Characterization of benzene, toluene, naphthalene and acenapthene degrading bacteria isolated from petroleum oil contaminated soil sediments located in Chhattisgarh and evaluation of their antibiotic and heavy metal resistance

THESIS

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

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DECLARATION

I hereby declare that thesis entitled **"Characterization of benzene, toluene, naphthalene and acenapthene degrading bacteria isolated from petroleum oil contaminated soil sediments located in Chhattisgarh and evaluation of their antibiotic and heavy metal resistance"** is my own research work carried out under the supervision of Dr. D. R. Modi, Associate Professor, Department of Biotechnology, Babasaheb Bhimrao Ambedar University, Lucknow (India). I have submitted this thesis to Babasaheb Bhimrao Ambedkar University, Lucknow (India), for the award of the degree of Doctor of Philosophy (Ph.D.) in Biotechnology and all the resources quoted in the thesis have been indicated and acknowledged by complete references.

Date:

Place: Lucknow (India) **(Jai Godheja)**

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CERTIFICATE

This is certify that the thesis **"Characterization of benzene, toluene, naphthalene and acenapthene degrading bacteria isolated from petroleum oil contaminated soil sediments located in Chhattisgarh and evaluation of their antibiotic and heavy metal resistance"** submitted by **Jai Godheja** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or other university.

The thesis is submitted to Babasaheb Bhimrao Ambedkar University Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) regulation – 1999 as amended in 2008/2010 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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IT IS OUR LIFE WE CALL IT SOIL

IT IS THE STUFF IN WHICH WE TOIL

FROM SOIL WE'VE SPRUNG TO SOIL WE'LL GO

PROTECT THE SOIL OF THIS EARTH SO WE CAN GROW

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ABBREVIATIONS

AMR	-	Antimicrobial resistant
AR	-	Analytical reagant
ATP	-	Adenosine Triphosphate
BBH	-	Bacto Bushnell Hass
BTEX	-	Benzene Toluene Ethylbenzene Xylene
$C_8H_4K_2O_{12}Sb_2.3H_2O$	-	Potassium Antimony Tartarate
$CdCl_2$	-	Cadmium Chloride
CFP	-	Cefoperazone
CLSI	-	Clinical and Laboratory Standards Institute
CTC	-	5-cyano-2,3-ditolyl tetrazolium chloride
CTX	-	Cefotaxime
$CuSO_4.5H_2O$	-	Copper Sulphate
CXM	-	Cefuroxime
DNA	-	Deoxyribonucleic Acid
EDTA	-	Ethylenediaminetetraacetic Acid
ELISA	-	Enzyme linked immunosorbent assay
EPA	-	Environment Protection Agency
ERH	-	Electrical Resistance Heating
EU	-	European Union
FAAS	-	Fluorescence Atomic Absorption Spectrophotometer
$FeCl_3$	-	Ferric Chloride
$FeSO_4.7H_2O$	-	Ferrous Sulphate heptahydrate
FTIR	-	Fourier Transform Infra Red
g/kg	-	gram per kilogram
HCl	-	Hydrochloric Acid
$HgCl_2$	-	Mercuric Chloride
HPLC	-	High Pressure Liquid Chromatography
IITR	-	Indian Institute of Toxicology Research
INT	-	2-(4- iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H- tetrazolium

		chloride
IPM	-	Imipenem
ISU	-	Insitu Vitrification
K	-	Potassium
$K_2Cr_2O_7$	-	Potassium Dichromate
KBr	-	Potassium Bromide
Kg/ l	-	kilogram per litre
KH_2PO_4	-	Potassium Dihydrogen Phosphate
KNO_3	-	Potassium Nitrate
KOW	-	Octanol/Water Partition coefficient
Mg/kg	-	Milligram per kilogram
MJ/Kg	-	Milli Joule per Kilogram
MMT	-	Million Metric Tonnes
$MnSO_4.H_2O$	-	Manganous Sulphate mono hydrate
MPN	-	Most Probable number
MTS	-	5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)
MTT -	-	2-(4,5-dimethyl-2-thiazolyl)-3,5- diphenyl-2H-tetrazolium bromide
MW	-	Molecular Weight
NaCl	-	Sodium Chloride
NaOH	-	Sodium Hydroxide
NAPL	-	Non Aqueous Phase Liquid
NAM	-	Nutrient Agar Medium
NB	-	Nutrient Broth
NBRI	-	National Botanical Research Institue
NCBI	-	National Centre for Biological Information
$NiSO_4.6H_2O$	-	Nickle Sulphate
NOR	-	Norfloxacin
OC	-	Organic Carbon
OPEC	-	Organization of Petroleum Exporting Countries

PAH	-	Poly Aromatic Hydrocarbon
$Pb(NO_3)_2$	-	Lead Nitrate
PCR	-	Polymerase Chain Reaction
PRL	-	Piperacillin
RFH	-	Radio Frequency Heating
RNAase	-	Ribonuclease
RT	-	Room Temperature
SDS	-	Sodium Dodecyl Sulphate
SEE	-	Steam Enhanced Extraction
SET	-	Sodium Chloride EDTA Tris
$SnCl_2.2H_2O$	-	Stannous Chloride
TCA	-	Tricarboxylic Acid Cycle
TE	-	Tris EDTA
TGC	-	Tigecyclin
TTC	-	2,3,5-triphenyl-2H-tetrazolium chloride
TV	-	2,5-diphenyl-3-2-naphthyl tetrazolium chloride or tetrazolium violet
UK	-	United Kingdom
UPLC	-	Ultra Pressure Liquid Chromatography
UV	-	Ultra Violet
WST	-	1- sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3- (4-nitrophenyl)-2H-tetrazolium
XTT	-	sodium 2,3-bis(2-methyloxy- 4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium
$ZnSO_4.7H_2O$	-	Zinc Sulphate

CHAPTER 1

Introduction

Production, refinery, storage and transportation of crude oil and its derivatives may lead to contamination of soils with BTEX compounds (benzene, toluene, ethyl-benzene and xylenes), polycyclic aromatic hydrocarbons (PAHs), as well as aliphatic hydrocarbons. Leakage from crude Oil wells, refineries, petroleum product industries, distribution and storage devices, transportation equipments are the main sources of the contaminations (Wolicka et al., 2009). Because trace heavy metals are common constituents of crude oil (Osuji and Onojake, 2004) and of petroleum derivatives (Pb^{2+} leaded gasoline, lubricating oils and greases; Zn^{2+}, Cd^{2+} compounds amended engine oil etc.) during an increased, long term pollution with hydrocarbons the heavy metal contamination of the respective areas should also be taken into account (John, 2007). Furthermore, within huge industrial areas (mining, metallurgical, oil distribution industry etc.) where hydrocarbon spills are very common the co-occurrence of hydrocarbon and heavy metal pollutants is observed (Abdullah et al., 2011).

All the above mentioned compounds in the environment cause serious health problems due to their carcinogenic and mutagenic effects (Mrozik and Piotrowska-Seget, 2009; Mishra et al., 2010). Because heavy metal and antibiotic resistance genes are often found on the same mobile genetic element, metal pollution often promotes the emergence of antibiotic resistances in exposed organisms, which fact has also a growing concern in natural and clinical settings (Knapp et al., 2011). Thus, the remediation of these sites has been of great interest.

Several treatment methods, applying physical, chemical and biological processes have been developed in the past decades. Physicochemical measures are capable of removing most of the contaminants, but the main disadvantages of these methods lie in the increased energy consumption and the need of additional chemicals. Furthermore, with physico-chemical treatments, such as incineration, the transfer of pollutants from one environmental compartment to another may occur (e.g. from soil to atmosphere). During bioremediation, metabolic activity of micro-organisms is involved in breakdown of

contaminants into non-toxic compounds. This technique is cost-effective, applicable over large areas and (usually) leads to the complete breakdown of the organic contaminants, potentially ending in their mineralization. Although bioremediation is a relatively time consuming process and the degree of success depends on a number of factors (pH, temperature, availability of O_2 and nutrients, etc.) aerobic bacterial biodegradation of aliphatic, simple aromatic hydrocarbons, like BTEX and low molecular weight PAHs is well characterized (Sarkar et al., 2004; Trindade et al., 2004).

As economic aspects are getting more considered in remediation processes, the more inexpensive bioremediation methods e.g. in situ biostimulation and bioaugmentation techniques are preferred. However, to evaluate the applicability of these bioremediation techniques in a petroleum hydrocarbon contaminated environment, it has to be known whether the microbial community of the contaminated environment has the metabolic potential to eliminate the contamination.

Due to the fact that Chhattisgarh has a notable oil collection centres, renowned steel production industries, aluminum production plants and power plants there is lot of hydrocarbon and heavy pollution around these industries. Since remediation of these sites is considerably costly, development of a microbial soil inoculant for bioaugmentation purposes could be an appropriate approach to treat the contaminated sites in Chhattisgarh. Therefore, the major goal of this study was to obtain a strain collection of hydrocarbonoclastic bacteria able to exert an outstanding degradation potential against high levels of hydrocarbon pollutants even within heavy metal impacted environments. In addition, our aim was to investigate the heterotrophic and fuel degrader counts among ten geographically close petroleum hydrocarbon contaminated sites. Furthermore, we determined the heavy metal tolerance and antibiotic resistance of isolated and identified aerobic hydrocarbon-degrading bacterial strains.

CHAPTER 2
Review of Literature

2.1 Background

The petroleum industry today is one of the fastest growing industries over the past 50 years with a predicted increase in world petroleum consumption from 85 million barrels in 2006 to 106.6 million barrels by 2030 (Igunnu and Chen, 2012). Many countries are totally dependent on this industry for their economic growth. They have contributed immensely to the global economy and industrialization. But the uncontrolled consumption has also led to increased pollution levels in the environment including soil (Cervantes et al., 2011). A study claims that between 1.7 and 8.8 million metric tonnes of oil were released annually into the global environment with more than 90 % being directly related to human activities (Dadrasnia and Agamuthu, 2013).

Soil is an essential component which provides the environment for the growth of plants, cycling of nutrients and a living base for microbes, insects, animals and humans (Harrison, 1983). Soil is a heterogeneous assembly of materials that include solid, liquid, and gaseous phases of mineral particles, organic matter, vapour, and water moisture. Soil pollution occurs due to oil spills, inadequate transportation, pipeline rupture, tank failures and production and storage problems. Crude oil is a complex mixture of petroleum hydrocarbons (PHC), mainly composed of aliphatic, aromatic and asphaltene components along with sulfur, nitrogen and oxygen containing compounds. PHC contamination exists in the form of petroleum vapours in the soil pore space, as residual petroleum trapped between or adsorbed into soil particles, or as petroleum dispersed in soil moisture (Friend et al., 1992). Petroleum toxicity could be a hazard to plants, animals, and a threat to human health through direct exposure.

2.2 Global Scenario

During last two centuries, petroleum has been reported to be found predominantly in surface soils, deep or shallow aquifers, aquitards, ocean sea-beds and oil seepage globally with the first oil exploration from an oil well in Pennsylvania in 1859 by Colonel Drake (Alloway and Ayres, 1993). Subsequently, oil exploratory activities have increased exponentially worldwide over the decades with global continental reserves represented in Figure

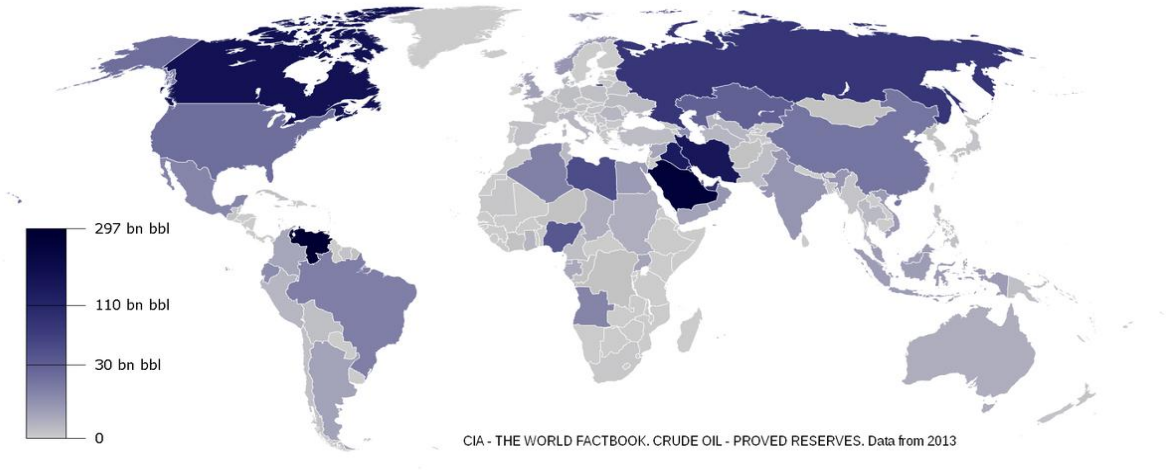

Figure 1: Oil reserves worldwide (Image adapted from wikipedia).

World's proven crude oil reserves at end of 2015 are 1697.6 thousand million barrels. Middle East with reserves of 803.5 thousand million barrels has the highest share (47.3%), followed by Central & South America (19.4%). Asia Pacific region constitute a mere 2.5% of the total basket. World Production of Crude Oil was 4361.9 Million Tonnes during 2015, which is 3.2% more compared to the data in 2014 (4228.68 Million Tonnes). Saudi Arabia had the largest share in crude oil production with 568.5 million tonnes (34.5%) for OPEC countries in 2015, followed by Iraq & Iran at 197 million tonnes (10.9%) and 182.6 million tonnes (10.1%) respectively (Indian Petroleum and Natural Gas Statistics, 2015-16).

2.3 Indian scenario

India is a big market for consumption of petroleum products and a significant part of them are imported from other countries. Petroleum products rank second after coal as a major source of energy in India. Oil constitutes over 35% of the primary energy consumption in India. Crude oil reserves in India are estimated at 757 million metric tons. Crude oil production during 2009-2010 was 33.69 million metric tons which has increased by 11.8 % to 37.46 million metric tons in 2014-2015. The consumption of petroleum products during 2009-2010 was 138.19 million metric tons which increased to 184.67 million metric tons during 2015-2016 (Indian Petroleum and Natural Gas Statistics, 2015-16). Thus demand is far more than supply. India imported 202.85 MMT of crude oil in 2015-16 which was 159.25 MMT during 2009-10 (Indian Petroleum and Natural Gas Statistics, 2015-16). The rise in automobile industry has increased the demand for petroleum products and is expected to rise to more than 240 million metric tons by 2021-22 which will further increase by 51.61% in 2031-32 (Garg, 2012). Indian government has established many new refineries and also has increased the refining capacity of existing refineries. Thus, India is a major petroleum consuming country thereby predisposing the environment to the drastic effects associated with its exploration and exploitation. Recently, in August 2010 two ships collided off the Mumbai coast leaking >2000 tons of oil into the sea. The large oil spill in the open ocean may do less harm to marine ecosystem than the relatively small spill near the shore. The consequences of these oil spills include widespread, long-term, and serious damage to human health, natural resources, marine ecosystems and terrestrial life.

2.4 Diesel

Production of petroleum diesel is carried out by the fractional distillation of crude oil between 200 °C and 350 °C at atmospheric pressure, resulting in a mixture of carbon chains that typically contain between 8 and 21 carbon atoms per molecule. Diesel consists of 75% saturated hydrocarbons (primarily paraffins including *n*-, *iso*-, and cycloparaffins) and 25% aromatic hydrocarbons (including naphthalenes and acenapthenes). The average chemical formula for common diesel fuel is $C_{12}H_{23}$, ranging approximately from $C_{10}H_{20}$ to

$C_{15}H_{28}$. The density of petroleum diesel is about 0.832 kg/l. About 86.1% of the fuel mass is carbon, and when burned, it offers a net heating value of 43.1 MJ/kg (Demirel, 2012). Many studies have shown various effects of diesel especially small chain hydrocarbons (*n*-C_{10} and *n*-C_{11}) pollution on plants such as delayed/reduced germination, less plant height, lower leaf and biomass production. Diesel pollution also leads to reduction in soil microflora population. Due to its relatively high mobility, the possibility of contamination of surface waters and groundwaters as well as soils is high (Gallego *et al.* 2001).

Therefore it is necessary to treat these soils for removal of petroleum pollutants. Physical and chemical means for remediation are very costly and impractical. In recent years, there has been increasing interest in developing on site and *in situ* techniques especially bioremediation for reclamation of petroleum oil-contaminated soils. Methods like natural attenuation, biopiling, bioaugmentation, phytoremediation or rhizoremediation, singly or in combination are commonly used to remediate contaminated sites.

Table 1: Composition of diesel oil

Element	Weight %	Hydrocarbon	Weight %
Carbon	83-87	Paraffins	30
Hydrogen	10-14	Naphthenes	49
Nitrogen	0.1-2	Aromatics	15
Oxygen	0.1-1.5	Asphaltics	6
Sulfur	0.5-6		
Metals	< 0.1		
The hydrocarbon weight % values are averages.			

2.5 Classification of petroleum hydrocarbons

Petroleum hydrocarbons can be classified into three major classes: aliphatics, aromatics and heterocyclics.

2.5.1 Aliphatic hydrocarbons

Aliphatic hydrocarbons are straight, branched or cyclic compounds which may be either saturated or unsaturated. They are subdivided into alkanes, alkenes and cycloalkanes.

Alkanes (or paraffins) are saturated hydrocarbons with a general formula C_nH_{2n+2} and have carbon and hydrogen atoms linked together by single bonds only. They are either straight-chained or branched and form structural isomers which different physicochemical properties from straight-chain ones (Nathanail and Bardos, 2005). They are odourless, colourless and are of generally low reactivity though they can be easily oxidized by microorganisms at the terminal carbon position. Cycloalkanes (commonly known as naphthenes) are different from alkanes in that their carbon atoms are bonded together to form one or more ring-like structures. Cycloalkanes share similar physical and chemical properties though cycloalkanes with only one ring are susceptible to nucleophilic attack, hence less stable and a little more reactive than normal alkanes (Mulligan, 2002). However, as the number of carbons and ring structure increases, they become more stable. Alkenes or olefins are unsaturated hydrocarbons containing at least one carboncarbon atom double bond. Alkenes also comprise straight-chain, branched-chain or cyclic configurations. Unlike alkanes and cycloalkanes, alkenes have shorter σ-bond length than alkanes and the presence of π-bonds makes alkenes more reactive chemically than alkanes and cycloalkanes (Patrick, 2000).

2.5.2 Heterocyclic hydrocarbons

Heterocyclic hydrocarbons are cyclic organic compounds containing at least two different elements as members of its rings. Examples of such elements include nitrogen, sulphur, oxygen and metals. The type of ring-like structure depends on the number of heteroatoms present in the ring and this varies from 3 to 8-membered ring compounds (Fig 2). Examples of heterocyclic compounds include pyrrole, dioxane, furan, pyridine, furfural etc. Depending on the type and nature of the heterocycles, they can be either be saturated or unsaturated. They are also very reactive and highly susceptible to both chemical and microbial attack (Nathanail and Bardos, 2005; Johnson et al., 2003).

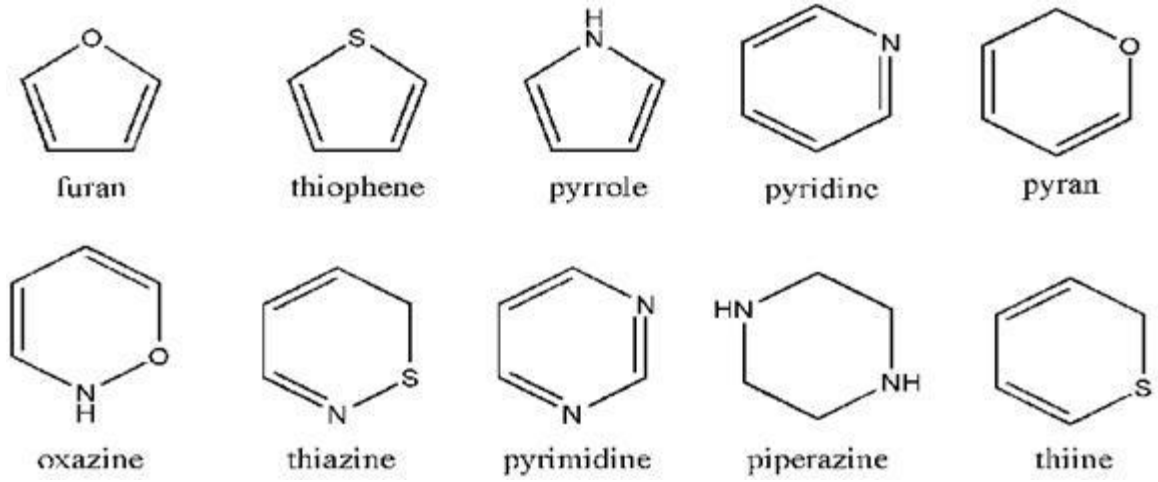

Fig 2: Heterocyclic Compounds

2.5.3 Aromatic hydrocarbons

Aromatic hydrocarbons differ significantly from aliphatic hydrocarbons due to the presence of ring structures solely. They are also unsaturated hydrocarbons due to the presence of two or more double bonds conjugated together to form a ring-like structure. The aromatics are the focus of regulatory agencies due to their persistent and toxic effect in the environment. They are compounds that contain one or more benzene rings. They can be further divided into two categories namely; monoaromatics and polyaromatics.

2.5.3.1 Monoaromatics - BTEX

Benzene, toluene, ethyl benzene and xylene (BTEX) are aromatic hydrocarbons with only one parent benzene ring (Figure 3). They are components of gasoline, aviation fuels and other major petrochemical products. They are carcinogenic, neurotoxic and have been blacklisted by the Environmental protection Agency (EPA), UK (Juana et al., 1998; Foght, 2008).

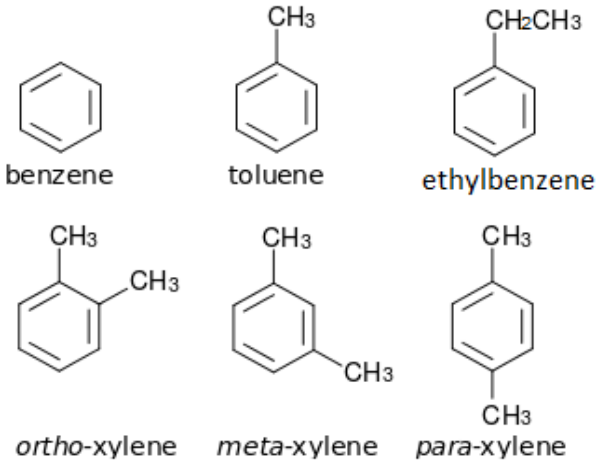

Figure 3 : BTEX compounds

Out of the BTEX compounds, benzene has been shown to be the most recalcitrant compound and highly mobile in matrix owing to higher solubility relative to other BTEX compounds (Johnson et al., 2003; Coates et al., 2002).

2.5.3.2 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are a class of fused ring aromatic compounds with the parent ring mainly of benzene. Structures of the 16 most potent toxic PAH is shown in Fig 4. PAHs are naturally found in crude oil, coal deposits, marine sediment, soil and in groundwater (Juana et al., 1998). They are lipophilic compounds with low aqueous solubility and thermodynamically unstable due to their relatively large negative resonance energies. Thus, they tend to be adsorbed on particles' surfaces in the environment or form non-aqueous phase liquids (NAPLs). As the molecular weight and number of fused rings of PAHs increases, the solubility and volatility decreases. In addition, they are generally persistent in the environment and have been reported to be carcinogenic (Johnson et al., 2003). They posses intrinsic chemical stability, high resistance to various transformation processes and toxicity (Juana et al., 1998).

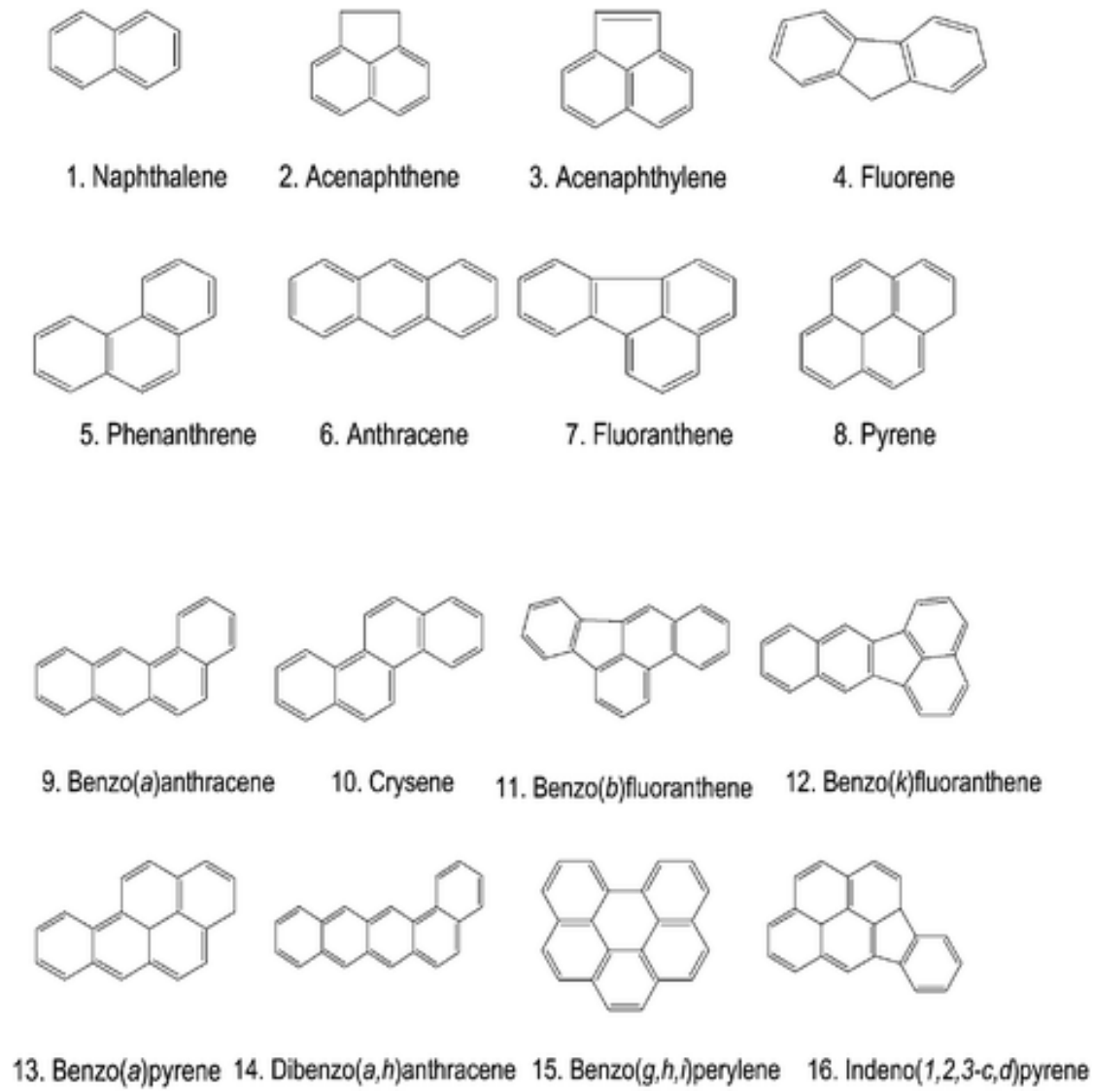

Figure 4**:** Structure of 16 priority toxic PAHs (EPA)

2.6 Toxicity of PHC

Bioacumulation of petroleum hydrocarbons by plants and animals in soil and aquatic matrices via biomagnification occurs slowly and contaminate the food chains. If the pollutant concentration is higher than its threshold concentration (i.e. the levels at which there are acute effects) then severe impairment of the metabolic activities or death of plants and/or animals can result (Johnson et al., 1996; Mandal et al., 2007). Some petroleum

hydrocarbons (especially the aromatics such as benzene, toluene, naptahlene and acenapthene) are carcinogenic and therefore listed among the EU priority pollutants (Hincapié et al., 2005). Hence, soil and groundwater pollution by petroleum hydrocarbons is an increasingly sensitive issue. In the European Union (EU) alone, there are about 3.5 million contaminated sites with an estimated cost of soil contamination in the range of $2.6-18.9 billion annually (Majone et al., 2015).

The toxic effects of the petroleum hydrocarbon spill mainly depend on the composition of the polluting petroleum product. Alkanes and aromatic hydrocarbons exhibit relatively low solubility in water with benzene being the most soluble at 1780 mg/L. The most biologically active substances, in terms of toxicity and bioaccumulation, have log octanol/water partition coefficient (KOW) values between 2 and 6, and are referred to as lipophilic compounds (Trapp *et al*., 2001) Many hydrocarbons fall into this group e. g. log KOW for *n*-hexane and *n*-decane are 2.91 and 5.58, respectively. The concentration of individual components really matters for the ecological impact. For instance, n-alkanes are less toxic and persistent than aromatic compounds. In particular, the PAHs are carcinogenic and have been implicated in many a wide range of human health problems and also disease problems with aquatic organisms (Grimmer, 1991).

