatic hydrocarbons contaminants (Wang et al., 2011), but these methods are expensive and non-environmentally friendly. For instance, incineration causes air pollution and contamination of water bodies. In incineration method in which spilled oil is burnt, the atmospheric carbon dioxide, nitrogen oxide and sulfur oxide levels are raised, and these contribute to already existing global warming problem (Nedaa et al., 2020). This research focuses on Anthracene and Naphthalene biodegradation ability of different Bacteria that are normal Microbial flora in PAH contaminated soils in Nigeria and USA (United States).

CHAPTER 2

LITERARY REVIEW

Polycyclic Aromatic hydrocarbon (PAH) like Naphthalene and Anthracene are important raw materials in the industry. Naphthalene is an important raw material in insecticide producing companies. Anthracenes are found in products that contain coal tar like paints, waterproof surface coating and roofing board. Despite their usefulness to human, they are also responsible for human health deterioration because they are mutagenic, genotoxic, and carcinogenic. PAH are Synthetic Organic Compounds (SOCs) that are listed worldwide as a priority pollutant because of the adverse effect they have on human health and the environment. Bioremediation which involves the use of microbes to degrade these environmental pollutants or their conversion to non-toxic intermediate products has been found to be safe and environmentally friendly pollutant removal (Prashant, 2021) than physical and chemical methods like dichlorination, solvent extraction and fixation. These methods have the disadvantage of high cost, complex process, inefficiency, and safety problem (Sarkar et al., 2020).

Enzymes Involved in Naphthalene and Its Derivatives Degradation

PAH are environmental pollutants of concern. They have a skeleton of Carbon with aromatic rings of Hydrogen atoms. Examples of PAH are anthracene, naphthalene,

phenanthrene etc., Low molecular weight PAH can resist natural degradation because they are hydrophobic and sparingly soluble. Aerobic microbes use enzymes of the class oxido-reductase to oxidize the PAH (Phale et al., 2020). The oxidation leads to aromatic -ring hydroxylation (mono or di-), aromatic ring cleavage and dehydrogenation. Naphthalene dioxygenase enzymes produced by some bacteria degrade naphthalene by attacking the aromatic ring. This leads to the formation of dihydrodiol product. CIS-dihydrodiol dehydrogenase enhances dehydrogenation of cis-naphthalene dihydrodiol to 1,2-dihydroxynaphthalene. The latter product is metabolized by ring operation reaction that has been enhanced by dioxygenase to produce 2-hydroxychromene 2-carboxylate which is metabolized to salicylate. Catechol or gentisate metabolizes the salicylate to central carbon metabolites. Oxygen hydrolase dehydrogenase and oxidase are involved in the metabolism of Naphthalene and its derivatives. This metabolic pathway is described in the table 1 below (Phale et al., 2020).

Table 1: Biochemical Details of the Enzymes Responsible for Degradation of Naphthalene and Its Derivatives (Prashant and Kamini, 2019)

Enzymes	Genes	Mol. wt. (in kDa)* and organization$	Co-factors	Gene Ontology function
Naphthalene 1,2-dioxygenase	*nah*Aa	35.5	2Fe-2S	Two iron, two sulfur cluster binding
reductase (ferredoxin reductase)		Trimer	FAD	Dioxygenase activity
EC: 1.14.12.12			FMN	Electron transfer activity
			NADH	Metal ion binding
Naphthalene 1,2-dioxygenase	*nah*Ab	11.4	2Fe-2S	Two iron, two sulfur cluster binding
(ferredoxin component)		Trimer	NADH	Dioxygenase activity
EC: 1.14.12.12				
Naphthalene 1,2-dioxygenase (large	*nah*Ac	55.0	2Fe-2S	Two iron, two sulfur cluster binding
subunit) EC: 1.14.12.12		Dimer	Fe2 + NADH	Dioxygenase activity Ion binding
				Cellular metabolic process
Naphthalene 1,2-dioxygenase (small subunit)	*nah*Ad	20.0	Fe-S	Dioxygenase activity
		Dimer	NADH	Cellular metabolic process

EC: 1.14.12.12				
cis-Dihydrodiol dehydrogenase EC: 1.3.1.29	*nah*B	27.5 Tetramer	$NADP^+$ NAD^+	Cellular metabolic process
1,2-Dihydroxynaphthalene dioxygenase EC: 1.13.11.56	*nah*C	33.9 Monomer	Fe2 +	Dioxygenase activity Cellular metabolic process
2-Hydroxychromene-2-carboxylate	*nah*D	23.1	Glutathione	Isomerase
Isomerase		Dimer		Protein disulfide oxido-reductase
EC: 5.99.1.4				Metabolic process
Trans-*O*-hydroxybenzylidene pyruvate	*nah*E	36.9	–	Aldehyde-lyase activity
hydratase-aldolase		Monomer		Hydratase-aldolase activity
EC: 4.1.2.45				
Salicylaldehyde dehydrogenase EC: 1.2.1.65	*nah*F	52.0 Monomer	NAD^+	Dehydrogenase activity Cellular metabolic process
Salicylate hydroxylase	*nah*G	46.83	NADH	FAD binding
EC: 1.14.13.1		Monomer		Monooxygenase
				Metabolic process
Catechol 2,3-dioxygenase EC: 1.13.11.2	*nah*H (*xyl*E)	35.2 Tetramer	Fe2 +	Dioxygenase Ferrous ion binding
2-Hydroxymuconic semialdehyde dehydrogenase	*nah*I (*xyl*G)	51.8 Dimer	NAD^+	Hydroxylation Oxido-reductase
EC: 1.2.1.85				
2-Hydroxymuconic semialdehyde	*nah*N (*xyl*F)	30.6	–	Hydrolase
Hydrolase		Monomer		
EC: 3.7.1.9				
2-Oxopent-4-enoate hydratase	*nah*L (*xyl*J)	23.9	–	Hydrolase
EC: 4.2.1.80		Dimer		
4-Hydroxy-2-oxovalerate aldolase EC: 4.1.3.39	*nah*M (*xyl*K)	37.4 –	Mn2 +	Aldolase Metal ion binding
Acetaldehyde dehydrogenase	*nah*O	33.1	CoA	Dehydrogenase activity
EC: 1.2.1.10	(*dmp*F	Dimer	NAD^+	NAD binding
4-Oxalocrotonate decarboxylase EC: 4.1.1.77	*nah*K (*xyl*L)	27.456 Dimer	NAD^+	Catalytic activity Metal ion binding
4-Oxalocrotonate tautomerase	*nah*J (*xyl*H)	6.8	–	Isomerase
EC: 5.3.2.6		Hexamer		Metabolic processes
Catechol 1,2-dioxygenase EC: 1.13.11.1	*cat*A	34.2 Dimer	Fe3 +	Oxido-reductase Metal binding
Muconate cycloisomerase EC: 5.5.1.1	*cat*B	41.1 Octamer	Mn2 +	Cycloisomerase Metabolic process
				Metal binding

Microbial Reactions That Help Efficient Degradation

Apart from metabolic efficiency, microbes display some responses like chemotaxis, cell surface alterations, and biosurfactant production that help in metabolizing aromatic pollutants. Chemotaxis responses promote organic pollutants in heterogenous contaminated system. For instance, it has been shown by a study that *pseudomonas 1putida* G7's chemotactic response to naphthalene enhanced degradation of Naphthalene in aqueous solution. Grade G-7 degradation rate is higher than that of chemotaxis-deficient strains (Hanzel et al., 2011). This knowledge of the chemotaxis being responsible for degradation has been employed in engineering some microbes to display higher biodegradation rate.

Mohapatra et al., 2019 reported that vital changes were observed in the structure of some microbes in response to the effect of naphthalene and other PAHs. Hydrophobic interaction of the microbes with the naphthalene was found to distort the acryl chain reaction and resulted in increase in membrane swelling and fluidity. Certain bacteria withstand the bactericidal effect by altering the proportion and composition of fatty acid between saturated and unsaturated fatty acids. *P. stutzeri* changed this ratio from 1:1 to 2:1 when subjected to growth in naphthalene, *Pseudomonas specie* JS 150 changed the ratio from 7.5 to 12, Achromobacter sp. displayed changes in surface charge from -22.5 to -2.5mV (Mohapatra et al., 2019)

Different bacteria show different preference for the carbon source they biodegrade. Microbes prefer to use simple carbon source over the complex carbon source. This is responsible for non-biodegradation of complex PAH if the simple PAHs are present.

