PAH degradation in artificially lubricant contaminated soil.

A Dissertation

submitted in partial fulfilment of the requirement
for the award of degree of

Master of Technology

in

Environmental Science and Technology

Submitted
By
IPSITA
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Under Supervision of

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CERTIFICATE

This is to certify that the thesis entitled **"PAH degradation in artificially lubricant contaminated soil "**submitted by **Ms. Ipsita** in partial fulfillment of the requirement for the award of **Masters in technology** degree in **Environmental science & technology at Thapar University, Patiala** is an authentic work carried out by her under our supervision and guidance.

To the best of our knowledge the matter embodied in this thesis has not been submitted in any other university/institute for award of any degree or diploma.

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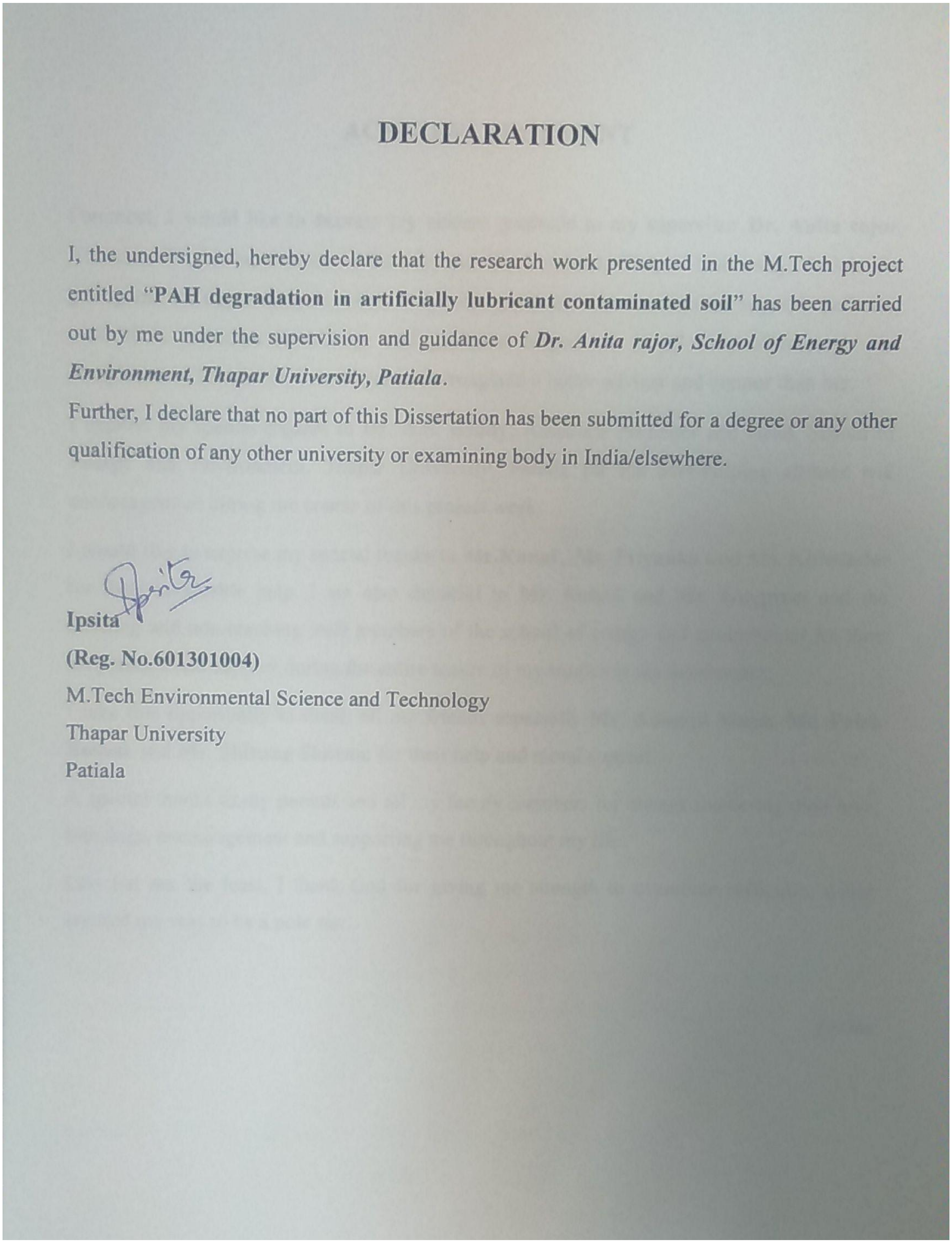

DECLARATION

I, the undersigned, hereby declare that the research work presented in the M.Tech project entitled "**PAH degradation in artificially lubricant contaminated soil**" has been carried out by me under the supervision and guidance of ***Dr. Anita rajor, School of Energy and Environment, Thapar University, Patiala***.

Further, I declare that no part of this Dissertation has been submitted for a degree or any other qualification of any other university or examining body in India/elsewhere.

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ACKNOWLEDGEMENT

Foremost, I would like to express my sincere gratitude to my supervisor **Dr. Anita rajor**, Associate Professor, School of Energy and Environment, Thapar University, Patiala for giving me an opportunity to work under his guidance. Her continuous support, patience, motivation, immense knowledge and guidance helped me in all time of research and completion of this thesis. I could not have imagined a better advisor and mentor than her.

I express my special regards to **Dr. A.S. Reddy,** Associate Professor and Head, School of Energy and Environment, Thapar University, Patiala for his ever-helping attitude and encouragement during the course of this project work.

I would like to express my special thanks to**Mr.Kunal , Ms. Priyanka and Ms. Kulwinder** for their invaluable help. I am also thankful to **Mr. Suhail**and **Mr. Gurpreet**andthe teaching and non-teaching staff members of the school of energy and environment for their cooperation and support during the entire tenure of my studies in the department.

I take this opportunity to thank all my friends especially **Mr. Amanjit Singh, Ms. PalakBansal**and**Mr. Shivang Sharma**for their help and moral support.

A special thanks to my parents and all my family members for always showering their love, blessings, encouragement and supporting me throughout my life.

Last but not the least, I thank God for giving me strength to overcome difficulty, which crossed my way to be a pole star.

Ipsita

Abstract

Bioremediation is considered as one of the safer, cleaner, cost effective and environmental friendly technology for the treatment of oil contaminated sites. Various industrial and anthropogenic have activities resulted in contamination of sites due to unawareness regarding production, use and disposal of hazardous substances. Due to the persistence nature of crude oil it remains into soil and effects it's physical, physiological and biological properties. The spillage of oil leads to the introduction of PAH (polyaromatic hydrocarbons) into the soil. They are ubiquitous in nature and are carcinogenic in nature. Bioremediation of PAH-contaminated soils, sediments, and water can be accomplished in a variety of ways e.g. in situ treatment or ex-situ methods such as bio-piling and composting.

In the present study the bacterial isolates were screened for their capability of degrading PAH present in the lubricant contaminated soil. They were characterized through biochemical and physiological testing for their potential of degrading PAH. Two bacterial strains S3 and S4 were isolated on the basis of their growth in enriched as well as minimal media. The degradation of PAH was carried out using the composting method in which organic manure was utilized as well as using bacterial culture. The used lubricant oil was added to sterile soil to form the synthetic sample and compost was added in the different proportions ranging from 0-75% and incubated for a period of 15 days. PAH incubated with different ratio of manure showed 43.74 to 89.56% removal efficiency whereas pure culture S3 and S4 (not identified) showed 79% (S3) and 72 %(S4) degradation on 15th day. Degradation was also observed in samples of soil contaminated with naphthalene and with pure naphthalene 83.8% degradation was observed with S3 culture. Hence manure was considered to be an effective tool in removing contamination of soil with lubricant oil.

Keywords : Biodegradation , Organic manure , PAH, Lubricant oil

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CHAPTER 1

INTRODUCTION

1.1 General

The abandonment of crude oil drilling sites and accidental spillage from crude oil production unit has led to the contamination of environment. The crude oil remains in the soil for long periods of time due to its persistent nature. Hence, the soil quality is affected as its physical, physiological as well as biochemical properties are affected. Moreover the intrinsic heterogeneous microbial diversity is also reduced in number. Many of the components of crude oil including polar hydrocarbons, n-alkanes and unresolved complexes of branched and cyclo-alkanes, aromatics, resin and asphaltene residuals; many of which are persistent organic pollutants.

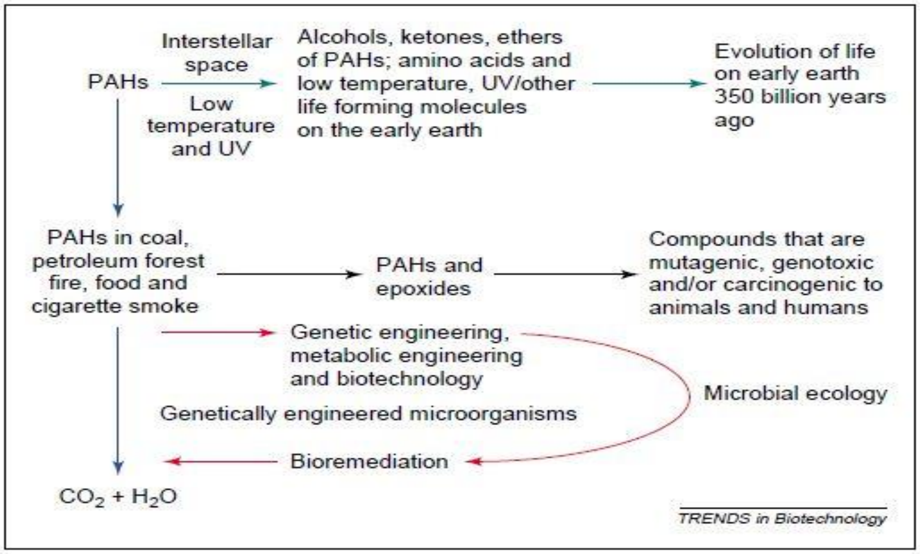


Fig.1.1 Fate, toxicity and remediation of polycyclic aromatic hydrocarbons (PAHs) from the environment.

Polyaromatic hydrocarbons (PAH'S) are the category of pollutants that are mutagenic, carcinogenic as well as toxic in nature, they often arise due to incomplete combustion of organic compounds and result from from burning of fossil fuels (coal, petroleum) & forest fire etc. These compounds are a class of pollutants of significant environmental concern due to their stability which delays their degradation and tendency to accumulate in food chains favored by their affinity for fatty tissues. Many individual PAHs, especially those with high molecular weight such as benzo[a]pyrene have been demonstrated to cause carcinogenic and mutagenic actions and . Some, while not carcinogenic, may act as synergists and lighter PAHs also have toxic equivalency factors. Most studies focused on 16 PAHs listed by the US Environmental Protection Agency (EPA) and the European Community as priority pollutants.

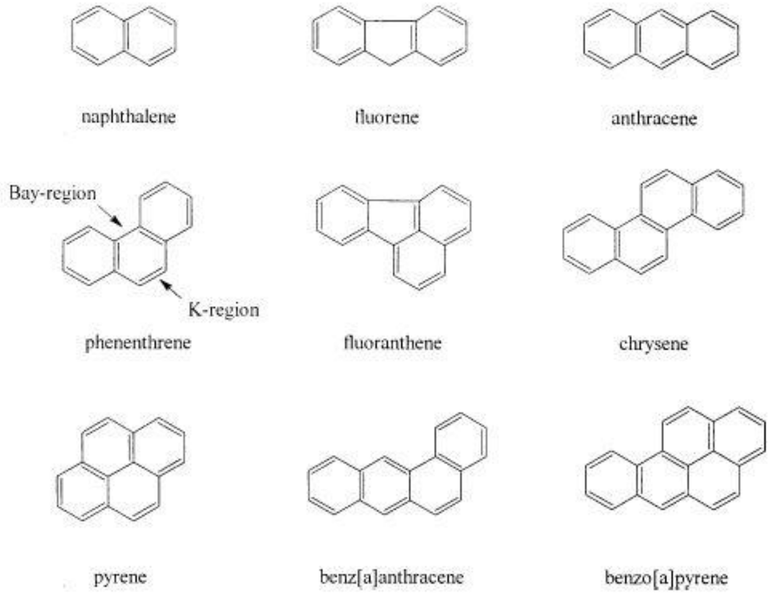


Fig. 1.2 Structure OfCommon Polyaromatic Hydrocarbon

1.2 Properties of Polyaromatic hydrocarbons (PAH'S)

Physical properties

Polyaromatic hydrocarbons fall under the category of hydrocarbons that have two or more fused benzene rings in linear, angular or clustered fashion. They are hydrophobic in nature and are low in solubility. Due to these properties they can persist in natural environment for longer period of time(Cerniglia,1992).With increase in the number of benzene rings infused the solubility and volatility decreases whereas the hydrophobicity would increase.

Chemical properties

PAHs exist as four-,five-,six-or seven membered rings but five and six are most common. The six membered rings are termed as alterant PAHs. Certain alterant PAHs are often called as benzenoid PAHs. The set of alterant PAHs are closely related to a set of mathematical entities called polyhexes (a planar figure that is composed by conjoining regular hexagons of identical sizes).

PAHs containing upto six fused rings are considered as “small” PAHs whereas those which contain more than six infused rings are “large” PAHs.Many PAHs contain a “bay-region” and a “K-region”. The bay- and K-region epoxides, which can be formed metabolically, are highly reactive both chemically and biologically.

Metabolism of PAHs occurs via the cytochrome P450-mediated mixed function oxidase system with oxidation or hydroxylation as the first step. The resultantepoxides or phenols might get detoxified in a reactionto produce glucoronides, sulfates or glutathioneconjugates. Some of the epoxides might metabolizeinto dihydrodiols, which in turn, could undergoconjugation to form soluble detoxification products orbe oxidized to diol-epoxides. (Stegeman, J.J. et al. 2001)

PAHs possess very characteristic UV absorbance spectra. These often possess many absorbance bands and are unique for each ring structure. Thus, for a set of isomers, each isomer has a different UV absorbance spectrum than the others. This is particularly useful in the identification of PAHs. Most PAHs are also fluorescent, emitting characteristic wavelengths of light when they are excited (when the molecules absorb light). The extended pi-electron electronic structures of PAHs lead to these spectra, as well as to certain large PAHs also exhibiting semi-conducting and other behaviors.

Table 1.1 Chemical And Physical Properties Of Selected PAH’S(Sikkema J. et al.1995)

Compound	No.of Carbon atoms	Molecular weight	Melting point (°C)	Boiling point (°C)	Solubility in water (mg/lt)
Naphthalene	10	128.2	80.2	218.0	30.6
Acenapthalene	12	154.2	96.0	278.0	3.9
Phenantherene	14	178.2	100.0	339.0	1.2
Anthracene	14	178.2	217.0	340.0	0.7
Pyrene	16	202.26	150.4	393.0	0.145
Fluoranthene	16	202.26	108.8	383.0	0.262

Chrysene	18	228.29	253.8	431.0	0.003

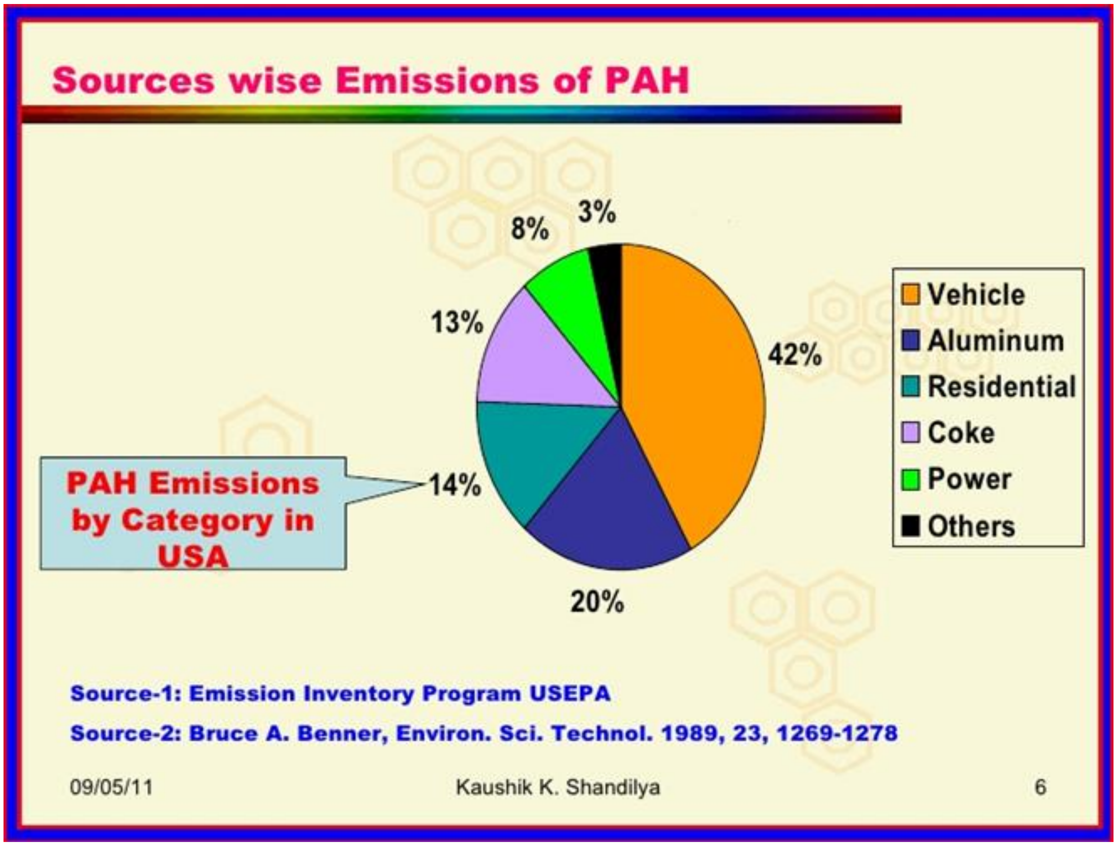
1.3 Sources and Occurrence of Polyaromatic Hydrocarbons (PAHs)

Polyaromatic hydrocarbons in sediments:

PAHs accumulate in the fine grain sediments due to their hydrophobic nature. The sources can be atmospheric deposition, marine seeps of petroleum hydrocarbons, off shore production or transportation of crude oil, sewage disposal or boating.