2.6.1 Toxicity to plants

PHC pollution leads to decrease in plant growth as well as in crop productivity. Diesel oil can cause long term or acute effects on the plants. Several studies show the effect of diesel oil pollution on plant growth in terms of reduced germination rate, root length, shoot length, plant weight etc. Many authors have reported a lower rate of seed germination in different plant species including grasses, legumes, herbs and commercial crops when planted in soil contaminated with 25 and 50 g/kg diesel fuel Adam and Duncan (2002).

Sharifi *et al*. (2007) observed the effect of 25, 50, 75, 100 g/kg of spent oil on seed germination, shoot height and biomass of six herbaceous plant species including one species of Fabaceae (*Medicago truncatular*), four species of Gramineae (*Bromous mermis,*

Secal seral, Triticum sativa and *Agropyron deserterum*) and one species of Linaceae (*Linum ussitasimum*). The authors reported only 16.2, 15 and 2.7% germination in *A. deserterum, B. mermis* and *L.ussitasimum*, respectively, while 63.5% germination was observed in *M. truncatular*. Reduction in seedling height and biomass was also observed in all plant species.

Houshmandfar and Asli (2011) evaluated the effect of 2 ml/kg (v/w) of mixed gasoline and diesel fuel on seed germination and seedling growth characteristics of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), alfalfa (*Medicago sativa* L.) and clover (*Trifolium resupinatum* L.). The authors reported delayed onset of germination due to gasoline and diesel fuel mixture stress. Siddiqui *et al.* (2001) also studied the effect of 0, 0.64, 1.6, 4.0 and 13.6% (w/w) diesel on the germination of perennial ryegrass. Inhibition of seed germination until 24 weeks was observed with 13.6% (w/w) diesel.

Saadoun and Al-Ghazawi (2010) studied toxicity of diesel towards plant seeds of *Atriplex halimus, Cochorus olitorius, Hordeum spontaneum* and *Triticum aestivum.* The authors reported 57.7 and 76.9% decline in seed germination of *C. olitorius* at 100 and 5000 mg/kg of diesel respectively after 10 days. While, seeds of *H. spontaneum, T. aestivum* and *A. halimus*showed a decline of less than 30 % at 100 mg/kg and 11.1, 20 and 29.4% decline at 5000 mg/kg of diesel, respectively. Akujobi *et al*. (2011) also studied the effect of 2, 4, 6, 8 and 10% diesel pollution and nutrient amendments on plant growth parameters of eggplant (*Solanum melongena*). Ogbo (2009) studied effects of 1, 2, 3, 4 and 5% of diesel contamination on four crop plants *Arachis hypogaea, Vigna unguiculata, Sorghum bicolor* and *Zea mays*. They observed that all the test plants tolerated diesel fuel contamination at 1-3% levels of contamination as seed germination was between 89-33%. The authors also observed reduction in seed germination with increase in levels of contamination (4 % and above) in the four test plants.

2.6.2 Toxicity to animals/birds/humans

PHC constitutes both of aliphatic and aromatic compounds, but the aromatics are known to be toxic, mutagenic or carcinogenic (Balba *et al.*, 1998). Birds and animals may face

problems such as blockage, pneumonia, emphysema and even death by inhaling the droplets of oil, or oil fumes or gas. Blockage may also lead to decreased absorption of nutrients and finally result in death of these birds and animals due to severe liver damage and anemia. Symptoms of crude oil toxicity include liver necrosis, blocking of the liver, fat disintegration and dissociation of hepatocytes (Sathishkumar *et al.*, 2008). Eventually food chain is also affected. Soil dwelling organisms may be affected by the reduced or total absence of aeration in hydrocarbon polluted soils. Few studies in humans (reviewed by Aguilera *et al.*, 2010) have linked PHC pollution to lower respiratory problems, long term mental health effects, genotoxic damage, hormonal imbalance, reproductive and developmental toxic effects, skin and lung cancer etc.

2.6.3 Toxicity to microorganisms

PHC's can be very toxic to the microorganisms living in the microenvironment. Gill and Ratledge (1972) reported that *n*-alkanes are toxic to microorganisms. Walker *et al.* (1975) demonstrated the toxicity of crude and refined oil to natural bacterial populations from pristine sediments with refined oil being more toxic. The aromatics in crude oils such as α-pinene, limonene, camphene, and isobornyl acetate were found to be toxic to the microorganisms (Andrews *et al.*, 1980). Even for bacteria surviving in presence of dissolved aromatic hydrocarbons like naphthalene, increase in lag phase and decreased growth rate were observed (Calder and Lader, 1976). There can be many reasons leading to inhibition of growth like impaired ATP synthesis and potassium uptake and accumulation of these compounds in the membrane of microorganisms. Hydrocarbon insertion alters membrane structure by changing fluidity and protein conformations and results in disruption of the barrier and energy transduction functions while affecting membrane-bound and embedded enzyme activity (Van Hamme *et al.*, 2003). This toxicity to microorganisms exerts selective pressure changing the microbial diversity of the polluted soil.

2.7 Existing methods for treating petroleum hydrocarbons

As a result of established risk to public health and the natural environments, treatment of petroleum hydrocarbons has increasingly become inevitable. However, a number of methods are currently used to remediate petroleum hydrocarbons, ranging from physicochemical to biological methods. Following tables briefly discuss about the biological, non biological (thermal) and non biological (non-thermal) methods.

Table 2: List of biological techniques used in remediation of contaminants with their advantages and disadvantages

Remediation Techniques	Types	Advantages	Disadvantages	References
Natural attenuation • Uses natural processes to reduce the concentration and spread of contamination at contaminated sites.		• Generation of less remediation waste and less impact on the environment; • Ease to use when combined with other technologies.	• Public may not perceive the effectiveness of the process correctly •Site Characterization can be more costly and complex	Khan and Husain, 2002; 2003).
Phytoremediation • Uses plants to clean up contaminated soils and groundwater.	Phytoextraction • Uptake of contaminants from the soil	• Minimal environmental disturbance. • Aesthetically pleasing and passive, solar energy driven technology. • Used on a large range of contaminants. • It is cost-effective for large contaminated sites	• Requires more than one growing season • Limited to soils less than one meter from the surface. •Food chain contamination possible through animals which eat the plants used in these projects	Marchiol et.al 2007; Wang et al., 2002; Meagher, RB 2000
	Phytotransformation • Involves the degradation of contaminants through plant metabolism			Kvesitadze et al, 2006; Burken, J.G. 2004; Subramaniam et al., 2006
	Phytovolatalisation • Contaminant is taken in by the plant tissue and then volatlalised in the environment			Danika et al., 2005; Zhang et al., 2001b
	Rhizofiltration • A water remediation technique in which contaminants are taken up by the plant's roots			Miller, R. 1996; Cooney, C M. 1996
	Phytostimulation • Stimulation of microbial degradation through the activities of plants in the root zone			Rupassara, S. I. 2002
	Phytostabilisation • Reduction of migration of contaminants through the soil medium			Mendez M.O. 2008
	Phytoscreening • Plants act as biosensors of subsurface contamination helping quickly to delineate contaminant plumes			Burken et al., 2011; Sorek et al., 2008;

Table 2(Cont.): List of biological techniques used in remediation of contaminants with their advantages and disadvantages

Remediation Techniques	Advantages	Disadvantages	References
Bioventing • Injection of air into the contaminated media at a rate designed to maximize in situ biodegradation and minimize the off-gassing of volatilized contaminants to the atmosphere	• Uses readily available equipment, easy to install • Easily combinable with other technologies	• High concentrations may be toxic for microorganisms • Not applicable for certain site conditions (e.g., low soil permeability) • Only treats unsaturated zones of soils, and needs other methods to treat saturated zones of soils and groundwater	Mihopoulos et al. 2002; Diele et al. 2002
Bioreactors/ Bioslurry • Uses bioreactors and selected bacteria to biodegrade the contaminants.	• Rapid degradation kinetic Optimized environmental parameters Enhances mass transfer • Effective use of inoculants and surfactant	• Soil requires excavation • Relatively high cost capital • Relatively high operating cost	Zhang et al., 2001.
Composting • Uses cow manure and mixed vegetable waste to remove the toxicants upto 90 % from the contaminated soil.	• Low cost Rapid reaction rate, • Inexpensive, self heating	•Extended treatment time •Requires nitrogen supplementation, • Incubation periods months to years	Atagana, 2004b
Biopiling • Piling up of petroleum-contaminated soils into piles or heaps and then stimulating aerobic microbial activity by aeration and the addition of minerals, nutrients, and moisture.	• Can be done on site	• Need to control abiotic loss • Mass transfer problem • Bioavailability limitation	Filler et al., 2001; Li et al., 2002.
Land Farming • Spreading of excavated contaminated soils in a thin layer (about 1.5 m) on the ground surface of a treatment site with stimulation of aerobic microbial activity.	• Relative simple design and implementation • Short treatment times (six months to two years under optimal conditions).	• The required area is high; • Dust and vapors arising from the land may cause some air quality problems.	Hejazi, 2002.
Bioslurping • Combines elements of bioventing and vacuum-enhanced pumping to recover free product from the groundwater and soil while promoting the aerobic bioremediation of hydrocarbon contaminants	• Applied at sites with shallow groundwater and with the groundwater below 30 m of soil surface. • Bioslurping recovers free product, thus speeding remediation	• Bioslurping decreases in low permeability soils. • Too much soil moisture reduces the air permeability of soil and decreases its oxygen transfer capability • Too little moisture inhibits microbial activity. • Low temperatures slow remediation	Yen et al., 2003.

Non Biological (Thermal) remediation strategies

Table 3: List of non-biological thermal techniques used in remediation of contaminants with their advantages and disadvantages

Remediation Techniques	Types	Advantages	Disadvantages	References
Thermal treatment (In Situ) • Five technologies are grouped under the in situ thermal treatment classification. With the exception of vitrification, all of these treatment technologies rely on the addition of heat to the soil to increase the removal efficiency of volatile and semivolatile contaminants.	Electrical resistance heating (ERH) • Electrical current is passed through moisture in the soil between an array of electrodes. As the current flows through the moisture in soil pores, the resistance of the soil produces heat.	• Contaminant toxicity, as well as volume reduction is addressed by this technology • Widely used and available commercially	• Metals are not destroyed and end up in the flue gases or in the ashes. • Certain types of soils such as clay soils or soils containing rocks may need screening.	Beyke and Fleming, 2002
	Steam injection and extraction / steam enhanced extraction [SEE]) • Steam is injected into injection wells and the recovery of mobilized groundwater, contaminants, and vapour is done.			Schmidt et al. 2002; Kaslusky and Udell 2002
	Conductive heating • Either an array of vertical heater/vacuum wells or, when the treatment area is within about six inches of the ground surface, surface heater blankets are used.			Baker and Heron, 2004
	Radio-frequency heating (RFH) • Uses a high frequency alternating electric field for in situ heating of soils.			Halliburton, 1995
	Thermal Desorption • An innovative treatment technology where contaminated soil is excavated, screened, and heated to release petroleum from the soil			Wait and Thomas, 2003
	In situ vitrification (ISV) • A thermal treatment process that converts contaminated soil to stable glass and crystalline solids.			Dermatas and Meng, 2003; U.S. EPA 1995

Table 3(Cont.): List of non-biological thermal techniques used in remediation of contaminants with their advantages and disadvantages

Remediation Techniques	Types	Advantages	Disadvantages	References
Thermal treatment (Ex Situ) • Contaminants are destructed through exposure to high temperature in treatment cells and combustion chambers used to contain the contaminated media during the remediation process	Hot gas decontamination The temperature of the contaminated area is raised to 260ºC for a specified period of time. The gas effluent is then treated in an after burner system to destroy all volatilized contaminants	• Eliminates a waste that currently is stockpiled and requires disposal as a hazardous material • Permit reuse or disposal of scrap as nonhazardous material • Used to remediate soils contaminated with explosives and hazardous wastes • OB/OD operations can destroy many types of explosives, pyrotechnics, and propellants • The target contaminant groups for pyrolysis are SVOCs and pesticides. • Has shown promise in treating organic contaminants in soils and oily sludges.	• Costs are higher than open burning. • Possible explosions from improperly demilitarized mines or shells • Slow rate of decontamination • Specific feed size and materials required • Heavy metals can produce a bottom ash that requires stabilization. • Minimum distance requirements for safety purposes mean substantial space is required. • Emissions are difficult to capture for treatment • The technology requires drying of the soil • Highly abrasive feed can potentially damage the processor unit. • High moisture content increases treatment costs	Hyman and Dupont, 2001
	Incineration Aerobic and high temperature treatments (8700C to 1200ºC) are used to volatilize and combust halogenated and other refractory organics in hazardous wastes.			Soesilo and Wilson 1997.
	Open Burn (OB) and Open Detonation (OD) • Explosives or munitions are destroyed by self sustained combustion, which is ignited by an external source, such as flame, heat, or a detonation wave (that does not result in a detonation).			Teer et al., 1993
	Pyrolysis • Anaerobic chemical decomposition induced in organic materials by heat.			Soesilo and Wilson, 1997

Non Thermal Strategies

Table 4: List of non-biological non thermal techniques used in remediation of contaminants with their advantages and disadvantages

Remediation Techniques	Advantages	Disadvantages	References
Soil Vapor Extraction (Soil Venting) • Vertical and/or horizontal wells are installed in the area of soil contamination and vacuums are applied through the wells to evaporate the volatile constituents which are subsequently withdrawn through an extraction well.	• Proven performance, readily available equipment, easy to install • Short treatment times (6-48 months).	• Effectiveness decreases when applied to sites with low permeability • Only treats the unsaturated zone	Zhan and Park, 2002; Halmemies et al., 2003.
Soil Washing • Uses liquids (usually water, occasionally combined with solvents) and mechanical processes to scrub soils.	• Reduces the volume of contaminant, therefore, further treatment or disposal is less problematic • Commercially available	• Contaminant toxicity is unchanged, although volume is reduced • Less effective when soil contains a high percentage of silt and clay • Costs associated with the disposal of the subsequent waste generated.	Feng et al., 2001; Chu and Chan, 2003; Urum et al., 2003.
Soil Flushing • An innovative remediation technology that 'floods' contaminated soils with a solution that moves the contaminants to an area where they can be removed	• Applies to all types of soil contaminants and is generally used in conjunction with other remediation technologies • Reduces the need for excavation, handling, or transportation of hazardous substances.	• Low permeability or heterogeneous soils are difficult to treat • Remediation times are usually lengthy • This technology requires hydraulic control to avoid the movement of contaminants off-site	Otterpohl, 2002; Logsdon et al., 2002; Di Palma et al., 2003.
Encapsulation • Contaminant source is covered with low permeability layers of synthetic textiles or clay caps designed to prevent leaching and migration of contaminants away from the site and into the groundwater	• Comprise of the physical isolation and containment of the contaminated material.	• Highly dependent on the lithology of the site. • Efficiency of encapsulation decreases with time and cannot be considered a permanent remedy. • Implemented only with shallow contaminated soils.	Anderson and Mitchell, 2003; Robertson et al., 2003.

Table 4(Cont.): List of non-biological non thermal techniques used in remediation of contaminants with their advantages and disadvantages

Remediation Techniques	Advantages	Disadvantages	References
Stable Isotope Probing • Identifies active microorganisms without the prerequisite of cultivation which has been widely applied in the study of pollutant degrading microorganisms.	• Provides the potential to establish the identity of microorganisms involved in biodegradation in situ	• Weaknesses of molecular methods (nucleic acid recovery, PCR bias, etc.) and incubation time may result in cross-feeding if too long or insufficient labelling if too short.	Boschker et al. 1998 Morris et al., 2002
Photo catalytic Degradation • It is the alteration of contaminant by light. Typically, the term refers to the combined action of sunlight and air.	• Complete Mineralization • No waste disposal problem • Low cost	• Limited to surface contaminants	Burrows et al., 2002; Boreen et al., 2003; Walter Simmler, 2011
Ultraviolet Oxidation • Uses an oxygen-based oxidant (e.g. ozone or hydrogen peroxide) in conjunction with UV light.	• Chemicals used do not add to the system's pollutant load.	• Low turbidity and suspended solids are necessary for good light transmission. • Free radical scavengers may interfere with the reactions.	Brillas et al., 2003; Liang et al., 2003.
Precipitation/ Flocculation • Non-directed physico-chemical complex cation reaction between dissolved contaminants and charged cellular components (dead biomass)	• Cost-effective	• Yet to be exploited commercially	Natrajan, KA, 2008
Microfiltration • Separates dispersed oil phase from water using porous membranes.	• Removes dissolved solids rapidly	• Yet to be exploited commercially	T. Darvishzadeh et al., 2013
Analytical Biosensors • Relies on analysis of gene expression typically by creating transcriptional fusions between a promoter of interest and the reporter gene expression serves as a measure of the availability of specific pollutants in complex environments	• Used for nutrient monitoring • Used for degradation metoblites monitoring	• Bio elements and chemicals used in the biosensors need to be prevented from leaking out of the biosensor over time (serious issue for non-disposable ones	Liu et al., 2004; Nouri et al., 2009; 2011

2.8 Factors affecting biodegradation rates of petroleum hydrocarbons

A number of limiting factors affect the process of bioremediation and have to be controlled and optimized. The nature and physico-chemical properties of the petroleum hydrocarbon is of foremost consideration followed by availability of the contaminant to the microorganism, cometabolism, environmental factors such as soil type, temperature, pH, nutrients and oxygen (Chikere et al, 2011; Yuodono et al., 2011; George-Okafor et al., 2009). Lighter hydrocarbons (the aliphatics), due to their relatively low molecular weight and structural complexity, have higher degradation rates than the aromatic hydrocarbons such as BTEX and PAH compounds.

As molecular weight increases, water solubility decreases thus making hydrocarbons less biodegradable. Other factors such as the molecular structure & spatial arrangement, chemical reactivity, non-polarity, increasing number of aromatics rings, among others also contribute to the low degradability of PAHs (Johnson et al., 1994). Borden et al (1997) studied the biodegradation of BTEX compounds in a contaminated site (under anaerobic conditions).

Most of the studies have emphasized on anaerobic degradation of BTEX compounds but but aerobic pathways are more prominent as the oxygen leads to the activation of the enzymes oxygenases.

2.9 Mechanisms of petroleum hydrocarbons degradation by microbes

Biotransformation of BTEX and PAHs proceeds either by aerobic or anaerobic Mechanisms. Aerobic transformation by BTEX and PAHs is achieved by ring oxidation mediated by enzymes (e.g. oxygenase) followed by ring cleavage (Johnsen et al., 2005). The enzymes use molecular oxygen to oxidise these substrates leading to ring hydroxylation. Fungi tend to biodegrade most aromatic hydrocarbons at faster rates than bacteria (Johnsen et al., 2005). They form intermediate oxidised products that can be

further oxidised by bacteria (Levina et al., 2003). However, fungi possess low competitive capabilities (especially when introduced into a natural ecosystem) and have longer acclimation times than bacteria (Gao et al., 2010). Petroleum hydrocarbon-degrading fungi species are also mainly limited to oxic and soil environments.

2.10 **Degradation pathways of mono and polyaromatics**

2.10.1 **BTEX Degradation**

The BTEX chemicals (Benzene, Toluene, Ethylbenzene, and Xylenes) are volatile monoaromatic hydrocarbons which are commonly found together in crude petroleum and petroleum products such as gasoline. They are also produced on the scale of megatons per year as bulk chemicals for industrial use as solvents and starting materials for the manufacture of pesticides, plastics, and synthetic fibers (Harwood et al.,1997).

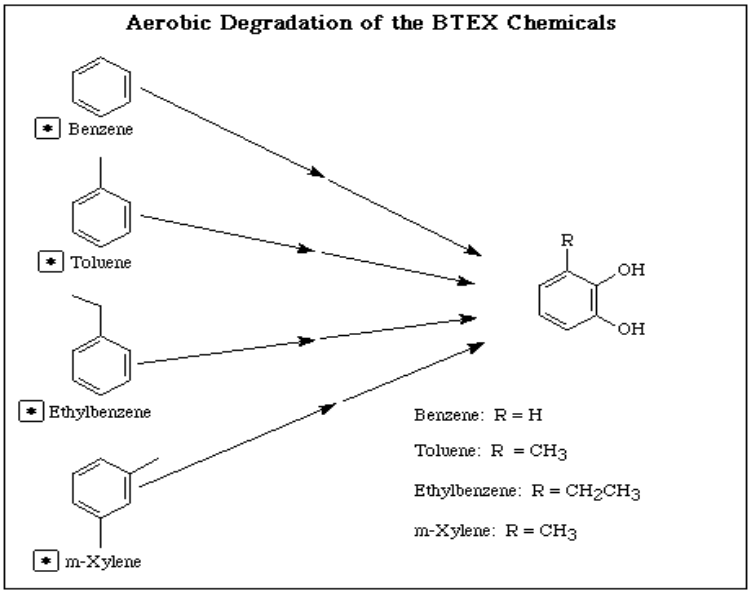

Fig 5. Aerobic and anaerobic pathways of BTEX degradation

Anaerobic pathways of BTEX biodegradation are important because these compounds are frequently found under conditions where the use of oxygen quickly exceeds the supply. These conditions are often found in such places as the sediments of all natural bodies of water, groundwater, and sometimes soil (Heider et al., 1997).

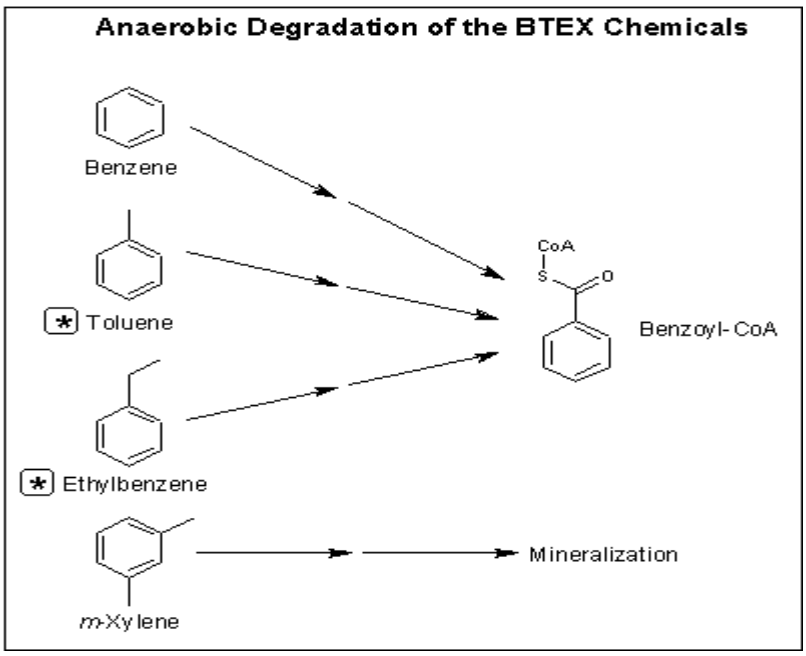

Fig 6. Anaerobic pathways of BTEX degradation

2.10.2 Naphthalene Degradation

Naphthalene is a simplest Polycyclic Aromatic Hydrocarbon (PAH) containing two fused benzene rings. It also serves as a model for understanding the properties of a large class of environmentally prevalent polycylic aromatic hydrocarbons (PAHs). It is commonly found in crude oil and oil products.

Common naphthalene-degrading bacteria include Pseudomonas spp, Vibrio spp, *Mycobacterium* spp, *Marinobacter* spp, *and Sphingomonas* spp. *Micrococcus* spp [Karl et. al]

Several *Pseudomonas* species can degrade naphthalene starting wtih 1,2 dioxygenation, shown here in the middle branch (Eaton et al., 1992). Naphthalene dioxygenase, the enzyme which initiates this route of bacterial naphthalene metabolism, is used in a biotechnological process to synthesize the blue jean dye indigo.

Bacillus thermoleovorans Hamburg 2 initiates naphthalene degradation with dioxygenation at the 2,3 position (Annweiler et al., 2000). The pathway also includes formation of

naphthalene as part of the degradation of 1,2-dihydronaphthalene. This is a minor part of 1,2-dihydronaphthalene degradation; the major route is dioxygenation to 1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene (Torok et al., 1995).

2.10.3 Acenapthene Degradation

Acenaphthene (Acenapthalene) a polyaromatic hydrocarbons is a constituent of coal tar and tobacco smoke. Its degradation serves as a model for bridged PAH metabolism.

Acenaphthalene is degraded through acenaphthenequinone, though the inital metabolites are different. *Beijerinchia* sp., *Pseudomonas* sp. BR, BC and A2279, and *Sphingomonas* sp. A4 can transform acenaphthalene to 1-acenaphthalenol. This compound is either hydroxylated to form 1,2-dihydro-1,2-acenaphthylenediol, or the hydroxyl group is oxidized to form 1-ketoacenaphthene. Both of these compounds are oxidized to 1-hydroxy-2-ketoacenaphthene and from there to acenaphthenequinone (Schocken & Gibson, 1984, Selifonov et al., 1998, and Kouzuma et al., 2006). Further acenapthequinone is oxidised to 1-Napthoic acid which then proceeds through naphthalene pathway for further oxidation.

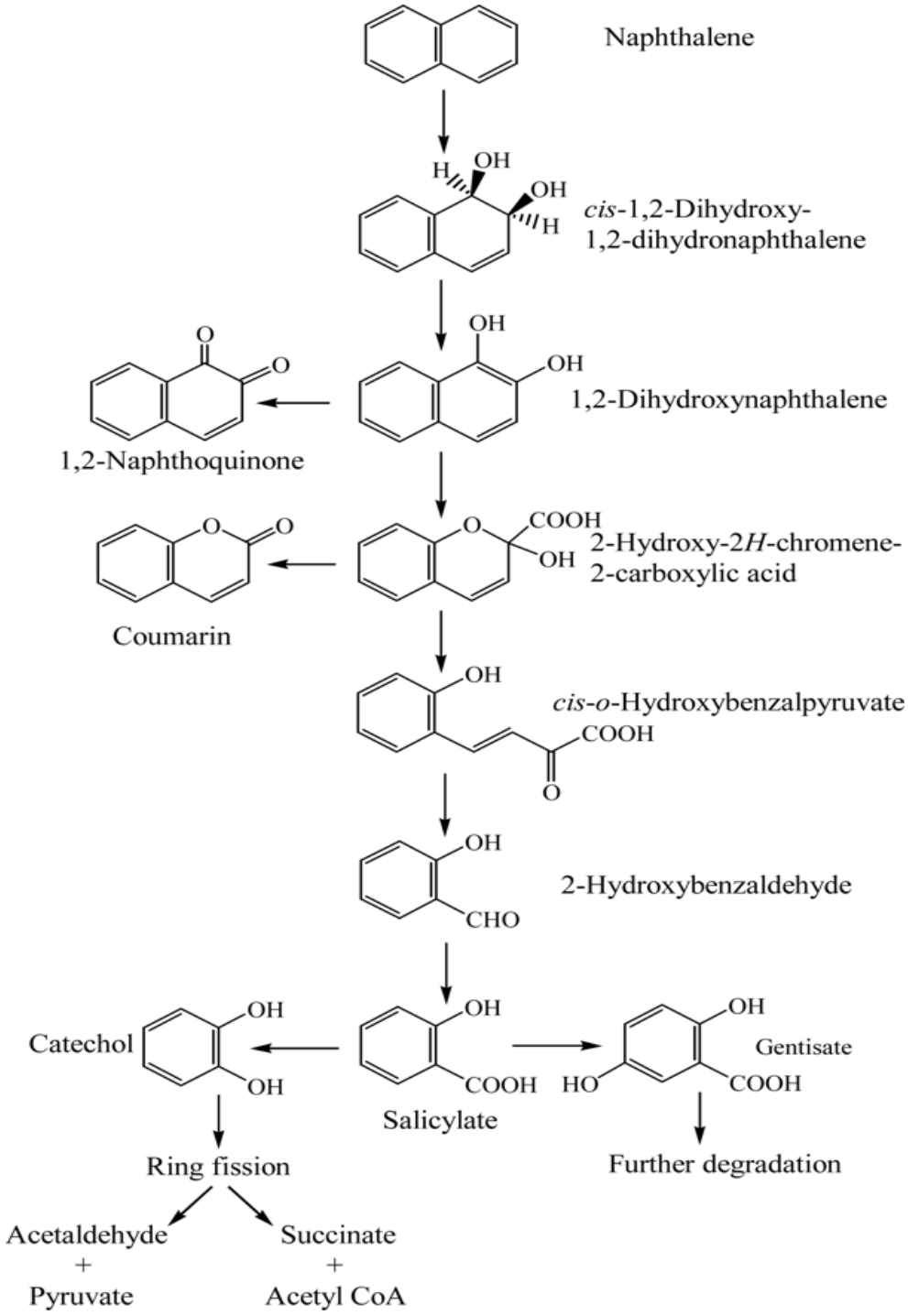

Fig 7. Napthalene degradation pathway

2.11 Bacterial strains involved in hydrocarbon degradation

Nicholson and Fathepure (2004, 2005) have reported the degradation of BTEX from oil contaminated soil samples in Oklahoma. Hassan et al. (2012) have reported the isolation of *Alcanivorax* sp. HA03 from soda lakes in Wadi E1Natrun capable of degrading benzene, toluene, and chlorobenzene as the sole sources of carbon. A multitude of catabolic pathways for the degradation of aromatic compounds have been elucidated; for example, toluene is degraded by bacteria along five different pathways. On the pathway encoded by the TOL plasmid, toluene is successively degraded to benzyl alcohol, benzaldehyde and benzoate, which is further transformed to the TCA cycle intermediates (Haryama, 1999). The first step of toluene degradation with *P. putida* F1 is the introduction of two hydroxyl groups to toluene, forming *cis*-toluene dihydrodiol. With *Pseudomonas mendocina* KR1, toluene is converted by toluene 4-monooxygenase to *p*-cresol, this being followed by *p*-hydroxybenzoate formation through oxidation of the methyl side chain (Haryama, 1999; Suyama, 1996). With *Pseudomonas pickettii* PKO1, toluene is oxidized by toluene 3-monooxygenase to *m*-cresol, which is further oxidized to 3-methylcatechol by another monooxygenase (Haryama, 1999; Suyama, 1996). With *Bukholderia cepacia* G4, toluene is metabolized to *o*-cresol by toluene 2-monooxygenase, this intermediate being transformed by another monooxygenase to 3-methylcatechol (Haryama, 1999).