Escherichia coli has been reported to use glucose as carbon source before utilizing lactose. *Pseudomonas* have shown hierarchy in carbon source utilization in the order organic acid→glucose→aromatic compounds (Balaram and Prashant, 2021). *P. putida has a unique preference compared to other Pseudomonas* in that it prefers utilization of aromatics (Benzoate, naphthalene, etc.) over glucose. There is no suppression of aromatic degradation and transport genes in this bacterium when glucose and organic acid is available to be used as a source of carbon (Choudhary *et al.,,* 2017) Repression was observed in glucose and metabolic genes when grown on glucose and aromatics. When the bacterium was grown in a mixture that contained aromatic and organic acids, the expression of aromatic metabolism was not altered (Phale et al., 2020).

Biosurfactant production (amphiphilic in nature) ability of some bacteria has helped in pseudo-solubilization and uptake of aromatics for increased biodegradation (Balaram and Prashant, 2021). The knowledge has been widely used to increase the biodegradation rate of hydrocarbons by microorganisms in which chemical surfactants and biosurfactants has been added to bacterial culture. Examples of such biosurfactants are rhamnolipids from *Pseudomonas aeruginosa, viscosin from* P. fluorescens, and trehalose dimycolipids from *Rhodococcus* sp. etc. The surfactants can reduce the surface tension from 72 to less than 30dyn/cm. Because of the importance of biosurfactant in biodegradation, genetic engineering has been employed to increase the biosurfactant production of some bacteria. An example of this is the enhancement of *Bacillus* sp. to produce surfactin through exchange of the promoter (srfA operon) and manipulation of the surfactin exporter Yerp, regulator ComX and PhrC. (Jiao et al., 2017).

Factors Affecting PAH Degradation

It is important to observe the effect of different environmental conditions on the ability of microbes to biodegrade PAH. Microbes are exposed to different environmental factors at the contaminated ecosystem (Temperature, pH, Oxygen, nutrient availability etc.). These conditions vary in different environment, and they have different effects on the metabolism pathway of microbes.

Temperature affects the biodegradation abilities of microorganism. A study showed that increase in temperature will lead to decrease in dissolve oxygen and this affect the metabolism of aerobic bacteria because oxygen is needed for oxygenase that helps in better uptake and biodegradation of PAH. Increase in temperature changes PAH to a more harmful substances and this affect the biodegradation ability of the microbe (Muller et al., 1998). There is a temperature range above which a microbe can biodegrade optimally. Biodegradation ability is reduced above or below this temperature range. A *Pseudomonas sp. strain* ZJF08's best temperature for biodegradation was 30◦C, above this, a decrease in biodegradation was observed (Ying et al., 2007).

There is also a pH range at which biodegradation is optimal. Most PAH contaminated environment falls outside this range' For instance, acidic mine drainage impacted sites were reported to have a PH of 1-4 and the pH of alkaline leachate impacted gas sites was between 8-12 (Gupta and Sar, 2020). This extreme pH can be adjusted by the addition of ammonium nitrate for alkaline soil and liming with calcium or magnesium carbonate for acidic environment (Gupta and Sar, 2020).

Microbes require nutrients like Nitrogen, Phosphorous, Potassium and Sodium for growth. When these nutrients are supplemental (biostimulation) in the contaminated site, these enhance the growth of the microbes and increase the rate of bioremediation (Sakar et al., 2020).

Anthtacene Biodegradation by Bacteria

It has been reported by a study that some bacteria like *Bacillus, Escherichia coli and Mycobacterium* can efficiently degrade anthracene even when heavy metals are present. They can reduce the harmful effect of heavy metal (Chang et al., 2016). A study reported that *Mycobacterium sp*. strain PYR-1 had the ability to degrade 92% of anthracene after 2 weeks of inoculation (Deorge et al., 2001) 3-(2-carboxy vinyl) naphthalene-2 carboxylic acid and 6,7-benzocoumorin was the bye products after degradation. *Streptomonas* species which are widely distributed gram-negative bacteria has been reported as a biodegrader of phenanthrene, xylene, naphthalene and phenanthridine. The highest growth was on naphthalene. The sequencing of the genome showed that it has 145 genes that are associated with PAH degradation. Some of the genes are chloromuconate isomerase (PEM_00043 EC:5.5.1.7), carboxymethylenebutenolidase etc. (Temidayo et al., 2020).

Ying et al., (2007) reported that Pseudomonas sp. Strain ZJF08 could degrade 97.1% of phenanthrene in 7 days. The best temperature was 30 and the best pH in the first 4 days was 6.5. The report further stated that the effect of pH was studied because pH determines the microbial diversity and activity in terms of parameters like transport process, nutrient solubility, and transport process. It was found out that the best pH at the

end of the biodegradation process was 7.5 in which there was a biodegradation of 98.4% of phenanthrene.

Multi-Biodegradation Ability of Nocardia

Nocardia Specie has been reported as a degrader of PAH, phenol and sodium sulfate (Davood and Shojaei, 2020). *N. farcinica, N. kroppenstedtti and N. fluminea* were able to utilize PAH as carbon source. Mixed PAH that contains acenaphthene, acenaphthylene, anthracene, naphthalene, pyrene etc. were degraded. *N. fluminea* was found to be the best biodegradation bacteria among the *Nocardia sp.* It degraded 90% of the PAH in mineral salt medium within 6 days of inoculation. *N. farcinica* degraded 80% while *N. kroppenstedtii* degraded 70% of PAHs. Not all *Nocardia specie* can degrade PAHs. *N. sienata* and *N. carnea* could not degrade the PAH according to the study.

Anthracene Biodegradation by Fungi

The great success recorded in fungal degradation is because of biomass production and rapid growth of fungi. Biostimulation is not required in biodegradation by fungi. *Aspergillus* was reported to degrade 15% of anthracene at a pH range of 5.0-7.5 and temperature of 30ºC (He et al., 2011). Different enzymes are responsible for the biodegradation by fungi, and they give different PAH metabolites. Examples of these enzymes are Lignin from peroxidase *Phanerochaete chrysosporium* which gives 9,10 - Anthraquinone as the metabolite, Lignin peroxidase from *Aspergillusfumigatus* that gives Phthalic anhydride, anthrone and anthraquinone as the PAH metabolites.

Dawoodi et al., (2015) reported that *Aspergillus* could degrade hydrocarbons from soil samples collected from oil contaminated area in Iran. From the 13 heterotrophic fungi isolated, only 2 (15%) were oil utilizing Fungi. The number of isolated Fungi is determined by the year of exposure of the soil to the oil contaminant. For instance, a soil sample with higher organic compound of 20.51% (Longer time of exposure) has 378 heterotrophic and 228 oil utilizing fungi (Cfu/g soil) while another soil sample with organic carbon of 5.13% (shorter time of exposure) has 3 heterotrophic fungi and 2 oil utilizing fungi. Soil samples with high organic carbon of 20.51% and greater population have fewer fungal diversity than those with lower organic carbon. The reduction in the diversity of fungal population in heavily contaminated soil was explained to be because of the stress imposed by oil hydrocarbons (Obire and Anyanwu, 2009). Chaillan et al., in 2004 reported that *Aspergillus and Penicillium* are the most common hydrocarbon degraders among the genera of fungi. Aspergillus has been reported to be isolated from diesel samples from refineries, fuel, and injection pumps of cars as well as the storage tanks.

Effect of bacteria in the biodegradation has been widely studied and researched but most of them are at lower concentrations. This research focus is on the potentials of the normal microbial flora of soil with evidence of PAH contamination to biodegrade higher concentration of naphthalene and anthracene in the environment. The effect of pH on the potentials of the bacteria to use naphthalene and anthracene as carbon source were studied.

CHAPTER 3

DESIGN OF THE STUDY

Samples Collection

Soil samples with evidence of PAH contaminations were aseptically collected from 8 different sites in Nigeria and 2 different sites in USA (environment of the workshop of motor mechanics that do oil change for many years).

Sample Preparations and Bacterial Isolations

Mineral Salt Medium (MSM) was used to enrich the soil samples. The enrichment was done by the addition of 2g of each soil sample to 250ml Erlenmeyer flask that contains 100 ml sterile MSM medium (0.2 g·L-1 $MgSO_4 \cdot 7H_2O$, 0.02 g·L-1 $CaCl_2$,1.0 g·L-1 KH_2PO_4, 1.0 g·L-1 K_2HPO_4 and 0.05 g·L-1 $FeCl_3$, (Kahng et al., 2002). 5 mL Phenol was added as the sole carbon source. The culture was incubated with orbital shaking at 120rpm at 37°C for 21 days. The plates were incubated at 37°C after which pure colony was isolated based on the colony morphology.