Polyaromatic hydrocarbons in soil

PAH concentration in soil of industrialized nations have shown a peak increase from the period ranging from 1950/1960’s. Anthropogenic combustion of fossil fuels and long range atmospheric transport has led to dispersion of PAHs throughout the environment. Some of the other sources can be accidental spillage of oil by oil tanker, leakage of oil from automobiles.



Sources wise Emissions of PAH

PAH Emissions by Category in USA

Source-1: Emission Inventory Program USEPA

Source-2: Bruce A. Benner, Environ. Sci. Technol. 1989, 23, 1269-1278

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Fig1.3 Various Sources of Emission of PAH’s

Polyaromatic hydrocarbons in air

Results from ambient air monitoring programs have shown that PAH concentrations are usually of the order of a few Nano grams per cubic meter of air. Motor vehicles, including spark emission and diesel automobiles, trucks and buses, also contribute to atmospheric PAH pollution through exhaust condensate and particulates, tyre particles and lubricating oils and greases.

Polyaromatic hydrocarbons in marine organisms

The uptake of PAH by the organism is dependent on the availability of the compound in the marine environment as well as the physiology of the organism. The organism size, ingestion rate, growth rate, membrane permeability, ventilator rate, gut residence time and osmoregulation are biological processes that influence the organism's uptake of PAHs.

1.4. Biodegradation pattern of PAHs

Biodegradation is one of the forms of bioremediation applied to treat soils, water or sediments contaminated with PAHs. It is the use of microorganisms to degrade or detoxify environmental pollutants (Bamforth& Singleton, 2005). Biodegradation is a clean-up method that presents the possibility to eliminate organic contaminants with the help of natural biological activity available in the substrate (Zeyaullah et al., 2009). The products of complete mineralization of the pollutant by biodegradation process include; CO_2, H_2O and cell biomas.

$$\textbf{PolyAromaticHydrocarbons + micro-organism} \longrightarrow \textbf{CO}_2 + \textbf{H}_2\textbf{O}$$

The optimization of biodegradation process involves many factors among which are the existence of a microbial consortia capable of degrading the pollutant, the bioavailability of the contaminant to microbial attack and certain environmental factors (soil type, temperature, soil pH, oxygen level of soil, electron acceptor agents, nutrient content of soil) contributing to microbial growth.

Abd-Elsalam, Hafez, and Hussain (2009), concluded that *Escherichia coli*, *Alcoligenes*sp., and *Thiobactersubterraneus*were efficient isolates for degrading anthracene and phenanthrene. A number of studies have reported extensively on a variety of other bacterial species that have been isolated and noted to possess the ability to utilize PAHs as energy source. It is also evident that anthracene could be completely mineralized by *Sphingomonas*, *Nocardia*, *Beijerinckia*, *Paracoccus*, and *Rhodococcus* with dihydriol as the initial oxygenated intermediate (Evans, Fernley, & Griffiths, 1988; Dean-Ross, Moody & Freeman,2001; Moody, Freeman, Doerge, &Cerniglia, 2001). Biosurfactants are produced to increase the solubility of insoluble substrates or solubilized (emulsify) insoluble substrates in order to enhance their direct contact between microorganisms and contaminants. Evidence have been accumulating to suggest that certain microorganisms namely; *Bacillus subtilis*, *Pseudomonas*, *Aeruginosa*and *Torulopsisbombicola*could produce bioremediation surfactants such as surfactin, rhamolipid and sophorolipid capable of improving bioremediation by solubilizing PAHs into the aqueous medium and enhance their bioavailability for degradation (Cottin& Merlin, 2007; Kuyukina et al., 2005).

There is a great diversity of organisms capable of degrading the low molecular weight PAH, such as naphthalene, acenaphthene and phenanthrene. It was suggested that nocardioform bacteria (e.g. *Rhodococcus, Nocardia, Mycobacterium and Gordona*) may play a crucial role in the degradation of high molecular weight PAHs in soils. This suggestion was based on results reported by the authors for screening pyrene-degrading bacteria (from soil samples) and on the observation that there are very few reports of bacteria outside the *nocardioformactinomycetes*group capable of growing on high molecular weight PAHs.Algae and cyanobacteria have also been shown to oxidise PAHs. While the oxidation of naphthalene by a number of algae and cyanobacteria has been reported, relatively few studies have demonstrated the degradation of high molecular weight compounds by these organisms.

Bioremediation of PAH-contaminated soils, sediments, and water can be accomplished in a variety of ways, eg*in situ* treatment or *ex-situ* methods such as bio-piling and composting. Waste can also be treated in bioreactors, though this can be more costly than *in situ* technologies. It is important for bioremediation to be comparable in cost and success to physical and chemical treatments of contaminated land, such as landfilling, incineration and soil washing.The applicability of bioremediation can be variable, but this is generally due to unfavorable siteconditions therefore a thorough understanding of site conditions will allow optimization of bioremediation and subsequently more effective result

CHAPTER 2

LITERATURE REVIEW

2.1 Hydrocarbon Pollution

Hydrocarbon contamination has been regarded as an increasingly serious international concern for environmental and health reasons. The exploitation of offshore oil resources, the use and transportation of petroleum products, wastes emission and the frequently occurring oil spill accidents incur detrimental impacts to the marine ecosystems. Methods using physical skimming and chemical dispersants to solve this problem are both costly and limited in effectiveness. Expectations for innovative and sound technologies for the removal of petroleum contaminants have therefore increased. The technique of microbial remediation has played an important role in the treatment of petroleum contaminants. Compared with the conventional methods, which have relied on human labor, microbial remediation has the advantage of both low cost and high efficiency without secondary pollution.(Beolchini et al.2010; Murado et al. 2011)\

Bioremediation of oil pollution is an efficient way to solve the problem. Crude oil is a complex mixture of hydrocarbons containing n-alkanes, branched alkanes, aromatics, cycloalkanes, isoprenoids, asphaltene and many others. Different sources of crude oil may also contain different components. Various microorganisms have the potential to use different hydrocarbons as their energy source. It has been found that 70 genera, more than 200 types of microorganisms, could oxidize one or more petroleum hydrocarbons. Previously native bacteria or exogenous microorganisms were usually applied during the bioremediation process in petroleum contaminated environment (Venosa and Zhu, 2003).

2.2 Degrative studies of polyaromatic hydrocarbons(PAH's) at National scale

India being a developing country is a hub of various industrial units that release various hazardous effluent discharge containing large amounts of polyaromatic hydrocarbons which lead to the contamination of soil and water. (Mallick&Dutta , 2008) studied the novel metabolic pathway involving 2-hydroxy-1-naphthoic acid (2H1NA) involved in phenanthrene degradation by *Staphylococcus* sp. strain PN/Y. The *Staphylococcus* sp. strain PN/Y was isolated from cretosol contaminated soil and utilized phenanthrene as their sole carbon source.At a

concentration of 1 g l−1 of phenanthrene, the maximum accumulation of 2H1NA was found to be 323 mg l−1. In addition, 2H1NA utilized by *Staphylococcus* sp. PN/Y as sole carbon source.
In the oil fields of Gujarat a *pseudomonas putida*strain was isolated , it could use naphthalene as sole source of carbon for obtaining energy and degraded naphthalene via salicylate pathway. The genes responsible for degradation were found in a 83KB plasmid and the strain was labeled as 67.The plasmid was transferred into a plasmid-free strain of *P. putida*KT2442 which resulted in the attainment of chemotaxis and degradation properties by this strain, thus establishing the role of this plasmid in chemotaxis associated with complete mineralization of these two compounds 68. (Roy et al.2007) carried out degradation studies on polyaromatic hydrocarbons using the mixed cultures. The collection process for strains were carried out throughout the petrol stations in different Indian cities and the strain which showed the best performance in degradation was selected for carrying out studies in mixture of anthracene and naphthalene which acted as carbon source in the solution of methanol.The cell growth curve and substrate depletion time history curve obtained from batch fermentative process show that the reaction engineering behavior of the systems under study can well be represented by classical substrate uninhibited Monod's model and in a separate attempt the intrinsic kinetic parameters μmax and KS were evaluated following differential analysis of experimental data.(Janbhandu&Fulekar, 2011) was conducted, where microbial consortium from 3 decade old petrochemical refinery field located in Nagpur, Maharashtra with history of PAH disposal was collected. Based on biochemical tests and 16S rDNA gene sequence analysis the consortium was identified as *Sphingobacterium*sp., *Bacillus cereus* and a novel bacterium *Achromobacterinsolitus*witheffective phenanthrene-degrading ability. The biodegradation data of phenanthrene indicates about 100%, 56.9% and 25.8% degradation at the concentration of 100 mg/l, 250 mg/l and 500 mg/l respectively within 14 days.One more organism, *Arthrobactersulfureus*(Jain et al.2005) which was also isolated
from Gujarat oil fields, could utilize phenanthrene as the sole source of carbon and energy. The
pathway for degradation of this compound was determined in this strain along with that in three
other strains, *Acidovoraxdelafieldi*P4-1, *Brevibacterium*sp. HL4 and *Pseudomonas* sp. DLCP11. Novel pathways for phenanthrene degradation in *Brevibacterium*HL4 and *Pseudomonas* DLC-P11 were proposed. These organisms could utilize several other PAHs such as acenaphthene, anthracene, fluorine, fluoranthene, naphthalene and pyrene, making them potentially useful in cleaning up petroleum contaminated soils containing a mixture of different PAH. Roy et al.2014

isolated 39 native crude oil degrading bacteria from different crude oil contaminated soils and identified by 16s rDNA technique. They checked bioremediation potential of hydrocarbon degrading bacterial strains in crude oil contaminated soil under microcosm study. Vineetha et al. 2012 conducted study on oil contaminated soil from a motor workshop site in kollam (kerala) to evaluate the effect of nutrients and bulking agent in the remediation of oil contaminated soil. The above said experiments were carried out in two reactors one with amendments and the other as a control plot. Vegetable waste and saw dust were added to the contaminated soil as the nutrient and bulking agent respectively in the ratio of 0.6:1 and 0.5:1. Neutral pH and 50% moisture content were maintained in both the reactors throughout the experiment. Oil content of the bioremediated soil was measured every 2 days during the period of study. Results indicated that the supplement of amendments significantly increased the indigenous microbial populations in soils, and thus enhanced the oil degradation. Maximum remediation occurred in the reactor with the amendments, where the oil removal efficiency was found to be 74%.

2.3 Degrative Studies of Polyaromatic Hydrocarbons on International scale

Microorganismswere isolated from hydrocarbon contaminated soils of Shadegan wetland located in southwest of Iran. The degradation experiments were conducted in liquid cultures. PAH or PAHsconcentration was 100 mg/L at the beginning of degradation experiments. After ten daysincubation, the mixed culture was capable of degrading phenanthrene completely and anthracene 80%, pyrene 60%, fluorene 30%, and fluoranthene 20%, individually. Optimal Temperature and pH were as 30 °C and 7.0 respectively. Results showed that in samplescontaining a mixture of the five PAHs, fluoranthene could degrade co metabolically and the rate of degradation increased from 20 to 44%. It was observed also that PAHs degradation could be enhanced by the individual addition of yeast extract or glucose. The isolated mixed culture is valuable in bioremediation of PAH-contaminated environments (Shafiee et al. 2006). Bacterial strainswere also isolated from different contaminated sites in middle delta, Egypt and screened for PAHs degradation. Enrich media was used to isolate the anthracene and phenanthrene degrading bacteria. Fourteen bacterial isolates showed high degradation for both anthracene and phenanthrene. For genotyping, these isolates were subjected to RAPD-PCR using four different primers. The data showed that the fourteen isolates were not related to each other. Only four isolates showed the highest ability for degradation was subjected to 16S rDNAsequence for identification (Hassan et al. 2009). Another bacterial strain for degrading phenanthrene, anthracene and pyrene was isolated from the polluted soil in the Jinan Oil Refinery Factory, Shandong Province of China (Ling et al 2011). The isolate was identified as *Bacillus vallismortis* with respect to its 16S rDNA sequence, DNA-DNA relatedness and fatty acid profiles, as well as various physiological characteristics. The strain was Gram-positive, motile, endospore forming, aerobic, oxidase and catalase-positive. *Bacillus vallismortis* strain JY3A could utilize naphthalene, phenanthrene, anthracene, pyrene, fluorene, benzene, toluene, phenol, methanol, ethanol, Tween 80, cyclohexane or catechol as sole carbon source. The strain alone removed 90.5% of pyrene at an initial concentration of 150ppm in 15days in the presence of 0.5% (w/w) Tween 80. However, in co-culture with *Phanerochaetechrysosporium*, JY3A reduced the concentration of pyrene by nearly 55.4% after 7days of incubation.Quian et al. 2011 isolated a bacterial strain, named KL2-13 by means of enrichment culture from soil contaminated by heavy oil from Karamay of Xinjiang Oilfield. The strain KL2-13 was identified as Bacillus according to its morphological, physiological, biochemical, and 16S rDNA sequence characteristics. The biodegradation experiments were made to research whether the bacterial strain KL2-13 could accelerate the biodegradation progress. The influence of inoculum concentration, N, P sources and the amount of wheat bran on the degradation ability of the strain was investigated. The petroleum polluted soil collected from Karamay was treated by the strain KL2-13 for 90 days at the definite water content. The results showed that the petroleum hydrocarbon pollutants degraded faster. The bioremediation effect was best when the inoculum concentration of the strain KL2-13 was 10%. There was an optimum matching value between the growth of microbe and the amount of the nutrients added, the optimum content of N was 0.2%, and P 0.05%. The degradation rate of hydrocarbon pollutants enhanced along with the increasing amount of wheat bran, when the amount of wheat bran was 25% of the soil volume, the degradation rate of oil arrived at 56.8% after 90 days' bioremediation.

2.4 Microbial remediation of Polyaromatic Hydrocarbons

Every year on an average of about 35 million barrels of petroleum is transported across the seas around the world and found at least 1.82 million (77% of the total spill)was not recovered. (kadafa 2012). Petroleum contamination in terrestrial as well as marine environments has persisted as a result of the increasing demand of liquid petroleum. This spilled petroleum required proper cleaning process which needs to be ecofriendly. Of all the cleanup techniques the microbial remediation proved to be cost effective and sustainable in nature (Macaulay 2014).

Microbial remediation technology is a practice that utilizes the metabolic potential of microorganisms such as bacteria, fungi, protozoans in degrading liquid petroleum spilled on terrestrial and marine environments, into harmless compounds. These oil-degrading microbes are not living in isolation. Hence, referred to as microbial consortia. (Watanabe, 2001). These oil-degrading bacteria are sometimes, referred to as Hydrocarbonoclastic bacteria (HCB) Bacteria's reproductive potential and ubiquity may have made them the most preferred agent of hydrocarbon degradation for bioremediation (McKewet al.2007).

The number of micro-organisms capable of utilizing the carbon in crude oil as their sole source of energy is relatively few compared to the general number of microbes available in the environment . It is important to have a good understanding of the available oil-degrading micro-organisms found both on land and water. Oil-eating micro-organisms produce enzymes which break down hydrocarbon compounds through single or multiple metabolic pathways. The enzymes produced are incapable of breaking down all forms of hydrocarbon compounds; as a result, most oleophilic microbes are hydrocarbon-specific, although a few are physiologically-versatile and can degrade a wide-range of hydrocarbons. For example, toluene, an aromatic hydrocarbon, can be broken down via multiple metabolic pathways by the enzyme, toluene 2-monoxygenase produced by a toluene degrading microbe (such as *Rhodococcus* spp.) (Ramos et al. 2011).Themetabolic pathways include five aerobic pathways and at least one anaerobic pathway(Pazoset al.2004). This biochemical diversity and hydrocarbon-specificity displayedby oleophilic microbes, if expressed in synergy by microbial consortia, will enhancebioremediation .Besides the catabolic potential of oil-degrading microbes, other strategies have been adopted by these microbes to enhance the detoxification of pollutants, such as the modification of their cell membrane to preserve their structural integrity when in contact with pollutants (De Carvalhoet al.2005).The production of surface active compounds known as bio surfactants helps to partially weather the pollutant thereby rendering them vulnerable to microbial degradation and the release of toxic pollutants from within the microbe's protoplasm via efflux pumps, active transporters located in the cytoplasmic membrane (van Hammeet al.2003).Some oleophilic microbial consortia develop synergistic relationships whilst few others showcompetitive relationships. For example, *Cycloclasticus*and *Alcanivorax borkumensis*show a synergistic association because *A. borkumensis*produces an extracellular lipidbiosurfactantwhich render PAHs readily available for digestion by *Cycloclasticus*andthe competition for hydrocarbon resources is absent because *A. borkumensis*feeds onanother hydrocarbon (alkanes) (McKewet al.2007). It is not necessary that oil degrading bacteria are capable of utilizing carbon as sole source of energy so it is essential to have good knowledge of all available oil degraders found in land as well as water. Most of the oil degrading microbes produce enzymes which help in breakdown of hydrocarbon through various metabolic pathways. But all the produced enzymes aren't capable of mineralizing all forms of hydrocarbons. Bacosa et al. 2012 used bacterial consortium to degrade aromatic hydrocarbons and got better results than the single bacteria.

To successfully exploit the microbial degradation of PAHs, it is imperative that we understand and master the mechanisms needed in order to manipulate the microbial activities. Microbial bioremediation of PAHs from oily sludge wastes are very much dependent on
these three factors:

2.4.1. The choice of microbial consortium.

Many microbial strains are capable of degrading only specific hydrocarbon compounds. However, oily sludge wastes are complex mixtures of different PAHs members, not to mention, the alkanes, NSO (nitrogen-, sulfur-, and oxygen-containing compounds) and resins fractions. A single bacterial species has only limited capacity to degrade all the fractions of hydrocarbons presents. Hence, a mixture of outside bacterial armies that can degrade a broad range of the hydrocarbon constituents of the oily sludge waste should be employed.