Archae bacteria have also been known in degradation of benzene. For example, the crude oil degrading *Haloferax*, *Halobacterium*, and *Halococcus* isolated from a hypersaline Arabian Gulf coast degraded benzene as the sole source of carbon (AlMaleim, 2010). The strain D15-8W degrades naphthalene, phenanthrene or anthracene as the sole source of carbon. Bonfa et al. (Bonfa 2011) have isolated several strains of *Haloferax* that degrade a mixture of the PAHs including naphthalene, anthracene, phenanthrene, pyrene and benzo[a] anthracene.

Table 5: Bacterial species characterized for benzene, toluene, naphthalene and acenapthene degradation

Hydrocarbon	Degrader	References
Benzene	Enrichment, Oilfield Oklahoma Enrichment, Great Salt Plains, Oklahoma *Planococcus* sp. strainZD22 *Arhodomonas* sp. Strain Seminole Enrichment, Rozel Point, Great Salt Lake, Utah *Arhodomonas* sp. strain Rozel *Marinobacter vinifirmus, M. hydrocarbonoclasticus* *Haloferax* sp.*Halobacterium* sp. *Halococcus* sp. *Alcanivorax* sp.HA03 *Marinobacter sedimentalis* *Marinobacter falvimaris*	Nicholson and Fathepure Nicholson and Fathepure Li et al. Nicholson and Fathepure , Dalvi et al. Sei and Fathepure Azetsu et al. , Dalvi et al. Berlendis et al. Al-Mailem et al. Hassan et al. Al-Mailem et al.
Toluene	Enrichment, oilfield soil, Oklahoma Enrichment, Great Salt Plains, Oklahoma *Planococcus* sp. strainZD22 *Arhodomonas* sp. Strain Seminole Enrichment, Rozel Point, Great Salt Plains, Utah *Arhodomonas* sp. Strain Rozel *Marinobacter vinifirmus, M.hydrocarbonoclasticus* *Haloferax* sp. *Halobacterium* sp. *Halococcus* sp. *Alcanivorax* sp.HA03 3–15	Nicholson and Fathepure Nicholson and Fathepure Li et al. Nicholson and Fathepure , Dalvi et al. Sei and Fathepure Azetsu et al. , Dalvi et al. Berlendis et al. Al-Mailem et al. Hassan et al.
Napthalene	*Micrococcus* sp. *Pseudomonas* sp. *Alcaligenes* sp. *Pseudomonas* sp. *Rhodococcus* sp. *Arthrobacter* sp. *Bacillus* sp. *Bacillus* sp strain DHT *Haloferax* sp. *Halobacterium* sp. *Halococcus* sp. *Haloferax* spp. *Arthrobacter* spp. SN17 Mixed culture (Qphe-SubIV) *Marinobacter sedimentalis Marinobacter falvimaris* *Marinobacter nanhaiticus* *Halobacterium piscisalsi, Halorubrum ezzemoulense, Halobacterium salinarium, Haloarcula hispanica Haloferax* sp. *Halorubrum* sp. *Haloarcula* sp.	Ashok et al. Plotnikova et al. Kumar et al. Al-Mailem et al. Bonfá et al. Plotnikova et al. Dastgheib et al. Al-Mailem et al. Gao et al. Erdogmus et al.
Acenapthene	EH4 (*Haloarcula vallismortis*) *Bacillus* sp. PD5 *Halomonas* sp.PD4;	Bertrand et al. Sikdar et al.

2.12 Detection of Microbial Growth by Resazurin and Tetrazolium

Growth on various PHCs as sole sources of carbon and energy cannot easily be determined with standard growth assays because of the low aqueous solubility and bioavailability of the PHCs, which lead to slow bacterial growth and low cell yields. Degradation tests have been done in three ways: (i) by mineralization of radioactive tracers (Willumsen 1997), (ii) by formation of clearing zones around colonies growing on agar plates covered with an opaque layer of PAH crystals (Kastner 1994; Kiyohara 1982), and (iii) by detection of accumulated colored PAH metabolites (Steiber, 1994; Wrenn, 1996).

This thesis consists of the miniaturized and sensitive microtiter plate method for detection of bacterial growth on PHCs. Resazurin, which is also referred to as “Alamar blue” is a non toxic dye reduced intracellularly to resofurin by enzymes in the electron transport system (Byth et. al. 2001). Initially a blue coloured resazurin is converted to a pink colored resorufin by the irreversible loss of one oxygen atom. The pink colored resorufin is further reduced to colorless hydroresorufin in the second stage of reduction. This reaction is reversible by atmospheric oxygen (Mariscal et al. 2009) and does not apply to rapid toxicity evaluation studies. Resazurin is often used to measure the growth of bacteria (O’Brien et al. 2000), indicator of bacterial cell numbers (Shiloh et al. 1997), for estimating a range of contaminant degrading bacteria (Guerin et al. 2001), for food safety measurements (Smith and Townsend 1999) and in microbial viability studies Srinivasan et al. 2003).

The reduction of tetrazolium salts to coloured formazans is commonly used as an indicator of cell metabolism during microbiology studies, although the reduction mechanisms have never clearly been established in bacteria. Tetrazolium salts are used in two ways in the context of microbiological studies, either in nutritive media where cells are growing or in non-nutritive media where cells do not grow and are thus in a resting state. The first type includes MTT, INT, CTC, TTC and TV, which form water insoluble formazans. These salts are positively charged and considered as cell permeable (Berridge et al., 2005). Their positive charge is thought to facilitate cellular uptake due to the membrane potential. They are generally used as indicators of cell redox activity in order to test resistance to solvents (Hayashi et al., 2003), drug susceptibility (Raut et al., 2008) or specific substrate utilization (Bochner & Savageau, 1977; Lin et al., 2008). They also enable the discrimination of viable cells in populations of respiring bacteria (Rodriguez et al., 1992) and anaerobic bacteria (Bhupathiraju et al., 1999; Smith & McFeters, 1997).

Tetrazolium salts of the second type, which include XTT, MTS, WST-1 and WST-5, form water-soluble formazans. They are negatively charged and largely cell impermeable. They are generally used in conjunction with intermediate electron acceptors (quinones or their derivatives) that facilitate reduction reactions. These salts have been used in microplate

assays to measure bacterial proliferation (Tsukatani et al., 2008) and in colorimetric assays to test antimicrobial susceptibility (Tsukatani et al., 2008; Tunney et al., 2004).

2.13 Heavy metal and antibiotic tolerance in bacteria

Heavy metal contamination is widespread throughout the world. Heavy metals are defined as a group of metals whose atomic density is greater than $5g/cm^3$. Naturally there are about 50 heavy metals of special concern because of their toxicological effect to human beings and other living organisms. Some of them like Zn, Cu, Co, Ni, Mn, and Fe are essential for bacterial survival in trace conditions but when in excess concentrations, the toxic effects of these dual functional ions are revealed such as membrane and DNA damage along with loss of enzyme activity. Heavy metals cannot be degraded or destroyed because they are stable and so persistent environmental contaminants [Gochfeld, 2003]. The sources of heavy metals include metal smelting, metalliferous mining, power plants, corrosion of metals in use, application of pesticides containing metal and petroleum exploration. By affecting the growth, morphology and biochemical activities, heavy metals influence the microbial population resulting in decreased biomass as well as diversity. Therefore microbes have evolved new mechanisms to tolerate the metals either by presence of heavy metals through efflux, complexation, or reduction of metal ions or to use them as terminal electron acceptors in anaerobic respiration [Gadd, 1999]. Most mechanism reported involves the efflux of metal ions outside the cell, and genes for tolerance mechanisms have been found on both chromosomes and plasmids. Bacteria that are resistant to and grow on metals play an important role in the biogeochemical cycling of those metal ions.

Many studies have examined the heavy metal sensitivity or resistance of bacteria isolated from different habitats and its mechanisms to adapt the toxic metal during expossure (Rajbanshi, 2008; Thippeswamy et. al., 2012).

In contaminated environment the concentrations and availability of metals as well biological factors such as the type of metal, nature of medium and microbes leads to succesfull remediation (Nwuche and Ugoji, 2008; Soboley and Begonia, 2008).

Furthermore, the frequency of appearance of resistant bacteria to specific heavy metals may be correlated with increasing loads of metals in the environment. As a result heavy metal resistant bacteria may be used as bioindicators of environmental contamination (Li et. al., 2010).

Bacteria that demonstrate heavy metal tolerance have been isolated from different sources (Basu et. al., 1997; Castro et. al., 2003; Choudhary et. al., 1998; Duscbury, 1986; Haefeli et. al., 1984; Lima et. al., 2007 and Otth et. al., 2005). Concerning heavy metals, terms such as “resistance” and “tolerance” are arbitrary and they are often used as synonymous in literature. Gadd (1992) suggested using the term "resistance" when it is possible to characterize a specific mechanism of bacterial detoxification for a metal. Therefore, the term tolerance seems more appropriate to refer to the ability of a bacterial strain to grow in the presence of high concentrations of a metal. An increasing problem for the treatment of different infectious diseases is bacterial resistance to antibiotics and other antimicrobial agents. Many studies have proved the transfer of antibiotic resistance in bacteria as a phenomenon of selection pressure.

The mechanism of heavy metal tolerance go along with antibiotic resistance of microorganisms [Davidson, 1999]. Strong corelations between these two patterns have been strongly correlated[Bell et. al]. The number of antimicrobial resistant (AMR) bacteria in the environment increases exponentially with the use of antimicrobials, as a result of increasing selective pressure on bacterial populations [Nel et. al., 2004; Samalla et. al., 2000 and Tsiodras et. al., 2008]. Further its spread between different bacterial strains in different habitats has also been demonstrated [Turnidge, 2004 and SVARM, 2006]. The resistant to antibiotics and heavy metal are found to be plasmid mediated. The genetic determinant responsible for the heavy metal resistance often resides on plasmids which mediate antibiotic resistance. The resistance development may be due to nonspecific mechanism with gene regulation of plasmids and chromosomes, which may be heritable or transferable due to the presence of a resistance (R-factor) factor [Silver and Walderhang, 1992].

CHAPTER 3

Materials and Methods

3.1 Site description and soil sampling

Soil samples were collected from ten different areas in Chhattisgarh (Table 6). The selected areas were from Raipur (21.2514^{0}N, 81.6296^{0}E), Bhilai (21.1938^{0}N, 81.3509^{0}E), Bilaspur 22.076^{0}N, 82.139^{0}E, Korba (22.3595^{0}N, 82.7501^{0}E) and Raigarh (21.8974^{0}N, 83.3950^{0}E).

Table 6: Soil sampling labels and physicochemical characteristics

Korba Thermal Power Plant	KTPP
Raigarh Steel Plant	RSP
Bhilai Steel Plant	BSP
Bhilai Steel Plant Oil Collection centre	BSPOCC
Coal Field Korba	CFK
Aluminium Plant Raigarh	APR
Sponge Iron Plant Urla	SIPU
Bilaspur Railway Oil Depo	BROD
HP Refilling Raipur	HPRR
Indian Oil Refilling Raipur	IORR

Petroleum hydrocarbon contaminations (mainly diesel-oil and fuel oil) in all the cases resulted from spillages during distribution of the products from storage tanks. Samples were collected during the month of June. The top 15 cm of soil was collected using sterile spatula into sterile plastic bags for further transportation and microbiological analysis. Samples were stored at 4^{0}C until further processing.

3.2 Physico Chemical Properties of soil samples

Soil pH of samples was determined by following the SR ISO 10390-1999 standard (Muntean and Rusu, 2011). Moisture content of soils (expressed in %) was determined according to Damian et al. (2008); available potassium and organic carbon content was analyzed by Soil testing Kit (HiMedia) while the amount of phosphate and nitrate content was analyzed according to (Hooda and Kaur, 1999).

3.3 Soil Temperature

Soil temperature were taken at the sample sites with a thermometer inserted at 7 cm depth and left in the same position until the temperature became stable. (Ramakrishna 2006)

3.4 Soil pH

A 10 % w/v suspension of air dried soil sediments was prepared in double distilled water. It was then allowed to settle for 1 hr and filtered through Whatmann filter paper no. 42. The pH of all the soil filtrates was checked using a pH meter.

3.5 Soil Moisture content

10 g of soil was weighed (W1) and dried at 105^0C in hot air oven for 24 hrs. The final weight (W2) of samples was determined and the moisture content (W1-W2/W1*100) was estimated percentage moisture.

3.6 Inorganic phosphate content

A 0.5% (w/v) suspension of air-dried soil was prepared in 100 ml of 0.002 N sulphuric acid. The suspension was filtered and the soil extract was processed in following manner. To the 5 ml of soil extract, 0.2 ml ammonium molybdate solution and 1 drop of stannous chloride were added. After 10 min, A_{690} was observed to determine the intensity of colour developed on UV visible Spectrophotometer against double distilled water as blank. Inorganic phosphate contents in the samples were determined using a standard curve of KH_2PO_4 at 690 nm

3.7 Nitrate Content

A 10% (w/v) soil suspension in double distilled water was filtered and treated with 0.4 ml of aluminium hydroxide. The suspension was then swirled and allowed to settle for 5 min and filtered. Afterwards, 0.1 ml of 1N HCl was added to 5 ml clear filtrate and absorbance was measured at 220 nm. The nitrate contents were determined using standard nitrate calibration curve prepared by using various concentration anhydrous potassium nitrate (KNO_3).

3.8 Potassium Content

Procedure:

1) A clean test tube was taken.
2) 10ml of water was poured in the test tube.
3) One small spoonful of reagent K/R1 and 5-6 drops of reagent K/R2 was added.
4) 5 g soil was mixed in the test tube. Closed the test tube with the cork.
5) It was shaken well for 1 minute. Mixture was filtered with the filter paper.
6) Another test tube was taken.
7) 2ml filtered solution was put in the test tube.
8) 1 spoonful (0.3g) of reagent K/R3 was added.
9) 2ml of reagent K/R4 was added.
10) Wait for 1 minute. Shake it well.
11) Wait for another 3 minute.
12) Yellow muddy color was developed. During the experiment, the temperature should be maintained below 20°C, ice bath was arranged at that time of experiment.
13) Took the color chart no.4. Put the test tube in front of line chart. Tried to see line chart through the solution of the test tube:

- If you do not see any line of the chart no.4 then the available Potassium of the soil is very high.
- If you see the deep line color chart only and not the other two, then Potassium is high.

- If the first deep line & 2nd middle line are visible and the other two, then Potassium is high.
- If the all the three lines are clearly visible then the Potassium is very low.

3.9 Oxidizable organic carbon

Procedure:

1) Clean Test tube was taken and 2.0 g soil added.
2) 1 small spoon full (0.5g) of reagent OC/R1 was added and mixed.
3) 10ml of water and one small spoonful of reagent OC/R2 was added and mixed.
4) 2.0 ml of reagent OC/R3 was added with dropper. The time of addition of reagent OC/R3 was noted.
5) The content of test tube was shaken intermittently for 5 minutes.
6) The test tube was kept in standing condition for net 5 minutes, total test period was 10 min. counted from the time of addition of Reagent OC/R3.
7) After 10 min. sharp, the test strip was dipped into the upper supernatant liquid portion and it was taken out immediately and the colour of test strip was matched with the colour chart provided.
8) Nearest colour or same colour of test strip colour and the colour of chart no. 2 would be the oxidizable organic carbon of test soil.

3.10 Estimation of total heterotrophic and hydrocarbon degraders by MPN (ELISA Method)

Each soil sample (100 g) was dissolved in 250 ml of deionised water and kept to stand for 30 mins after vigorous shaking in sterile conditions. The sediments were collected and air dried for all the future experiments. Aerobic heterotrophic and hydrocarbon degrading bacterial counts were estimated using the most probable number (MPN) method using 96 well microtitre plates using two different media. For aerobic heterotrophic count Bacto Bushnell Haas broth + Glucose was used and for hydrocarbon degrading count BBH broth + Tetrazolium Violet (2,5-diphenyl -3- α napthyl tetrazolium chloride) was used as media. Tetrazolium is reduced to a dark purple colored derivative formazan on microbial respiration.

The base medium for the assay was prepared from two solutions. Solution A contains a BBH medium (Bacto Bushnell Haas Broth) modified to contain 0.005 g/L $FeCl_3$ and filtered through a 0.22 μm filter (HiMedia). Solution B contains 0.025 g tetrazolium violet (HiMedia) dissolved in 100 mL of water. The 100 mL of solution B is then added to 400 mL of solution A, and the resulting solution is sterilized by autoclaving. Napthalene and acenapthene were prepared in dichloromethane. Plates are prepared in the same manner for either fuel-degraders or heterotrophic microorganisms. For hydrocarbon degraders, each well was poured with 7 μl (filter-sterilized through a 0.22 μm) hydrocarbon solution and the solvent was evaporated (for napthalene and acenapthene) in sterile conditions. Cooled, autoclaved medium (100 μL) was then added in each well of a 96-well microtiter plate. Wells containing only solvent was used as control and uninoculated well used as blank. Prepared plates were stored at 4°C in a humidified chamber up to 1 week prior to inoculation. For the heterotrophic medium, the plates were incubated for 1 to 2 days at room temperature prior to use to ensure sterility.

For the dilution series, 24-well culture plates are used to prepare the inoculum for the 96-well MPN plates (Figure 8). With the 24-well plate oriented so that there are 4 rows and 6 columns, each well except the first, third, and fifth in row 1 is filled with 2.25 mL of Ringer solution (Collins et al., 1989). For soil sediment, the sample (10 g wet weight) is first diluted in 90 mL of Ringer solution in a bottle. To remove microorganisms loosely associated with particles, the bottles are shaken by hand for 1 minute before further dilutions are made. Three samples can be diluted in each 24-well plate. Dilutions were done from 10^{-1} till 10^{-7} .

To dilute the first sample, 2.5 mL of soil slurry was pipetted into the first empty well in row 1. Using a pipetter, 0.25 mL was then transferred from the first well in row 1 to the first well in row 2, and so on, discarding the tips between transfers. Serial dilutions were performed using columns 1 and 2 for the first sample. A second sample can then be diluted in a similar manner using columns 3 and 4 and a third sample using columns 5 and 6. Once a dilution series is completed for a sample these aliquots are then dispensed into the previously prepared 96-well plate, oriented so that there are 8 rows and 12 columns.

Samples from the highest dilutions are transferred first so the same pipette tips can be used for the lower dilutions. This procedure is repeated two more times, so that a 6-tube MPN series from each dilution factor is created. The plates were stored in tightly sealed plastic bags during incubation to prevent carbon loss and drying. The prepared plates are incubated without agitation at room temperature for 10 to 14 days or until microbial growth is maximal. At the end of this period, each plate is scored visually by purple color development (indicating reduction of the tetrazolium dye via respiration) for fuel degraders or by turbidity for heterotrophs.

Fig 8: ELISA plates kept in incubator

3.11 FTIR analysis of soil samples

3.11.1 Sample Preparation

The infrared spectra were recorded on Thermo Scientific USA. The spectra were scanned in the 400–4000 cm^{-1} range. The spectra were obtained using potassium bromide (KBr) pellet technique. Potassium bromide (AR grade) was dried under vacuum at 100 °C for 48 h and 100 mg of KBr with 1 mg of sample was taken to prepare KBr pellet. The spectra were plotted as intensity versus wavenumber.

3.12 Enrichment, purification and culturing of hydrocarbon degrading bacteria

Diesel-oil degrading bacteria were isolated using enrichment containing: 40 ml BBH mineral broth medium supplemented with 1 ml diesel and 4.0 g of contaminated soil sediment. After 2 weeks of incubation at 32^0C, the enriched cultures were serially diluted and inoculated onto BBH agar plates. The lid of Petri-dishes contained 250 ml of sterile diesel-oil as sole source of carbon and energy. Colonies with different morphologies were selected as candidate petroleum hydrocarbon degrading strains and were maintained on standard Nutrient Agar (HiMedia).

3.13 Selection of most potent hydrocarbon degraders

3.13.1 Qualitative analysis (Dye method)

Out of 25 different looking colonies, 10 isolates were used for checking the degradation of benzene, toluene, naphthalene and acenapthene. The setup was prepared in 250 ml sterilized conical flasks.

3.13.1.1 For Napthalene and acenapthene

Solid hydrocarbons naphthalene and acenapthene (10 g/l) were dissolved in dichloromethane and filtered sterilized through 0.22 μm membrane. 2 ml of naphthalene and acenapthene solution at a final concentration of 100 ppm were added in different conical flasks and left open in sterilized conditions so that the solvent gets evaporated. After that 100 ml of BBH media with trace elements and 100 μl of resazurin was added in the flasks. Finally 500 μl of culture (OD_{600}=0.5) previously grown in BBH broth was added in the flasks and kept in shaking incubator at 120 rpm and 30^0 C. Flask with solvent, media and resazurin was used as control 1 and flask with inoculant and media was used as control 2.

Resazurin, which is also referred to as “Alamar blue” is a non toxic dye reduced intracellularly to resofurin by enzymes in the electron transport system. Initially a blue coloured resazurin is converted to a pink colored resofurin by the loss of one oxygen atom. The pink colored resofurin is further reduced to colorless hydroresofurin in the second stage of reduction.

3.13.1.2 For Benzene and Toluene

Filtered liquid hydrocarbons (0.1%) benzene and toluene were used while maintaining the same procedure mentioned above.

3.14 Optimization of temperature, pH and initial concentration of benzene, toluene, napthalene and acenapthene on selected bacterial species

3.14.1 Optimization of temperature

Inoculated the flasks containing BBH media supplemented with 200 ppm of hydrocarbons with bacterial cultures and incubated them in different temperatures (15^0C, 25^0C, 30^0C, 35^0C and 45^0C) for 48 hrs in a rotary shaker at 150 rpm. Examined the cultures after two days of incubation for the presence or absence of growth by turbidity method using a spectrophotometer at wavelength 600 nm.

3.14.2 Optimization of pH

Inoculated the flasks containing BBH media supplemented with 200 ppm of hydrocarbons with bacterial cultures and incubated them in different pH such as 6.0, 7.0, 8.0, 9.0 and 10.0 using either HCl or NaOH at 35^0C for 48 hrs in a rotary shaker at 150 rpm. Examined the cultures after two days of incubation for the presence or absence of growth by turbidity method using a spectrophotometer at wavelength 600 nm.

3.14.3 Optimization of initial concentration of benzene, toluene, napthalene and acenapthene on selected bacterial species

Modified BBH amended with different concentrations of benzene and toluene (0.025% - 0.15 % or 250 ppm to 1500 ppm), naphthalene and acenapthene (50 – 300 ppm) separately were inoculated with bacterial species and incubated at 32^0C at 150 rpm. Examined the cultures after two days of incubation for the presence or absence of growth by turbidity method using a spectrophotometer at wavelength 600 nm.

3.15 Quantitative analysis (by UPLC / HPLC method)

The second method was setup for four time intervals for each identified strain and

hydrocarbon. The test media consisted of 20 ml BBH supplemented with one of the filter sterilized (0.2 µm) hydrocarbons at a final concentration of 200 mg/l for naphthalene and acenapthene whereas 0.1 % for benzene and toluene set after optimization experiments. Incubation was done at 35^0C for 10, 20, 30 and 40 days at 150 rpm. Samples were collected at regular intervals of time and the residual hydrocarbons were extracted according to Vilas Patel et.al. (2012) and analyzed by HPLC (Benzene and Toluene) and UPLC (Napthalene and acenapthene).

Extraction Procedure (Vilas Patel et al.)

- The entire content (20 ml) in the test tube was taken for residual hydrocarbon
- Ethyl acetate (5 ml) was added in the test tube and then incubated at 37^0C at 150 rpm for 1 hr so that residual hydrocarbon dissolves completely.
- Content was then centrifuged at 5000 g for 10 min
- Supernatant was dried using anhydrous Sodium Sulphate (activated at 150^0C for 3 hrs) to remove the water molecules
- The content was then passed through 0.2 µm membrane to get a sterile solution.
- The solvent was evaporated completely by passing nitrogen gas.
- HPLC/UPLC was used to check the residual hydrocarbon

HPLC program (For Benzene and Toluene)
(In collaboration with Dept. of Env. Microbiology, BBAU, Lucknow)

Analysis by HPLC (Waters) Column program (Reverse Phase)
Solvent used: 100 % Acetonitrile
Flow rate: 0.5 ml / min
Time period: 15 min and 10 min
Volume Injected: 20 ul
UV Wavelength: 254 and 262 nm

UPLC program (For Napthalene and Acenapthene)
(In collaboration with IITR, Lucknow)

Analysis by UPLC (Shimadzu) Column program (Reverse Phase)
Solvent used: 100 % Acetonitrile
Flow rate: 0.5 ml / min
Time period: 06 min
Volume Injected: 5 ul
UV Wavelength: 227 nm (Acenapthene) and 221 nm (Napthalene)

Calculations

Formula used to calculate the residual concentration and % degradation are given below:

Residual concentration (in ppm)

$$= \frac{\text{Sample Area}}{\text{Standard Area}} \times \frac{\text{Amount of std. injected (ng)}}{\text{Vol. of sample taken (ml)}} \times \frac{\text{Sample make up volume (ml)}}{\text{Volume of sample injected}} \times \text{DF}$$

$$\textbf{\% Degradation} = \frac{\text{Initial Concentration} - \text{Residual Concentration}}{\text{Initial Concentration}} \times 100$$

3.16 Microbial identification by ribosomal sequencing of selected isolates (In collaboration with NBRI, Lucknow)

Isolation of DNA

→ Bacterial cells were grown in NB media

→ Cells were harvested by centrifugation (10 min, 8000 rpm) and cell pellet was resuspended in 200 µl of SET

→ Lysozyme was added to a conc. Of 5-10 mg/l and incubated at 37^0C for 1-2 hrs

→ 25 µl of 10 % SDS + 5 µl RNAase A (10 mg/ml) was added and incubated at 37^0C for 15 min.

→ 20 µl of proteinase K (20 mg/ml) was added and incubated at 55^0C overnight.

→ Samples were heated to 70^0C for 15 min to inactivate the proteinase K.

→ 100 µl of 5 M NaCl and 800 µl of chloroform : isoamylalcohol (24:1) and phenol were added and incubated at RT for 30 min with frequent inversion

→Centrifuged at 8000 rpm for 15 min and the aqueous layer was transferred to a new tube using a blunt end pipette tip to reduce shearing of DNA

→ The DNA was precipitated with the addition of an equal volume of ethanol (400 µl) and gently inverted the tube to finally keep at RT for 10-15 min.

→ DNA was pelleted by centrifugation in a microfuge at 12,000 rpm for 10 min

→ Pellet was washed with 70 % ethanol (180 µl) and centrifuged again at 12,000 rpm for 5 min

→ Washings were done twice

→ Pellets were air dried for 2 hrs and 100 µl of sterilized distilled water was added.

→ To dissolve the DNA pellet, samples were incubated at 50-55^{0}C for 1 hr.

Reagents Used

1. SET i) NaCl: 250 µl
   ii) EDTA : 20 mM
   iii) Tris : 100 mM
2. SDS : 10 %
3. NaCl: 5 M
4. Chloroform: Isoamylalcohol (24:1)
5. Phenol
6. Isopropanol
7. Ethanol: (70 %)
8. Sterile DW
9. Sodium Acetate: 3 M
10. TE Buffere i) Tris: 10 mM
    ii) EDTA: 1 mM

PCR programme and methods

→ The PCR was carried out with 50-90ng of pure genomic DNA.

→ The primers eubacterial primers 27f (5'-AGAGTTTGATCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'), located respectively, at the extreme 5' and 3' ends of the ribosomal rDNA sequence, enable the amplification of nearly the entire gene.