PAH Degrading Bacteria Screening

Each isolated pure culture was checked for catechol 2,3-dioxygenase enzyme production using Franklin method (Ying et al., 2007). One drop of 100mMcatechol was added to the growing colony on the plates and the plates were left undisturbed for 1 minute

to observe if there will be any color change. A color change of reddish brown within 1 minute was an evidence of 2,3-dioxygenase enzyme formation after the PAH metabolism.

Growth Response During PAH Degradation Determination

Isolated bacteria with evidence of PAH degradation and enzyme production tolerance to high concentration of naphthalene and anthracene were checked. To do this, each of the selected bacteria were inoculated into sterile MSM that contained 50mg/L of naphthalene, 100mg/L of naphthalene, 50 mg/L of anthracene and 100mg/L of anthracene in 2 different bottles. The pH of the culture in the first bottle was made acidic and adjusted to 4.7 by the addition of 10 $mol \cdot L^{-1}$ Hydrochloric acid solution, while the PH of the second bottle was made to be basic and adjusted to 11.7 by the addition of 10 mol·L-1 ammonium solution. The bottles were incubated and the absorbance reading of the samples were taken at 24 hours interval by Spectrophotometer at wavelength 600nm.

BIOCHEMICAL TESTS

Gram Stain

A smear of the selected bacteria was prepared on a clean slide with a loopful of the culture. The slide was air dried, and heat fixed. Crystal violet was used to stain the slide for 1 minute after which the slide was rinsed with the water. Gram's iodine was used to stain the slide for 1 minute after which it was rinsed with water. The slide was decolorized with 70% alcohol in a fume chamber. The slide was counterstained with safranin, blot dried and viewed under the microscope.

Catalase Test

A loopful of the bacterial sample was aseptically collected and placed on a clean microscope slide. A drop of 3% hydrogen peroxide was added to the slide with the aid of a dropper. The slide was observed for the formation of bubbles.

Oxidase Test

Two-three drops of oxidase reagent were placed on a filter paper. Small portions of the culture were added on the filter paper with the aid of a sterile glass rod. The filter paper was observed for any color change within 30 seconds. If the color changes to dark blue within 30 seconds, it is oxidase positive, absence of coloration or delayed coloration indicated a negative result.

Nitrate Reduction Test

The selected isolates were inoculated in nitrate broth and incubated at 37°C for 24 hours. The culture was observed for formation of Nitrogen gas.6-7 drops of nitrogen reagent A and B were added and the culture was observed for any color change. Zinc powder was added and the set up was observed for any color change in 3 minutes.

Bacterial Identification with BIOLOG GEN III Technology

A day culture was prepared. The culture was inoculated into IFA GEN III fluid at optical density 0.01. Pipette was used to load the bacterial sample into GEN III plate, and this was incubated for 24 hours. After 24 hours of incubation, the plates were read by the Biolog system, and the names of the bacteria displayed were recorded.

CHAPTER 4

RESULTS AND DISCUSSION

The result shown on Table 2 indicates that out of the 15 distinguishing bacteria that could use phenol as carbon source, only 2 (13.3%) were able to produce catecholase. This indicates that not all bacteria that can degrade any carbon source can be a good isolate for PAH biodegradation. Some were weak in their biodegradation potential while some were strong. The 2 best isolates: 2A2 and 7 A1 were identified as *Pseudomonas aeruginosa and Bacillus thuringiensis* by the Biolog identification method and so were coded as *Pseudomonas aeruginosa* 2A2 and *Bacillus thuringiensis* 7A1.

Table 2: Distinguishing Bacteria that Can use Phenol as Carbon Source and Can Produce Catechol Enzymes

SOIL SAMPLE	SITES OF THE SAMPLE	NUMBER OF PHENOL DEGRADER	BACTERIAL IDENTIFICATION NUMBER	CATECOL PRODUCTION
1	NIGERIA	1	1A	NEGATIVE
2	NIGERIA	3	2A1 2A2 2A3	NEGATIVE POSITIVE NEGATIVE
3	NIGERIA	2	3A1 3A2	NEGATIVE NEGATIVE
4	NIGERIA	1	4A	NEGATIVE
5	NIGERIA	2	5A1 5A2	NEGATIVE NEGATIVE
6	NIGERIA	2	6A1 6A2	NEGATIVE NEGATIVE
7	USA	2	7A1 7A2	POSITIVE NEGATIVE
8	USA	2	8A1 8A2	NEGATIVE NEGATIVE

As shown in Figure 3 *pseudomonas aeruginosa* was able to adapt to the environment and use naphthalene as carbon source faster than *Bacillus thuringiensis* as revealed by the OD measurement at concentration 50mg/L. *Bacillus thuringiensis* was able to start significant biodegradation of naphthalene at this concentration after 48 hours while significant biodegradation of *Pseudomonas aeruginosa* started after 24 hours. *Pseudomonas aeruginosa* has the higher biodegradation effect. Naphthalene biodegradation reduced at basic pH at the same concentration of 50mg/L when compared to the acidic medium (Figure 4)

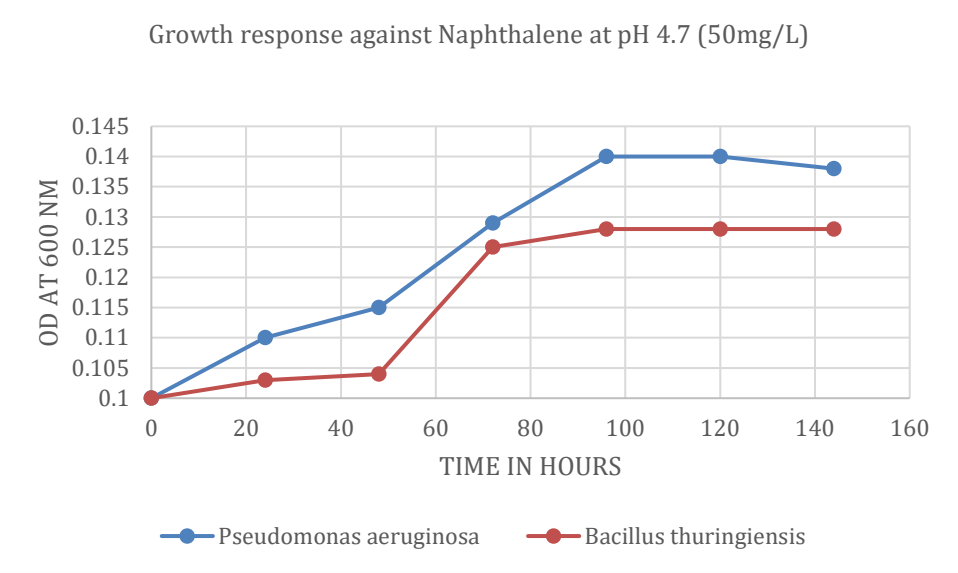

Figure 3: Influence of 50mg/L naphthalene, an acidic pH (4.7) on the growth of *Pseudomonas aeruginosa* 2A2 and *Bacillus thuringiensis* 7A1, Incubated over 144 Hours

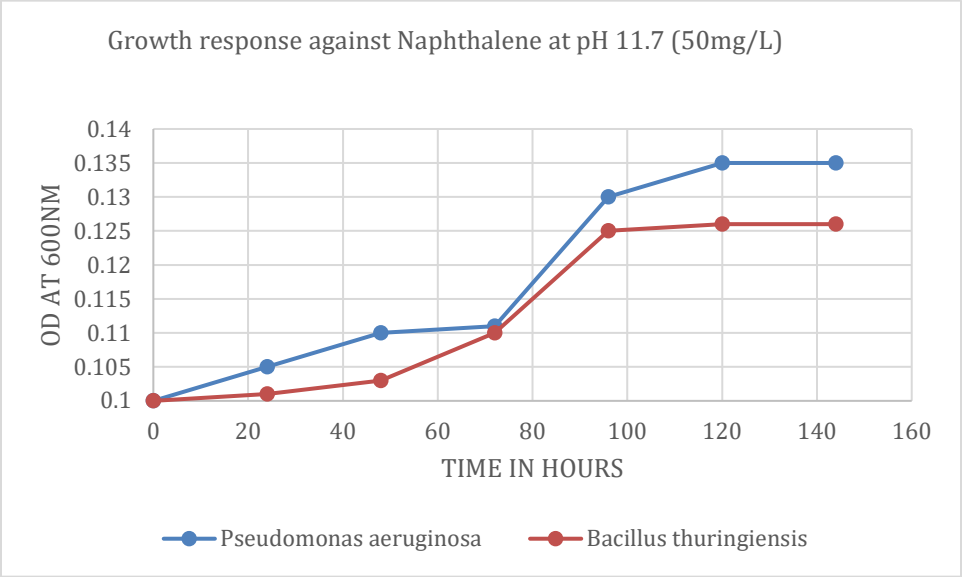

Figure 4: Influence of 50mg/L Naphthalene, a Basic pH (11.7) on the Growth of *Pseudomonas aeruginosa* 2A2 and *Bacillus thuringiensis* 7A1 Incubated Over 144 Hours