2.4.2 Factors affecting the biodegradation mechanism.

There are many factors (physical, chemical and biological) that will ultimately determine the effectives of strategies of choice for microbial bioremediation of PAHs. According to(van Hamme et al. 2003), these factors include:

- **Biosurfactant.** Biosurfactants are important agents in the effective uptake of PAHs by bacteria and fungi. The formation of emulsions in the presence of biosurfactants is reported to be in 96% of hydrocarbon metabolizing freshwater bacteria.

- **pH.**Most important PAHs degrading heterotrophic bacteria and fungi perform best when pH is neutral. At pH 7, the mineralization of oily sludge in soil is also improved, thus, enhancing the overall biodegradation process **.**

- **Nutrients.** The nitrogen and phosphorus contents greatly affect the microbial degradation of hydrocarbons. It was observed that adjustment of the ratios of these two elements ratios by the addition of nitrogen and phosphorus in the form of slow releasing fertilizers stimulated thebiodegradation of crude oil and individual PAHs.
- **Salinity.** Studies have shown that there are generally positive correlations between salinity and rates of mineralization of PAHs such as phenanthrene and naphthalene However, hyper salinity will result in the decrease in microbial metabolic rates.
- **Oxygen.** Aerobic biodegradation is the most effective pathway for bioremediation, which means the presence and concentration of oxygen is the rate-limiting parameter in the biodegradation and catabolism of cyclic and aromatic hydrocarbons by bacteria and fungi This is because PAHs break-down processes involve the utilization by oxygenases, for which molecular oxygen is required. Although anaerobic degradation of PAHs by microorganisms has been shown to occur, the rates are somewhat negligible and limited to halogenated aromatic compounds such as the halobenzoates, chlorophenols and alkyl-substituted aromatics.
- **Temperature.** Temperature is another important variable that influences petroleum biodegradation. Optimum temperature dictates the rate of PAHs metabolism by microorganisms and also the pattern of the microbial community. Temperature also has direct effect on the physical nature and chemical composition of the PAHs constituents. When temperatures are low, PAHs tend to be more viscous and their water solubility is greatly reduced (Leahy and Colwell, 1990).The optimum temperature is typically in the range of 30 to 40°C. At temperature above this norm, enzymatic activities are inhibited as proteins denature .
- **Genetic enhancement/mechanisms.**Genetic compatibility and readiness is probably one of the most important determining factors in the success of microbial catabolism of PAHs. Bacterial species with either chromosomal or plasmid-borne genes capable of PAHs of hydrocarbon metabolisms are well documented. The most extensively characterized gene is encoded by the *Pseudomonas putida Gpo1*. The role of plasmid is also well documented in the bacteria communities, especially in the *Pseudomonads*. The metabolic pathways for compounds such as naphthalene, salicylate, camphor, octane, xylene, and toluene have been shown to be encoded on plasmids in *Pseudomonas spp*.

It has been well established that the presence of microbial consortia rather than individual microbes will enhance bioremediation; therefore, the antagonistic potential of some oil degrading microbes could limit the efficiency of microbial remediation. The activities of these antagonistic oil-degrading bacteria have to be controlled in order to encourage the continued development of viable oil-degrading microbial consortia.

Table 2.1: Bacterial genera involved in PAHs degradation

Bacterial specie strain	PAH Involved	References
*Achromobacter*sp. NCW	Carbazole	Guo*et al*., 2008
*Arthrobacter*sp. P11	Phenanthrene, Carbazole,Dibenzothiophene	Seo*et al*., 2006
*Bacillus subtilis*BMT4i (MTCC9447)	Benzo[a]pyrene	Lily *et al*., 2009
Geobacillusstearothermophilus“AAP7919”	Anthracene	Kumar *et al*., 2011
*Terrabacter*sp.DBF63	Fluorene, Dibenzofuran, Chlorinated dibenzo*p*dioxin, Chlorinated dibenzothophene	Habe*et al*., 2001, 2002, 2004.
*Polaromonas naphthalenivorans*CJ2	Naphthalene	Pumphrey*et al*., 2007
*Cupriavidus*spp.	Decane	Bacosa*et al*., 2012

2.4.3 Mechanism of microbial bioremediation

Microorganisms degrade PAHs either via metabolism or cometabolism. Cometabolism is especially relevant for the degradation of mixtures of PAHs. Both aerobic and anaerobicmetabolism exist for PAH degradation.The aerobic aromatic metabolism is characterized by the extensive use of molecular oxygen as cosubstrate for oxygenases that introduce hydroxyl groups and cleave the aromatic ring. The aerobic PAH catabolism is mediated by the enzymatic activity of dioxygenase/monooxygenase. It incorporates atoms of molecular oxygen into the aromatic nucleus and as a result aromatic ring is oxidized(Goyalet al.1997). On the basis of the substituents on the original molecule, two hydroxyl groups may be positioned either *ortho*(catechol and protocatechuate) or *para*to each other (gentisate and homogentisate). The cisdihydrodiols that are formed in this reaction are further oxidizedto the aromatic dihydroxy compounds (catechol's). These compounds are further oxidized through the ortho or meta cleavage pathways (Denomeet al. 1993; Baboshin et al. 2008).Hybrid type aerobic metabolism is used by facultative aerobes eg. aerobic metabolism ofbenzoate, phenylacetate, and anthranilate. This pathways uses coenzyme A thioesters of the substrates and do not require oxygen for ring cleavage. An oxygenase/reductase leads to dearomatization of the ring.In the presence of oxygen, facultative aerobes and prototroph use a reductive aromatic metabolism. The reduction of the aromatic ring of benzoyl coenzyme A is catalyzed by benzoylcoenzyme A reductase. This reduction is led by the hydrolysis of 2 ATP molecules. It has been documented that a little characterized benzoyl coenzyme A reductase operates in strict anaerobe as they can not afford the costly ATP dependent ring reduction (Georg, 2008).

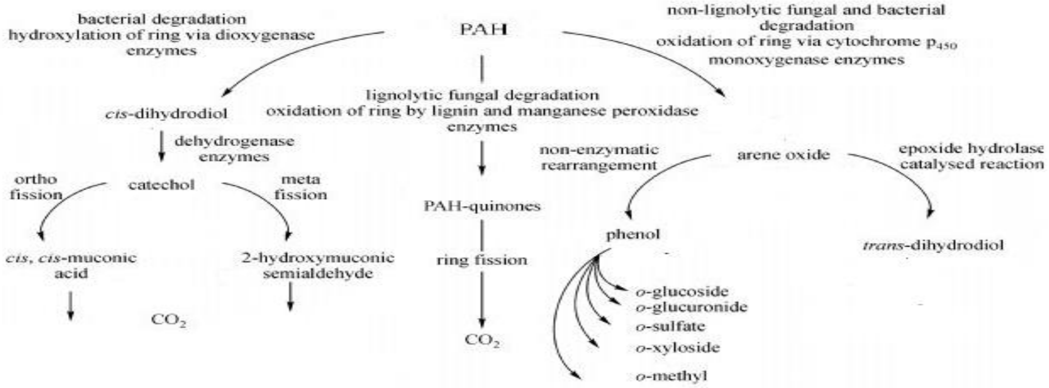

Fig 2.1 Microorganisms mediated pathways for degradation of PAH

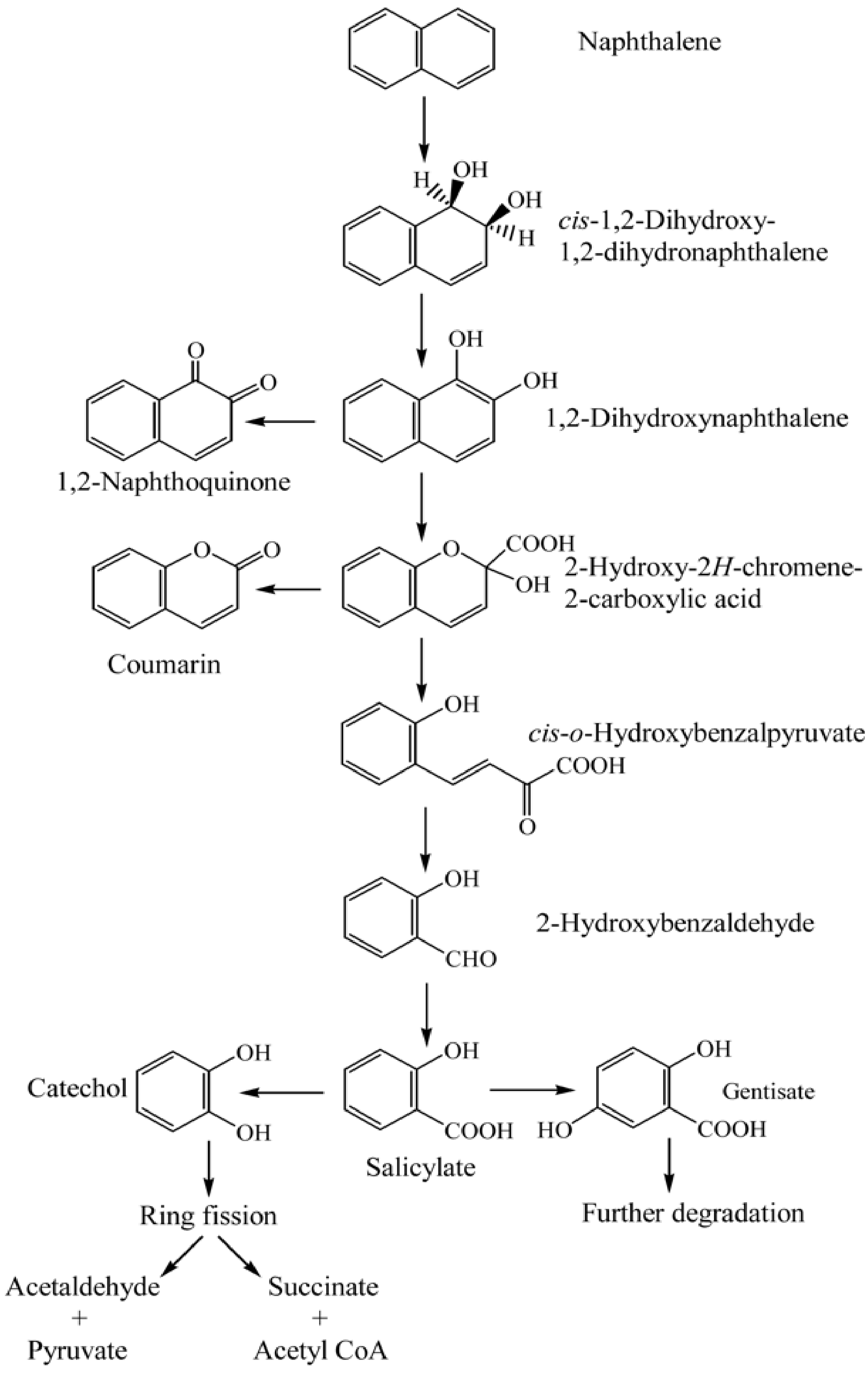

Fig 2.2 The proposed pathway for naphthalene oxidation by some bacteria from the genus *Pseudomaonas.*

2.5Effects Of Polyaromatic Hydrocarbons on soil chemistry

2.5.1Chemical properties of soil.

In general the oil spills lead to substantial increase in soil carbon content. The normal soil carbon content may vary between 0.2%-0.12% whereas the contaminated soil has shown an increase of 5%. Moreover there was depletion in the nitrate levels of contaminated soil which could be a possible effect of microbial activity. Total phosphorous however, remained unchanged. The pH Of the contaminated soil were found out to be lower due to the accumulation of acidic microbial metabolites being derived from hydrocarbons (Vincent et al., 2011; Kaakineen et al., 2007) studied biological oxygen demand (BOD) / theoretical oxygen demand(ThOD) for normal forest soil with a value of 45.1%. After period of 30 days the values were recorded for a typical model substance sodium benzoate and there was a value increase of 76.2%. It was observed in study that crude oil pollution caused a reduction in pH, conductivity and phosphorous.

2.5.2 Soil biota and profile

In a study done on Antarctic soil it was observed that the hydrocarbon degraders are quiet low or negligible in natural soil environment whereas $>10^5$ hydrocarbon degraders have been cultivated from contaminated soils. The hydrocarbon degrading bacteria isolated from contaminated site have been assigned number of genera including *Rhodococcus, Acinetobacter, pseudomonas and Sphingomonas.* All these bacteria reported are psychrotolerant rather than psychrophilic. They grow over low temperature ranges 15^0C being the optimum range. One of the isolates *Pseudomonas stutzeri*5A showed growth on toluene, benzene and m-xylene whereas *Pseudomonas sp.* 5B utilized hexane and dodecane vapors. At various drilling sites where the diesel oil has been spilled is rich in ammonifers and hydrocarbon degraders.

2.5.3 Soil temperature and moisture pattern

The temperature profiles of a hydrocarbon contaminated soil is often on a warmer side (by up to 10^0C). The elevated temperature profiles were attributed to decreased soil surface albedo due to surface darkening by hydrocarbons. In contrast to subsurface contamination no considerable temperature difference was determined. The contaminated soils tend to be usually hydrophobic in nature but no major difference in moisture level have been recorded between normal and contaminated soil (Balks et al. 2002).

2.6 Analytical technique for detection of polyaromatic hydrocarbons (PAH's)

Many analytical techniques have been applied in the determination of PAHs and their metabolites in contaminated systems. The use of solid-phase extraction (SPE) in measuring freely dissolved concentrations of PAHs in soil pore water has been examined and proved out to be quite efficent.(Ter-Laak et al 2006). Some researchers have suggested that solid-phase microextraction (SPME) with gas chromatographic/mass spectrometric (GC/MS) analysis can achieve detection limit as low as nanograms per litre of PAHs and alkyl PAHs in sediment pore water (Hawthorne et al 2005). In soil samples, high resolution gas chromatography-mass spectrometry (HRGC-MS) and liquid chromatography-fluorescence detection (LC-FD) have been applied successfully in measuring the final concentrations of PAHs). It was revealed that HRGC-MS has a high linear range compared to LC-FD. Hence, under practical considerations, HRGC-MS was regarded as a superior analytical technique than LC-FD. (Berset et al 1999).

A recent study had noted that laser-induced fluorescence (LIF) spectroscopy is a mature technique for PAHs determination in terrestrial sediments (Grundl et al 2003). In a related study, SPME coupled with LIF was applied successfully in in-situ sampling in an effort to develop a simple field-portable method to determine total dissolved PAHs concentrations in sediment pore water (Hawthorne et al 2008). It was evident that PAHs undergo a variety of processes resulting from chemical, biological or photochemical reactions. Hence, environmental conditions usually affect intermediate products of PAHs formed during degradation processes. A notable study has revealed that different degradation pathways of PAHs require specialized analytical tools in their degradation studies. (Dabroska et al.2005). GC-MS has been used in analyzing sediment samples in a study aimed at characterizing the distribution of PAHs in sediment samples. (Jiang et al.,2007).

The requirements of any extraction technique are that it can produce valid data, rapidly, with minimum operator involvement, be cost effective and satisfy safety considerations for both the operator, and other personnel and its location within the operating environment. Each technique has its own merits and the choice of extraction depends on several factors including capital cost, operating cost, sample matrix, simplicity of operation, sample throughput and the availability of a standardized method. The conditions would be optimized and the best technique would be chosen according to the accuracy of the results obtained.

CHAPTER-3

MATERIAL AND METHODS

In the following chapter the various methodologies adopted for the completion of study will be discussed along with the material required.

3.1 Chemicals

Analytical grade chemicals were used for the experiments.

3.2 Selection of PAH Degrading Bacteria

3.2.1 Culture media

Nutrient agar and Minimal medium (M9) was used for initial screening.Media was autoclaved at 121^0C at 15 psi for 15 minutes.

Table 3.1: M9 media Composition

Component	Concentration (g/L)
Sucrose	10.0
K_2HPO_4	2.50
KH_2PO_4	2.50
$(NH_4)_2HPO_4$	1.00
$MgSO_4.7H_2O$	0.20
$FeSO_4.7H_2O$	0.01
$MnSO_4.7H_2O$	0.007
Distilled water	1000mL
Agar	15

3.2.2 Collection of Soil Sample

The contaminated soil sample was collected from nearbypetroleum service station, Jammu. The sample was collected after removing 1-2 cm soil from the top and then collected in a plastic bag.

3.2.2.1 Screening of PAH Degrading Bacteria

The screening of the PAH degrading bacteria was done by dissolving 1 g of contaminated soil in 10 ml of distilled water. Shake it for about 30 minutes. The supernatant of the sample was inoculated in the nutrient broth (NB) and incubated for 24-48 h at 37^0C on rotary shaker at 150 rpm. After growth of consortia in NB, serial dilutions were made and 100µl of the liquid was surface spread on nutrient agar plates and plates were incubated at 37^0C for 24-48 h. The colonies of interest were streaked on NB plates and incubated at 37^0C for 24h. Out of these the best grown isolates were selected and were inoculated in M9 medium and then transferred to M9 agar and stored at 4^0C for further experimentation.

3.2.3 Identification of selected bacterial isolates

3.2.3.1 Morphological and biochemical characterization

Selected isolates were characterized by colony morphology on nutrient agar, gram staining and morphological characteristics. Additional biochemical test were performed for taxonomic characterization which included gelatin liquefaction, H_2S production, lipase test, methyl red-voges proskauer (MR-VP), citrate utilization, carbohydrate utilization and amino acids utilization.

A) Biochemical tests

1) Gelatin agar medium

Table 3.2: Composition of Gelatin Agar Medium

Concentration	Composition(g/L)
Gelatin	40
Tryptic soy broth	30
Distilled water	1000

Procedure:

Stab a small inoculum of the bacterium about ¾ of the way to the bottom of a tube of deep agar with the inoculating needle. The procedure is repeated with separate stab tubes for each of bacteria and incubated at 37_0C for 24-48 h. The control and the inoculated tube were kept in the refrigerator for about 30 minutes. The inoculated stab is compared with that of control by tapping gently the tubes.

2) H_2S production test

Table 3.3: Media Composition for test of H2S Production	
Composition	Concentration (g/L)
Peptone	30
Beef extract	3
FAS	0.2
Sodium thiosulphate	0.02
Agar	15
Distilled water	1000

Procedure:

An inoculating needle was used to stab a small inoculum of each of the bacteria approximately ¾ of the way to the bottom into separate agar tubes. Incubated the deep stab at 37^0C for 24-48 h. The inoculated deep stab was compared with the control.