→ The amplification reactions were performed in a 50 µL volume by mixing template DNA with the polymerase reaction buffer (10x); primers PA and PH (100 ng each) and 0.5 U Taq polymerase.

PCR Cocktail: For each tube

Taq Buffer: 4.5 µl
F' primer: 2.5 µl
R' primer: 2.5 µl

d NTP's: 4.5 µl
Taq: 1.5 µl

→The cyclic condition for the amplification reaction was as follows:

- 96^{o}C for 2 min. (Initial denaturation).
- 96^{o}C for 45 sec. (30 cycles).
- 56^{o}C for 30 sec. (annealing).
- 72^{o}C for 2 min. (extension).
- 72^{o}C for 5 min. (final extension).

→Amplified genomic DNA was taken and run on 1.2% agarose gel with PCR ladder (Hind III MW marker) at a constant voltage of 55 V and visualized under UV light**.**

PCR product purification:

PCR products (approx. 1500 bp) were purified by PCR purification kit (Qiagen, Valencia, CA)

Sequencing and phylogenetic analysis:

→Sequencing was being performed by Miniprep kit (Qiagen) and sequenced by using Big Dyeterminator with an automated capillary sequencer (Applied Biosystems) in NBRI, Lucknow

→Inserts will be sequenced using vector-specific and a suite of 16S rRNA-specific primers to generate an overlapping set of sequences which will be assembled into one contiguous sequence.

→Later the sequences obtained were submitted to NCBI for identification.

→ Finally a phylogenetic tree was constructed using MEGA 7.02.

3.17 Heavy metal analysis of soil sediments by FAAS

Procedure for acid digestion of soil sample: (Followed from EPA3050b)

1. Weighed 1 gm of homogenized soil and took in 100 ml beaker
2. Added 10 ml of 1:1 (Conc. HNO_3: Perchloric Acid) and refluxed for 15 min at 95^{0}C

3. Allowed the sample to cool and again refluxed for 30 min at 95^0C after adding 5 ml of Conc. HNO_3.
4. Repeated the steps till no brown fumes are given off
5. Allowed the remaining solution to evaporate till 5ml at 95^0C.
6. Again added 10 ml of conc. HCl to the digest and refluxed for 15 min at 95^0C
7. Filtered the digest with Whatman No. 1 filter and made upto volume
8. Analyzed by Flame Atomic Absorption Spectrometry (FLAA)

3.18 Testing heavy metal and antibiotic resistance of strains

Heavy metal resistance of strains was tested in nutrient broth tubes containing different concentrations (0.25, 0.5, 1.0, 2.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 mM) of $CdCl_2$, $CuSO_4.5H_2O$, $Pb(NO_3)_2$, $ZnSO_4.7H_2O$, $MnSO_4.H_2O$, $C_8H_4K_2O_{12}Sb_2.3H_2O$, $SnCl_2.2H_2O$, $K_2Cr_2O_7$, $NiSO_4.6H_2O$, $HgCl_2$, $FeSO_4.7H_2O$ (HiMedia). Stock solutions were prepared in deionized water and were also filter sterilized before inoculation. All tests were done in duplicates and the results were evaluated visually for growth against heavy-metal free control cultures and blank tubes.

Sensitivity of selected strains against antibiotics was assayed by the Kirby Bauer's disc diffusion method using Muellere Hinton agar (Bauer et al., 1996; CLSI, 2013). Used antibiotic discs (HiMedia) belonged to the following antibiotic groups: cephalosporins (cefuroxime sodium-CXM30, cefoperazone-CFP75, cefotaxime-CTX30,); tetracyclines (tigecycline-TGC15); penicillins (penicillin G-10U, piperacillin-PRL100, amoxyclav-30); quinolones (norfloxacin-NOR10); carbapenems (imipenem-IPM10). Regarding to antibiotic resistance, strains were divided into three groups (resistant-R, intermediate resistant-I, susceptible-S) according to the diameter of the inhibition zone taking into account CLSI interpretive standards (CLSI, 2013). All the experiments were done in triplicates.

Chapter 4

Results

4.1 Physico chemical properties of soil samples

The pH of the soil samples varied from 7.57 (HPRB) to 8.5 (KNPP, RJSP, BSP) which is alkaline. The soil temperature varied from 36.2 (KNPP) to 37.5 (CFK). Moisture content was found to be most for HPRB (7.5 %) and least for APR (0.2 %). Inorganic phosphate , nitrate and potassium contents were less when compared to uncontaminated soil standard values. The organic carbon content was high or medium for all the samples except CFK (Table 7)

Table 7: Physico chemical properties of soil samples

Parameters	**pH**	**Temp. (^{0}C)**	**Moisture content (%)**	**Inorganic Phospahte Content (mg/L)**	**Nitrate Content (mg/L)**	**Available Potassium**	**Organic carbon**
Uncontaminated Agricultural soil	7.7	35.1	9.0	922	89	No line visible very high above 392Kg/ha	Low
Korba Power Plant (KTPP)	8.5	36.2	5.0	680	44	No line visible very high above 392Kg/ha	Medium High
Raigarh Steel Plant (RSP)	8.5	37.1	2.1	660	41	No line visible very high above 392Kg/ha	Medium High
Bhilai Steel Plant (BSP)	8.5	36.4	2.5	640	47	No line visible very high above 392Kg/ha	Medium
Bhilai Steel Plant Oil Collection centre (BSPOCC)	7.57	36.5	7.5	590	52	No line visible very high above 392Kg/ha	High
Coal Field Korba (CFK)	8.18	37.5	1.4	450	49	No line visible very high above 392Kg/ha	Low
Aluminium Plant Raigarh (APR)	8.36	37	0.2	650	46	No line visible very high above 392Kg/ha	Medium
Sponge Iron Plant Urla (SIPU)	7.84	36.5	0.3	480	49	Last two line visible: Medium 112 to 280 Kg/ha	Medium High
Bilaspur Railway Oil Depo (BROD)	7.79	37.5	0.5	680	40	Last two line visible: Medium 112 to 280 Kg/ha	Medium Low
HP Refilling Raipur (HPRR)	8.4	37	0.3	600	54	No line visible very high above 392Kg/ha	Low
Indian Oil Refilling Raipur (IORR)	8.1	37.2	1.5	620	49	Last two line visible: Medium 112 to 280 Kg/ha	Medium

4.2 Total aerobic heterotrophic and hydrocarbon degrading bacteria counts of soil samples

The total heterotrophic count of bacteria was highest in sample sites BROD, HPRR and BSPOCC and least in CFK (Fig 12). Hydrocarbon degrading bacterial count was less than the total heterotrophic count in most of the samples. In case of benzene degraders the count was maximum for BROD, HPRR, and APR and least for CFK whereas in case of toluene it was highest for BROD, HPRR and BSP and least in CFK (Fig 13 and 14). In case of naphthalene degraders the count was maximum for BROD, HPRR, and APR and least for IORR which followed the same trend in case of acenapthene degraders (Fig 15 and16).

Fig 9: Soil Sedimentation process

Fig 10: Soil Sedimentation process

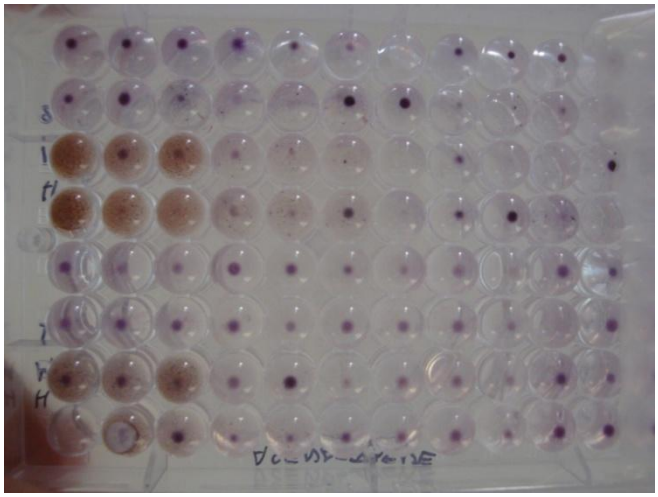

Fig 11: ELISA plate showing tetrazolium reduction

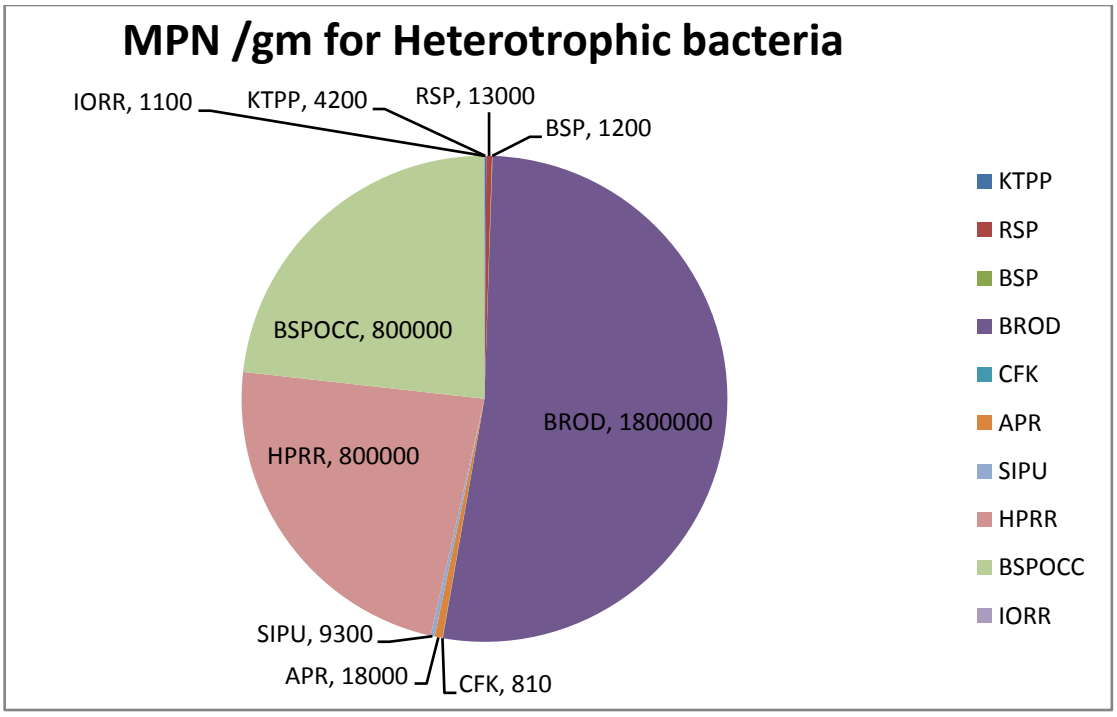

Fig 12**:** Most probable number analysis for the 10 soil samples

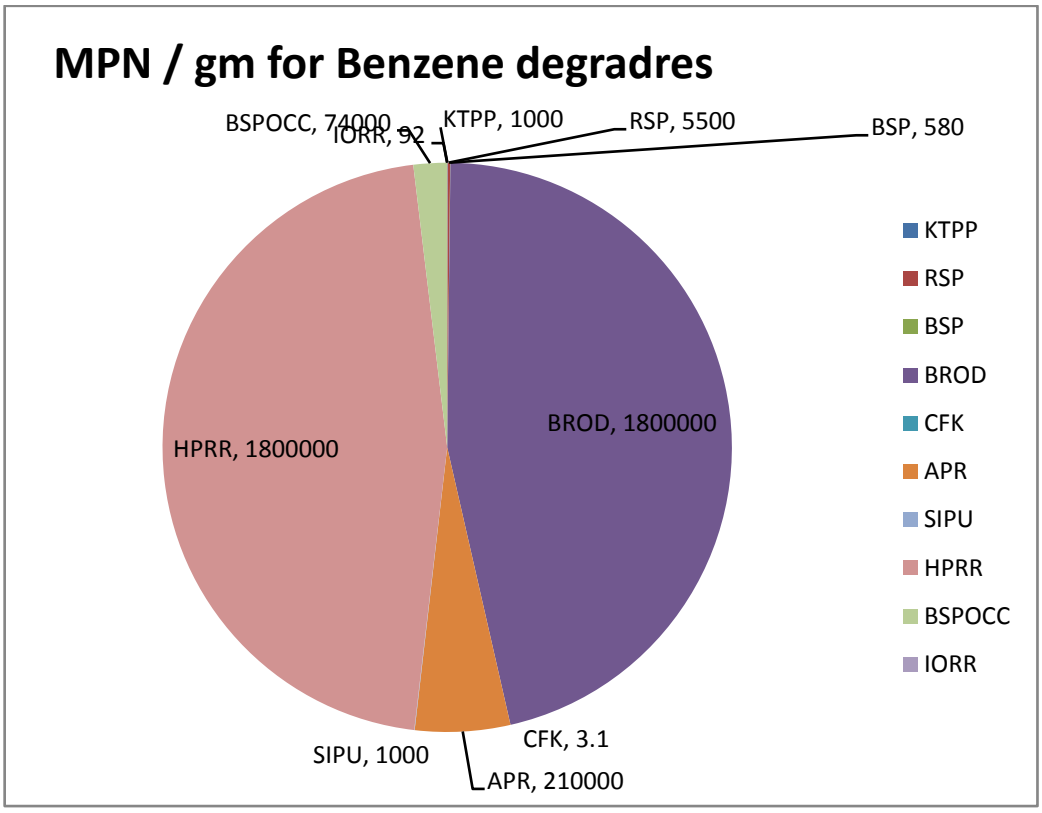

Fig 13: MPN pie chart for benzene degraders

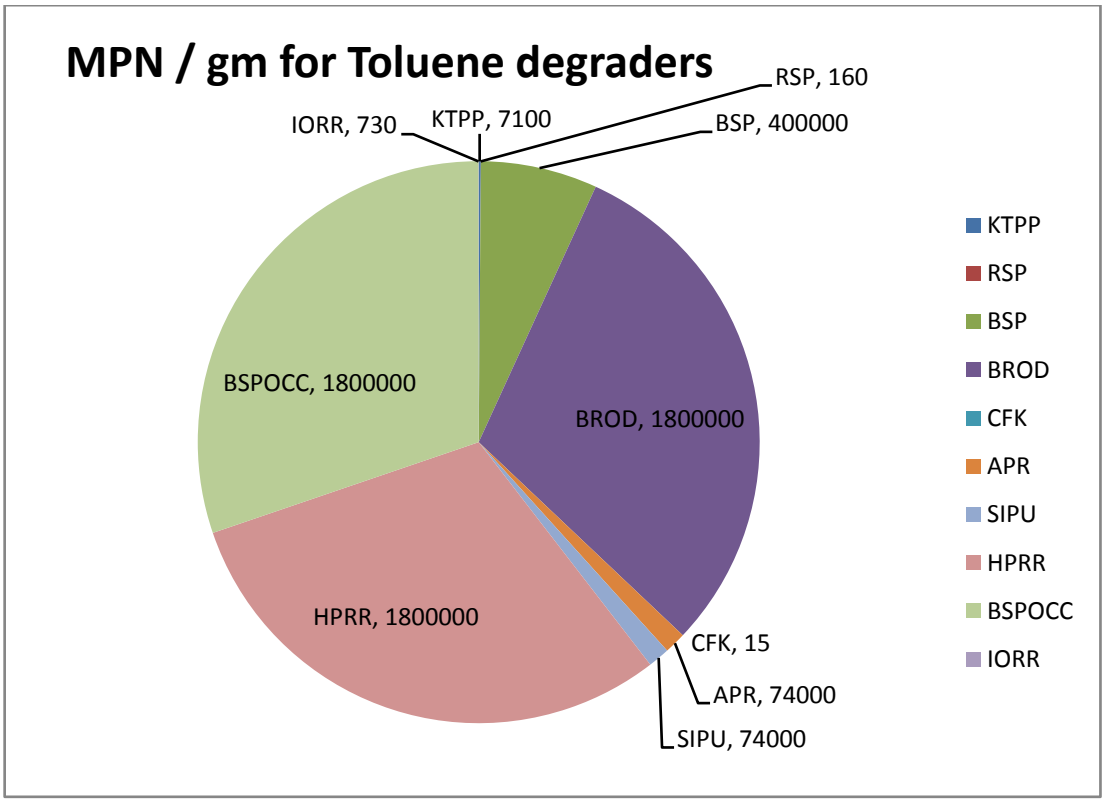

Fig 14: MPN pie chart for toluene degraders

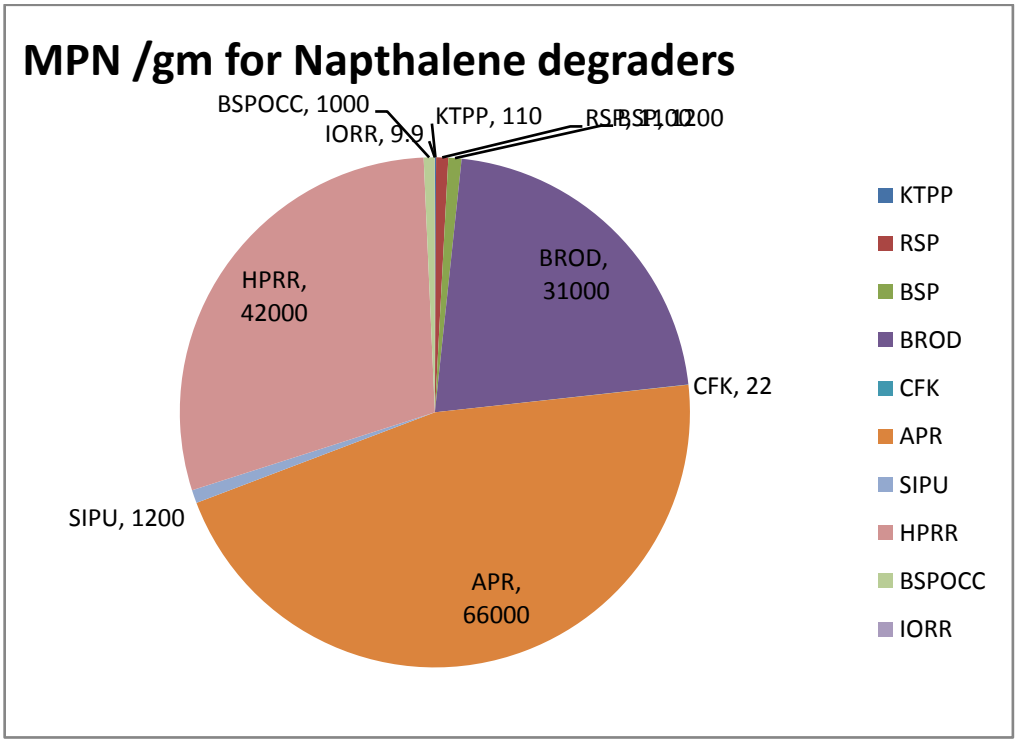

Fig 15: MPN pie chart for naphthalene degraders

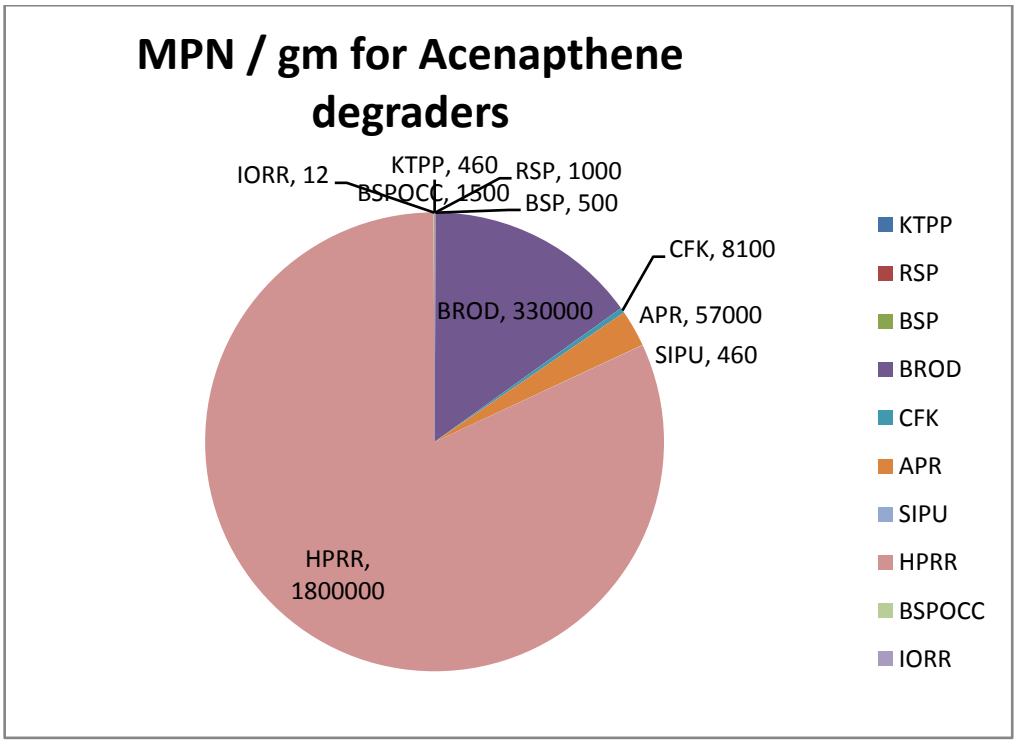

Fig 16: MPN pie chart for acenapthene degraders

4.3 FTIR analysis of soil samples

From the analysis of FTIR peaks it is clear that all the soil samples contain aromatic compounds other than alkanes, alcohols, phenols, acids, esters and ethers (Table 8). The following figures (Fig 17-26) gives an idea about the IR spectra of the soil samples.