In all the days, *Pseudomonas aeruginosa* can biodegrade Naphthalene at concentration 100mg/l than *Bacillus thuringiensis*. Naphthalene Biodegradation at the concentration of 100mg/L is less than that of 50mg/L even under the same acidic condition for the 2 bacteria. This is an indication that the ability of the bacteria to biodegrade Naphthalene depends on the concentration and that it reduced with increasing concentration under constant Ph (Figure 5). At 100mg/L of Naphthalene concentration, the biodegradation abilities of the 2 bacteria are lower in Basic medium than acidic medium. This is an indication that the acidic pH is favorable for the bacteria for better biodegradation at this concentration (Figure 6).

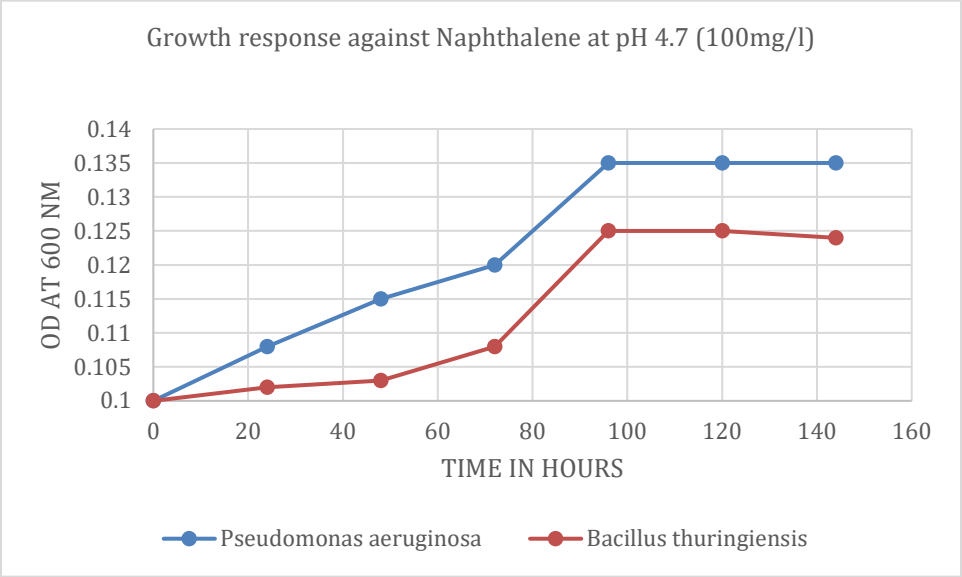

Figure 5: Influence of 100mg/L Naphthalene, An Acidic pH (4.7) on the Growth of *Pseudomonas aeruginosa* 2A2 and *Bacillus thuringiensis* 7A1 Incubated Over 144 Hours

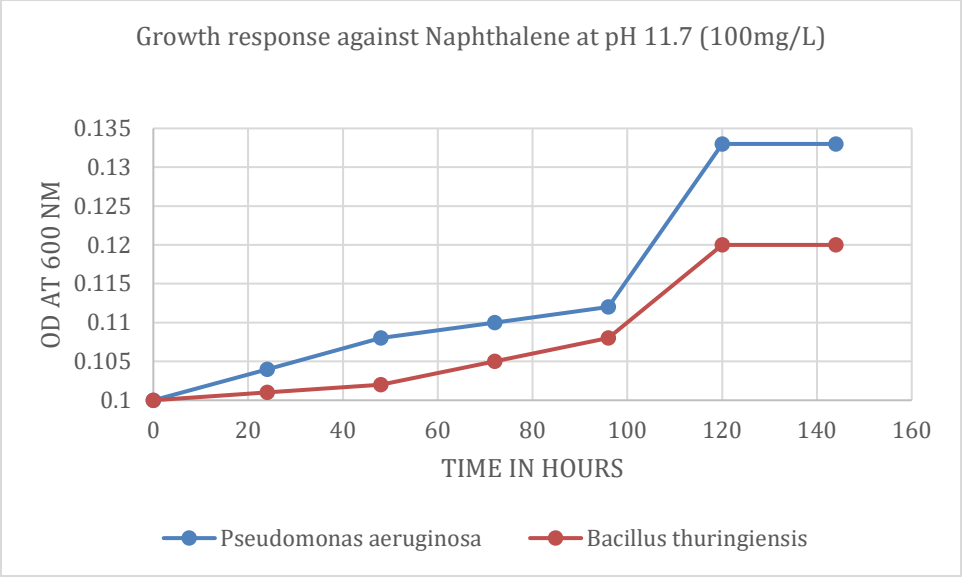

Figure 6: Influence of 100mg/L Naphthalene, a Basic pH (11.7) on the Growth of *Pseudomonas aeruginosa* 2A2 and *Bacillus thuringiensis* 7A1, Incubated Over 144 Hours

Figure 7 shows the overall biodegradation rate of the 2 bacteria is lower at concentration 150mg/L than that of 50mg/L and 100mg/L. This is an indication that the concentration of the PAH had an inverse relationship with the biodegradation of naphthalene for the 2 bacteria. At this concentration, naphthalene biodegradation is lesser under basic pH. This suggested that while using the 2 bacteria for environmental clean-up, it is better to supplement the contaminated soil with acidic solution if better biodegradation is to be achieved (Figure 8).

In Figure 9 the biodegradation rate of the 2 bacteria at the same concentration and pH is lesser in anthracene than in naphthalene. This is an indication that *Pseudomonas aeruginosa* and *Bacillus thuringiensis* have better potentials to degrade Naphthalene than anthracene even under the same concentration and pH.

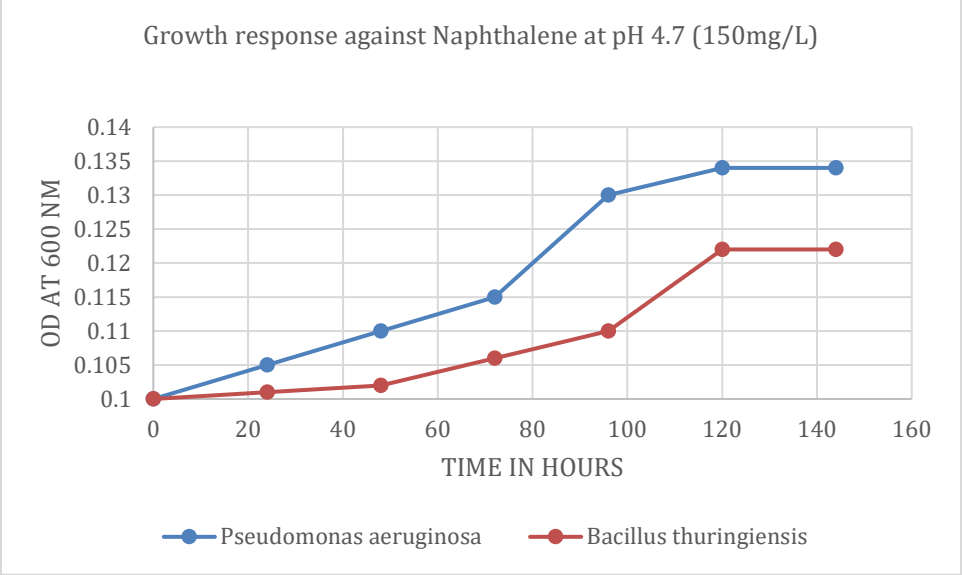

Figure 7: Influence of 150mg/L Naphthalene, an Acidic pH (4.7) on the Growth of *Pseudomonas aeruginosa* 2A2 and *Bacillus thuringiensis* 7A1 Incubated Over 144 Hours