3) Citrate utilization test

Table 3.4: Media Composition for Citrate Utilization Test	
Composition	Concentration (g/l)
$(NH_4)_2HPO_4$	0.1
K_2HPO_4	1
NaCl	5
Sodium Citrate	2
$MgSO_4$	0.2
Bromothymol blue	0.08
Agar	2
Distilled Water	1000ml

Procedure:

The surface of slants of Citrate agar was inoculated with the bacteria's by using a small amount of inoculum and incubated at 37^0C for 48 h. The inoculated slant was then compared with the control.

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

Table 3.5: Media Composition for MR-VP Test

Composition	Concentration(g/l)
Peptone	7
Glucose	5
Potassium Phosphate	5
Distilled Water	1000ml

Procedure:

Inoculated separate tubes of MR-VP broth with the bacteria's and incubated the tubes at 37^0C for 24-48 h. After incubation, transferred approximately 1/3rd of each culture into empty glass test tubes and set aside for VP test. Five drops of the methyl red indicator was added to the remaining broths in the original tubes and colour change was observed. Added 12 drops of VP reagent 1 (5% napthol solution in absolute ethanol) to each of the broths that were set aside for the VP test. Each culture was shacked to mix the reagent with the rest of the broth. Immediately add 2-3 drops of VP reagent 2 (40% KOH in water) and shake the cultures again. Shake the cultures again every 3-4 minutes until approximately 15 minutes had passed. Results were compared with the control.

5) Carbohydrate Utilization

Table 3.6: Media Composition for Carbohydrate Utilization Test

Composition	Concentration (g/L)
Peptone	10
NaCl	15
Sugar *	5
Distilled water	1000 ml

*glucose, sucrose, fructose, dextrose, maltose

Procedure:

Transferred 0.1 ml of inoculum of each of the bacteria's into broths (glucose, sucrose, fructose, dextrose, matlose) and incubated the inoculated broths at 37°C for 24-48 h in a rotary shaker at 150 rpm. Results were observed for each broth and compared to the uninoculated controls.

6) Lipase Screening

Table 3.7: Media Composition for Lipase Screening Test(spirit blue agar)

Composition	Concentration (g/L)
Casein enzymic hydrolysate	10
Yeast extract	5
spirit blue	0.150
Agar	17
Distilled water	1000ml

Procedure:

Suspend 3.21 grams in 100 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs. pressure (121°C) for 15 minutes. Cool to 50°C and add 3 mllipase substrate (contaminated oil) slowly while agitating to obtain an even distribution. Add two drops of tween 80 to uniformly spread oil over plates. The plates were surface spread and observed after 48 hrs.

7) Lipase test (conformation)

Table 3.8 Media composition for lipase conformation test (Tributyrin agar)

Composition	Concentration (g/L)
Peptic digest of animal tissue	5.00
Yeast extract	3.00
Agar	15.00

Procedure :

Suspend 2.3 gm. in 90 ml of distilled water. Add 1 ml of tributyrin (FD081). Mix and heat to boiling in order to dissolve the medium completely. Sterilize by autoclaving at 15 lbs. pressure (121^0C) for 15 minutes. The media was poured into glass petri plates and surface spread. The plates were observed after 48hrs for results.

8) Amino acid utilization test

Table 3.9: Media Composition for Amino acid utilization Test

Composition	Concentration (g/L)
Sucrose	10.0
K_2HPO_4	2.5
KH_2PO_4	2.5
$MgSO_4.7H_2O$	0.2
$FeSO_4.7H_2O$	0.01
$MnSO_4.7H_2O$	0.007
Amino acids*	2
Distilled water	1000 ml

Amino acids*: Tyrosine, Valine, Glycine, Arginine, Alanine, Leucine, Threonine.

Procedure:

0.1ml of inoculum was transferred to broths supplemented with amino acids (Tyrosine, Valine, Glycine, Arginine, Alanine, Leucine, and Threonine) and incubated for a period of 48-72 h in a rotatory shaker at 150 rpm at 37^0 C. The inoculated broths were compared to the control for results.

b) Physiological Testing

various factors like optimum temperature , pH and salinity were evaluated for the growth of the bacterial strains.

i. Effect of Incubation Temperatures on growth.

Temperature is one of the most important physical factors affecting microorganisms. Bacteria may be divided into three major groups with respect to their temperature requirements: i) psychrophiles, those with optimum temperature between 0^0C to 20^0C; ii) mesophiles, those with optimum temperature between 20^0C to 40^0C; and iii) thermophiles, with optimum temperature between 40^0C to 80^0C.

Procedure:

The flasks containing the M9 media were inoculated with different bacterial strains and incubated at different temperatures (20, 25, 30, 35, 40, 50) for a period of 24-48h in a rotatory shaker at 150 rpm. The cultures were observed for growth after 2 days. The degree of growth was determined using spectrophotometer at 600nm.

ii. Effect of pH on growth.

Each species has the ability to grow within a specific pH range, that may be broad or limited, with the most rapid growth occurring within a narrow optimum range.

Procedure:

Series of tubes maintained at a pH ranging from (6 -10) were inoculated with 0.1 ml bacterial strain were incubated in a rotatory shaker at 150 rpm for a period of 24-48h. The degree of growth was determined using spectrophotometer at 600nm.

iii. Effect of salt concentrations on growth.

Bacteria can be divide into four groups depending on their ability to grow at various sodium chloride concentration; non halophilic (grow at less than 2% sodium chloride concentration), halophilic (grow above 2% sodium chloride concentration). Halophilic may further be of three types: slightly halophilic (grow between 2 to 5% NaCl), moderately halophilic (grow upto 10% NaCl) and extremely halophilic (grow at more than 20% NaCl).

Procedure:

Flasks containing the M9 media were supplemented with different concentrations ranging from (4%, 8%, 12%, 16% & 20% of NaCl). Each flask was inoculated with 0.1ml of culture and incubated for a period of 24-48h in a rotatory shaker at 150rpm. Growth was examined after 2 days with spectrophotometer at maximum wavelength of 600 nm.

C) Morphological studies

i. Gram staining:

Thin smear of the cultures was made on separate glass slides. Smear was air dried and heat fixed. Covered the smear with crystal violet for 30 seconds then washed with distilled water.After that covered the smear with gram’s iodine solution for 60 seconds and washed withdecolorizer and after that with distilled water. Applied safranin for 30 seconds and washed with distilled water. Stained slides were air dried and examined microscopically.

ii. Spore staining:

Smears were made on separate clean slides. Smears were air dried and heat fixed. Flooded the smears with malachite green and heated the slides to steaming and steamed for 5 minutes, more stain to the smear was added from time to time. Washed the slides with distilled water and counterstained with safranin for 30 seconds. Washed smear with distilled water andexamined microscopically.

3.3 Biodegradation Studies of PAH (polyaromatic hydrocarbons)

3.3.1 Biodegradation using manure

The soil was collected from a healthy environment without any traces of contamination by automobile oil. It was dried, meshed and sieved in a 2mm size sieve. Sterilize the soil by autoclaving at least 2-3 times for complete sterilization and killing of microbes. The soil was amended with manure in different ratios as 5%, 10%, 50% &75%. The 5% amend contained 285g soil and 15gm manure , 10% amend contained 270g soil and 30g manure, 50% amend contained 150g soil and 150g manure and 75% amend contained 75g soil and 225g manure. The amend were mixed in a ratio such that the sample should be 300g.

The samples were mixed thoroughly and were artificially contaminated by introducing known quantity of automobile oil to create a synthetic sample. The samples were monitored for a period of 15-20 days. The control sample composed of soil and known amount of automobile oil. Moisture in the pots were maintained by sprinkling water occasionally.

3.3.2 Chemical Analysis of Soil and Manure

The various test applied for checking the chemical properties of soil are pH, conductivity, moisture, organic matter in soil and total nitrogen in soil.

3.3.2.1**pH**

10 gm of soil and oil samples were dissolved in 100 ml of distilled water and flasks were shaken in incubator shaker for 2 hrs at 130 rpm. After 2hrs sample were kept at stationary condition until sediments settled down.

3.3.2.2 **Moisture**

Soil and manure samples were dried at 105^0C till constant weights were obtained. Percentage moisture of the mixtures were as per the eq.

$$\text{Moisture \%} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

3.3.2.3**Conductivity**

1. Weigh 10 gm. of sample and add 100 ml of distilled water to it
2. Keep the suspension with continuous stirring for 15 min. and leave it overnight.
3. Connect the instrument and caliberate the cell constant of the cell with 0.01 (745.6 mg KCl/L distilled water) and (0.1 M KCl) to instrument. Put the temperature knob also in KCL solution.
4. After calibration dip the conductivity electrode into the suspension and record the results.

3.3.2.4**Organic Matter**

1. Take 2gm of soil sample in a conical flask and add 10 ml of 1N of $K_2Cr_2O_7$ solution.
2. Add 20 ml of concentrated H_2SO_4, swirling the flask during the addition, cool the contents of the flask for 30 minutes to complete the reaction at room temperature.
3. Add approximately 2gm of sodium fluoride or 10 ml of orthophosphoric acid, 100 ml of distilled water and shake vigorously to mix.
4. Add 10 drops of the indicator diphenyl amine and titrate it with 0.5 M ferrous ammonium sulfate till the color changes from violet to bright green.
5. calculate %carbon and % organic matter using the formulas in eq. (1) and (2).

<u>% Easily Oxidizable Organic C</u> **:**

$$\%C = \frac{(B-S) \times M \text{ of } Fe^{2+} \times 12 \times 100}{\text{g of soil} \times 4000} \ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(1)}$$

Where:

B = mL of Fe^{2+} solution used to titrate blank

S = mL of Fe^{2+} solution used to titrate sample

12/4000 = mill equivalent weight of C in g.

To convert easily oxidizable organic C to total C, divide by 0.77 (or multiply by 1.30).To convert total organic C to organic matter use the following equation:

<u>%Organic Matter</u>

$$\% OM = \frac{\% \text{ total C} \times 1.72}{0.58} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(2)}$$

3.3.2.2 **Total Nitrogen**

Acid digestion of sample:-

1. Take 5 g of soil and wrap it into a filter paper and put it into a 500ml of kjeldahl flask. Add 30 ml of conc. H_2SO_4 and shake the flask in swirling movement for 1 minute.

2. Add 10 g of Hibbardi's mixture ($K_2SO_4.CuSO_4$), 1gm of salicylic acid and 5g of sodium thiosulfate. Heat slowly till there is no frothing.

3. Raise the heat and continue digestion until the contents of the flask turn grey or greenish yellow in color. Cool and add about 250 ml ofdistilled water. Swirl well and transfer the contents and decant in a 500 ml kjeldahl flask .

Distillation:-

250 ml of the digested sample was transferred into a distillation flask and 25 ml of 40% NaOHwas added. Take exactly 20 ml of 0.1 N H_2SO_4 into a conical flask , add two drops of methyl red indicator and place under the delivery tube of the condenser in the distillation apparatus. Collect about 100 ml of distillate and when the distillation is over, remove the receiver and put off the burner.Titrate the excess of acid in the receiver against 0.1 N NaOH until the color changes from pink to yellow. Note the volume of NaOH used.

Formula used :

<u>% of N in the soil</u>

$$\% N = (20-x) \times 0.00014 \times \underline{250} \times \underline{100}$$

V W

V= Volume of the filtrate used for distillation

W= Weight of the soil taken for digestion

3.3.3 **Microbial Monitoring**

Microbial monitoring was done from the amended sample (soil + manure) and the sample was removed after shaking properly from each pots. studywas done for days 0,5,10 and 15. 10g of soil sample was removed from pots and a 10 ml saline solution was taken and shaked properly for 15 minutes. Kept the sample in stationary condition for half an hour. Make the serial dilution from 10^{-1} to 10^{-6}. From the each dilution 0.1 ml of sample was taken and surface spread on nutrient agar plates.The total aerobic heterotrophic bacterial population was estimated using the plate count technique.

3.3.4 **Estimation of total petroleum hydrocarbon by chloroform**

The automobile oil is extracted from the soil samples using chloroform and quantitatively determined by taking absorbance at 460 nm by using UV-VIS spectrophotometer. In this method the soil samples were air dried to a constant weight and 5g of the sample were placed in beaker and 10 ml of chloroform was added. The beaker was gently shaken for about two minutes and residual oil was extracted. Each extract was filtered with whattsmann filter paper and analyzed for lubricant oil content. A standard curve of absorbance (460 nm) against varying concentration of lubricant in chloroform was drawn after taking readings from spectrophotometer. The lubricant oil concentration was calculated from the standard curve.

3.3.5 Biodegradation of PAH Using Bacterial Culture

a) **For Oil**

Biodegradation of PAH using S3 and S4 bacterial strains was evaluated in soil. Clean uncontaminated soil was collected, dried, sieved and sterilized by autoclaving. 30 ml of used automobile oil was introduced into the uncontaminated soil and thoroughly mixed. The mixture was inoculated with bacterial strains S3 and S4 with O.D. 1 (at 600 nm) and kept in pots and monitored for period of 15-20 days.The samplewas extracted at 0 time, fifth day, tenth day and fifteenth day for the purpose of measuring residual concentration of PAH.

B) **For Naphthalene**

Clean uncontaminated soil sample was collected, dried, sieved and sterilized used autoclave. Solutions of naphthalene dissolved in methanol with 100 ppm and 200 ppm concentrations were prepared. The solution were added to the sterilized soil and mixed thoroughly. The sample was inoculated with 1, 0.5 , 0.7 O.D. of bacterial culture (S3) and monitored for a period of 15-20 days. The whole sample was extracted at 0 time, fifth day, tenth day and fifteenth day for the purpose of measuring residual concentration of PAH.

The degradation efficiency can be calculated using the formula:

$$\textbf{Biodegradation efficiency (\%)} = [(C_0 - C_e / C_0)] \times 100$$

Where,
Co initial concentration of naphthalene (μg/g).
Ce final concentration of naphthalene (μg/g).

3.3.6 Analytical method -GC/MS-(gas chromatography/mass spectra analysis)

Aliquots (5 ml) were centrifuged at 10,000 rpm for 10 minutes and supernatant was filtered by 0.2 µm filter and kept for analysis.For the analysis conditions given in table 3.10. A gradient elution was used. Solvent A (deionized water) and solvent B (chloroform) was used as mobile phase.

Table 3.10: analysis conditions used for GC/MS

GC-MS model	GCMS-QP2010Ultra
column	Elite-5MS(length25mt.*0.20mm I.D.,*df=0.33µm)
Vaporizing chamber temperature	300^{0}C
Column oven temperature	90^{0}C(2min) $\longrightarrow$ (5^{0}C/min) $\longrightarrow$ 320^{0}C(12min)
Carier gas	Helium
Purge flow rate	3ml/min
Injection rate	1.0µl
Control mode	Constant linear velocity(43.7 cm/sec)
Conditions for MS	
Interface temperature	250^{0}C
Ion source temperature	200^{0}C
Solvent elution time	3.5 min
Data sampling time	4.5-50 min.
Mass range	*m/z*45-450

CHAPTER - 4

RESULTS AND DISSCUSSION

4.1 Selection of PAH (Polyaromatic Hydrocarbon) Degrading Bacteria

A total of four strains were isolated from the oil contaminated soil. Two strains showed best and one moderate growth on nutrient agar plates. These strains were further transferred on Minimal media (M9) where PAH is the sole source of carbon and energy. Bacterial strains S3 and S4 showed rich growth and were selected for further experimentation.

4.1.1 Identification of selected bacterial isolates

a) Morphological characterization

Differential staining required the use of at least three chemical reagents, which makes it an essential tool for classification of microorganisms.Gram staining test was performed on the selected isolates. On observing under microscope both were found to be gramnegative and capable of producing spores.

Table 4.1 Morphological Characterization of Bacterial Isolates

Strain	Gram strain	Spore stain
S3	Negative	Capable of producing spore.
S4	Negative	Capable of producing spore.

b) **Biochemical Characterization**

1. **Gelatin liquefaction test:**

   Gelatin is a protein produced by hydrolysis of collagen, a major component of connective tissue and tendons in human and other animals. Below temperature of 25^0C gelatin will maintain its gel properties and exist as a solid, at a temperature above 25^0C gelatin is liquid. Liquefaction is accomplished by some microorganism capable of producing a proteolytic extracellular enzyme called gelatinase, which acts to hydrolyze this protein to amino acids. Once this degradation occurs even very low temperature of 4^0C will not restore the gel characteristics. Both the strains S3 and S4 showed positive results for this test as they produced gelatinase enzyme for liquefaction of gelatin.(Table 4.4)

2. **Carbohydrate utilization test:**

Most organisms obtain their energy through a series of orderly and integrated enzymatic reactions leading to the bio oxidation of a substance. Organisms use carbohydrates differently depending on their enzyme complement aerobically and anaerobically.In case of carbohydrate utilization test S3 strain showed growth in all sugar supplemented media except the one with fructose whereas S4 showed growth in all. Maltose supplemented as carbon source in media was recorded with the maximum growth of O.D.$_{600}$ of 1.502 and 1.693 for S4 and 1.689 fructose, 1.598 sucrose and 1.579 in glucose whereas S3 ranges from 0.706 to 1.502 respectively (Table 4.2).

Table 4.2 Carbohydrate Utilization test for bacterial isolates S3 and S4.

Bacterial Strain	Fructose	Maltose	Sucrose	Glucose
S3	-	1.502	0.706	0.762
S4	1.689	1.693	1.598	1.579

Control (O.D.) – 0.065

From the table it was observed that S4 is having good affinity with all the carbohydrates than S3.

3. **Amino acid utilization test**

Every biologically active protein is composed of the 20 essential amino acids. Structurally amino acids are composed of an alpha carbon (-c-), an amino group (NH_2), a carboxyl group (-COOH-) and a hydrogen group (-H-). It depends which amino acid is utilized by which bacteria. The strain S3 and S4 utilized almost all amino acids used.In the test performed growth was observed in all the media supplemented with different amino acids (Leucine, Tyrosine, Serine, Valine, Glycine, Arginine and Threonine) but maximum growth was recorded in case of tyrosine followed by Leucine with O.D.of 1.985 and 1.585 respectively. Whereas least growth was observed in glycines with O.D. of 0.136 for strain S3. In case of strain S4 maximum growth was recorded in Leucine followed by Valine with O.D.of 1.369 and 1.145 respectively whereas least growth was recorded for threonine with O.D.of 0.546 (Table 4.3).