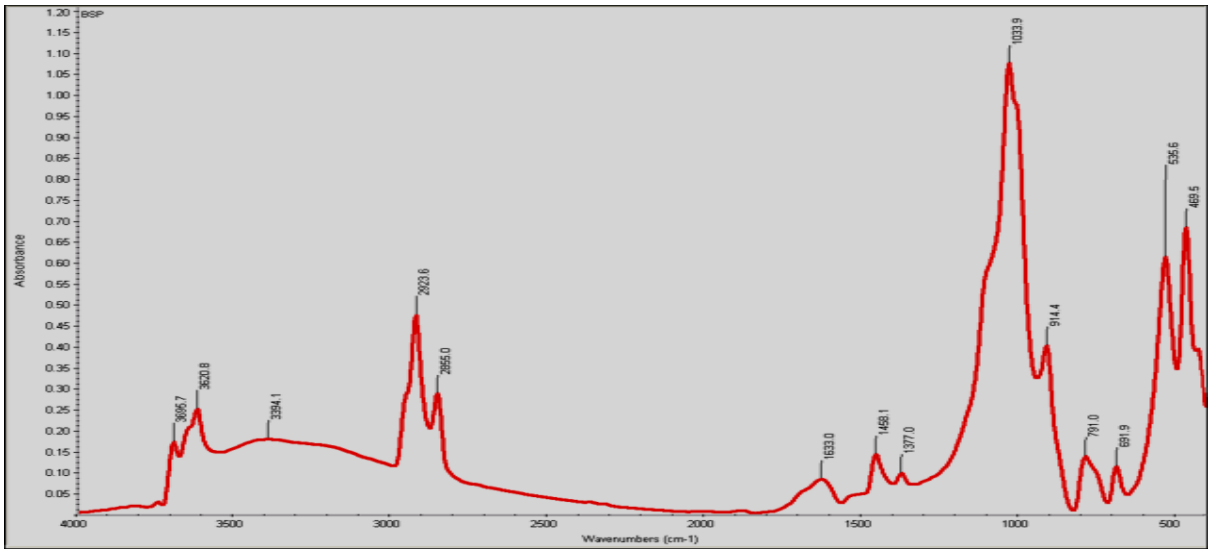

Fig 17: FTIR peaks for sample BROD

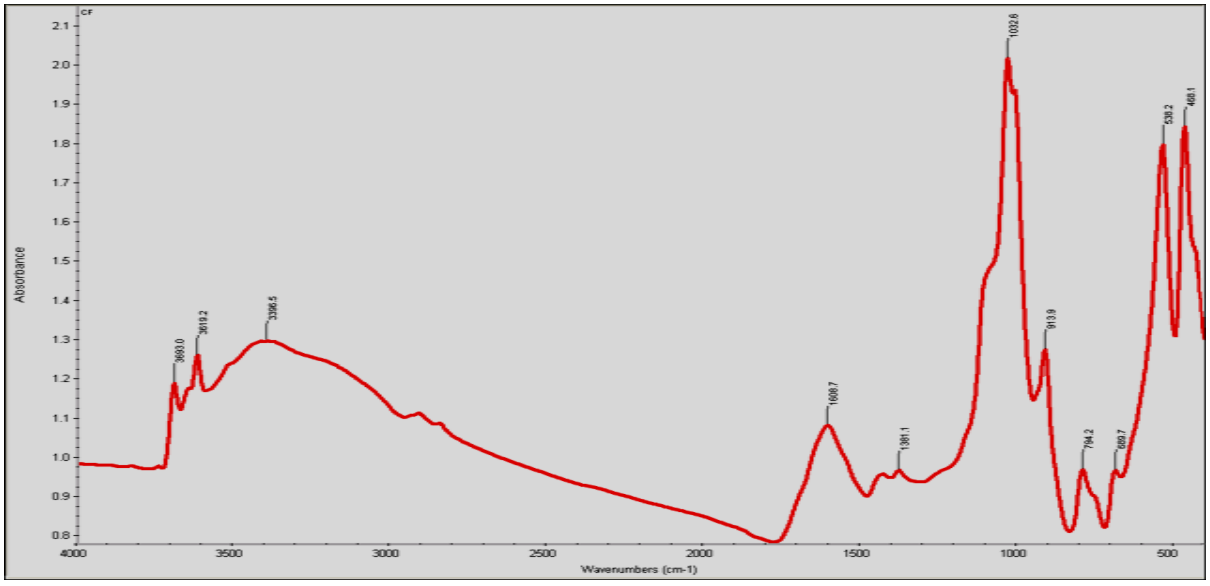

Fig 18: FTIR peaks for sample CFK

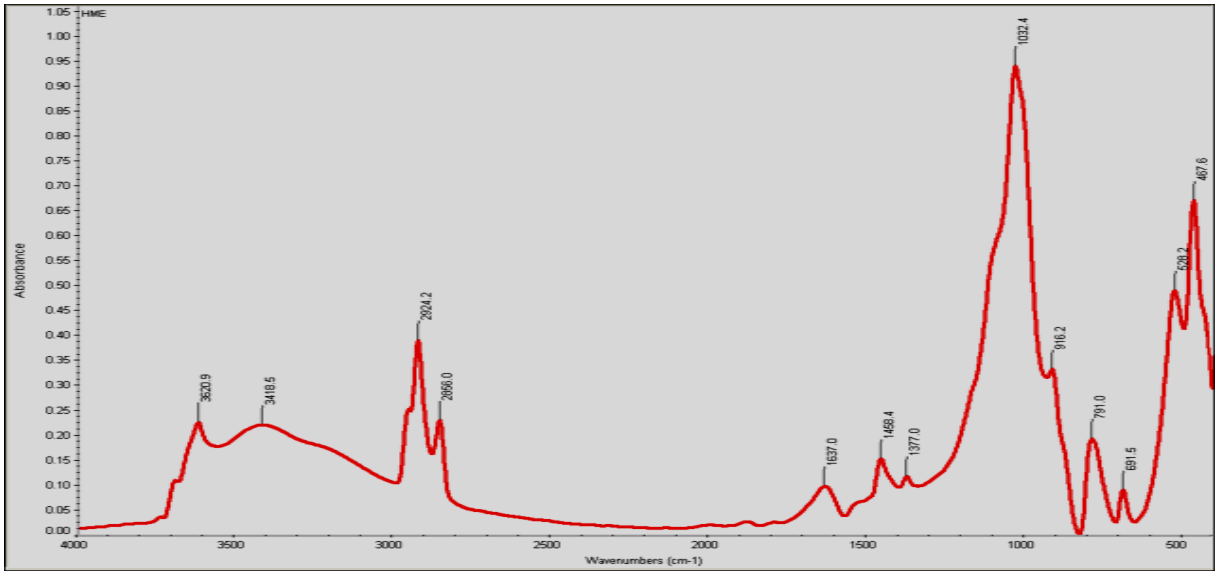

Fig 19: FTIR peaks for sample SIPU

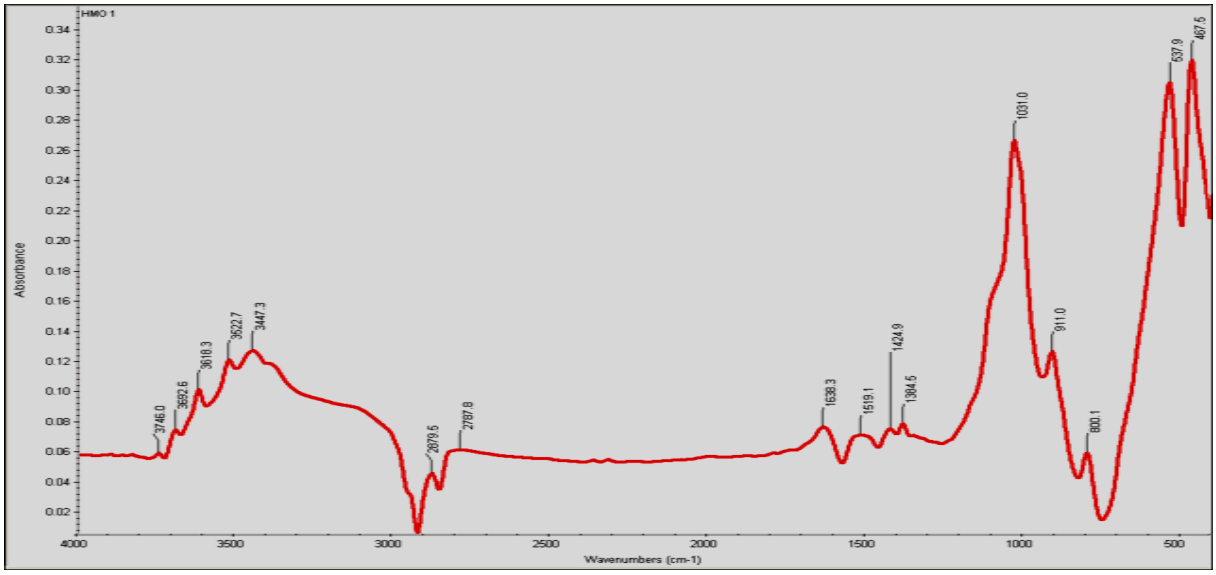

Fig 20: FTIR peaks for sample APR

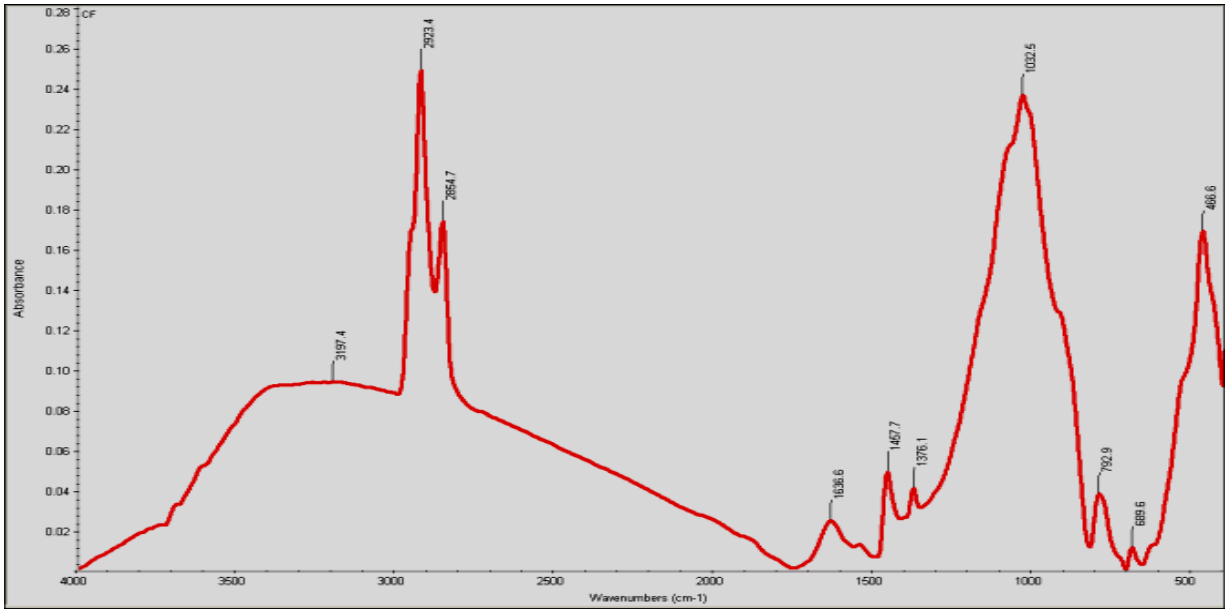

Fig 21: FTIR peaks for sample UOS

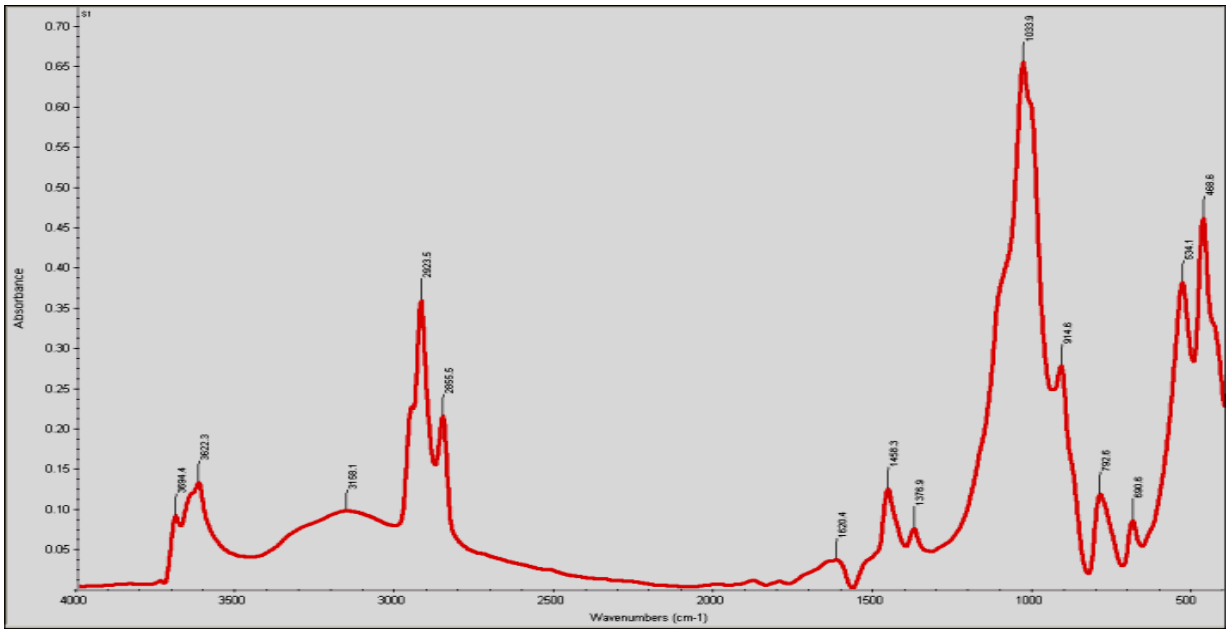

Fig 22: FTIR peaks for sample HPRR

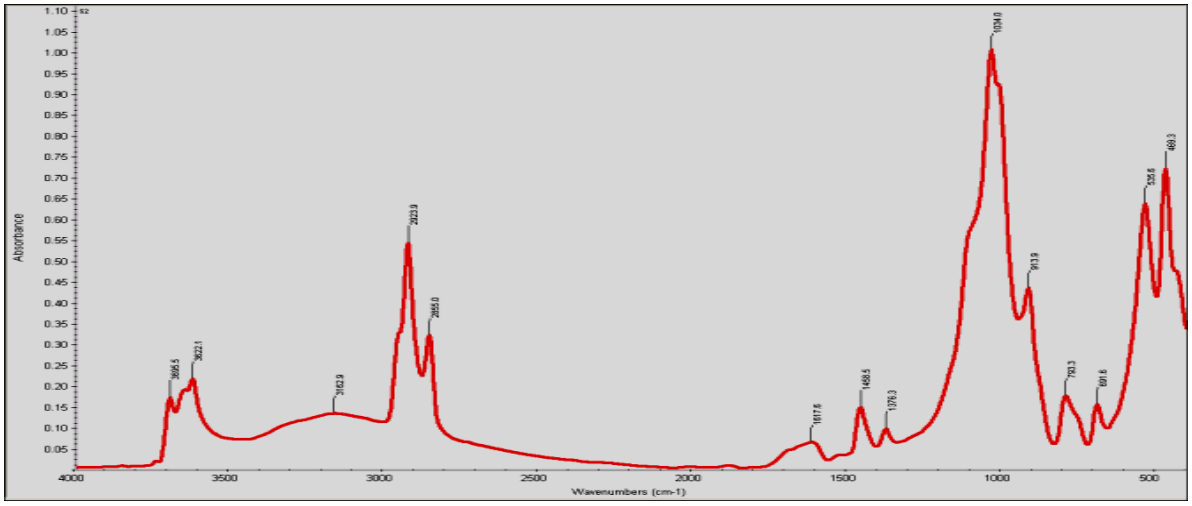

Fig 23: FTIR peaks for sample KTPP

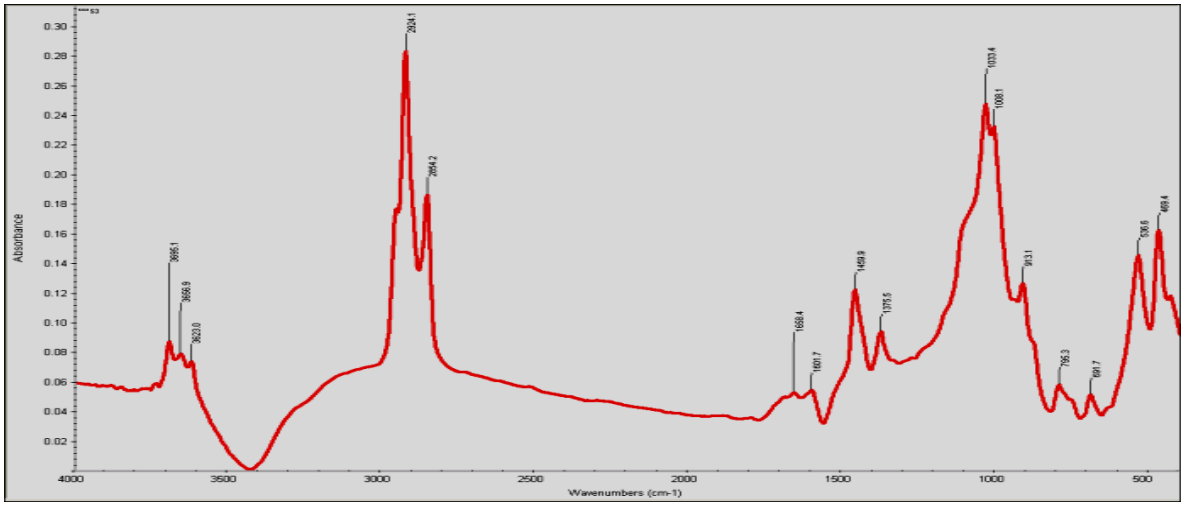

Fig 24: FTIR peaks for sample RSP

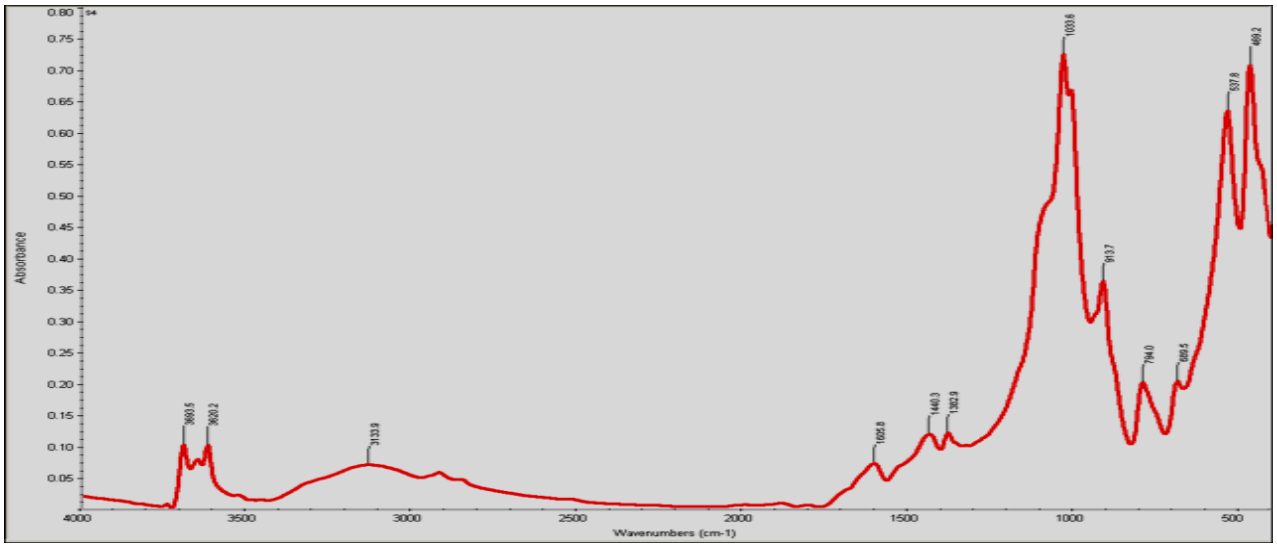

Fig 25: FTIR peaks for sample BSP

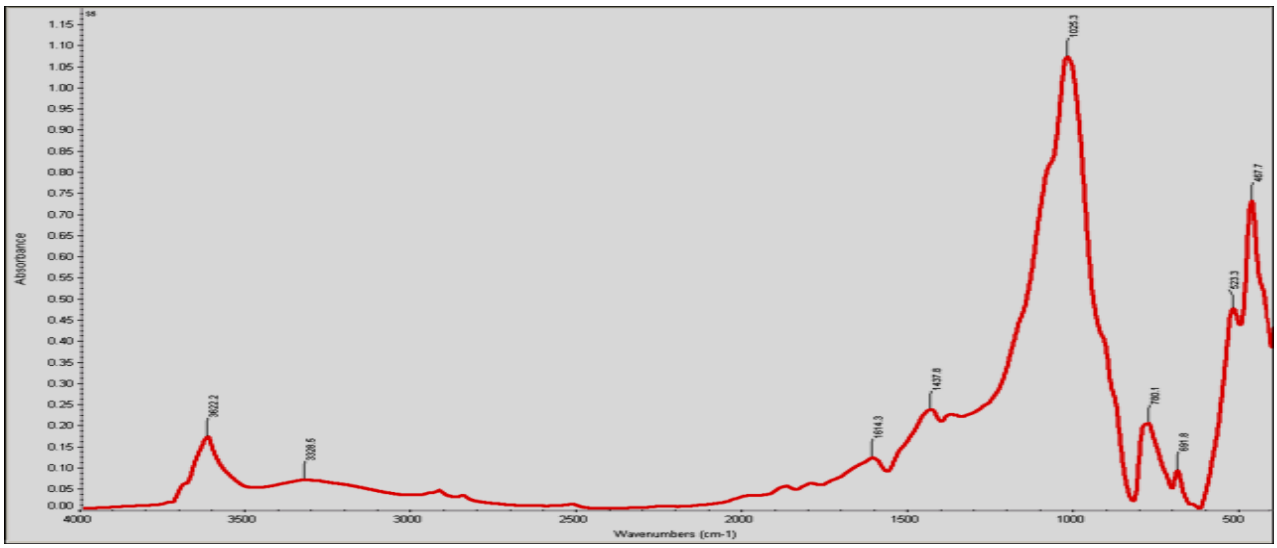

Fig 26: FTIR peaks for sample IORR

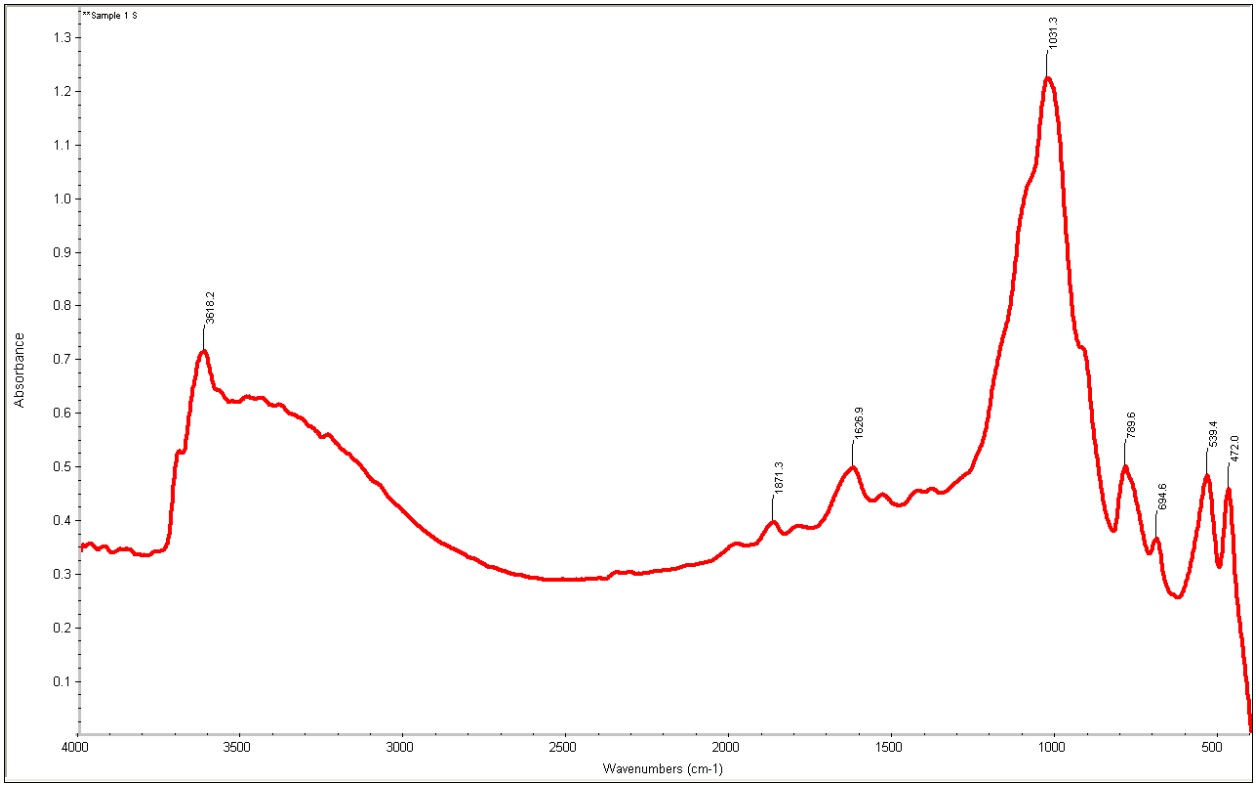

Fig 27: FTIR peaks for uncontaminated agricultural soil

Table 8: FTIR peak analysis

Frequency, cm^{-1}	**Bond**	**Functional Group**	**Soil Samples in which peak was present**
3600-3200	O-H stretch	Alcohols and phenols	BROD, CFK, SIPU, APR, HPRR, KTPP, RSP, BSP, IORR
3000-2850	C-H stretch	Alkanes	BROD, SIPU, BSPOCC, HPRR, KTPP, RSP
1500–1400	C–C stretch (in–ring)	aromatics	BROD, CFK, SIPU, APR, BSPOCC, HPRR, KTPP, RSP, BSP, IORR
1320–1000	C–O stretch	Alcohols, esters, acids and ethers	BROD, CFK, SIPU, APR, BSPOCCC, HPRR, KTPP, RSP, BSP, IORR
900–675	C–H “oop”	aromatics	BROD, CFK, SIPU, APR, BSPOCCC, HPRR, KTPP, RSP, BSP, IORR

4.4 Enrichment, purification and culturing of hydrocarbon degrading bacteria

At least 25 different looking colonies were identified which were maintained in Agar slants after enriching them from diesel oil contaminated soil samples.

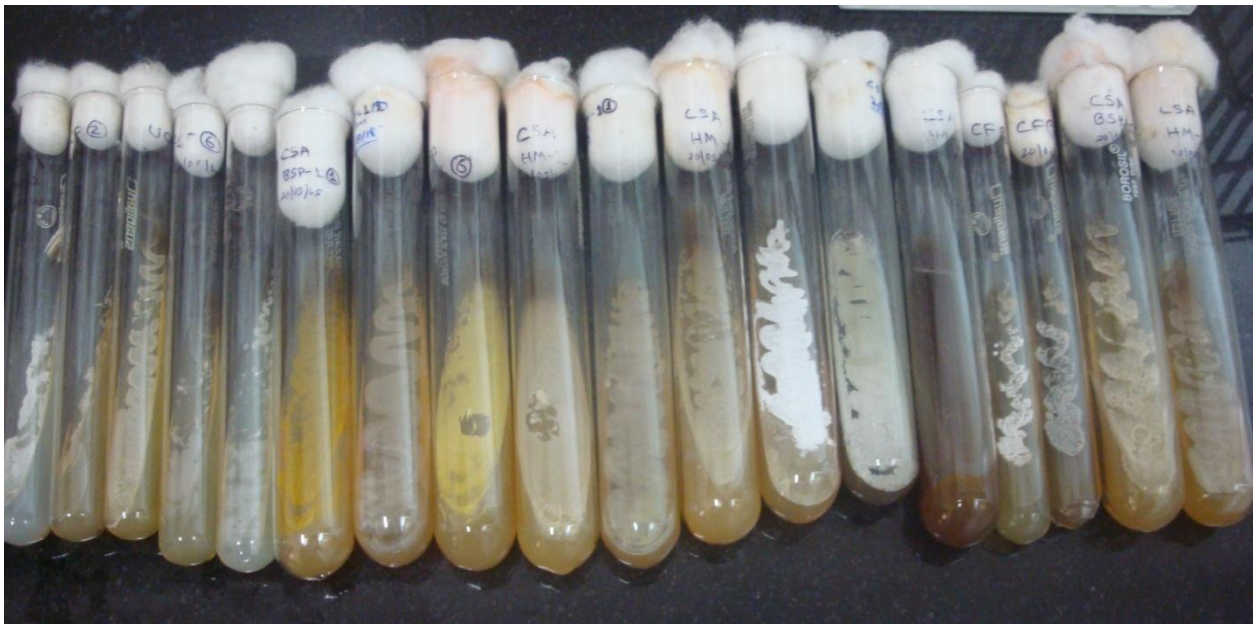

Fig 28: Bacterial cultures isolated from soil sediments

Fig 29: Bacterial cultures isolated from soil sediments

4.5 Qualitaive degradation potential of the isolates (Dye method)

The aromatic hydrocarbon degradation potential of the 10 isolates by dye method is shown in table 9. Bacterial cultures obtained from KTPP, RSP, BSP, BROD, BSPOCC showed maximum microbial activity by reducing resazurin to pink colored resofurin. These five isolates were further used for molecular identification, quantitative hydrocarbon degradation, antibiotic and heavy metal resistance studies. Isolate from CFK was not able to grow on any of the hydrocarbons whereas isolates from SIPU, HPRR, IORR and APR showed intermediate response to hydrocarbons.

Fig 30: Resazurin (purple)and resorfurin (pink) formation

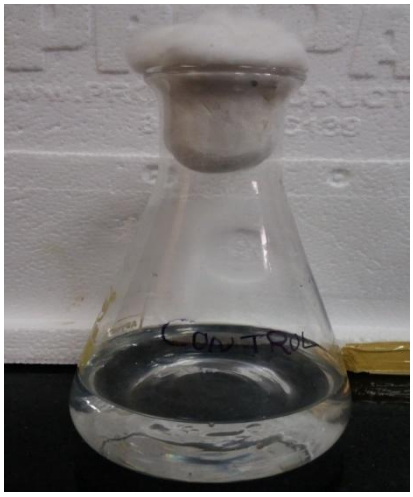

Fig 31: Control

Table 9: Qualitative hydrocarbon degradation (Dye Method)

Parameters	Aromatic hydrocarbon degradation potential			
	Benzene	Toluene	Napthalene	Acenapthene
B1-KTPP	++	++	++	++
B2-RSP	++	++	++	++
B3-BSP	++	++	++	++
B4-BROD	++	++	++	++
B5-CFK	_	_	_	_
B6-APR	+	+	++	++
B7-SIPU	+	++	+	++
B8-BSPOCCC	++	++	++	++
B9-HPRR	++	++	+	+
B10-IORR	++	+	++	+

+++" results were not obtained as the reaction is reversible in aerobic conditions

4.6 Taxonomic Identification of the bacterial strains

Results after PCR are shown in figures

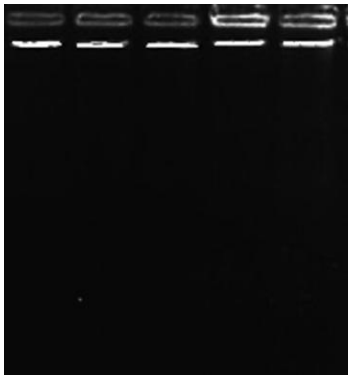

Fig 32: DNA bands after DNA Isolation

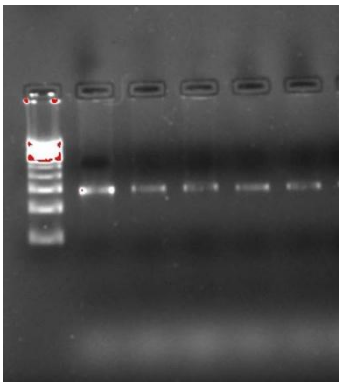

Fig 33: DNA bands after PCR

The unknown bacterial cultures were identified as members of the phylum Firmicutes consisting predominantly of gram positive Bacillus and Aneurinibacillus species Table 10:

Table 10**:** Taxonomic identification of the isolated bacterial strains.

Strain	**Gene bank accession number**	**Sequence alignment**		**Nearest phylogenetic neighbor (Gene bank accession number)**
		No. of nucleotides	Identity, %	
Aneurinibacillus aneuriniliticus strain RSP	KX371250	730	97	*Aneurinibacillus aneuriniliticus* strain NBRC 15521 (NR112639)
Aneurinibacillus migulanus strain KTPP	KX371251	1187	97	*Aneurinibacillus migulans* strain NBRC 15520 (NR113764)
Aneurinibacillus migulanus strain BROD	KX371252	865	98	*Aneurinibacillus migulans* strain NBRC 15520 (NR113764)
Bacillus thuringiensis strain BSPOCC	KX371253	1384	99	*Bacillus thuringiensis* strain ATCC 10792 (NR114581)
Bacillus cereus strain BSP	KX371254	1370	99	*Bacillus cereus* strain ATCC 14579 (NR074540)

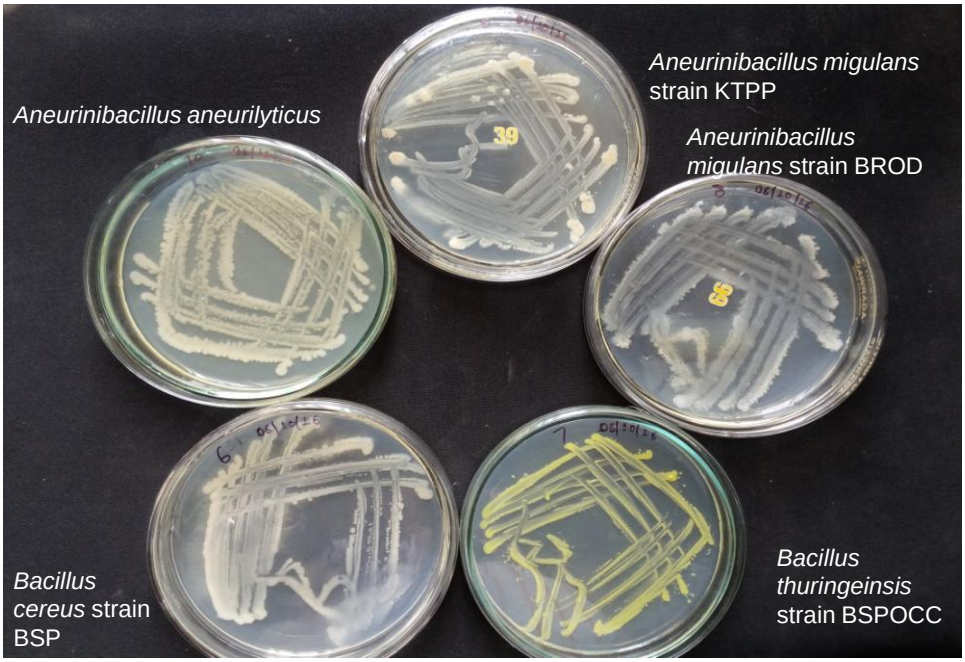

Fig 34: Identified bacterial species grown in NAM plates

Table 11: Morphological characteristics of isolates

Characteristics	Bacterial isolates				
	Aneurinibacillus aneuriniliticus strain RSP	*Aneurinibacillus migulanus* strain KTPP	*Aneurinibacillus migulanus* strain BROD	*Bacillus thuringiensis* strain BSPOCC	*Bacillus cereus* strain BSP
Colonial characteristics	Circular, white	Circular, white	Circular, white	Circular, yellowish	Circular, white
Morphological					
Gram's reaction	+	+	+	+	+
Shape	Circular	Circular	Circular	Circular	Circular
Spore staining	+	+	+	+	+
Motility test	+	+	+	+	+
Biochemical Indole	-	-	-	-	-
Methyl red	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+
Citrate utilization	+	+	+	+	+
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
Nitrate reduction	+	+	+	+	+
Urease test	-	-	-	-	-
Glucose fermentation	+	+	+	+	+
Lactose fermentation	+	+	+	+	+
Sucrose fermentation	+	+	+	+	+

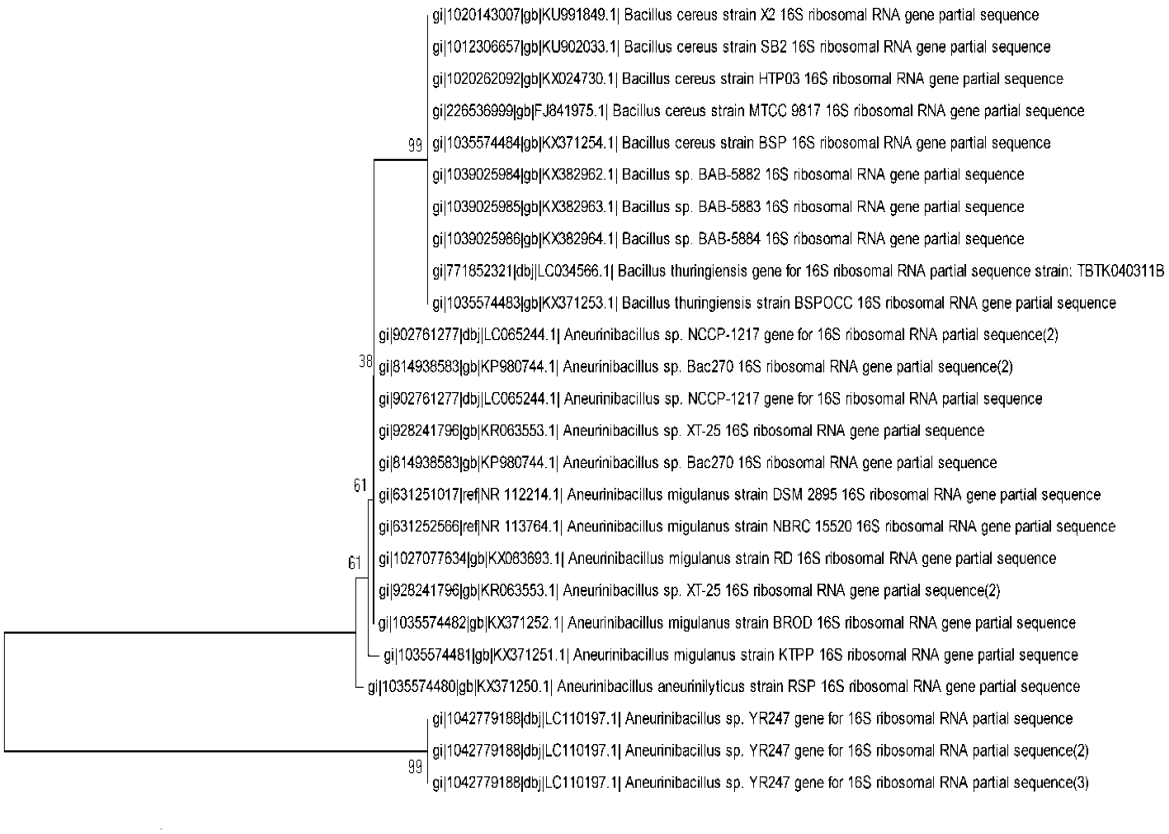

Fig 35: Phylogenetic tree derived from 16S rRNA gene sequences showing the relationship between isolates and other species belonging to the genus Bacillus constructed using the neighbor-joining method. Bootstrap values were expressed as percentages of 1000 replications.