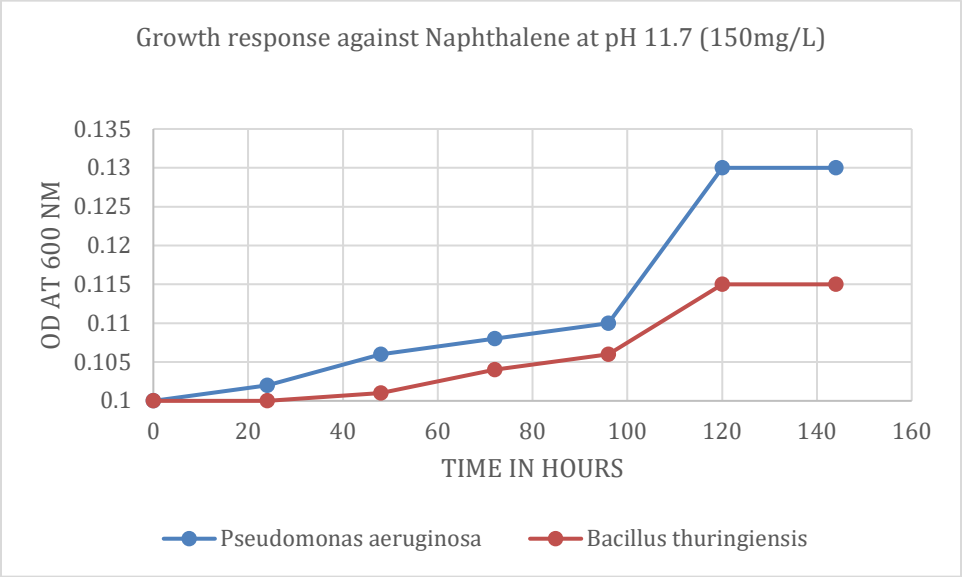

Figure 8: Influence of 150mg/L Naphthalene, a Basic pH (11.7) on the Growth of *Pseudomonas aeruginosa* 2A2 and *Bacillus thuringiensis* 7A1 Incubated over 144 Hours

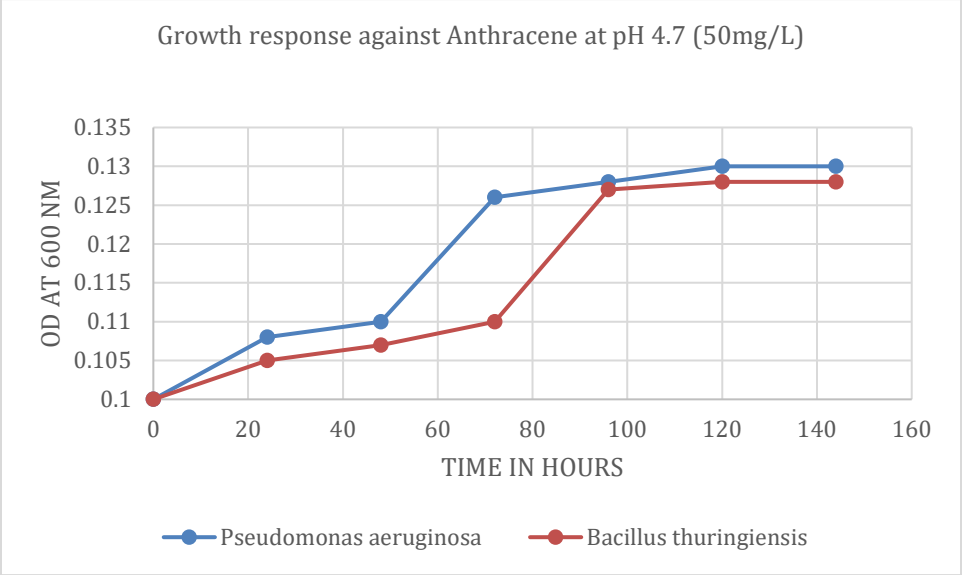

Figure 9: Influence of 50mg/L anthracene, an Acidic pH (4.7) on the Growth of *Pseudomonas aeruginosa* 2A2 and *Bacillus thuringiensis* 7A1 Incubated Over 144 Hours

As shown in Figure 10 the biodegradation rate of the 2 Bacteria under basic condition at concentration 50mg/l of anthracene is lower than under acidic condition. This is an indication that *Pseudomonas aeruginosa and Bacillus thuringiensis* required acidic condition for optimal degradation of anthracene at concentration 50mg/L. The potentials of the 2 bacteria to biodegrade anthracene is lower at concentration 100mg/L than 50mg/L under the same acidic condition. This means that the concentration of the medium determined the biodegradation potentials of anthracene by the 2 isolates. The higher the concentration of the medium, the lower the biodegradation potentials (Figure 11).

In Figure 12 The biodegradation ability of *Pseudomonas aeruginosa* at this concentration and pH is higher than that of *Bacillus thuringiensis.* This is an indication that apart from the concentration and pH of anthracene, another important factor that determined the biodegradation rate is the type of bacteria used for the biodegradation. It takes 96 hours before *Bacillus thuringiensis* could display significant potential of Anthracene biodegradation at this PH and concentration. The significant biodegradation is between day 4 and day 5 and the stationary phase started at day 5. This bacterium may not be useful as Anthracene degradation bacteria at a concentration that is higher than this under acidic medium (Figure 13).

The 2 bacteria in Figure 14 shows the potential of biodegradation of anthracene at this concentration and PH. The biodegradation effect of these 2 bacteria under basic pH is lesser than that of acidic pH for the same concentration. This indicate that the 2 Bacterial requires acidic medium for optimal biodegradation.

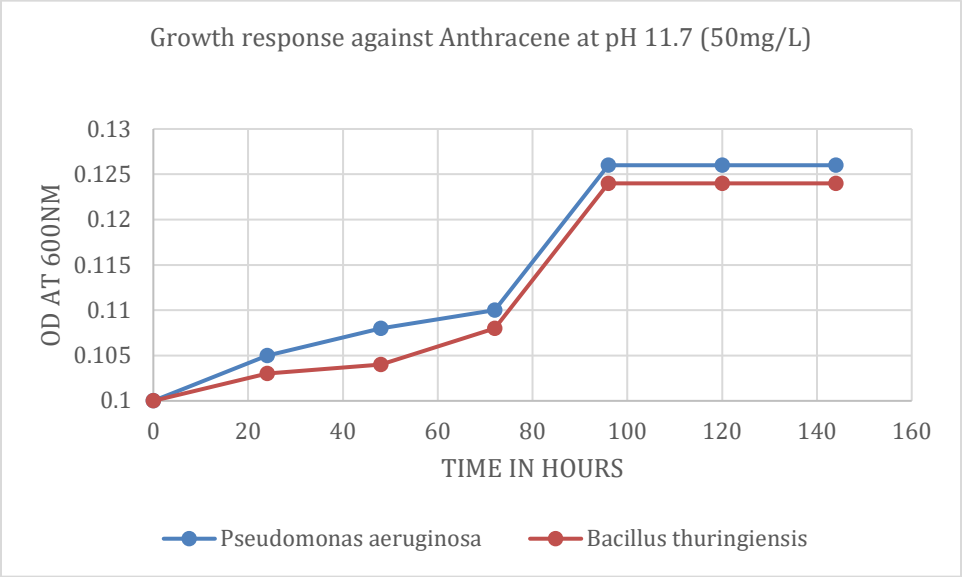

Figure 10: Influence of 50mg/L Anthracene, a Basic pH (11.7) on the Growth of *Pseudomonas aeruginosa* 2A2 and *Bacillus thuringiensis* 7A1 Incubated Over 144 Hours