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Table 4.3 Amino Acid Utilization for bacterial isolates S3 and S4

Bacterial Strain	Leucine	Tyrosine	Serine	Valine	Glycine	Arginine	Threonine
S3	1.585	1.985	1.321	0.289	0.136	0.197	0.411
S4	1.369	1.095	1.088	1.145	0.616	0.577	0.546

Control (O.D.) - 0.083

4. Methyl red test

The glucose is the major substrate oxidized by entire organism for energy production. The end products of this process will vary depending on the specific enzymatic pathways present in the bacteria. In this test the pH indicator methyl red detects the presence of large concentrations of acid end product.A yellow colour was observed as endpoint for both strains S3 and S4 which indicates that little or no acid remains in the medium. Hence , the test observed was negative. (Table 4.4) .

5. Voges Proskauer test

This test indicate the capability of some organisms to produce non acidic or neutral products such as acetylmethylcarbinol from the organic acids that are the results of glucose metabolism. This reaction will occur in the presence of the α-napthol catalyst and a guanidine group that is present in peptone of the MR-VP medium.In the test a deep rose color was recorded as end point for S3 strain showing a positive result while for S4 strain deep rose colour didn't appear which gives a negative result for S4 strain.(Table 4.4)

6. H_2S production test

There are two major fermentative routes by which some microorganisms produce H_2S.

I. Gaseous H_2S produced by reduction (hydrogenation) of organic sulfur present in the amino acids e.g. cysteine

$\text{Cysteine} \xrightarrow{\text{cysteinedesulferase}} \text{pyruvic acid} + H_2S + NH_3\uparrow$

II. By reduction of in organic sulfur compounds, such as thiosulfates ($S_2O_3^{2-}$), sulfates or SO_3^{2-}.

$S_2O_3^{2-} + 4H^- + 4e^- \xrightarrow{\text{thio sulfate reductase}} 2SO_3^{2-} + 2H_2S \uparrow$

In this test the medium contains peptone which is degraded by microbial enzymes to amino acids and also sodium thiosulfate acts as the sulfur substrate , ferrous sulfate ($FeSO_4$) which behaves as the H_2S indicator. Hydrogen sulphide production test showed negative results which mean that the strains are unable to reduce a sulphur metabolite and release H_2S gas (colorless gas generating black color in agar).(Table 4.4).

7. **Citrate utilization test**

In the absence of glucose or lactose, some microorganisms are capable of utilizing citrate as a carbon source for their energy. Citrate facilitates the transport of citrate in the cell. This product enzymatically converts to pyruvic acid and CO_2. CO_2 combines with sodium and water to form sodium carbonate an alkaline product which changes the color of indicator. The results for citrate utilization were positive for the strain S3 indicating that the strain can utilize citrate as sole source of carbon while it was negative for strain S4.(Table 4.4)

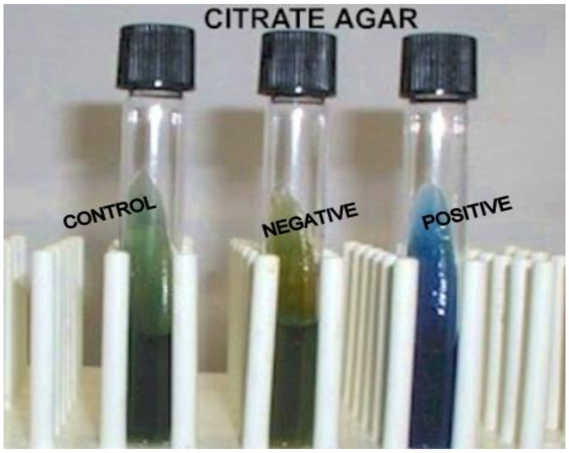

Fig 4.1 : Citrate utilization test indicating a positive test for S3 and negative for S4.

8. **Lipase test**

Lipids are high molecular weight compounds containing large amount of energy. The degradation of lipids such as triglycerides is accomplished by extracellular hydrolyzing enzymes called as lipases. In this test procedure tributyrin agar is used to demonstrate the hydrolysis activity of lipase. Both the strains showed positive results for lipase activity implying that they can carry out hydrolysis of lipids by releasing enzyme (appearance of clear halo surrounding the lipase producing organisms).(Table 4.4)

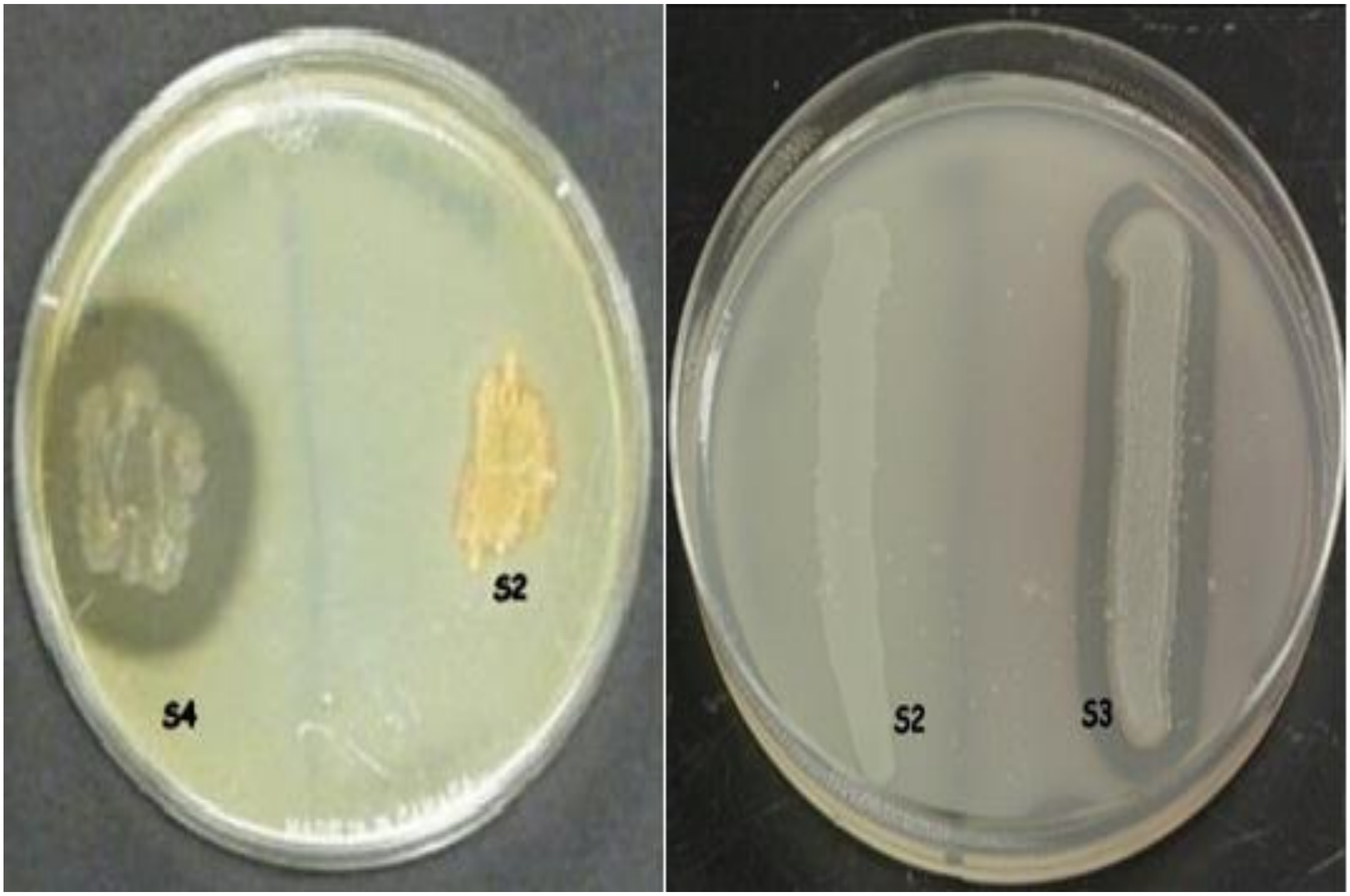

Fig 4.2:Appearance of clear halo around the lipase producing strains S3 and S4 confirms the test for lipase activity in the organisms.

Table 4.4 Biochemical Characterization of Bacterial Isolates

Bacterial strain	Gelatin agar test	H_2S production	Voges Proskauer	Citrate utilization	Lipase test	Methyl red
S3	+	-	+	+	+	-
S4	+	-	-	-	+	-

4.1.1.2 Physiological Characterization

i. Effect Of Temperature

a) The growth of strain S-3 was monitored in minimal media (M9). From the results the maximum growth was observed at 30^0C with O.D.of 1.005 after a period of 48 hours, while decrease in growth was observed at temperature 35^0C and 40^0C with respective

O.D. 0.178 and 0.735. At 20^0C the decrease in O.D. of bacteria was 0.42 which further decreased to 0.152 at 26^0C which could be due to experimental error. The growth at 35^0C and 40^0C were almost same except very minute changes.

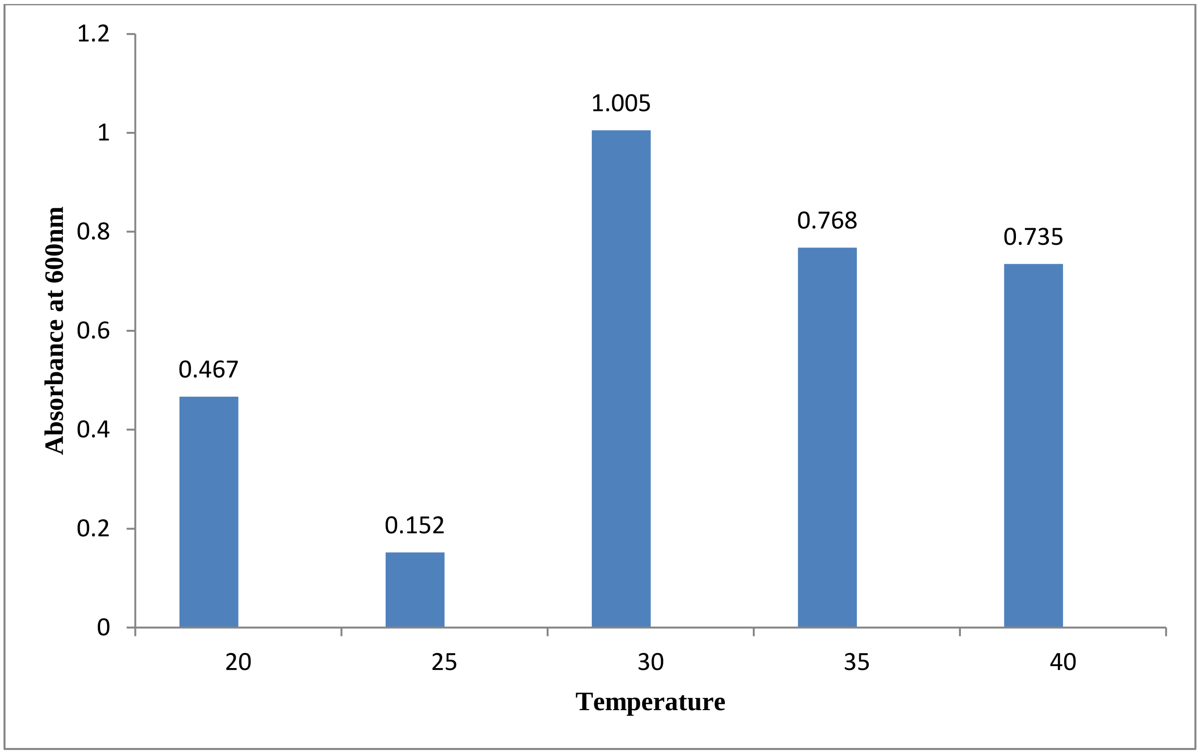

Fig 4.3 Growth of strain S-3 in M9 media at different temperature

b) The growth of S-4 strain was also evaluated in minimal media (M9) which was entirely different from S3. From the result the maximum growth was observed at 20^0C with O.D.of 1.222 after a period of 48hrs which decreased from 25^0C to 35^0C linearly uptil 40^0C. At 40^0C the value was minimum of 0.125. These results indicated that the strain S4 could grow better at low temperature rather than higher temperature but can tolerate temperature till 35^0C and after 35^0C growth of bacteria suddenly decreased. (Fig 4.2)

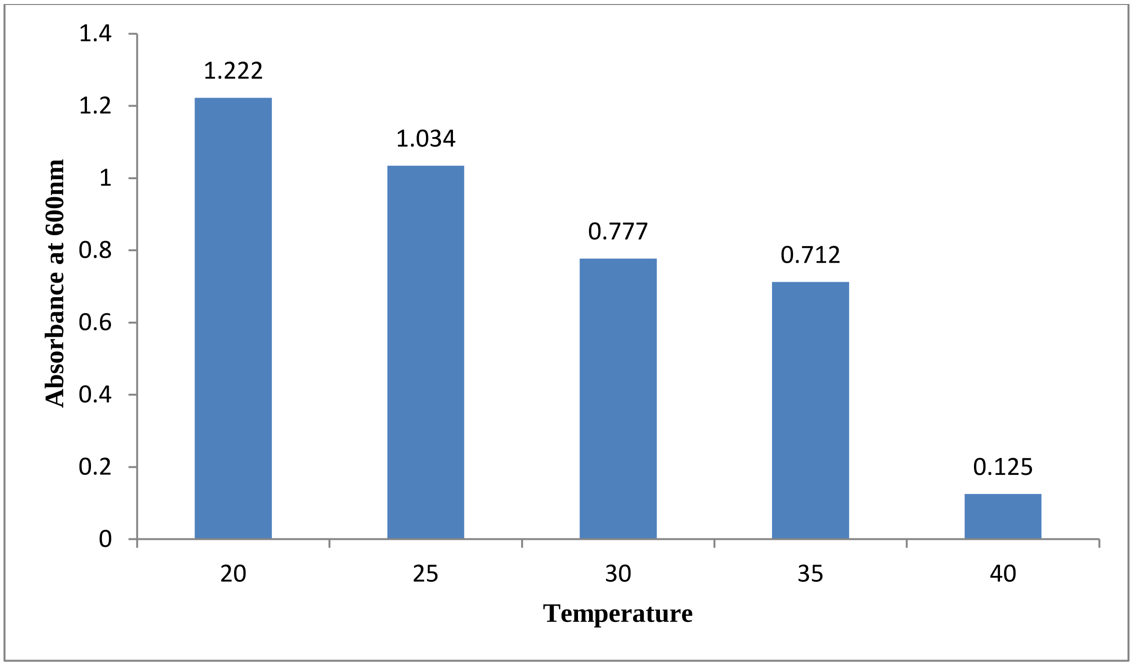

Fig 4.4 Growth of strain S-4 in M9 media at different temperature

Table 4.5 Effect of temperature on the growth of bacterial isolates

Temperature	O.D.(S3)	O.D.(S4)
20^{0}C	0.467	1.222
26 ^{0}C	0.152	1.034
30^0 C	1.005	0.777
38^{0}C	0.768	0.712
40^{0}C	0.735	0.125

Control O.D.-0.062

ii. Effect of pH

a) The effect of pH was observed on S-3 grown in minimal (M9) media. The maximum growth was observed around pH 8 with 0.D. of 1.508 after a period of 48 hrs followed by pH 9 and 10 with values 1.428 and 1.367 respectively. The growth was good around pH 9 and decreased at pH 10 with O.D.$_{600}$of 1.428 and 1.367 respectively. The growth of bacteria was 1.116 at pH 6 which increase linearly uptill pH 10. The growth recorded at pH 9 and 10 is more than at pH 6 and 7.

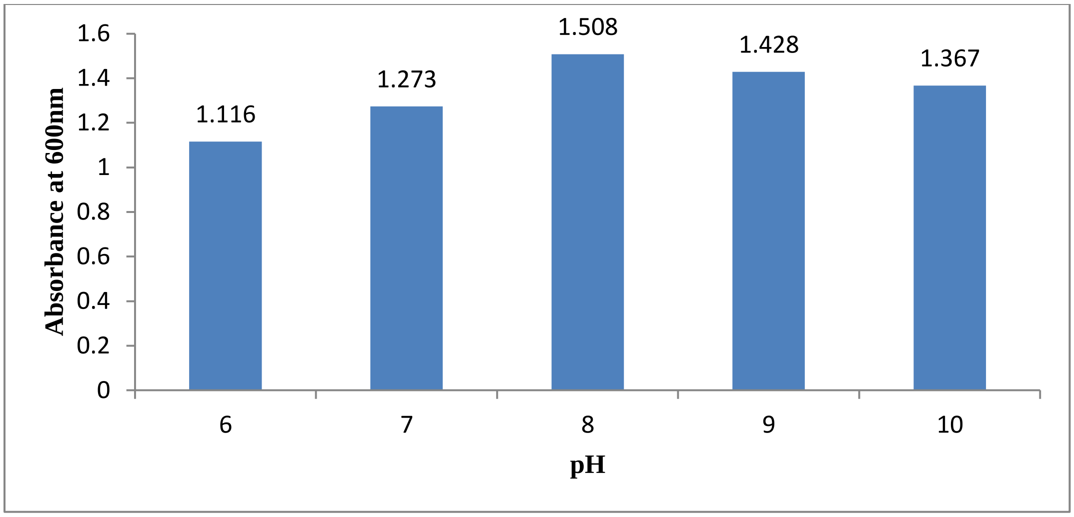

Fig 4.5 Growth of strain S-3 in minimal media (M9) at different pH

b) The effect of pH showed a different pattern in S4 as compared to S3. The maximum growth was observed around pH 8 with O.D.$_{600\ \mathrm{of}}$ 1.587 and goodgrowth was observed at pH 9 (1.558) and the growth decreased around pH 10(1.423) which is almost linear .The growth at pH 6 and 7 was comparatively less than pH 8, 9 and 10 which indicated that the bacteria is alkalophile which can grow better at pH 8 and 9 rather than 6 and 7. The overall growth at pH 6 and 7 is lesser than pH 10. (Fig.4.4). In both the strains pH 8.0 was found better as maximum growth was observed at this particular pH (Table 4.6).