4.7 Optimization of temperature, pH and initial concentration of benzene, toluene, napthalene and acenapthene on selected bacterial species

4.7.1 Optimization of temperature

Results obtained show that the most optimum temperature for bacterial growth is 35^0C.

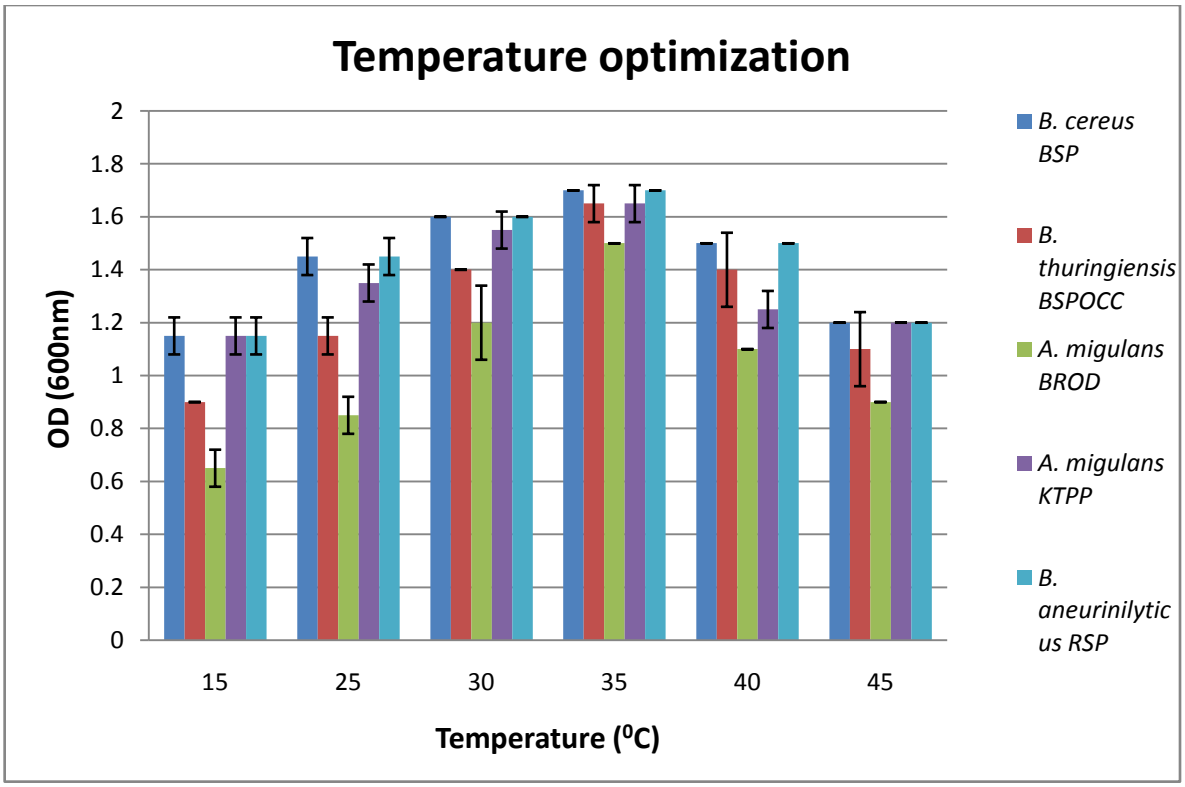

Fig 36: Temperature optimization chart for the identified bacterial species

4.7.2 Optimization of pH

Results obtained show that the most optimum pH for bacterial growth is 7.0.

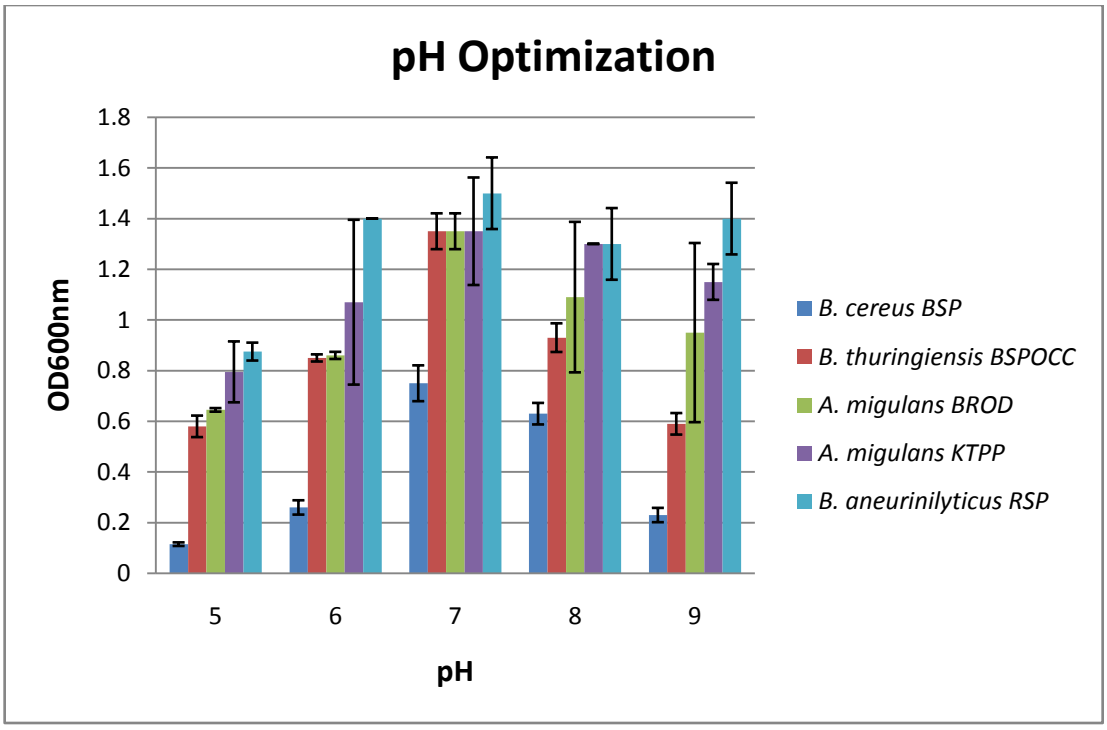

Fig 37: pH optimization chart for the identified bacterial species

4.7.3 Optimization of initial concentration of benzene, toluene, napthalene and acenapthene on selected bacterial species

All the bacterial species showed maximum growth in 200 ppm concentration of napthalene and acenapthene whereas the optimum concentration in case of benzene and toluene was found at 0.1 %. Fig:

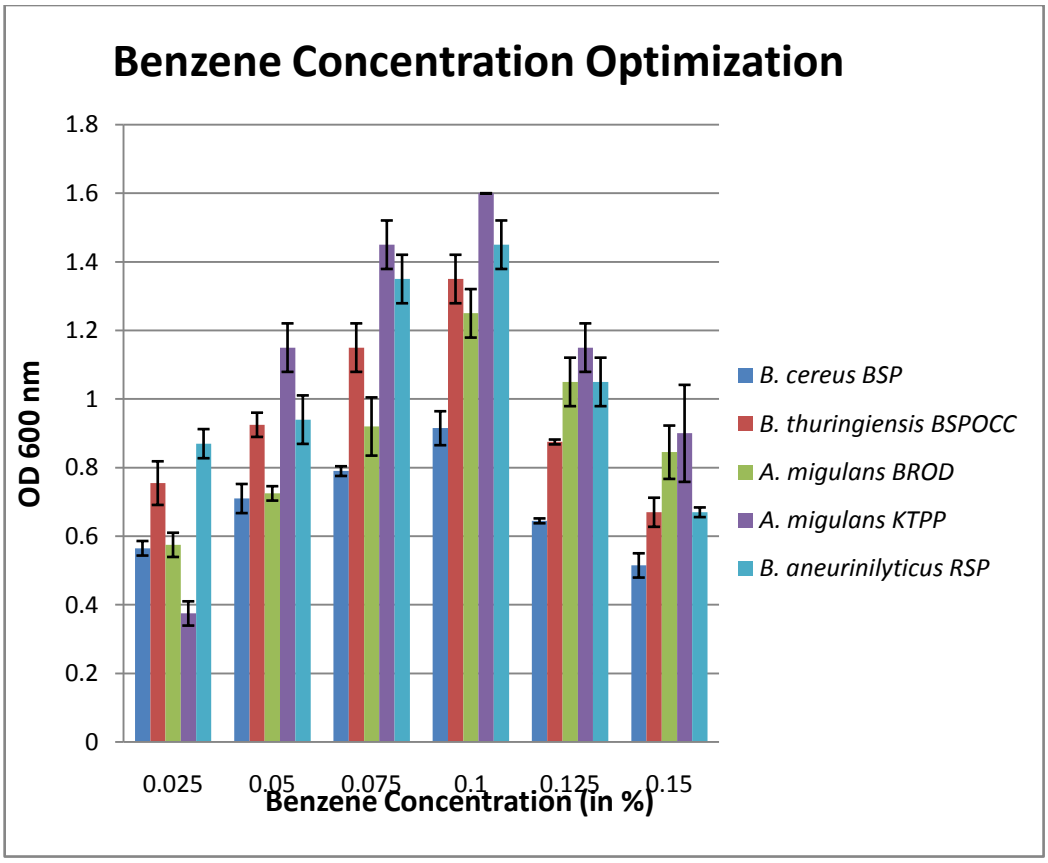

Fig 38: Benzene concentration optimization chart for the identified bacterial species

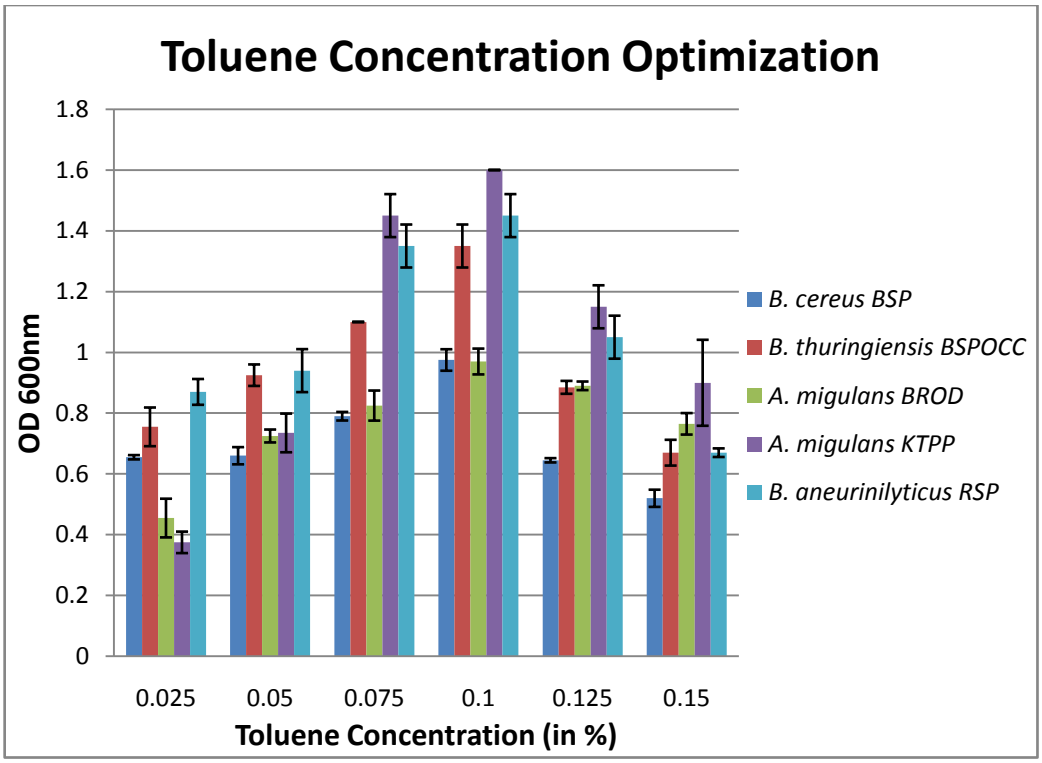

Fig 39: Toluene concentration optimization chart for the identified bacterial species

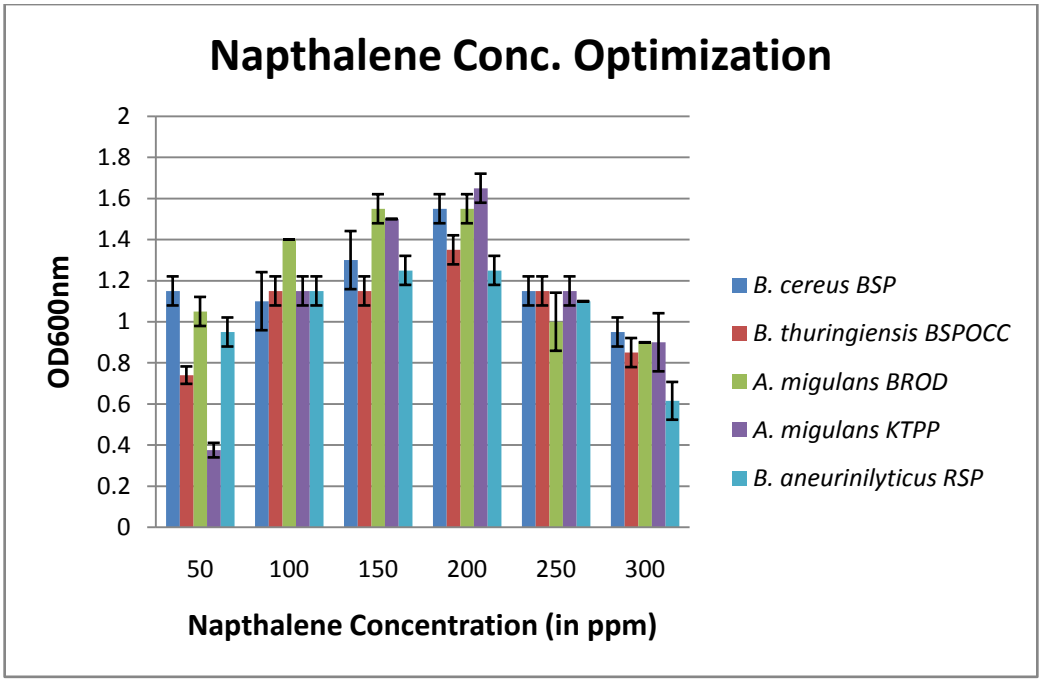

Fig 40: Napthalene concentration optimization chart for the identified bacterial species

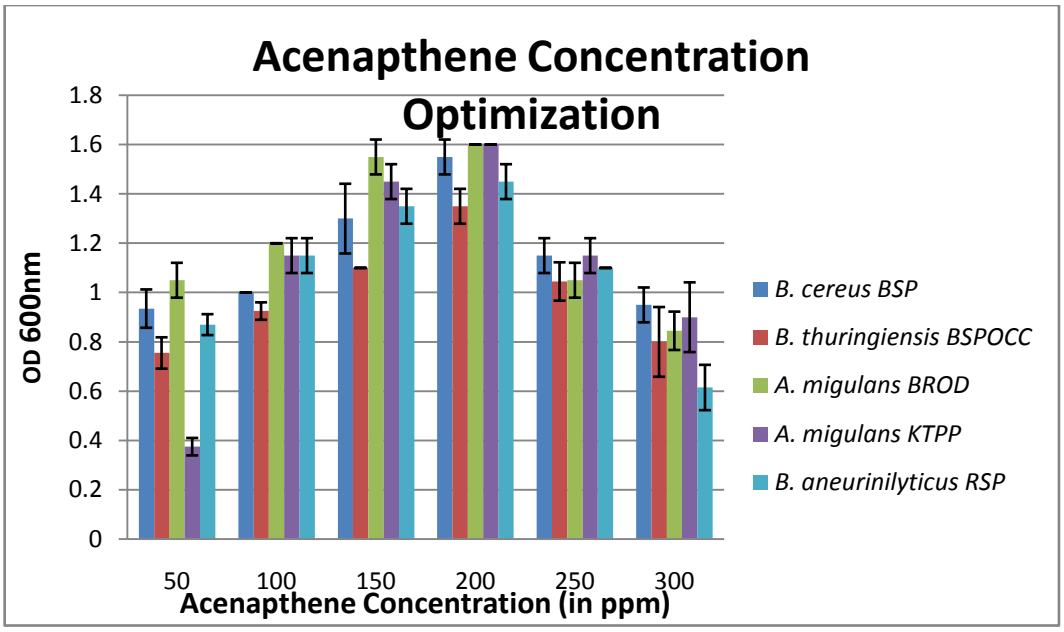

Fig 41: Acenapthene concentration optimization chart for the identified bacterial species

4.8 Quantitaive degradation potential of the isolates

The degradation potential of all the isolates was quantified by HPLC and UPLC. The calibration curves obtained for the four hydrocarbons (Figure 42 to 45) are given below based on which the residual concentration and % degradation was calculated.

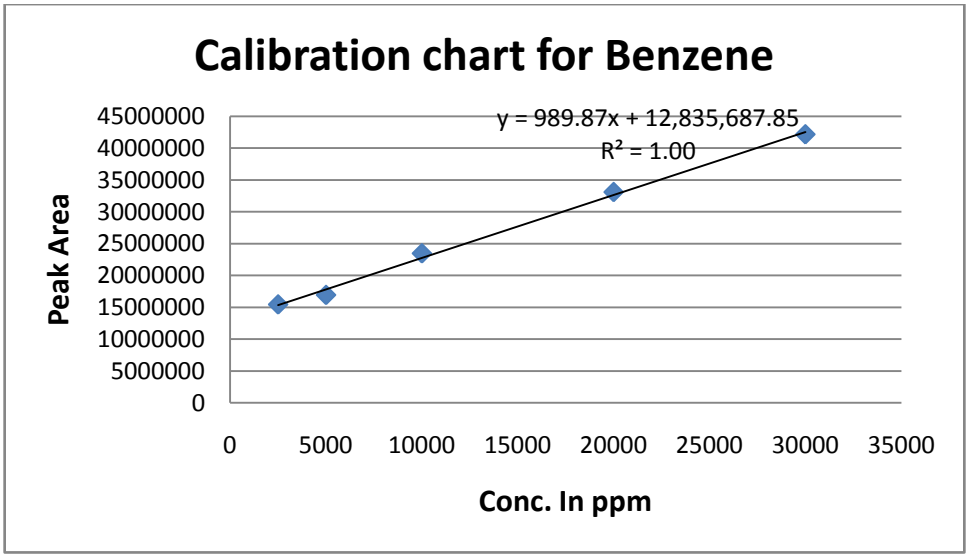

Fig 42: Standard calibration trend line for benzene

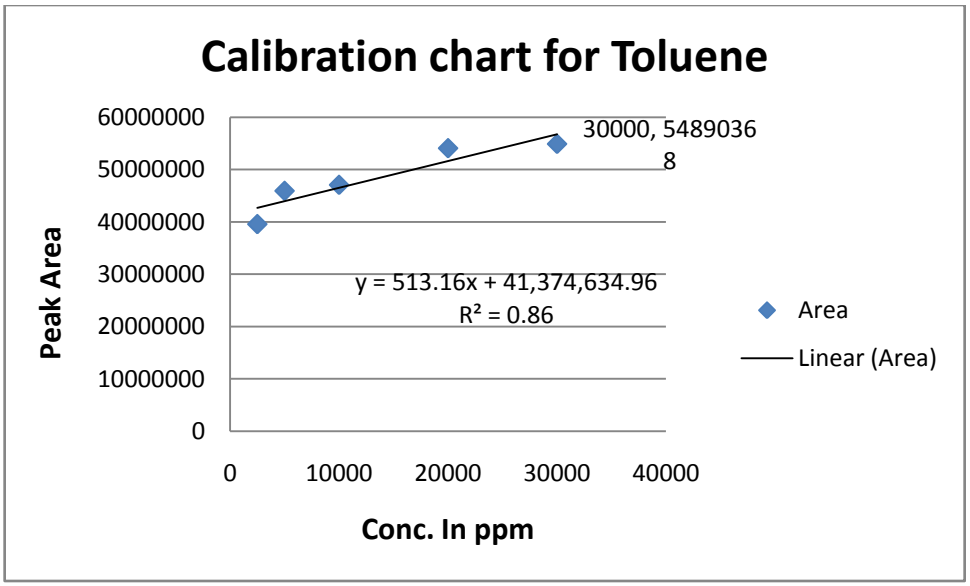

Fig 43: Standard calibration trend line for toluene

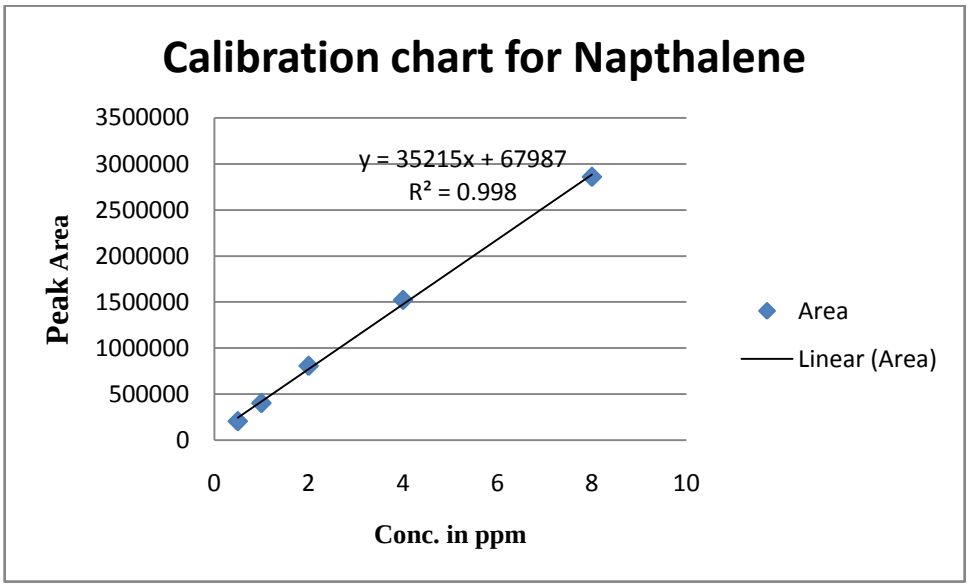

Fig 44: Standard calibration trend line for napthalene

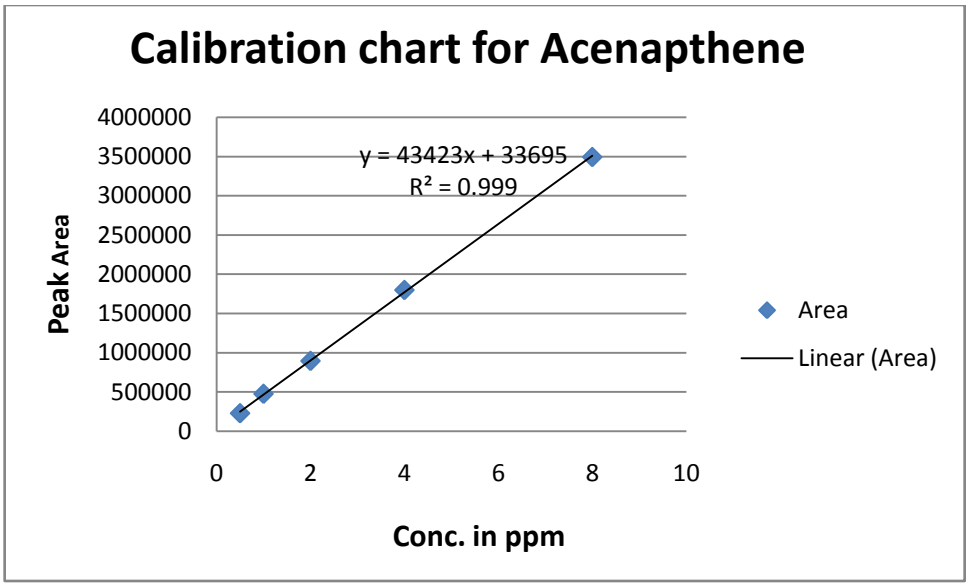

Fig 45: Standard calibration trend line for acenapthene

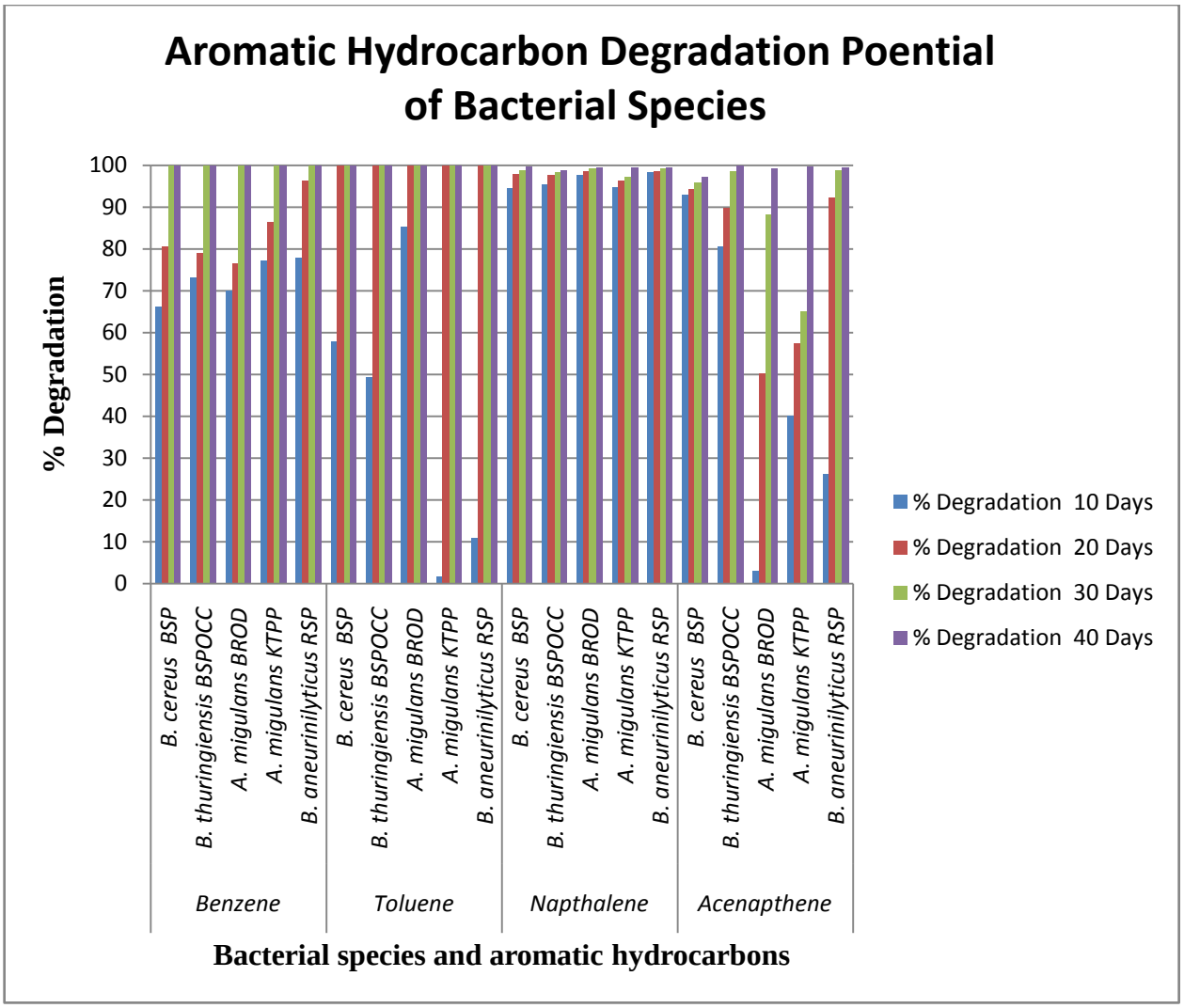

Fig 46: Quantitative hydrocarbon % degradation graph

B. cereus and *B. thuringiensis* were most potent in degrading naphthalene and acenapthene followed by their ability to degrade benzene and toluene in the first 10 days of incubation. *B. cereus* degraded 94.6 % naphthalene, 92.9 % acenapthene, 66.1 % benzene and 58 % toluene. *B. thuringiensis* degraded 95.5 % naphthalene, 80.6 % acenapthene, 73.1 % benzene and 41.4 % toluene. Results show that *A. migulans* BROD was not able to degrade acenapthene in the first 10 days with 3.1 % degradation but degraded naphthalene, benzene and toluene at 97.7 %, 69.9 % and 85.3 % respectively in the first 10 days. *A. migulans* KTPP on the other hand was able to degrade 1.7 % toluene and 40.1 % toluene in intial 10 days of incubation. *B. aneurinilyticus* RSP also showed very less activity when treated with toluene and acenapthene at 10.9 % and 26.2 % degradation respectively.