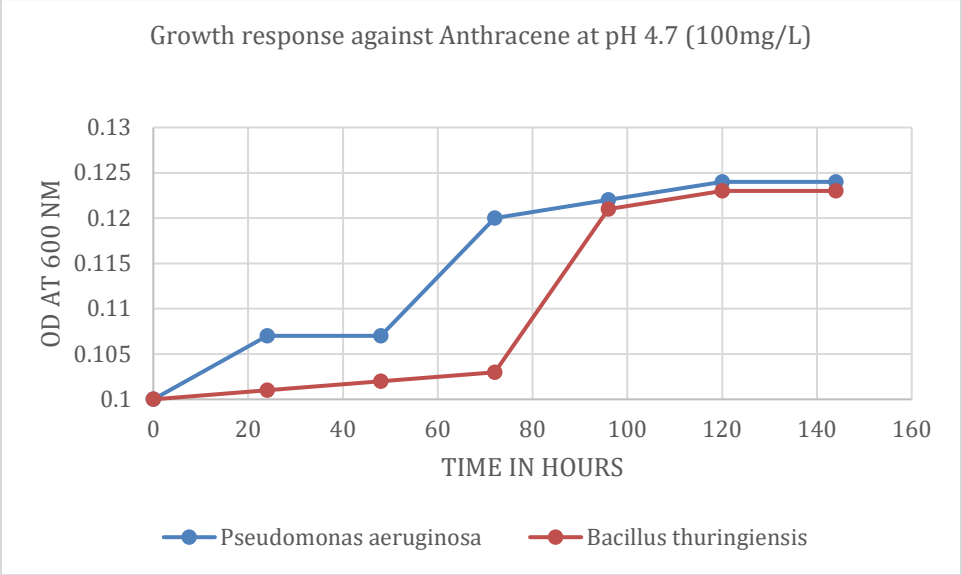

Figure 11: Influence of 100mg/L anthracene, an acidic pH (4.7) on the Growth of *Pseudomonas aeruginosa* 2A2 and *Bacillus thuringiensis* 7A1 Incubated Over 144 Hours

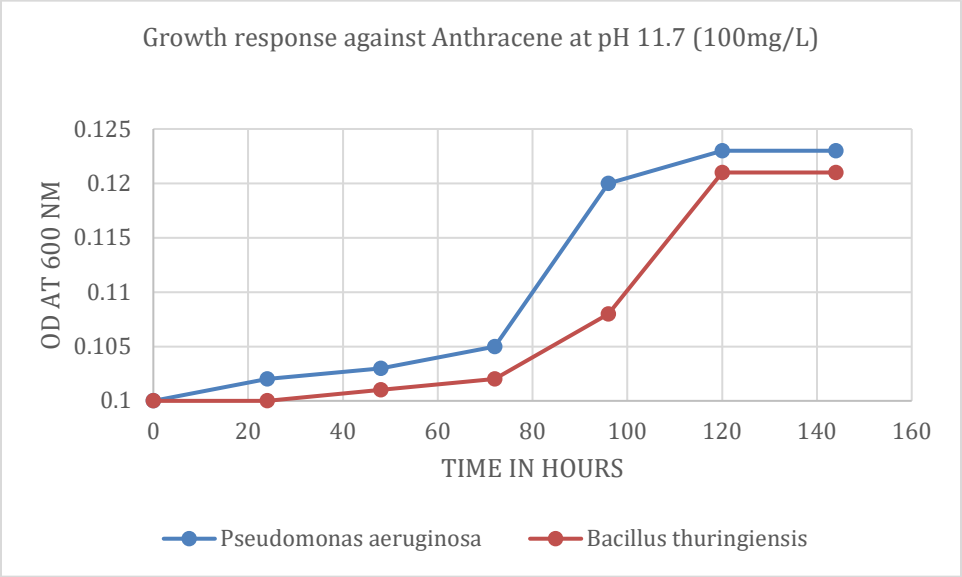

Figure 12: Influence of 100mg/L Anthracene, a Basic pH (11.7) on the Growth of *Pseudomonas aeruginosa* 2A2 and *Bacillus thuringiensis* 7A1 Incubated Over 144 Hours

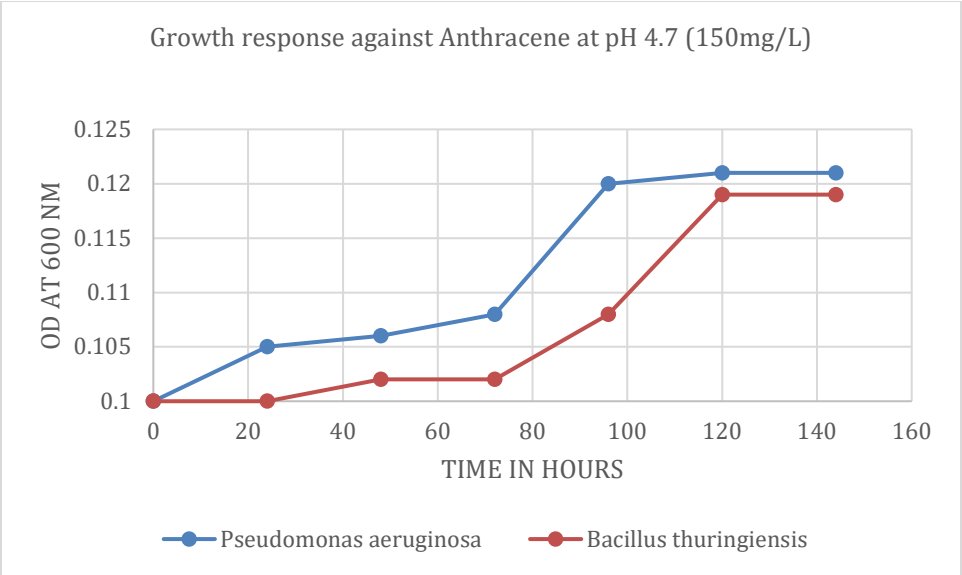

Figure 13: Influence of 150mg/L Anthracene, an Acidic pH (4.7) on the Growth of *Pseudomonas aeruginosa* 2A2 and *Bacillus thuringiensis* 7A1 Incubated Over 144 Hours

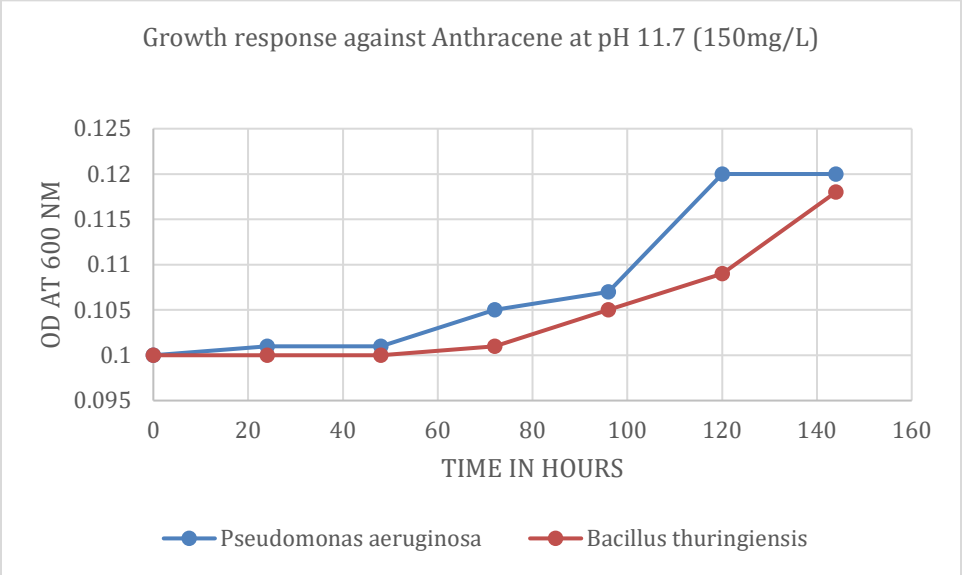

Figure 14: Influence of 150mg/L Naphthalene, a Basic pH (11.7) on the Growth of *Pseudomonas aeruginosa* 2A2 and *Bacillus thuringiensis* 7A1 Incubated Over 144 Hours

The best 2 isolates that were used in this study were found to be Gram positive and gram-negative bacteria (Table 3). This might suggest that the Peptidoglycan layers of Bacterial cell might not be the only determinant of the biodegradation, but the enzymes produced by the Bacteria.

Table 3: Identification and Biochemical Reactions of Best 2 Isolates for Catecol Enzyme Producer

BACTERIA	GRAM REACTION	CATALASE TEST	OXIDASE TEST	NITRATE REDUCTION TEST
Pseudomonas aeruginosa	Gram negative	Positive	Positive	Positive
Bacillus thuringiensis	Gram positive	Positive	Negative	Positive

The research showed that *Pseudomonas aeruginosa* displayed better degradation of naphthalene and anthracene than *Bacillus thuringiensis* even under the same pH at all concentrations. This is an indication that the PAH biodegradation of Bacteria varies even at the same concentration. This agrees with Ying et al., 2007 that reported that *Pseudomonas aeruginosa* strain ZJF08 is a better degrader of phenanthrene than Rhizobium specie ZJF05(97% against 95%).