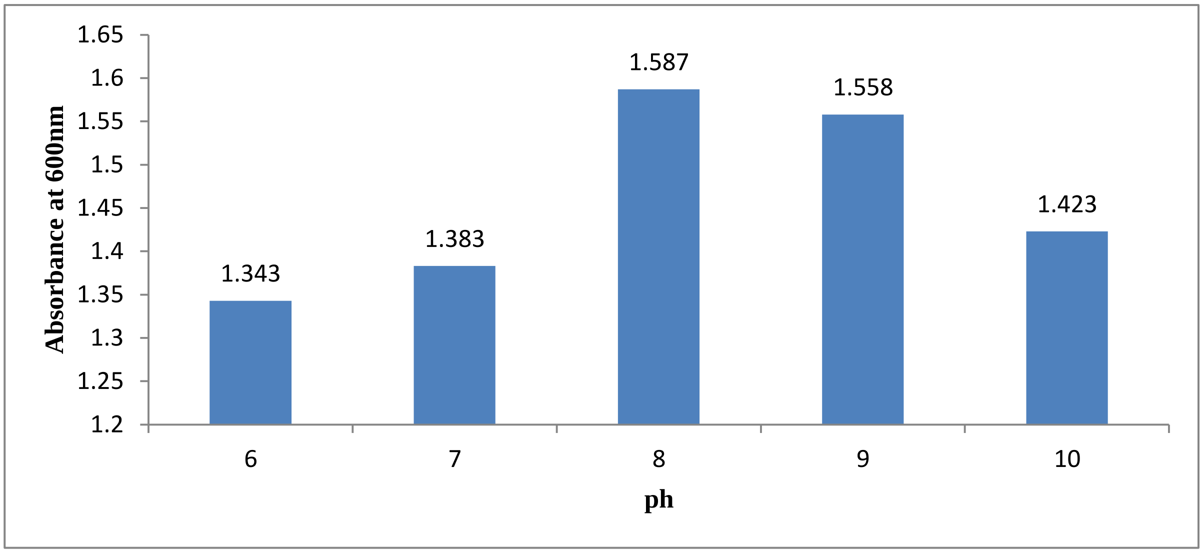

Fig 4.6 Growth of strain S-4 in minimal media (M9) at different pH

Table 4.6 Effect of pH on bacterial isolates		
pH	O.D. (S3)	O.D. (S4)
6	1.116	1.343
7	1.273	1.383
8	1.508	1.587
9	1.428	1.558
10	1.367	1.423

Control O.D. 0.511

iii. Effect of salt concentration

a) For strain S3 maximum growth was observed at 12% of the salt concentration with O.D.(1.354) whereas the minimum growth was recorded at 4% of the salt concentration with O.D.(0.611). The optimum growth was observed at 12 % of salt concentration and it decreases nominally in 16 and 20% salt concentration. The growth with 8% salt concentration was recorded with value of 0.768 which indicating that both 4% and 8% can be considered for minimum growth. But as soon as salt concentration reached 12% the growth increases approximately two folds which decrease thereafter but quite less compared to 4 and 8 % salt concentration. The results indicated thatbacteria are halophilic in nature.

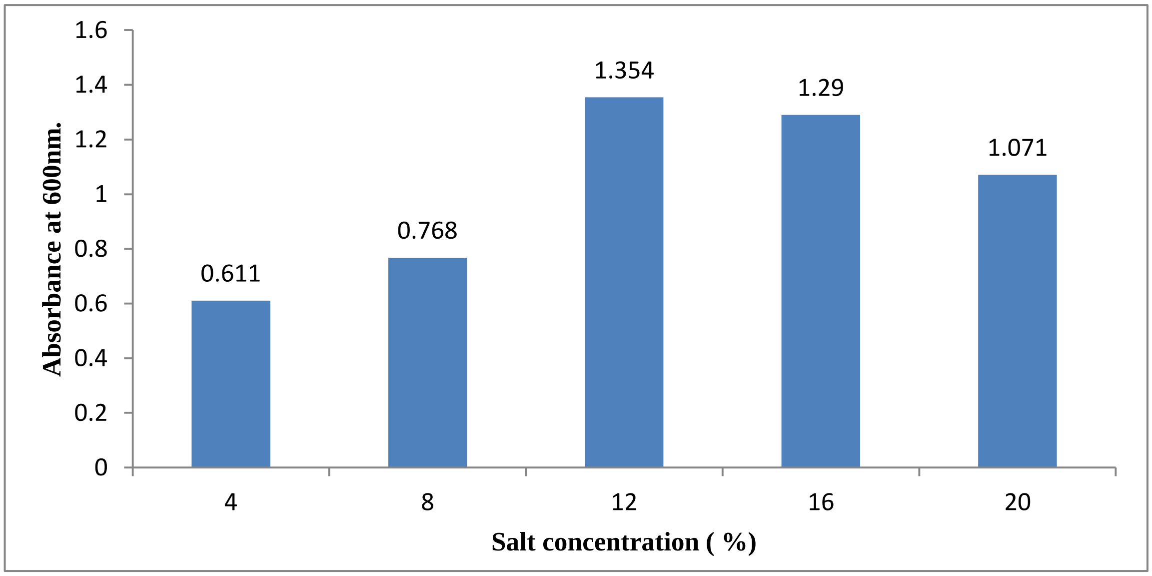

Figure 4.7 Growth of strain S3 in minimal media (M9) at different salt concentration

b) For strain S4 the maximum growth was observed at salt concentration of 8% with O.D.(1.476) whereas the minimum growth was observed at salt concentration of 20% with O.D.(1.019) and the optimum growth ranges between 4-12 % figure (4.6). After 12% of salt the bacterial growth decreases which is 1.123 and 1.019 respectively for 16% and 20% salt concentration. This growth is lesser than 4% and 8% salt concentration with O.D. of 1.325 and 1.476 respectively. Fig. 4.6 indicates that bacteria is moderately halophilic.

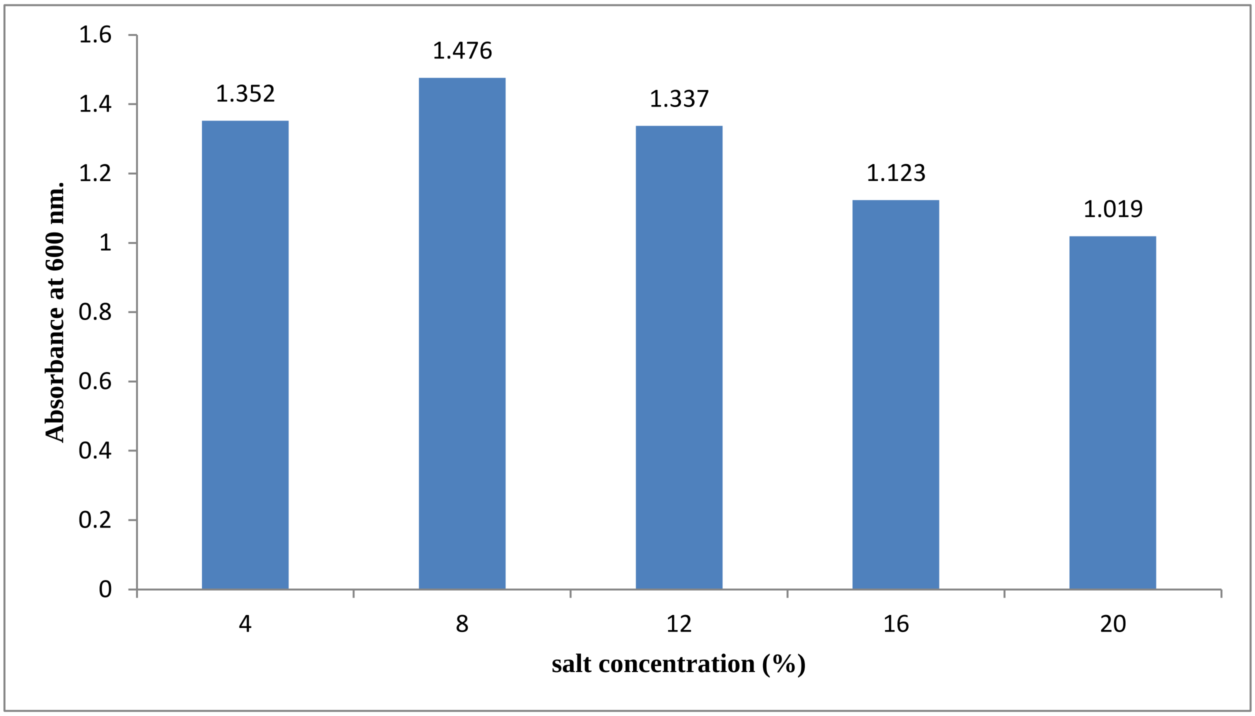

Figure 4.8: Growth of strain S4 in minimal media (M9) at different salt concentration

Table 4.7 Effect of different salt concentration on bacterial isolates		
Salt concentration (%)	O.D.(S3)	O.D.(S4)
4	0.611	1.352
8	0.768	1.476
12	1.354	1.337
16	1.290	1.123
20	1.071	1.019

Control O.D. -0.786

4.1.3 **Chemical analysis of soil**

(a) **pH**

The pH values of lubricant oil contaminated soil with organic manure are given in table4.8 .pH refers to the hydrogen ion concentration in soil which plays a vital role in the decomposition process. At the start of the experiment the pH of all amendments including control was neutral.

Table 4.8 Variation in the pH values with different concentration of organic manure in soil.

Manure in soil (%)	No. of days			
	1	5	10	15
0%	8.24	8.45	8.47	8.68
5%	8.33	8.37	8.54	8.59
10%	8.23	8.27	8.46	8.58
50%	8.76	8.45	8.18	8.89
75%	8.61	8.64	8.72	8.9

The pH was increased with the increase of amends upto 10% till day 15. At the higher concentration of amends (50%) the value of pH was reduced from 8.76 to 8.18. The increase in pH value of amends 5% and 10% could possibly be because of production of intermediate metabolites in the organic manure system due to high metabolic activity whereas the decrease in amend having 50 % manure could be due to release of acidic intermediates produced in the degradation process of hydrocarbons by organic manure. Generally pH range suitable for bacterial growth is 6.7-9.6.

b) Moisture

The values moisture content in lubricant oil contaminated soil with different concentration of organic manure are given in Table 4.8. The optimum microbial activity is observed by achieving maximum water content that does not restrict with oxygen diffusion process. Water is important for transportation of microbial cells and due to which the substrate mineralization occurs. Moisture is an important growth factor for microbes as nutrients in liquid form are easily available to them.

Table 4.9 Moisture content recorded in mixture of organic manure at different concentration with soil

Manure in soil (%)	Moisture (%)
0%	27.4
5%	36
10%	30
50%	35
75%	40

c) the values of conductivity , organic matter , total nitrogen and phosphate content are described in Table 4.10.

Table 4.10. Values of conductivity , organic matter , total nitrogen and phosphate content in soil and manure.

Chemical analysis	Manure	Soil
Conductivity	0.64 mho/cm^{-1}	0.53 mho/cm^{-1}
Organic matter	6.1%	5.6%
Total nitrogen	1.85%	1.53%
Phosphate content	0.085 mg/l	0.064 mg/l

4.2 Biodegradation study of PAH (poly aromatic hydrocarbons)

4.2.1 Biodegradation Using Manure

Biodegradation is one of the forms of bioremediation which can be utilized to treat soil as well as any environmental pollutants. It is also a cleanup method for eliminating organic contaminants. The microorganisms used for biodegradation should be indigenous to the contaminated area or site (Dass and chandran 2011). Now a days polycyclic aromatic hydrocarbons (PAH's) have major concern in our environment. PAH sticks to solid sediments, water and air. Their presence in these media creates problems because consuming products obtained from these sources could be deleterious to human health (uniwe et al. 2013).

The efficiency of the treatment option using organic manure amends depends upon the percentage reduction of oil in the soil samples. A variety of methods are available in the system to treat contaminants which are present in lubricant oil. Now a days stabilization and solidification is very important to control the toxicant from such type of hazardous materials. Other methods are vapor extraction, solvent extraction and thermal destruction also but they are costly and not ecofriendly. The biological method is good option for remediating oil contaminant and any type of hazardous material.

Sask et al 2003 also used compost for removal of polyaromatic hydrocarbons from contaminated soil. He found after composting, inhibition of bioluminescence decreased whereas no significant change was observed for earthworm survival and seed germination. With the use of bacterial culture of *E.coli k12* the genotoxicity decreased in composting pile.

Facundo et al. 2000 observed the enhancement of the microbial activity in hydrocarbon contaminated soil can be achieved with the combination of stepwise soil inoculation and nutrient addition. Hence, the above results show that nutrient supplementation would enhance the biodegradation process. By increasing the percentage of manure in mixture of soil and automobile oil there is increase in microbial consortia which would degrade more oil. In chemical characterization it was found that the nitrogen content in soil was observed 1.53 % and that in manure 1.85 %. The phosphorous content is 0.064 mg/lt and 0.085 mg/lt in soil and manure respectively. These results indicate that the mixture had sufficient nutrients which promote the bacterial growth and enhance the degradation rate.

Ling and lsa 2006 worked on bioremediation of oil sludge contaminated soil by co composting with sewage sludge. He used 5 Kg of soil and spiked with petroleum refinery oil sludge (10%, dry wt. basis). They found enhanced biodegradation of oil and grease due to sewage sludge addition. By increasing the percentage of manure in mixture of soil and automobile oil there is increase in microbial consortia which would degrade more oil. Macaulay 2014 studied remediation of spilled petroleum by microorganisms. His study aims to identify the factors responsible for the change in behavior towards the oil degrading microbes which might help to facilitate better petroleum spill management.

Table 4.8 (a) show the degradation pattern on Day5 of incubation. The maximum degradation was observed in 275 + 25 ratio of manure and soil with a degradation of 61.34% ± 3.14 and with 5% manure only degradation of 6.22 % ± 0.93 was observed. Similar trend was observed in table 4.8(b) showing results for 10 days of incubation. On 10th day the maximum degradation reaches to 71.42% ± 1.86 which indicates that the number of microorganisms present in manure has increased due to which degradation rate is increased whereas at 5% and 10% inclusion only 24.99% ± 4.17 and 27.78% ± 1.08 degradation was observed.

Table 4.11: Degradation values of PAH for day 0 at different concentration of organic manure

Percentage of manure+soil (w/w)	Absorbance (nm.) After PAH extraction	Concentration of PAH (µg/g)	Percentage degradation/ removal (%)
5% Manure	0.011	0.31 ± 0.01	0
10% Manure	0.010	0.28 ± 0.02	0
50% Manure	0.010	0.28 ± 0.08	0
75% Manure	0.009	0.25 ± 0.006	0

Table 4.11(a): Degradation values of PAH for day 5 at different concentration of organic manure

Percentage of manure+soil (w/w)	Absorbance (nm.) After PAH extraction	Concentration of PAH (µg/g)	Percentage degradation/ removal (%)
5% Manure	0.015	1.13 ± 0.115	6.22 ± 0.93
10% Manure	0.016	1.21 ± 0.293	11.06 ± 0.54
50% Manure	0.018	1.47 ± 0.455	26.74 ± 1.66
75% Manure	0.021	5.18 ± 0.180	61.34 ±3.14

Table 4.11(b): Degradation values of PAH for day 10 at different concentration of organic manure

Percentage of manure+soil(w/w)	Absorbance (nm.) After PAH extraction	Concentration of PAH (µg/g)	Percentage degradation/ removal (%)
5% Manure	0.012	0.90 ± 0.107	24.99 ± 4.17
10% Manure	0.013	0.98 ± 0.302	27.78 ± 1.08
50% Manure	0.050	4.19 ± 0.337	57.30 ± 0.89
75% Manure	0.014	1.06 ± 0.042	71.42 ± 1.86

Table 4.11(c): Degradation values of PAH for day 15 at different concentration of organic manure

Percentage of manure (w/w)	Absorbance (nm.)	Concentration of PAH (µg/g)	Percentage degradation/ removal (%)
5% Manure	0.009	0.68 ± 0.250	43.74 ± 1.81
10% Manure	0.008	0.67 ± 0.237	50.56 ± 2.63
50% Manure	0.006	0.45 ± 0.276	87.75 ± 3.79
75% Manure	0.012	2.42 ± 0.159	89.56 ± 3.17

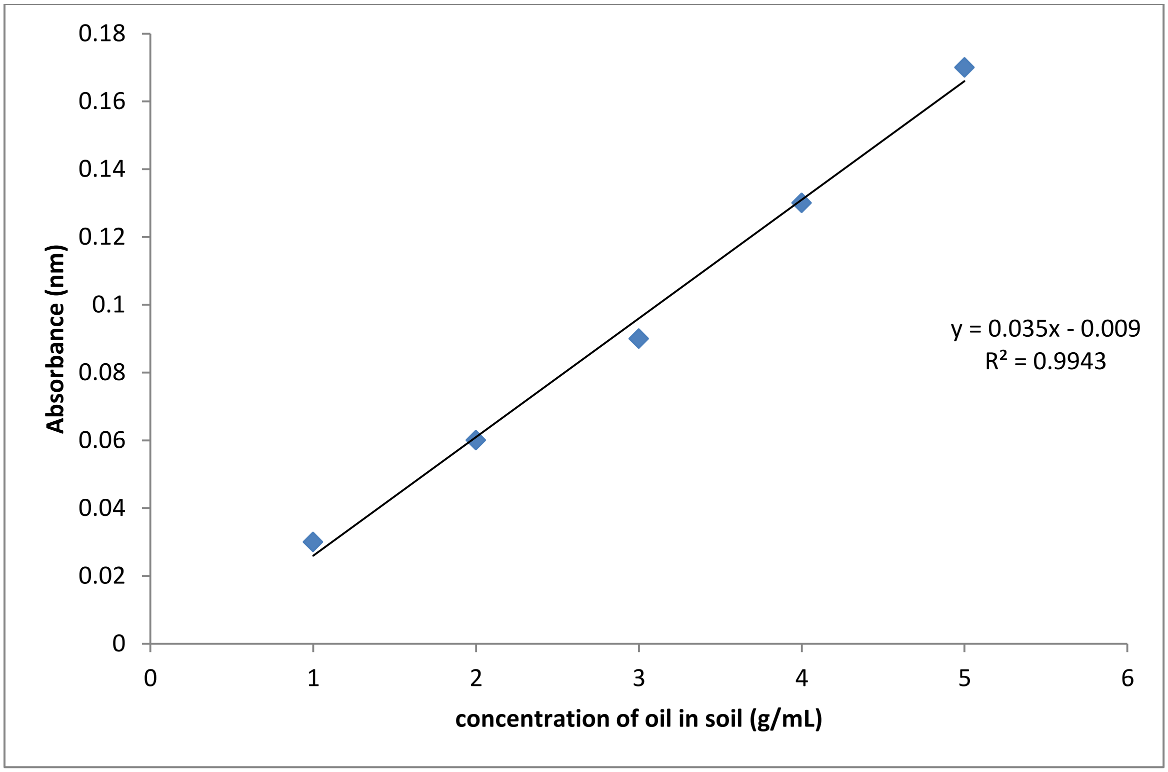

Fig 4.9 Standard curve of oil with chloroform (extraction solvent)