Increasing the days of incubation slowly increased the bacterial growth in all the bacterial cultures which reveals in the percentage degradation for 20, 30 and 40 days. All the bacterial cultures when treated with 0.1 % benzene showed 100 % degradation in 30 and 40 days of incubation. In case of toluene treatment the degradation was enhanced after 10 days and reached 100 % in 20 days. Degradation of naphthalene reached at a maximum of 99.4 % for *A. migulans* BROD, *A. migulans* KTPP and *B. aneurinilyticus* after 40 days of incubation. Highest degradation (99.9 %) was observed when *B. thuringiensis* was treated with acenapthene during 40 days of incubation. All the bacterial isolates showed maximum activity when treated with naphthalene as the % degradation reached above 90 % in the first 10 days itself.

4.9 Heavy-metal resistance of strains

4.9.1 Heavy metal analysis of soil sediments by FAAS

Heavy metal analysis was carried out in the conditions as shown in Table 12.

Table 12: Experimental conditions in FAAS

Element	**Pd**	**Cd**	**Cr**	**Zn**	**Ni**	**Cu**	**Fe**	**Mn**	**Hg**
Wavelength (nm)	217	228.8	357.9	213.9	248.3	324.7	248.3	279.5	253.7
Slit (nm)	1.0	0.5	0.2	1.0	0.2	0.5	0.2	0.2	0.5
Mode	AA	AA	AA	AA	AA	AA	AA	AA	AA
Flame	Air-Ac	Air	Ac	Air	Ac	Air	Ac	Air	Ac
Burner Head	10 cm	10 cm	10 cm	10 cm	10 cm	10 cm	10 cm	10 cm	10 cm
Lamp current, mA	5 mA	4 mA	7 mA	5 mA	5 mA	4 mA	5 mA	5 mA	4 mA
Standards (mg/L)	2.5, 5.0, 10.0	0.25, 0.5, 1.0	1.25, 2.5, 5.0	0.2, 0.4, 0.8	1.5, 3.0. 6.0	1.0, 2.0, 4.0	1.25, 2.5 5.0	0.5, 1.0, 2.0	35, 70, 140
Air Flow (L/min)	17	17	17	17	17	17	17	17	17
Acetylene flow (L/min)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5

Table 13: Heavy metal analysis of soil sediments by FAAS

Heavy metals →	Lead	Cadmium	Chromium	Zinc	Nickel	Copper	Iron	Manganese	Mercury
Soil Samples ↓	Concentration in ppm								
SIPU	59.22	0.6	75.3	270	23.4	80.1	558	167.4	3840
BROD	32.58	0.3	24.6	144	37.8	35.1	576	162	2760
BSPOCC	44.28	0.75	45.3	180	12.9	68.7	660	171	2040
KTPP	59.04	1.35	6.9	480	19.5	200.7	654	156	1500
BSP	53.4	0.36	113.1	63	22.8	40.8	384	108	1080
APR	48.24	0.98	29.1	189	6.9	96.6	702	82.2	2280
RSP	56.52	0.82	52.8	60	41.7	53.1	372	116.4	1440
Permissible Limits → Reference: (Sources and Impacts of past, current and future contamination of soil. Nicholson and Chambers, 2007)	200	0.01-0.7	5-100	10-200	2-80	2-30	7000-550000	20-10000	0.01-0.5

Results show that the cadmium concentration in BSPOCC (0.75 ppm), KTPP (1.35 ppm), APR (0.98 ppm) and RSP (0.82 ppm) was slightly more than the permissible limits. Chromium concentration at 113.1 ppm was also slightly higher in sample BSP. The concentration of zinc was found to be more than permissible limits in samples SIPU (270 ppm) and KTPP (480 ppm). Concentrations of copper was high and that of mercury very high in all the seven samples.

4.9.2 Heavy metal resistance of identified bacterial species

Maximum tolerance was shown by *Bacillus thuringiensis* BSPOCC and *Aneurinibacillus migulanus* BROD against antimony (15 mM) and least tolerance was shown by four bacterial species except *B. cereus* BSP with MTC values of 0 mM for cadmium salts. *B.*

cereus showed least tolerance against Hg^{2+} (0.25 mM) but was able to tolerate 10 mM of Mn^{2+} . The results for *B. thuringiensis* showed maximum tolerance for antimony (15 mM) and least tolerance for Cadmium salt (No microbial growth was observed). *A. migulans* BROD resisted the antimony concentration as high as 15 m M and also showed good resistence against Mn (10 mM). *A. migulans* KTPP showed maximum activity against Mn (8 mM) whereas *A. aneurinilyticus* resisted 6 Mm of Pd, Zn, Mn and Sb (Table 14).

Table14: Maximum tolerated concentration (MTC) values of tested heavy metals

Strains ↓	**MTC values of heavy metals (mM)**										
	Lead Nitrate ($PbNO_3$)	**Cadmium Chloride ($CdCl_2$)**	**Copper Sulphate ($CuSO_4.5H_2O$)**	**Stannous Chloride ($SnCl_2.2H_2O$)**	**Zinc Sulphate ($ZnSO_4.7H_20$)**	**Potassium Dichromate ($K_2Cr_2O_7$)**	**Manganese Sulphate ($MnSO_4H_2O$)**	**Potassium Antimony Tartarate ($C_8H_4K_2O_{12}Sb_2.3H_2O$)**	**Nickle Sulphate ($NiSO_4.6H_2O$)**	**Mercuric Chloride ($HgCl_2$)**	**Ferrous Sulphate ($FeSO_4.7H_2O$**
Bacillus cereus strain BSP	6	1	4	4	6	3	10	6	2	0.25	4
Bacillus thuringiensis strain BSPOCC	6	0	4	4	6	2	10	15	1	0.25	4
Aneurinibacillus migulanus strain BROD	6	0	4	4	6	2	10	15	2	0.25	4
Aneurinibacillus migulanus strain KTPP	6	0	4	4	6	2	8	6	6	0.25	4
Aneurinibacillus aneurinilyticus strain RSP	6	0	4	4	6	2	6	6	2	0.25	4

4.10 Antibiotic resistance of strains

All the identified bacterial species showed resistance against penicillin G and cefuroxime and were susceptible to impenem, amoxyclav and tigecycline. B. cereus showed resistance or intermediate resistance against 6 out of 9 antibiotics and the other four (*B. thuringiensis*

BSPOCC, *A. migulans* BROD, *A. migulans* KTPP and *A. aneurinilyticus* RSP) were resistant only to 2 out of 9 antibiotics used (Table 15).

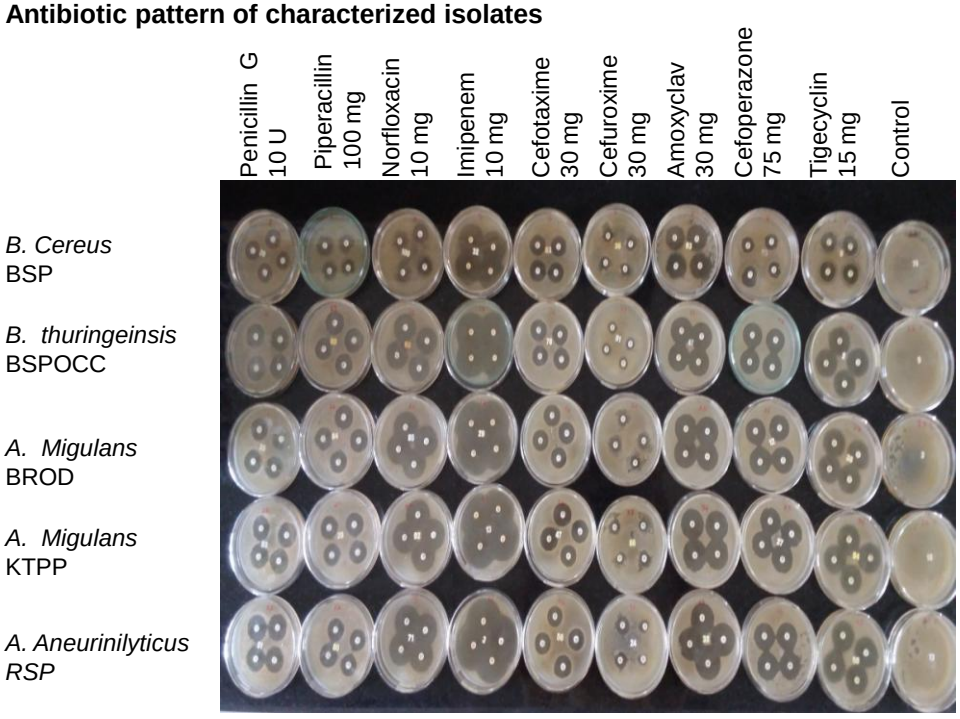

Fig: 47: Antibiotic sensitivity pattern of the bacterial species

Table 15: Susceptibility of identified strains against antibiotics

Antibiotic & concentration →	**Penicillin G**	**Piperacillin**	**Norfloxacin**	**Imipenem**	**Cefotaxime**
	10 units	**100 mcg**	**10 mcg**	**10 mcg**	**30 mcg**
Strains ↓	**The average diameter of the inhibition zones (mm)**				
Bacillus cereus strain BSP	(R) 18.3 ± 0.57	(R) 16.3 ± 0.58	(I) 16.3 ± 0.58	(S) 32 ± 0	(I) 18.6 ± 0.58
Bacillus thuringiensis strain BSPOCC	(R) 24 ± 0	(S) 23.6 ± 0.58	(S) 27.6 ± 0.58	(S) 38.3 ± 0.58	(I) 22 ± 1
Aneurinibacillus migulanus strain BROD	(R) 20.6 ± 0.58	(S) 23.3 ± 0.58	(S) 30.6 ± 0.58	(S) 43.3 ± 1.15	(I) 21.3 ± 0.58
Aneurinibacillus migulanus strain KTPP	(R) 21.6 ± 0.58	(S) 21.6 ± 0.58	(S) 28.6 ± 0.58	(S) 41.6 ± 0.58	(S) 24 ± 0
Aneurinibacillus aneurinilyticus strain RSP	® 25.3 ± 1.1	(S) 22 ± 0	(S) 30.3 ± 0.58	(S) 44 ± 0	(I) 20 ± 1

R- Resistant; S-Susceptible; I-Intermediate Resistance

Table 15 Cont.: Susceptibility of identified strains against antibiotics

Antibiotic & concentration →	**Cefuroxime**	**Amoxyclav**	**Cefoperazone**	**Tigecycline**
	30 mcg	**30 mcg**	**75 mcg**	**15 mcg**
Strains ↓	**The average diameter of the inhibition zones (mm)**			
Bacillus cereus strain BSP	(R) 10 ± 0	(S) 25.6 ± 0.58	(I) 15.6 ± 0.58	(S) 24.3 ± 1
Bacillus thuringiensis strain BSPOCC	(R) 10.3 ± 0.58	(S) 28.6 ± 0.58	(S) 25.6 ± 0.58	(S) 27.6 ± 0.58
Aneurinibacillus migulanus strain BROD	(R) 13.6 ± 0.58	(S) 30 ± 1	(S) 25.6 ± 0.58	(S) 28.1 ± 1.1
Aneurinibacillus migulanus strain KTPP	(R) 9.6 ± 0.58	(S) 30 ± 0	(S) 25.3 ± 1.1	(S) 29 ± 0.58
Aneurinibacillus aneurinilyticus strain RSP	(R) 11.7 ± 0.58	(S) 30 ± 1	(S) 25.6 ± 0.58	(S) 29.3 ± 0.58

Chapter 5

Discussion

In this study we aimed to obtain a strain collection of hydrocarbonoclastic bacteria, with an outstanding ability to degrade hydrocarbon pollutants and to resist elevated heavy metal concentrations at the same time, for further use in the development of a microbial soil inoculants for bioaugmentation purposes. Total ten PHC contaminated soil samples were collected from various locations in Chhattisgarh, India. To check the effect of contamination on different properties of soil, physicochemical analysis of the collected soil samples and one uncontaminated agricultural soil was carried out. PHC pollution exerts adverse effects on soil conditions, microorganisms and plants (Uche et al., 2011), leads to deterioration of soil structure, loss of organic matter contents, loss of soil mineral nutrients such as sodium, calcium, magnesium, nitrogen and sulphate, phosphate and nitrate (Akubugwo et al., 2009). There was no significant change in the pH of the contaminated and uncontaminated soil. When compared with pH of uncontaminated soil which was 7.7 the most alkaline were the soil samples from KTPP, RSP and BSP with pH values of 8.5. These results correlate with the findings of Margarita et al., (2014) and Efsun et al., (2015). All the samples were found to be slightly alkaline which is efficient for bioremediation (Vidali, 2001). As expected due to hydrocarbons from the petroleum, the organic carbon content in all the contaminated soil samples was significantly higher than normal soil. The nitrate and phosphate content of the soil samples was less than that of normal soil which has been reported by (Ahamefule et al., 2014). Lower concentration of nitrate and phosphate have been reported as limiting factors for the growth of microorganisms in PHC polluted environments (Rahman et al., 2002). The moisture content which determines the extent of water retention and aeration in the soil was also less in PHC contaminated soils as that of normal soil. These two properties are important for the growth of biotic components in the soil. Presence of PHC in the soil increases the soil hydrophobicity (Khamehchiyan et al., 2007, Bennett et al., 1993; Roy et al., 1999), reducing the water holding capacity of the soil (Osuji and Nwoye, 2007). Bundy et al. (2002) have also reported that nutrient balance (C and N), pH and moisture content of soil were usually affected as a result of contamination by hydrocarbons. The altered physico-

chemical properties of PHC contaminated soil makes it unfit for the growth of agricultural crops as well as the normal soil flora. Anoliefo and Vwioko (1995) observed that oil in soil created unsatisfactory conditions for plant growth, probably due to insufficient aeration of the soil. The soil temperature were slightly higher because of the components present in oil which absorbs light of both the visible and UV range (Yu et. al.,2006). Moreover the contaminants in soil form a dark coating which increases the subsurface soil temperature (Balk et. al., 2002).

Identification results of isolated hydrocarbon degrading strains suggested the dominance of the representatives of the Bacillus (Firmicutes) in all the samples. There have been fewer reports on the roles of *Bacillus cereus, Bacillus thuringiensis, Aneurinibacillus migulans and Aneurinibacillus aneurinilyticus* in hydrocarbon bioremediation although there are several reports of bioremediation of pollutants by the action of Bacillus sp. occurring in extreme environments. Sorkhoh et al. (1993) isolated 368 isolates belonging to the genus Bacillus from desert samples. In addition, Annweiller and co-workers (2000) described a *B. thermoleovorans* that degrades naphthalene at 60˚C. More recently, Ijah and Antai (2003) reported Bacillus sp. being the predominant isolates of all the hydrocarbon utilizing bacteria characterized from highly polluted soil samples. Nonetheless, the capacities that have been proven include survival on individual aliphatic and aromatic hydrocarbons (Verma et al. 2006; Ghazali et al. 2004; Das and Mukherjee, 2007), degradation of crude oil as well as oily sludge (Ijah and Antai, 2003).

The MPN count was always higher for heterotophic bacteria which were supplied with glucose as a sole source of carbon when compared to fuel degrader bacteria which were supplied with either benzene, toluene, naphthalene and acenapthene as sole source of carbon. MPN/gm was significantly lower in soil sediments as compared to the whole soil as most of the microbes were removed during the process of soil sedimentation. The presence of hydrocarbon further decreased the MPN/gm values in all soil samples. Nevertheless, microbial populations can be one of the measures for evaluating the level of petroleum contamination at these sites. The concentration of hydrocarbons is a major factor contributing to the number of microorganisms adapted to degrade that hydrocarbon. But

other factors like availability of different nutrients and terminal electro acceptors are also responsible for proper microbial growth (Leahy and Colwell. 1990). Similar studies have been done in gasoline contaminated aquifiers in the artic (Braddock and McCarthy, 1996)

From the degradation abilities obtained by quantification of residual hydrocarbons by HPLC and UPLC it is clear that the identified bacterial species are able to detoxify the hydrocarbons and present a suitable candidature for bioremediation programs. Very less reports were available which emphasize on microbial bioremediation of benzene, toluene, naphthalene and acenapthene when treated with either of the four identified bacterial species. Dolanchapa et al. 2016 have reported the degradation of acenapthene using two isolated micro-organism *Bacillus sp. PD5*. There has been reports of acenapthene degradation by other bacterial species as reported by Selinofov et al. 1993 and Komatsu et al. 1993. Napthalene degradation by Bacillus strains have been reported by Silva et al. (2009b). Similarly Lidija et al. (2011) have reported the bioremediation of toluene and naphthalene by B. cereus including three other bacterial species. Anaerobic degradation of benzene has been most widely studied (Anderson et al. 1998; Chakraborty and Coates, 2005; Da Silva and Alvarez, 2007) but fewer studies have been done on aerobic biodegradation of benzene.

Heavy metal resistance of strains was tested in nutrient broth containing different concentrations (0.25, 0.5, 1.0, 2.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 mM) of $CdCl_2$, $CuSO_4.5H_2O$, $Pb(NO_3)_2$, $ZnSO_4.7H_2O$, $MnSO_4.H_2O$, $C_8H_4K_2O_{12}Sb_2.3H_2O$, $SnCl_2.2H_2O$, $K_2Cr_2O_7$, $NiSO_4.6H_2O$ $HgCl_2$, $FeSO_4.7H_2O$ (HiMedia). Amongst the tested heavy metals Pb^{2+} , Cu^{2+} , $Mn^{2+,}$ Sb^{3+}, Sn^{2+} and Zn^{2+} were the most tolerated by the tested strains, and the upper limits of tolerance were rallied around a wide range of values (Sn^{2+} - 4 mM to Sb^{3+} - 15 mM). As the concentrations of some metals like Zn, Cu, Cd, Cr and Hg were found higher than the permissible limits, it can be said that these bacterial species have developed a mechanism to tolerate abnormal metal concentrations. The presence of Cd^{2+} was very toxic to all the isolates except B. cereus BSP which tolerated 1mM concentration. The presence of Hg^{2+} had an inhibitory effect on all the isolates with tolerance limit of only 0.25 mM inspite of high concentrations found in FAAS analysis. This is in contrast with

other findings that a microbial population living in heavy metal polluted site should have the ability to tolerate high metal concentrations (Chiu et al., 2007). Outstanding ability of *Bacillus* species in remediation of heavy metals has been demonstrated in various studies (Ferdag et al., 2011; Ersoy et al., 2009; Othman and Thoufeek 2015; Yogendra et al., 2013). Among the various possibilities of metal–microbe interactions (viz. bioaccumulation/ biosorption, enzymatic/extracellular transformations, etc.) that determine environmental fate of toxic cations, metal sequestration by bacterial cells is of great importance for the development of microbe based remediation strategies (Lloyd and Macaskie, 2000). *B. cereus* showed resistance / intermediate resistance against 6 of the 9 antibiotics whereas rest of the isolates showed resistance only against 2 antibiotics (Penicillin and Cefuroxime). Multiple resistance against heavy metals and antibiotics of *B. cereus* isolates has also been reported by Singh et al. (2010). Since several genes responsible for degradation of aromatics and for heavy metal/antibiotic resistances are located on plasmids which are key vectors of horizontal gene transfer, the members of the bacterial community gained opportunity to expand their chromosome encoded resistance and catabolic potential with those encoded on plasmids. This phenomenon may explain the strong correlation among hydrocarbon degradation ability and heavy metal/antibiotic tolerance among strains. In case of strains isolated from the solely PHC impacted sample the lack of correlation among foregoing capabilities might be linked to the lack or low rate of transmission of mobile genetic elements. In the absence of a strong driving force (e.g. presence of heavy metals or antibiotics) the endogenous micro biota is not actuated for the exchange of resistance carrying plasmids. Our findings justify the above statement that in the absence of antibiotic driving force in the environment around soil sample collected caused the partial gene transfer for heavy metal resistance only and not for antibiotic resistance. Furthermore, as several biodegradative pathways are located also on mobile genetic elements, a long term exposure to heavy metals/antibiotics may be linked to the widespread distribution of biodegradative capabilities as well (Roy et al., 2002). Nevertheless, the lack or complete/partial loss of these transmissible genetic segments may lead to the reduced degradative functions, as well as to the loss of multiple resistances (Amábile-Cuevas et al., 1991; Marqués and Ramos, 1993).

CHAPTER 6
SUMMARY AND CONCLUSION

This thesis presents a study of the bacteria bioremediation characters and their heavy metal and antibiotic tolerance from the petroleum contaminated soil located in industrialized areas of Chhattisgarh, India. Soil sediments were characterized by FTIR for the presence of aromatic and aliphatic compounds and were also characterized for heavy metal contamination by FAAS. Further soil sediments were analyzed for heterotrophic and fuel degrader counts by ELISA micro plate technique using tetrazolium violet as indicator for microbial respiration. Most potent strains characterized were predominantly from the Bacillus species which showed mixed resistance patterns against antibiotics and heavy metals. All the bacterial cultures when treated with 0.1 % benzene showed 100 % degradation in 30 and 40 days of incubation. In case of toluene treatment the degradation was enhanced after 10 days and reached 100 % in 20 days. Degradation of naphthalene reached at a maximum of 99.4 % for *A. migulans* BROD, *A. migulans* KTPP and *B. aneurinilyticus* after 40 days of incubation. Highest degradation (99.9 %) was observed when *B. thuringiensis* was treated with acenapthene during 40 days of incubation. All the bacterial isolates showed maximum activity when treated with naphthalene as the % degradation reached above 90 % in the first 10 days itself. *Bacillus cereus* strain BSP showed resistance against most heavy metals and also was the second most active bacteria to degrade the selected hydrocarbons. All the isolates except *Bacillus cereus* were resistance only to Penicillin G and Cefuroxime and sensitive to others which shows that the transfer of antibiotic resistance genes has not taken place.

CHAPTER 8

FUTURE PROSPECTS

1. Metal resistant microbes can be effectively used in detoxification or cleaning-up of the metal from that environment.

2. Cometabolism of heavy metal as well as hydrocarbons can be studied at molecular lever which can help to design an effective bioaugmentation process.