This research also revealed that *Pseudomonas aeruginosa* and *Bacillus thuringiensis* degraded naphthalene and anthracene better under acidic condition. This agrees with Ying et al.,,2007 that reported that pH has effect on the biodegradation potentials of *Pseudomonas aeruginosa*. It was found out from the research that in the first 72 hours, *Pseudomonas aeruginosa* was able to degrade 75% of phenanthrene at pH 6.5 while 60% was degraded at pH 7.5. The effect of pH on the degradation potential of *Bacillus thuringiensis* in this study also agrees with Gangireddygari et al., 2017 who reported that *Bacillus thuringiensis* displayed more degradation of quinalphos at pH 6.5 than 8.5 (85% against 78.95%).

CHAPTER 5

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

The objective of this study is to determine if normal Bacterial flora of soil samples with evidence of crude oil contaminations could degrade high concentrations of naphthalene and anthracene. This research showed that 2 out of the 15 different isolates of distinguishing bacteria (*Pseudomonas aeruginosa* and *Bacillus thuringiensis*) were good degraders of the 2 PAH. This suggests that not all bacteria isolated from crude oil contaminated environment can degrade naphthalene and anthracene. Some might be an opportunistic bacterium while some might be a good degrader of other PAH other than naphthalene and anthracene.

This study showed that the concentration of the PAH contaminants (naphthalene and anthracene) affects bacterial potentials to degrade the PAH. *Pseudomonas aeruginosa* and *Bacillus thuringiensis* degrading potentials reduced with increase in concentrations. The pH of the medium is also a factor that determines the PAH degradation. Optimal biodegradation potential of the studied isolates was achieved under acidic conditions.

The gram reactions of the 2 bacteria (*Pseudomonas aeruginosa*-positive and *Bacillus thuringiensis*-negative) are an indication that naphthalene and anthracene degradation potential of bacteria is not gram specific. This suggests that other factors like production of degrading enzymes are responsible for the degrading potentials of Bacteria.

The study revealed that *Pseudomonas aeruginosa* and *Bacillus thuringiensis* are good degraders of naphthalene and anthracene even at a very high concentration.

Due to the findings of this study, it is recommended that there should be large scale production, and inoculations of *Pseudomonas aeruginosa* and *Bacillus thuringiensis* to ecosystems that have been contaminated with naphthalene and anthracene because, these 2 bacteria have the potentials to cause bioremediation of naphthalene and anthracene polluted ecosystems. Future research should focus on genetic manipulations of PAH enzyme producing genes of the two bacteria to produce more PAH degrading enzymes to achieve better degradation.

APPENDIX A

FIRST READING FOR BACTERIAL GROWTH RESPONSES DURING PAH DEGRADATION DETERMINATION

TABLE 4: Growth response against Naphthalene at pH 4.7 (50mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.110	0.103
48	0.115	0.104
72	0.129	0.125
96	0.140	0.127
120	0.140	0.128
144	0.138	0.128

TABLE 5: Growth response against Naphthalene at pH 11.7 (50mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.105	0.101
48	0.110	0.103
72	0.111	0.110
96	0.130	0.125
120	0.135	0.126
144	0.135	0.126

TABLE 6: Growth response against Naphthalene at pH 4.7 (100mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.108	0.102
48	0.115	0.103
72	0.120	0.108
96	0.135	0.125
120	0.135	0.125
144	0.135	0.124

TABLE 7: Growth response against Naphthalene at pH 11.7 (100mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.104	0.101
48	0.108	0.102
72	0.110	0.105
96	0.112	0.108
120	0.133	0.120
144	0.133	0.120

TABLE 8: Growth response against Naphthalene at pH 4.7 (150mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.105	0.101
48	0.110	0.102
72	0.115	0.106
96	0.130	0.110
120	0.134	0.122
144	0.134	0.122

TABLE 9: Growth response against Naphthalene at pH 11.7 (150mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.102	0.100
48	0.106	0.101
72	0.108	0.104
96	0.110	0.106
120	0.130	0.115
144	0.130	0.115

TABLE 10: Growth response against Anthracene at pH 4.7 (50mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.108	0.105
48	0.110	0.107
72	0.126	0.110
96	0.128	0.127
120	0.130	0.128
144	0.130	0.128

TABLE 11: Growth response against Anthracene at pH 11.7 (50mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.105	0.103
48	0.108	0.104
72	0.110	0.108
96	0.126	0.124
120	0.126	0.124
144	0.126	0.124

TABLE 12: Growth response against Anthracene at pH 4.7 (100mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.107	0.101
48	0.107	0.102
72	0.120	0.103
96	0.121	0.121
120	0.124	0.123
144	0.124	0.123

TABLE 13: Growth response against Anthracene at pH 11.7 (100mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.102	0.100
48	0.103	0.101
72	0.105	0.102
96	0.120	0.108
120	0.123	0.121
144	0.123	0.120

TABLE 14: Growth response against Anthracene at pH 4.7 (150mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.105	0.100
48	0.106	0.102
72	0.108	0.102
96	0.119	0.108
120	0.121	0.119
144	0.121	0.118

TABLE 15: Growth response against Anthracene at pH 11.7 (150mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.101	0.100
48	0.101	0.100
72	0.105	0.101
96	0.107	0.105
120	0.119	0.109
144	0.120	0.117

APPENDIX B

SECOND READING FOR BACTERIAL GROWTH RESPONSES DURING PAH DEGRADATION DETERMINATION

TABLE:16: Growth response against Naphthalene at pH 4.7 (50mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.109	0.103
48	0.115	0.104
72	0.129	0.125
96	0.140	0.129
120	0.139	0.128
144	0.138	0.128

TABLE 17: Growth response against Naphthalene at pH 11.7 (50mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.105	0.101
48	0.110	0.103
72	0.111	0.110
96	0.130	0.125
120	0.134	0.126
144	0.135	0.126

TABLE 18: Growth response against Naphthalene at pH 4.7 (100mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.107	0.102
48	0.115	0.103
72	0.120	0.108
96	0.135	0.125
120	0.135	0.125
144	0.135	0.124

TABLE 19: Growth response against Naphthalene at pH 11.7 (100mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.103	0.101
48	0.108	0.102
72	0.110	0.105
96	0.112	0.108
120	0.133	0.120
144	0.133	0.120

TABLE 20: Growth response against Naphthalene at pH 4.7 (150mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.105	0.101
48	0.110	0.102
72	0.115	0.106
96	0.130	0.110
120	0.134	0.122
144	0.134	0.122

TABLE 21: Growth response against Naphthalene at pH 11.7 (150mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.102	0.100
48	0.106	0.101
72	0.107	0.104
96	0.110	0.106
120	0.130	0.115
144	0.130	0.115

TABLE 22: Growth response against Anthracene at pH 4.7 (50mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.108	0.105
48	0.110	0.107
72	0.126	0.110
96	0.128	0.127
120	0.130	0.128
144	0.129	0.128

TABLE 23: Growth response against Anthracene at pH 11.7 (50mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.105	0.102
48	0.108	0.104
72	0.110	0.108
96	0.126	0.124
120	0.126	0.124
144	0.126	0.124

TABLE 24: Growth response against Anthracene at pH 4.7 (100mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.106	0.101
48	0.107	0.102
72	0.120	0.103
96	0.122	0.121
120	0.124	0.123
144	0.124	0.123

TABLE 25: Growth response against Anthracene at pH 11.7 (100mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.102	0.100
48	0.103	0.101
72	0.105	0.102
96	0.120	0.108
120	0.123	0.121
144	0.123	0.121

TABLE 26: Growth response against Anthracene at pH 4.7 (150mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.104	0.100
48	0.106	0.102
72	0.108	0.102
96	0.120	0.108
120	0.121	0.119
144	0.121	0.119

TABLE 27: Growth response against Anthracene at pH 11.7 (150mg/L)

HOURS	*Pseudomonas aeruginosa* (OD at 600nm)	*Bacillus thuringiensis* (OD at 600nm)
0	0.100	0.100
24	0.100	0.100
48	0.101	0.100
72	0.105	0.101
96	0.107	0.105
120	0.120	0.109
144	0.120	0.117

REFERENCES

1. Balaram M., Prashant P. (2021). Microbial Degradation of Naphthalene and Substituted Naphthalenes: Metabolic Diversity and Genomic Insight for Remediation. Front. Bioeng. Biotechnol. https://doi.org/10.3389/fbioe.2021.602445.

2. Campbell W. L., Cerniglia C. E., Evans F. E., Freeman J .P., Fu P. P. (1990).Stereoselective fungal metabolism of methylated anthracenes. Applied and Environmental Microbiology, 56(3):661–668

3. Chaillan F., Le F.A., Bury E., Phantavong Y.H., Grimont P. (2004). Identification and biodegradation potential of tropical aerobic hydrocarbon- degrading microorganisms. Res Microbiol, 155: 587-595.