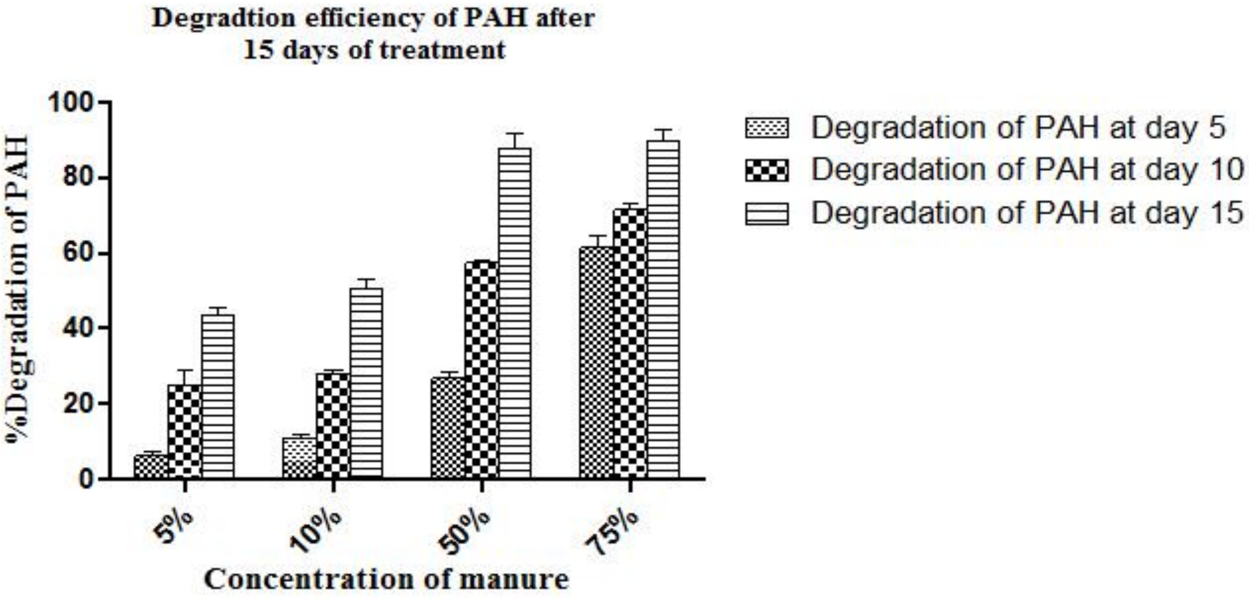

Fig 4.10: Degradation efficiency of PAH after treatment for a period of 15 days at different concentration of organic manure.

4.2.2 Microbial Monitoring

Total heterotrophic counts were observed for different concentration of organic manure and the result indicates that the soil amends supplemented with 75% organic manure gave the highest heterotrophic bacterial count. THC indicates that the microbial population is increasing over the period of time which would enhance the degradation of oil. The number of microorganisms increase due to the increase in amount of manure. The THC count for manure control was observed as 1123×10^{5}cfu/gm.

Table 4.12 Total heterotrophic count with respect to time of incubation at different concentration of organic manure

No. of days	5% manure	10% manure	50%manure	75%maure
Day 0	110×10^{5}cfu/gm.	130×10^{5}cfu/gm.	136×10^{5}cfu/gm.	150×10^{5}cfu/gm.
Day 5	148×10^{5}cfu/gm.	225×10^{5}cfu/gm.	280×10^{5}cfu/gm.	348×10^{5}cfu/gm.
Day 10	190×10^{5}cfu/gm.	389×10^{5}cfu/gm.	547×10^{5}cfu/gm.	689×10^{5}cfu/gm.
Day 15	238×10^{5}cfu/gm.	468×10^{5}cfu/gm.	589×10^{5}cfu/gm.	894×10^{5}cfu/gm.

4.2.3 Biodegradation of PAH's (poly aromatic hydrocarbons) using bacterial strains.

a) Serviced automobile oil

Petroleum hydrocarbons released into the environment can pose risk to ecosystems and human health. Some of the compounds are known to be mutagenic and carcinogenic. Nwaogu et al. 2008 studied degradation of diesel oil in a polluted area using *Bacillus subtilis*. He has isolated four bacteria but out of them *B. subtilis* had a higher potential to utilize diesel oil as carbon source .

Microbial degradation process aids the elimination of spilled oil from environment after critical removal of large amounts of oil by various physical and chemical methods. This is possible because microorganisms have various enzymes system that degrade and utilize diesel oil as a source of carbon and energy (Ezeji et al 2005).

Biodegradation study was conducted at optimized conditions with the help of bacterial isolates S3 and S4. 150 gm of sterilized soil samples artificially contaminated with 30 ml lubricant oil were thoroughly mixed and extracted at day 1 , 5th , 10th and 15th day of incubation. The extracted sample was filtered and O.D. was recorded at 330 nm. The maximum degradation of 79.96 % ± 2.69 and 72.54 % ± 1.99 for S3 and S4 respectively was recorded at the 15th day of incubation.

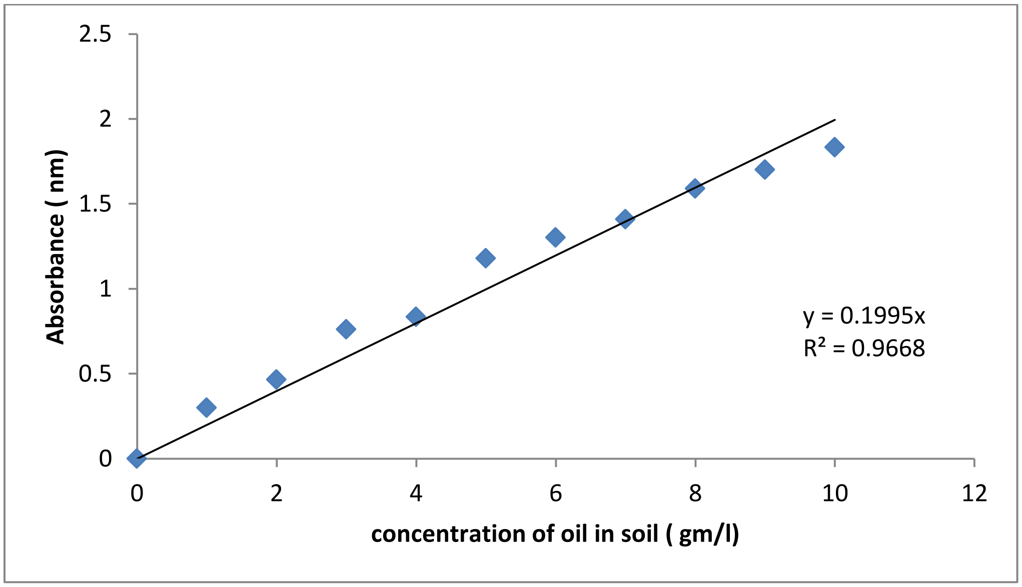

Table 4.13 Degradation value of PAH incubated with isolate S3 for a period of 15 days.

Period of incubation (days)	Absorbance (nm.)	Concentration of PAH (µg/g)	Percentage degradation/ removal (%)
0 Day	1.118	93.16 ± 7.08	0
5 Days	1.017	84.75 ± 2.54	9.03 ± 1.14
10 Days	0.567	47.25 ± 2.89	49.28 ± 3.26
15 Days	0.224	18.66 ± 3.61	79.96 ± 2.69

Table 4.14 Degradation value of PAH incubated with isolate S4 for a period of 15 days.

Period of incubation (days)	Absorbance (nm.)	Concentration of PAH (µg/g)	Percentage degradation/ removal (%)
0 Day	1.087	90.58 ± 2.54	0
5 Days	1.011	84.25 ± 3.01	6.99 ± 0.37
10 Days	0.695	57.91 ± 6.25	36.06 ± 3.65
15 Days	0.224	24.83 ± 2.68	72.58 ± 1.99

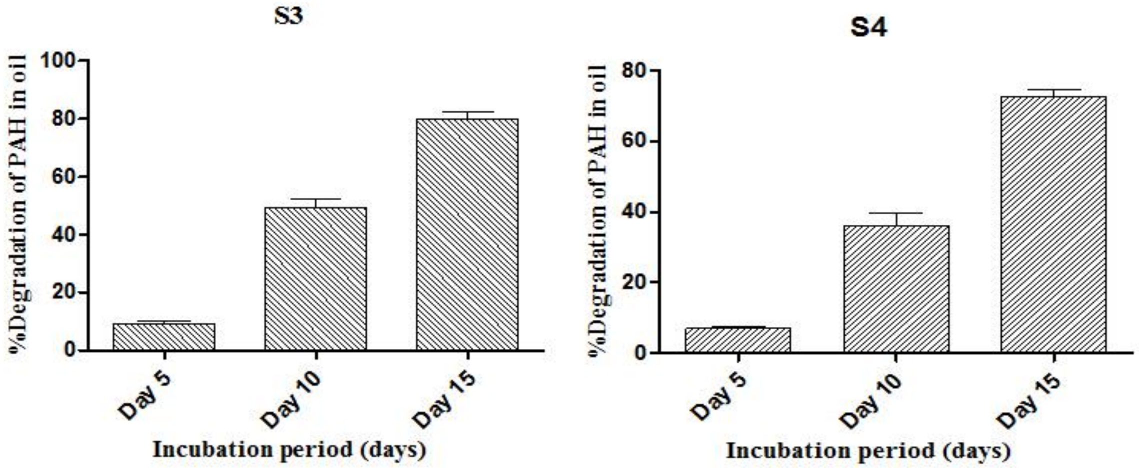

Fig 4.11: The percentage of degradation of PAH in contaminated soil sample by bacterial isolates S3 and S4 after incubation of 15 days.

b) Naphthalene

Biological decomposition of persistent organic pollutants by microorganisms is one of the most important and effective ways to remove the toxicants. Microbial degradation is an efficient and sustainable oil spill cleanup technique (Tyagi et al 2011). Jonathan et al 2003 published in recent advances in petroleum microbiology. The aerobes for PAH metabolism showed that a vast array of microbial species (bacteria, fungi, alga and cyanobacteria) that use low molecular weight three ring and high molecular weight (four rings) such as naphthalene, anthracene, fluoranthene and pyrene etc as sole carbon and energy source. The maximum utilization was in the form of carbon source. It is a technology that utilizes the metabolic potential of microorganisms such as bacteria, fungiand few protozoans (watanabe 2001).Brodkorb and legge 1992 studied the biodegradation of Phenanthrene in oil contaminated soil with fungi *Phanaerochaete chyrsosporium* and found full mineralization of 38 % in a period of 21 days.

In laboratory experiment we used naphthalene (100 ppm, 200ppm) amended with 150gm of sterilized soil sample and was incubated at room temperature for 3-4 hrs for proper adsorption of naphthalene. The contaminated sample was inoculated with strain S3 of different cell concentration (0.5, 0.7, and 1.0) and incubated at 35^0C for 15 days.The results for biodegradation study of naphthalene were recorded by extracting the samples at a period of day 1, 5th, 10th and

15th days. The O.D. of the extracted sample was recoded at 204 nm. The results of table 4.15-17 and fig.4.11 showed that at O.D. 0.5 where the bacteria are approximately 10^{-4}cells have the maximum degradation on 15th day (84.04% ± 5.01) and minimum on 5th day which is 55.13% ± 2.04. The same trend was observed with 0.7 and 1.0.O.D. culture. But if day wise comparison is done 0.7 O.D. (Approx.$10^{-6)}$ cells give only 31.36% ± 3.77 degradation which is lesser as compared to 1 O.D. (Approx. 10^{-8}cells) and 70.9% ± 3.45 as degradation. But overall degradation is maximum on day 15th with value 88.6% ± 4.58 for 0.7 O.D. followed by O.D.5 (84.04% ± 5.01) and then O.D. 1.0 (83.88% ± 2.65). (Fig.4.11)

From the results we concluded that if the cell concentration is increased the total degradation rate is faster as shown in table 4.15-17 and fig. 4.11. Degradation of 70% ± 3.45 was achieved within a period of 5 days for O.D. 1.0 and after day 5 marginal differences were observed. The same observations were recorded for O.D.0.5 and0.7. So, if cell concentration is increased degradation time can be decreased. It maybe because the carbon source which they are taking from naphthalene become less for increased cell concentration and rate of degradation decreases (degradation rate for O.D. 0.1 is 83.88% ± 2.65).

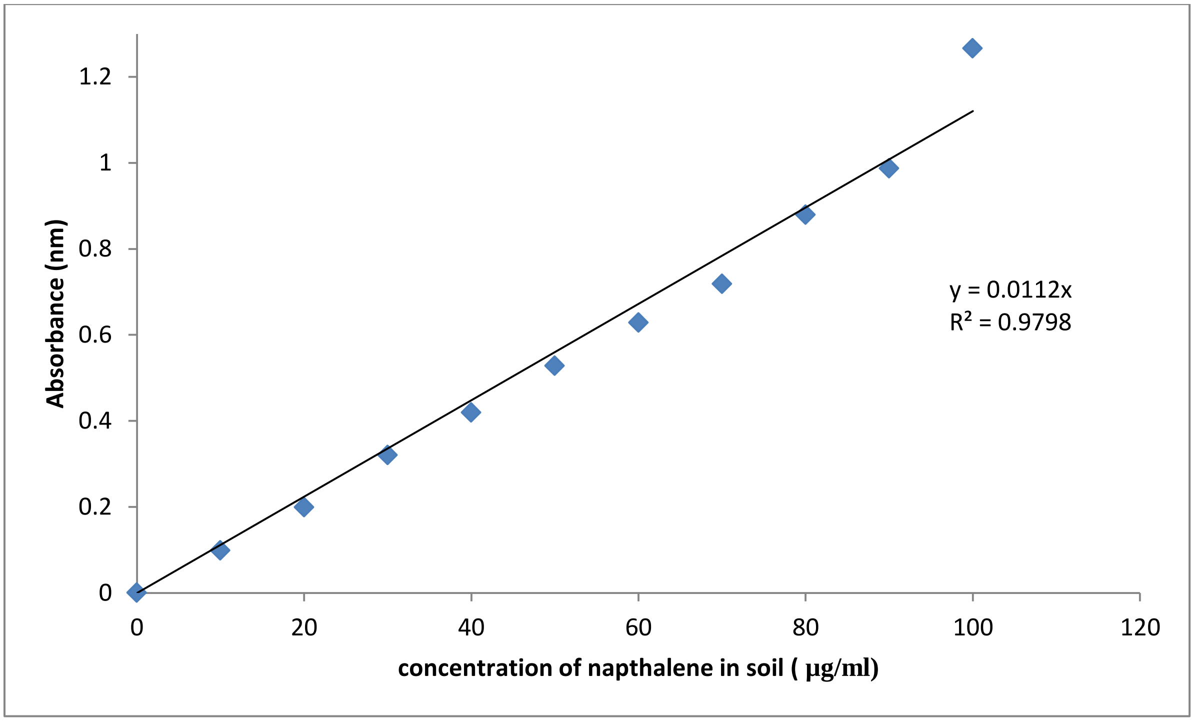

Fig. 4.12 Standard curve for naphthalene in extraction solvent chloroform

Table 4.15: Degradation values of naphthalene with O.D. 0.5* for an incubation period of 15

days

Period of incubation (days)	Absorbance (nm.)	Concentration of PAH (µg/g)	Percentage degradation/ removal (%)
0 Day	1.266	70.33 ± 2.06	0
5 Days	0.568	31.55 ± 2.35	55.13 ± 2.40
10 Days	0.217	12.05 ± 1.15	82.26 ± 4.69
15 Days	0.202	11.22 ± 1.21	84.04 ± 5.01

*approximately (1×10^{5}cells/ml)

Table 4.15(a) : Degradation values of naphthalene with O.D. 0.7* for an incubation period of 15 days

Period of incubation (days)	Absorbance (nm.)	Concentration of PAH (µg/g)	Percentage degradation/ removal (%)
0 Day	1.116	60 ± 0.55	0
5 Days	0.766	41.18 ± 2.10	31.36 ± 3.77
10 Days	0.368	19.78 ± 0.49	67.03 ± 5.08
15 Days	0.202	6.82 ± 1.70	88.62 ± 4.58

*approximately (1×10^{6}cells/ml)

Table 4.15(b): Degradation values of naphthalene with O.D. 1.0 for an incubation period of 15 days

Period of incubation (days)	Absorbance (nm.)	Concentration of PAH (µg/g)	Percentage degradation/ removal (%)
0 Day	0.856	46.02 ± 1.99	0
5 Days	0.249	13.38 ± 2.57	70.91 ± 3.45
10 Days	0.153	8.22 ± 1.53	82.13 ± 3.78
15 Days	0.138	7.41 ± 3.55	83.88 ± 2.65

*Approximately (1×10^{8} cells/ml.)

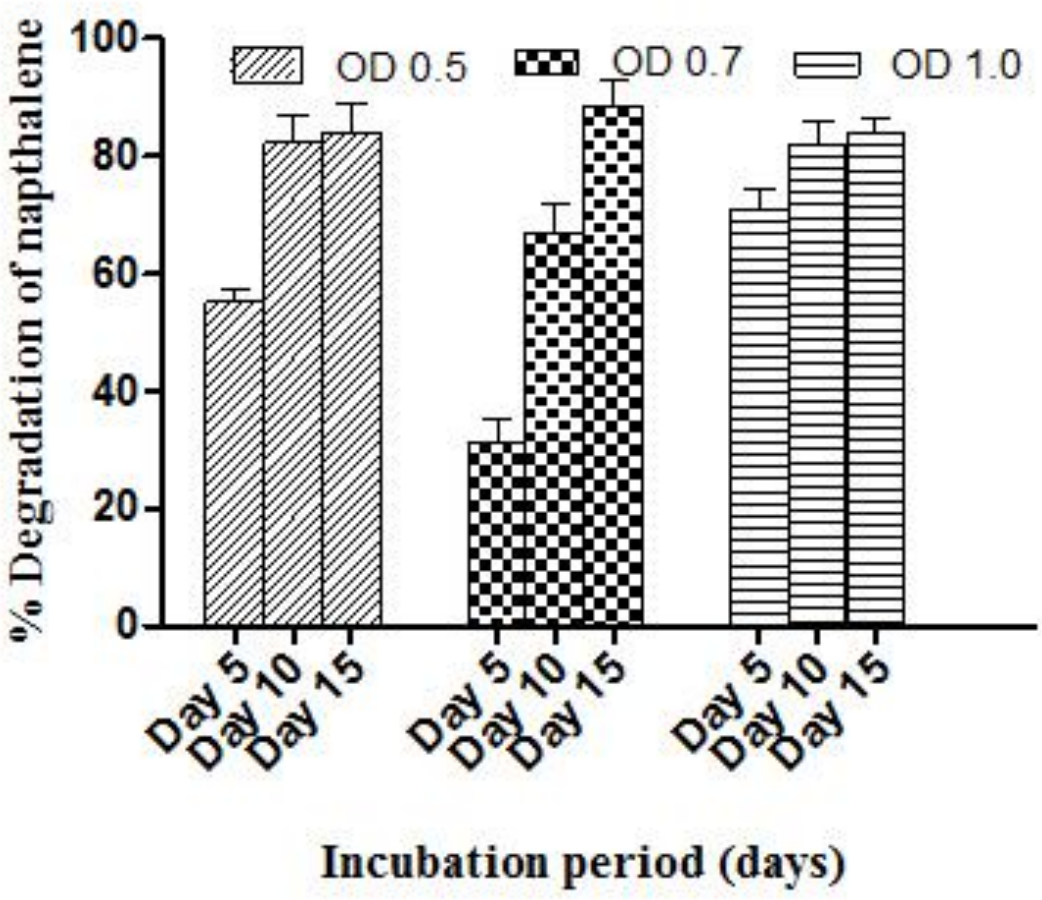

Fig 4.13: Degradation pattern of naphthalene by bacterial strain S3 at different cell concentration for an incubation period of 15 days.

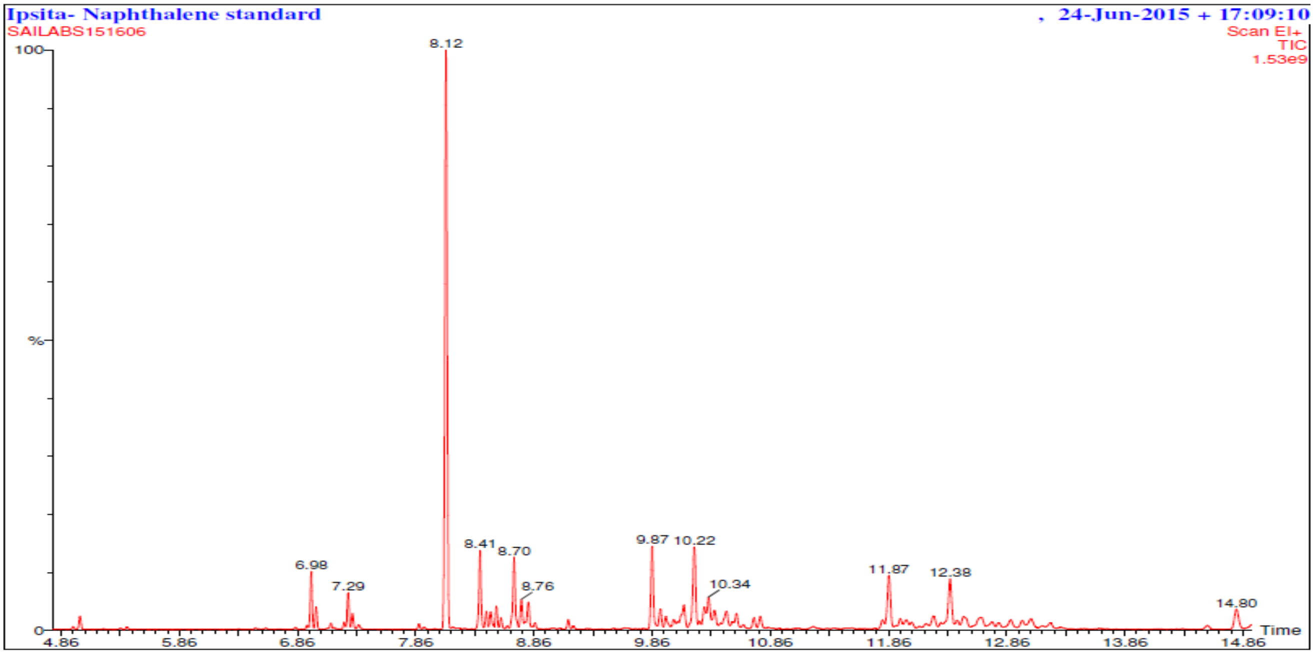

Fig 4.14 :chromatogram of standard(100 µg/gm) naphthalene

Peak area of chromatogram of standard (100µg/gm)naphthalene = 1.40 cm^2

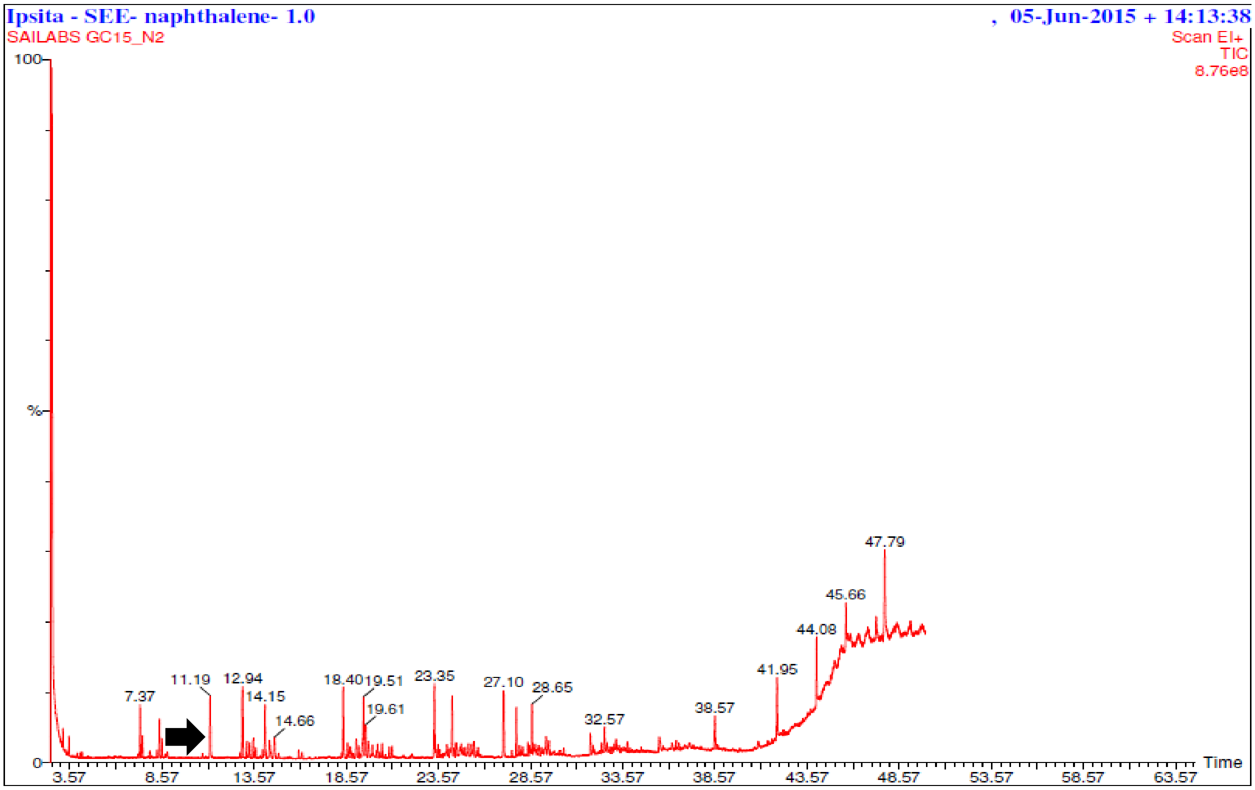

Fig 4.15: Chromatogram of naphthalene contaminated soil at day 0 inoculated with S3 (O.D=1.0)

Peak area of chromatogram of naphthalene contaminated soil at day 0 inoculated with S3 = 1.73 $cm^{2.}$

Concentration of naphthalene in sample (µg/gm) at 0 day of incubation = (1.73/1.40)*100

= 123.57µg/gm.

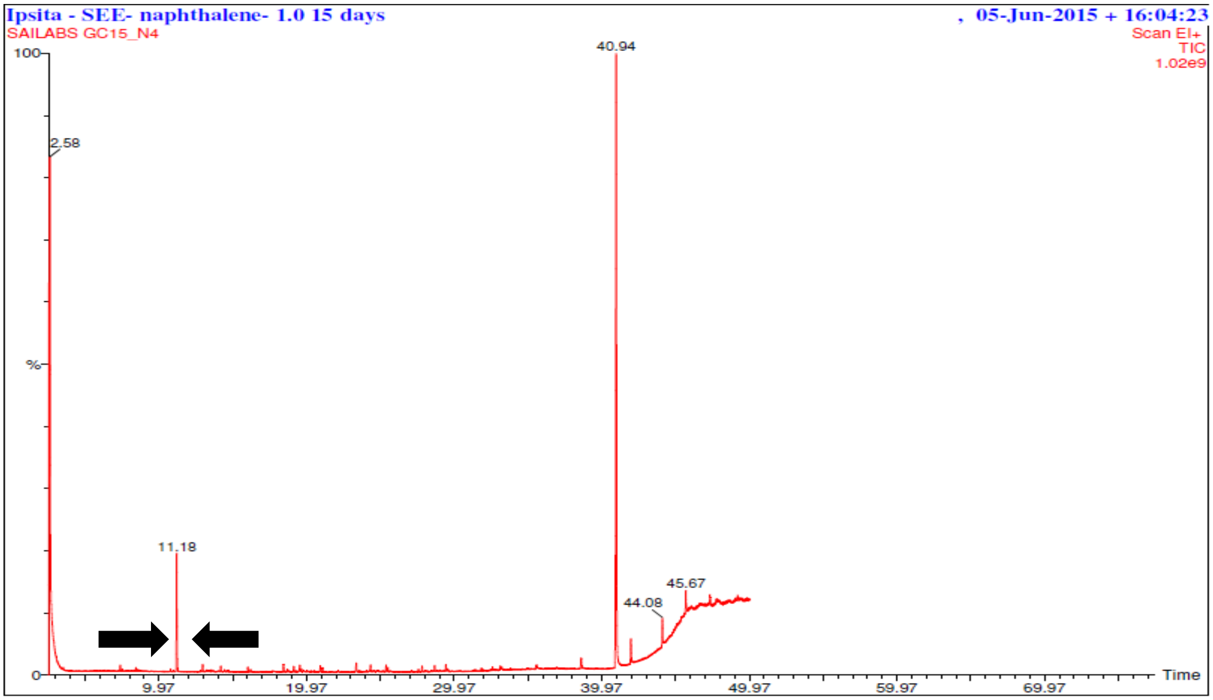

Fig 4.16: Chromatogram of naphthalene contaminated soil after 15 days of incubation with S3 (O.D. =1.0)

Peak area of chromatogram of naphthalene contaminated soil at day 15 inoculated with S3 = 0.51 cm^2

Concentration of naphthalene in sample (µg/gm) at 15th day of incubation = (0.51/1.40)*100

= 36.42µg/gm.

Biodegradation efficiency (%) of S3 (O.D.=1.0) = [(123.57-36.42)/(123.57)] *100

= 70.52 %

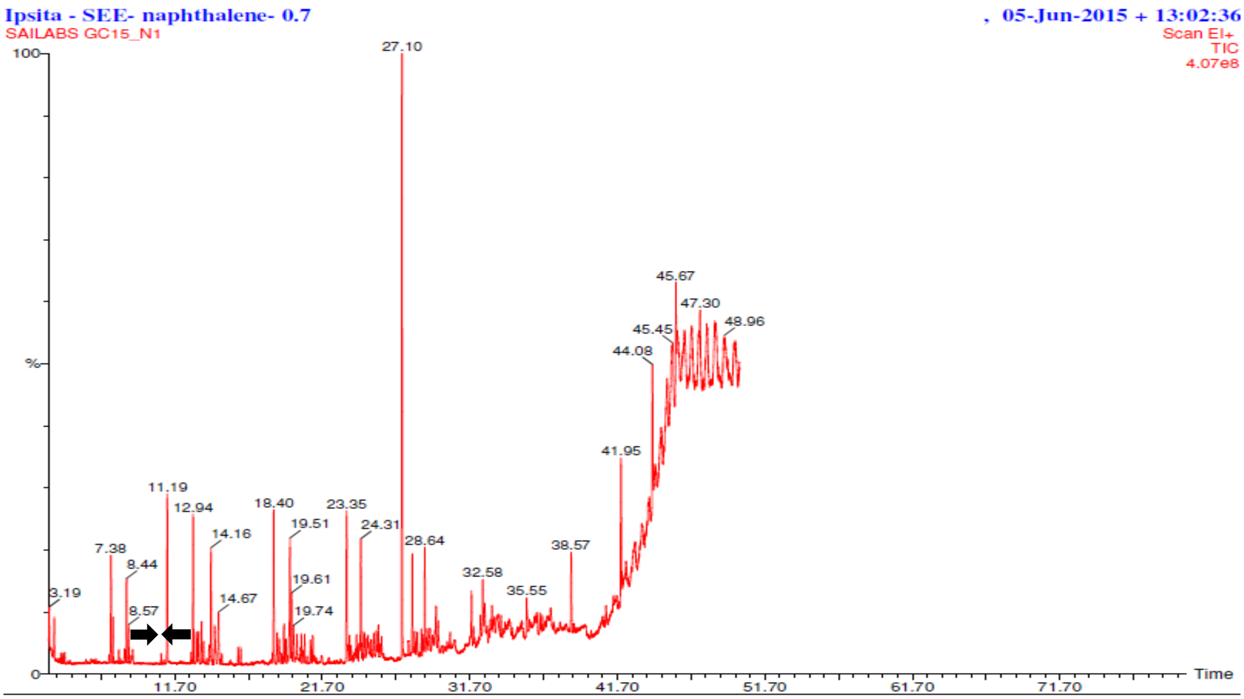

Fig4.17 : Chromatogram of naphthalene contaminated soil at 0 day incubated with S3 (O.D.=07)

Fig 4.18: Chromatogram of naphthalene contaminated soil after 15 days incubated with S3 (O.D. =0.7)

Peak area of chromatogram of naphthalene contaminated soil at day 0 inoculated with S3 (O.D. =0.7) = 0.43 cm^2

Concentration of naphthalene in sample (100 µg/gm.) at 15th day = (0.43/1.40)*100

= 30.71µg/gm.

Biodegradation efficiency (%) of S3 (O.D. =0.7) = [(123.57 - 30.71)/(123.57)] *100

= 75.14 %

The biodegradation efficiency of strain S3 with varying cell concentration(O.D. =1.0 and O.D. = 0.7) was analyzed using GC/MS technique. It showed that the efficiency of strain S3 with O.D. 0.7 to degrade naphthalene is more than that of O.D. 1.0. The degradation efficiency for S3 with O.D. 0.7 was calculated as 75.14% while that of O.D. 1.0 was calculated as 70.52 %. Thus from the results it is clear that strain S3 with O.D. 0.7 Showed better degradation of naphthalene after 15 days of incubation period.

CHAPTER 5
CONCLUSION

- PAH contaminated sediments or soil has strains of bacteria which are capable of degrading PAH and their potential can be exploited for the bioremediation of soil. Overall degradation time can be reduced if the cell concentration is increased. Enhancement of the microbial activity in hydrocarbon contaminated soil can be achieved with the combination of stepwise soil inoculation and nutrient addition. Hence, we conclude that nutrient supplementation would enhance the biodegradation process.

- The soil sample which was contaminated by serviced oil can be treated efficiently by using manure which can give approximately (90% reduction).

- Bacterial culture pure can also be utilized for reduction of toxicant (especially PAH) from the contaminated soil.

- Degradation of PAH with different ratio of manure showed 43.74 to 89.56% removal efficiency with 15 day.

- Pure culture S3 and S4 (not identified) showed 79%(S3) and 72 %(S4) degradation on 15th day.

- With pure naphthalene 83.8% degradation was observed with S3 culture.

- Hence, overall for mixed contamination (with lubricant oil) manure proved better for contamination removal.

CHAPTER 6

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In conferences:

1. "Comparative Studies on Bacterial and Fungal Degradation of Polycyclic Aromatic Hydrocarbons in Soil – An Overview". 7th National Conference on Recent Advances in Chemical, Biological and Environmental Sciences. Mulatani Mal Modi College, Patiala. January 30-31, 2015.
2. "Bioremediation of Polyaromatic Aromatic Hydrocarbon contaminated soil using manure". National Seminar on Sustainable Renewable Energy Generation- Current Scenario. Energy research centre, Punjab University, Chandigarh. March 20-21, 2015.