4. Das N., Chandran P. (2011). Microbial degradation of petroleum hydrocarbon contaminants: an overview. *Biotechnology Research International* ,1–13.

5. Cheng M., Jiang L.H., Liu S.H. Liu Y., Niu Q.Y., Tan X.F., Xu P., Zeng G.M., Zhang C., Zhou L. (2016). Bioremediation mechanisms of combined pollution of PAHs and heavy metals by bacteria and fungi: A mini review. Bioresource Technology,224:25–33

6. Das D., Baruah R., Sarma R.A., Singh A.K., Deka B.H.P., Kalita J., Bora T.C. (2015). Complete genome sequence analysis of *Pseudomonas aeruginosa* N002 reveals its genetic adaptation for crude oil degradation. *Genomics,* 105:182–190.

7. Doerge D.R., Freeman J.P., Moody, J.D. (2001). Degradation of phenanthrene and anthracene by cell suspensions of mycobacterium sp. strain PYR-1. Applied and Environmental Microbiology,67(4):1476–1483.

8. Domde P., Kapley A., Purohit H. J. (2007) Impact of bioaugmentation with a consortium of bacteria on the remediation of wastewater-containing hydrocarbons. Environ.Sci.Pollut.Res., 14:7-11

9. Davood A., Hasan S. (2020). Biodegradation of polycyclic aromatic hydrocarbons, phenol and sodium sulfate by Nocardia species isolated and characterized from Iranian ecosystems. Scientific Report,10:21860.

10. Elufisan T.O., Lozano L, Bustos P, Rodríguez-Luna IC, Sánchez-Varela A, Oyedara OO, Villalobos-López MÁ, Guo X. (2019). Complete genome sequence of stenotrophomonas maltophilia strain SVIA2, isolated from crude oil-contaminated soil in Tabasco, Mexico. *Microbiology Resource Announcements,* 8(30): e00529-19.

11. Gao Y., Goikavi C., Kang F., Ling W., Waigi M.G. (2015). Phenanthrene biodegradation by sphingomonads and its application in the contaminated soils and sediments: A review. International Biodeterioration and Biodegradation, 104:333–349.

12. Guo C.L., Zhao Y.H., Wang Y.F., (2016). Research on repairing oil pollution by hydrocarbon degradation microbes. Applied Chemical Industry, 45(6): 1156.

13. Hanze J., Thullner M., Harms H., Wick, L. Y. (2011). Microbial growth with vapor-phase substrate. Environ. Pollut. 159, 858–864.

14. Haritash A.K., Kaushik C.P. (2009). Biodegradation aspects of Polycyclic

Aromatic Hydrocarbons (PAHs): A review. Journal of Hazardous Materials, 169(1–3):1–15.

15. He B.Y., Peng H., Qiang J., Qin H.M., Ye J.S, Yin H., Zhang N. (2011). Biodegradation of anthracene by Aspergillus fumigatus. Journal of Hazardous Materials, 185(1):174–181

16. Hussein I. Mansour M., (2016). A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation.25(1), 107-123.

17. Jiao S., Li X., Yu H., Yang H., Li X., Shen Z. (2017). In situ enhancement of surfactin biosynthesis in *Bacillus subtilis* using novel artificial inducible promoters. Biotechnol. Bioeng., 114:832–842.

18. Juhasz A.L., Naidu R. (2000). Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: A review of the microbial degradation of benzo[a]pyrene. International Biodeterioration and Biodegradation, 45(1):57–88.

19. Kim S.J., Kweon O., Jones R.C., Edmondson R.D., Cerniglia C.E**.** (2008). Genomic analysis of polycyclic aromatic hydrocarbon degradation in Mycobacterium vanbaalenii PYR-1. *Biodegradation,* 19:859–881.

20. Li M., Zheng X. L. (2009). Tong L, et al., Influence of petroleum pollution on the permeability of soils. Acta Scientiarum naturalium Universitatis Sunyatseni, 48(2):122–123.

21. Liao J.Q., Wang J., Huang Y. (2015). Bacterial community features are shaped by geographic location, physicochemical properties, and oil contamination of soil in main oil fields of China. Microb Ecol., 70: 380–389.

22. Mohapatra, B., Kazy, S. K., and Sar, P. (2019). Comparative genome analysis of arsenic reducing, hydrocarbon metabolizing groundwater bacterium Achromobacter sp. KAs 3-5T explains its competitive edge for survival in aquifer environment. Genomics 111, 1604–1619.

23. Muller R., Antranikian G., Maloney S., Sharp R. (1998). "Thermophilic degradation of environmental pollutants," in Biotechnology of Extremophiles: Advances and Challenge, ed. P. H. Rampelotto, (Berlin: Springer), 155–169.

24. Nedaa A., Narjes D., Majida K., Hussain A., Samir R. (2020). Bioremediation of Soils saturated with spilled crude oil. Scientific Reports volume 10, Article number: 1116.

25. Obire O., Anyanwu E.C. (2009). Impact of various concentrations of crude oil on fungal populations of soil. Int. J. Environ Sci., 6: 211-218.

26. Phale P. S., Malhotra H., Shah, B. A. (2020). "Degradation strategies and associated regulatory mechanisms/features for aromatic compound metabolism in bacteria," in Advances in Applied Microbiology, eds G. M. Gadd, and S. Sariaslani, (Cambridge: Academic press), 1–65.

27. Pinedo J., Ibáéez R., Irabien Ã. A. (2014). comparison of models for assessing human risks of petroleum hydrocarbons in polluted soils. Environ Model Software,55:61.

28. Prashant S., Kamini G. (2019). Microbial degradation of xenobiotics like aromatic pollutants from the terrestrial environments. Pharmaceuticals and Personal Care Products.1-20.

29. Sarkar, J., Roy, A., Sar, P., and Kazy, S. K. (2020). ""Accelerated bioremediation of petroleum refinery sludge through biostimulation and bioaugmentation of native microbiome. Emerging Technologies in Environmental Bioremediation, eds M. Sh (Amsterdam: Elsevier).23-65.

30. Szulc A. (2014) The influence of bioaugmentation and biosurfactant addition on bioremediation efficiency of diesel-oil contaminated soil: Feasibility during field studies. J. Environ. Manag.132:121-128.

31. Tay C.C., Abidin N.Z., Talib S.A. (2017). Occurrence and bioremediation of anthracene in the Environment. J. Fundam. Appl Sci,9(6S) :214-226.

32. Temidayo O.E., Oyedara O.O., Rodriquez-Luna I.C. (2020)..The polycyclic aromatic Hydrocarbon (PAH) degradation activities and genome analysis of a novel strain *Streptomonas sp*. Pemsol Isolated from Mexico.PeerJ,8: e8102.

33. United States Environmental Protection Agency (USEPA)(1987). Health and environmental effects profile for anthracene. Washington DC.

34. Van L., H., Top, E. M., Verstraete W. (1998). Bioaugmentation in activated sludge: Current features and future perspectives. Appl. Microbiol. Biotechnol., 50:16–23.

35. Varjani S. J., Upasani V. N. (2017)A new look on factors affecting microbial degradation of petroleum hydrocarbon pollutants. Int. Biodeterior. Biodegr., 120: 71–83.

36. Vida D., Mahbobeh M., Arezoo T., Zeynab G. (2015). The study of heterotrophic and crude oil-utilizing soil fungi in crude oil contaminated regions. J. Biomed Biodeg,6:2.

37. Wang Q., Zhang S., Li Y., Klassen W. (2011). Potential approaches to improving biodegradation of hydrocarbons for bioremediation of crude oil pollution. J. Environ Prot.,2:47–55.

38. Wang B. (2014) Mechanism of growth and physiological response of three arbor species to oil contamination. Shaanxi: Northwest A&F University, 2014.

39. Xu Y H, Lu M. (2010) Bioremediation of crude oil-contaminated soil: Comparison of different biostimulation and bioaugmentation treatments. Hazardous Materials, 183:398–401

40. Zhang Y. F. (2006). The effects of oil field exploration to ecological environment. Environment protection of oil and gas field,12: 28–30.

41. Zhou Y., Wei J., Jiang T, Welfeng G. Ma Y., Wei D. (2007). Characterization of Thermostable catechol-2,3-dioxygenase from phenanthrene-degrading *Pseudomonas* sp. Strain ZJF08. Annals of Microbiology,57(4):503-508.