3. Biodegradation can be enhanced in the contaminated area by implementing other nutrients which can lead to more efficient bacterial growth

CHAPTER 7
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Appendix 1

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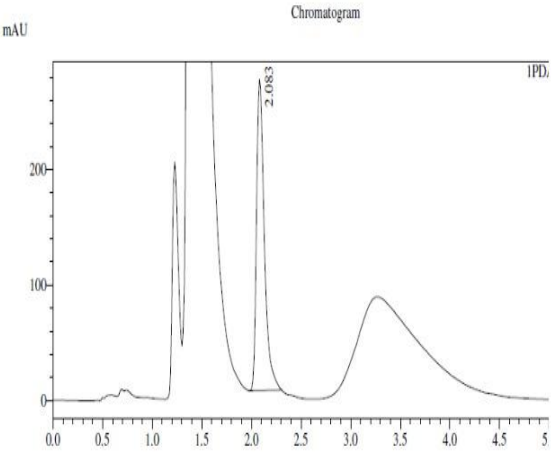

<Peak Table>

PDA Ch1 227nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
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Total		1478236	270052			

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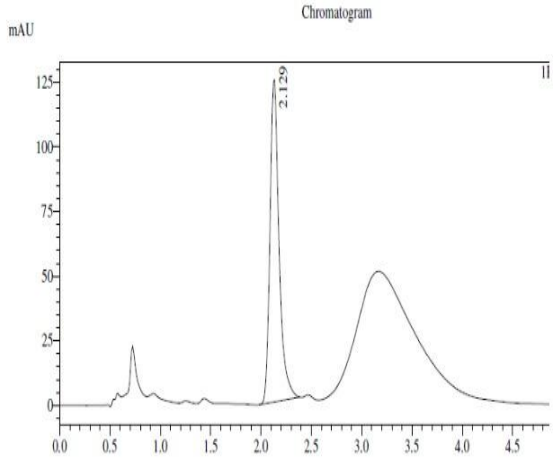

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PDA Ch1 227nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
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Total		781768	124595			

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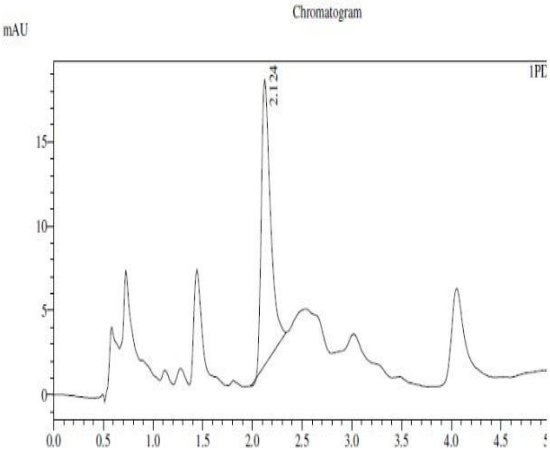

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PDA Ch1 227nm

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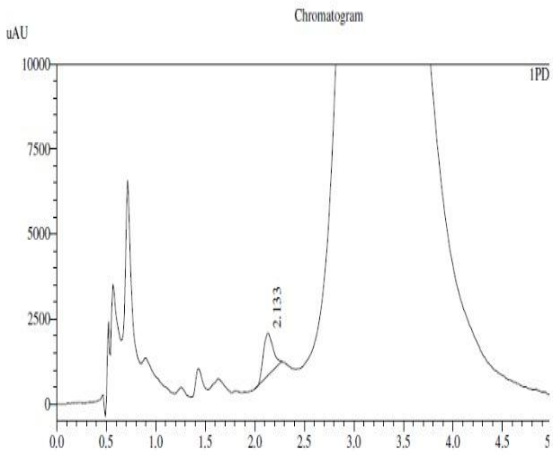

<Peak Table>

PDA Ch1 227nm

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1	2.133	8613	1244	0.000		M
Total		8613	1244			

Fig: UPLC Chromatograms for 10, 20, 30 and 40 days when B. cereus BSP was treated with Acenapthene (200ppm)

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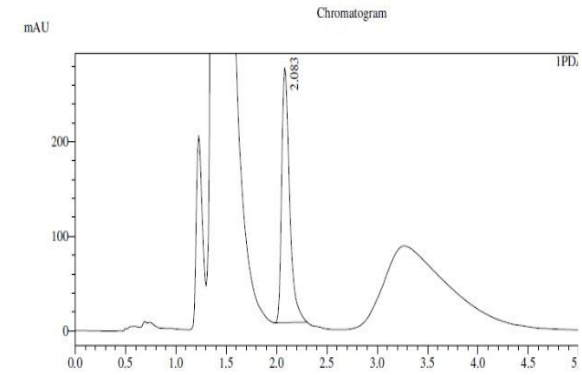

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Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
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Total		1478236	270052			

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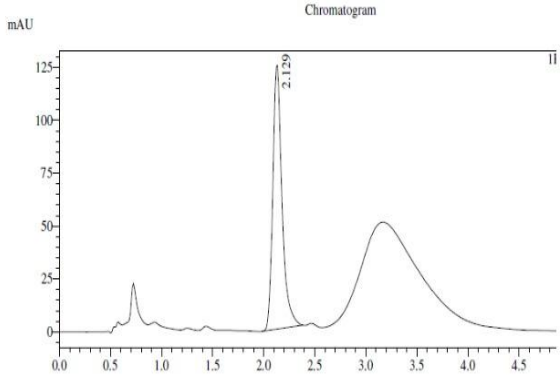

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PDA Ch1 227nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
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Total		781768	124595			

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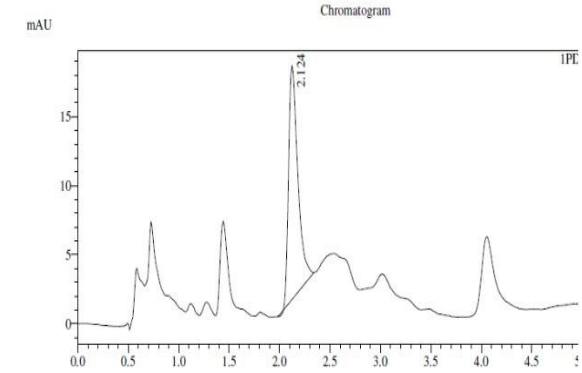

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Total		109234	16966			

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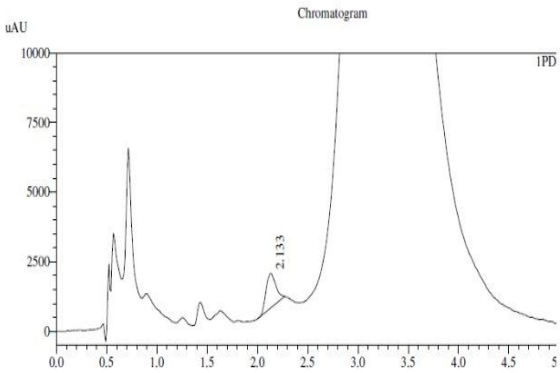

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PDA Ch1 227nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	2.133	8613	1244	0.000		M
Total		8613	1244			

Fig: UPLC Chromatograms for 10, 20, 30 and 40 days when B. thuringiensis BSPOCC was treated with Acenapthene (200ppm)

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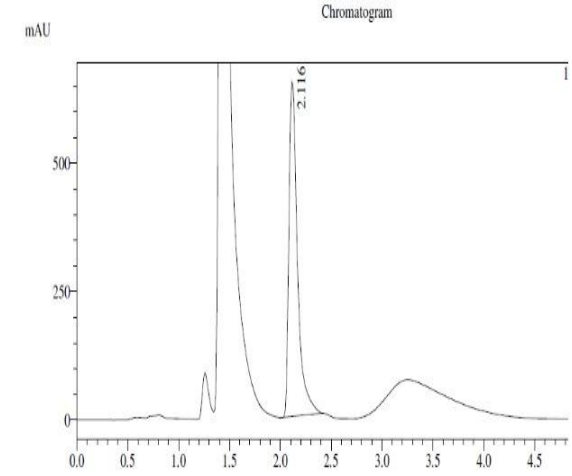

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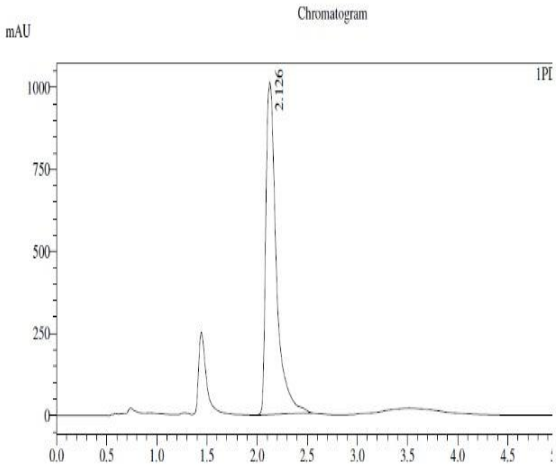

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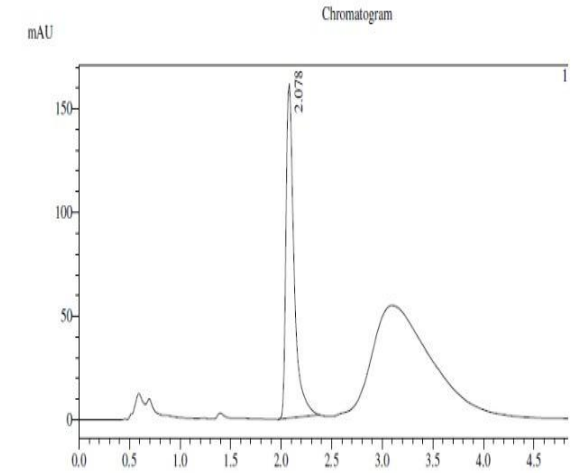

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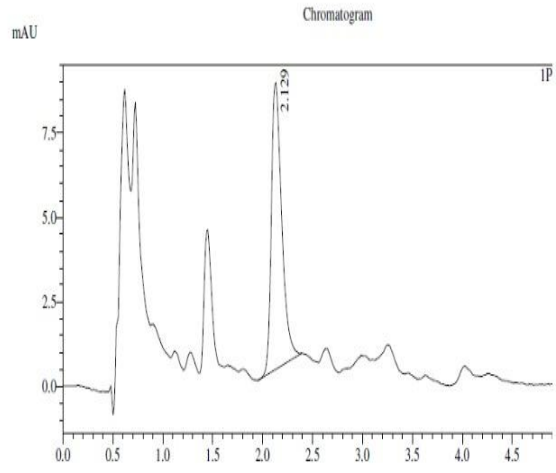

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PDA Ch1 227nm

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Fig: UPLC Chromatograms for 10, 20, 30 and 40 days when A. migulans BROD was treated with Acenapthene (200ppm)

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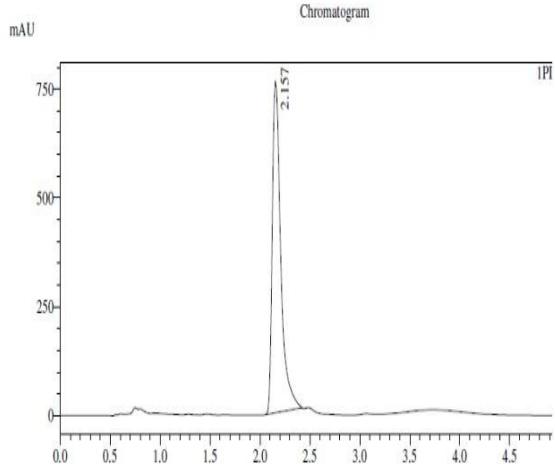

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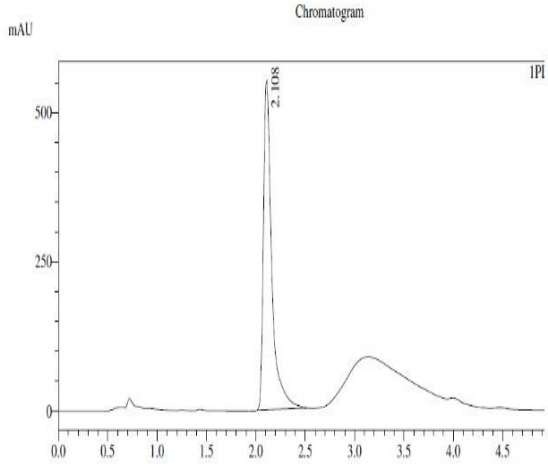

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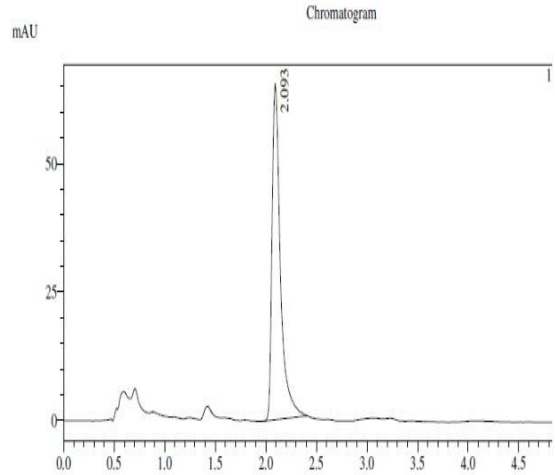

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PDA Ch1 227nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
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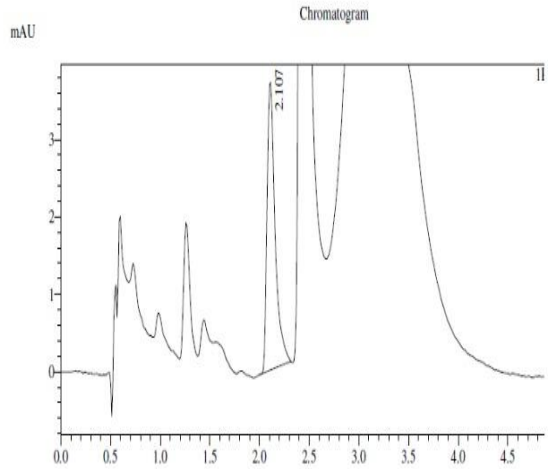

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PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	2.107	21020	3715	0.000		M
Total		21020	3715			

Fig: UPLC Chromatograms for 10, 20, 30 and 40 days when A. migulans KTPP was treated with Acenapthene (200ppm)

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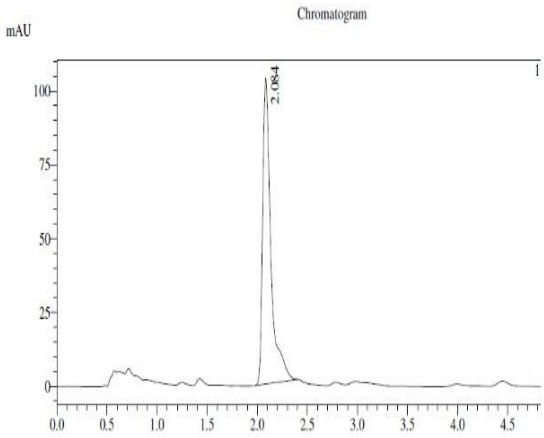

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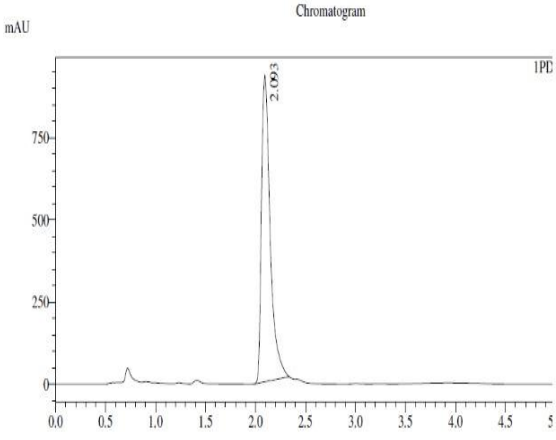

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PDA Ch1 221nm

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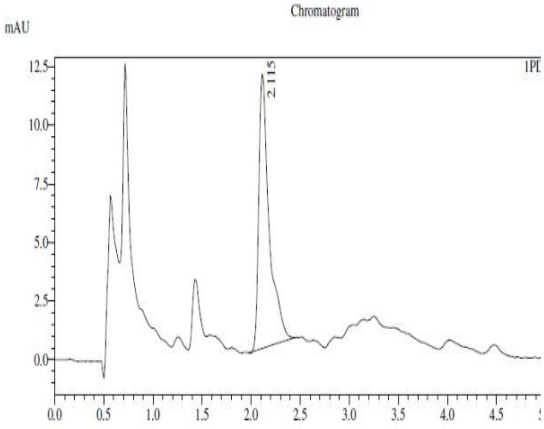

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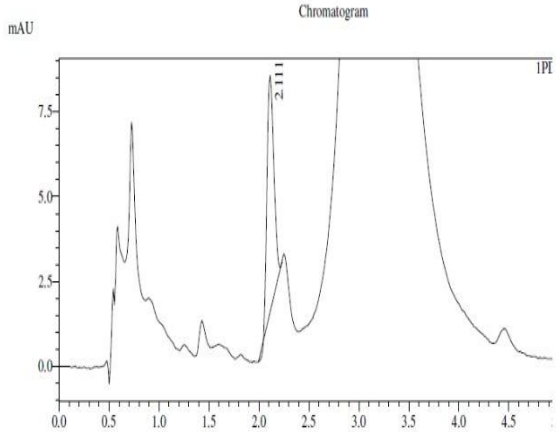

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PDA Ch1 227nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	2.111	33728	6977	0.000		M
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Fig: UPLC Chromatograms for 10, 20, 30 and 40 days when A. aneurinilyticus RSP was treated with Acenapthene (200ppm)

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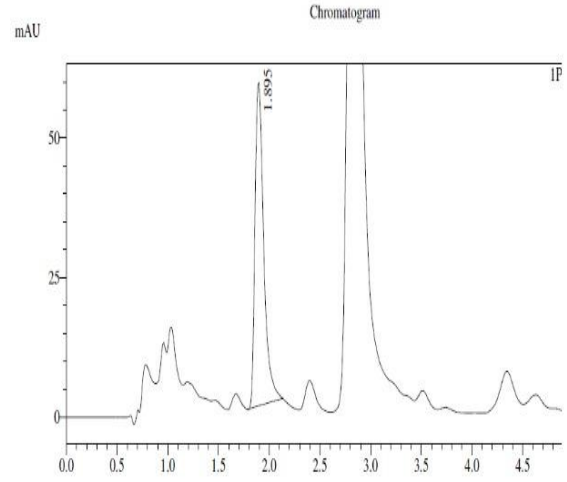

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Total		347269	57801			

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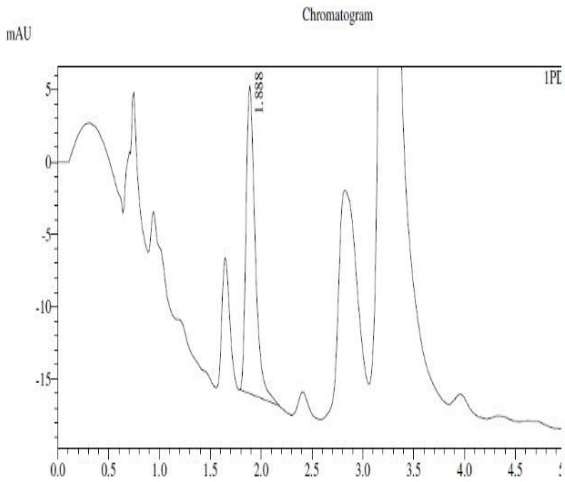

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Total		132554	21301			

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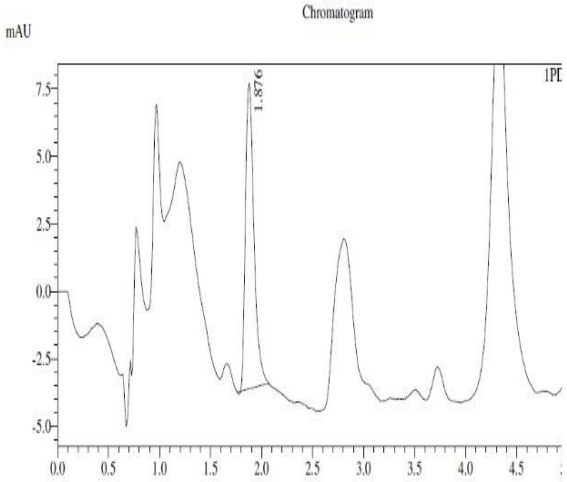

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PDA Ch1 221nm

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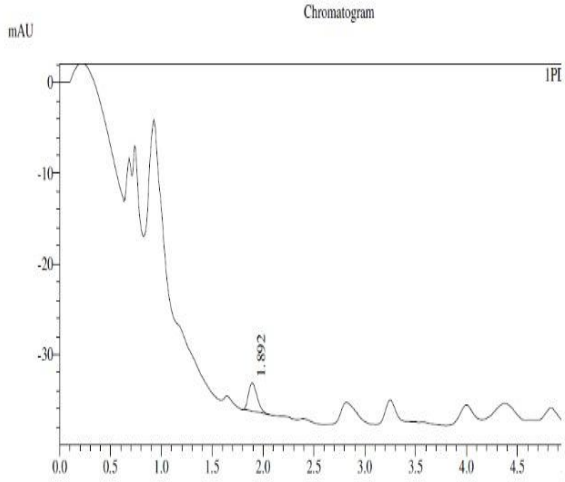

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PDA Ch1 221nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	1.892	18136	3088	0.000		M
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Fig: UPLC Chromatograms for 10, 20, 30 and 40 days when B. cereus BSP was treated with Napthalene (200ppm)

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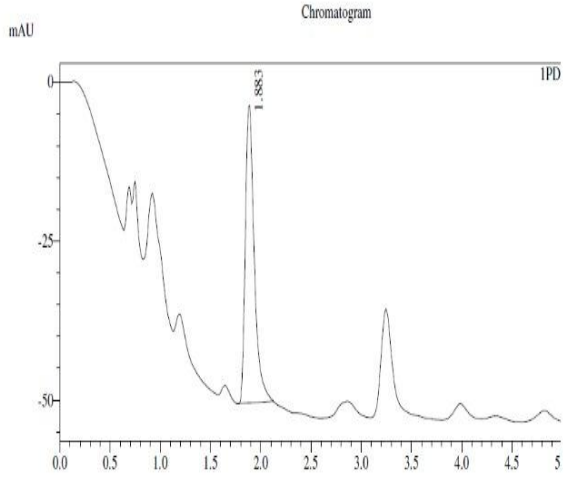

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PDA Ch1 221nm

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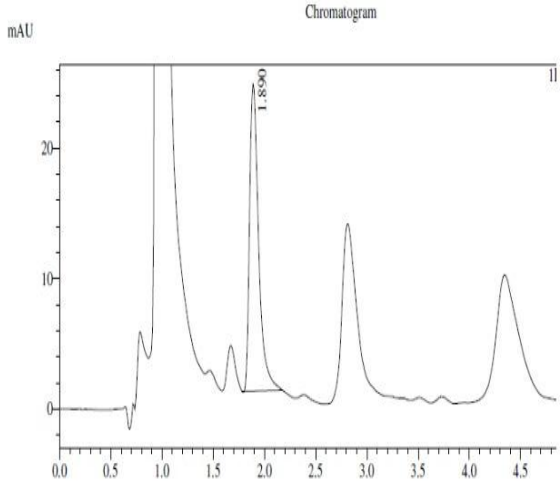

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PDA Ch1 221nm

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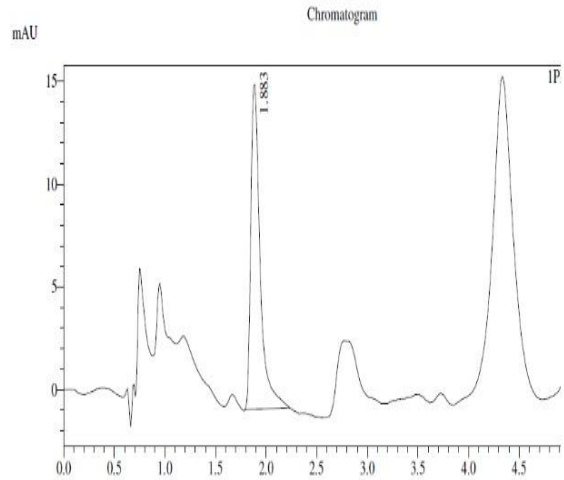

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PDA Ch1 221nm

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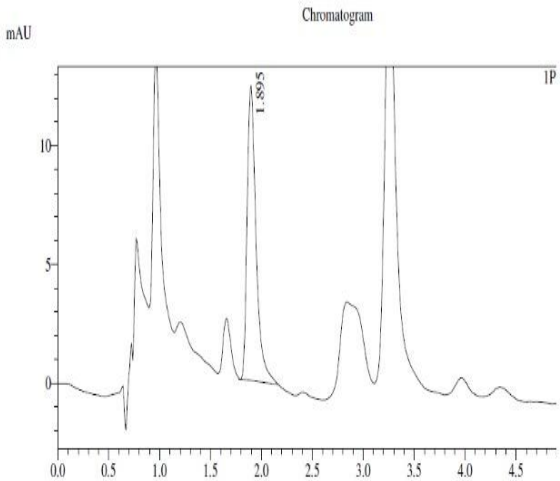

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PDA Ch1 221nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	1.895	75541	12414	0.000		M
Total		75541	12414			

Fig: UPLC Chromatograms for 10, 20, 30 and 40 days when B. thuringiensis BSPOCC was treated with Napthalene (200ppm)

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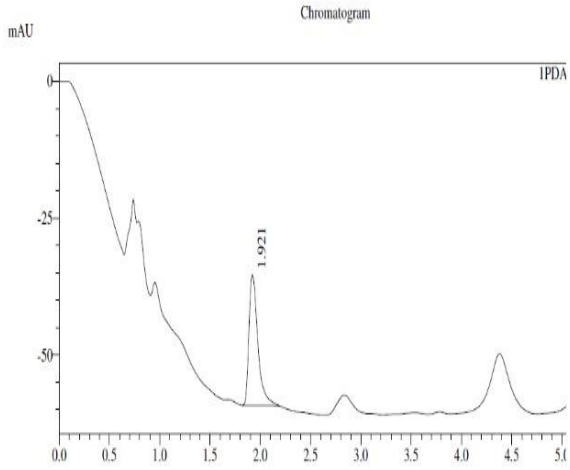

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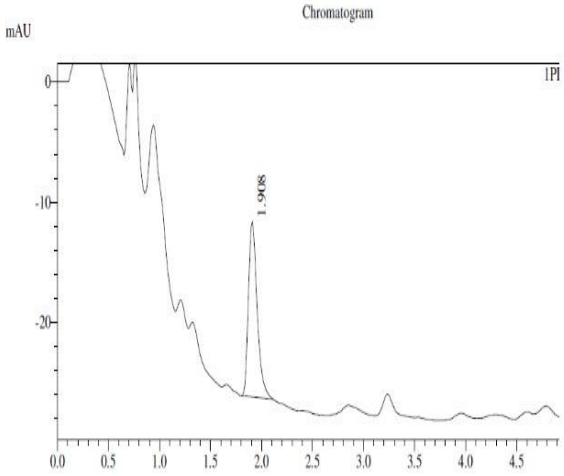
PDA Ch1 221nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	N
1	1.921	147823	23954	0.000		M	
Total		147823	23954				

<Chromatogram>

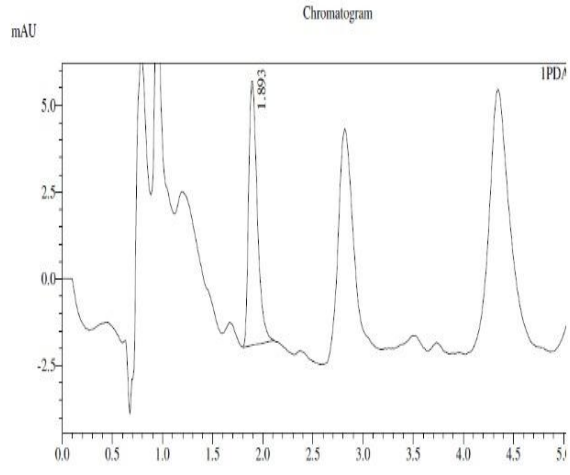


<Peak Table>

PDA Ch1 221nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	
1	1.908	87155	14579	0.000		M	
Total		87155	14579				

<Chromatogram>

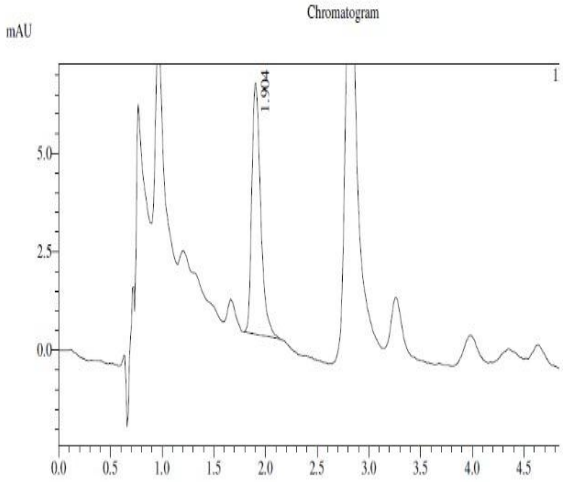


<Peak Table>

PDA Ch1 221nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	N
1	1.893	45711	7609	0.000		M	
Total		45711	7609				

<Chromatogram>



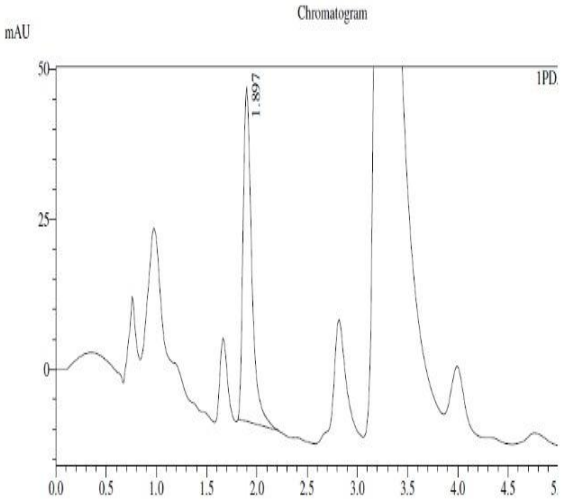
<Peak Table>

PDA Ch1 221nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	
1	1.904	38097	6381	0.000		M	
Total		38097	6381				

Fig: UPLC Chromatograms for 10, 20, 30 and 40 days when A. migulans BROD was treated with Napthalene (200ppm)

<Chromatogram>

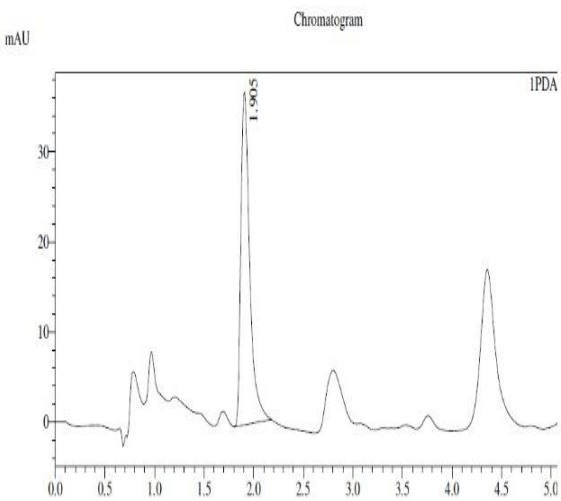


<Peak Table>

PDA Ch1 221nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	1.897	333375	55746	0.000		M
Total		333375	55746			

<Chromatogram>

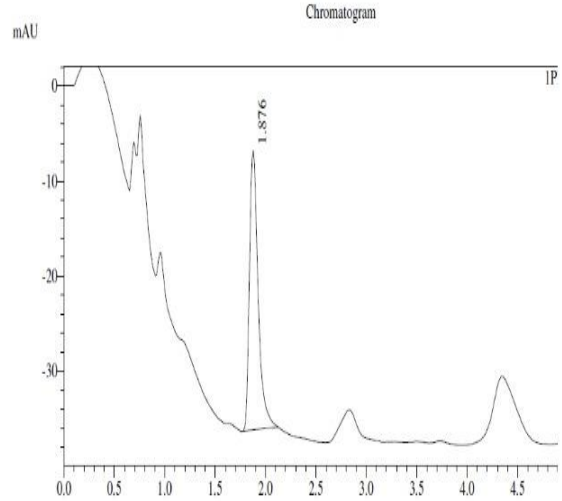


<Peak Table>

PDA Ch1 221nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	1.905	229195	36876	0.000		M
Total		229195	36876			

<Chromatogram>

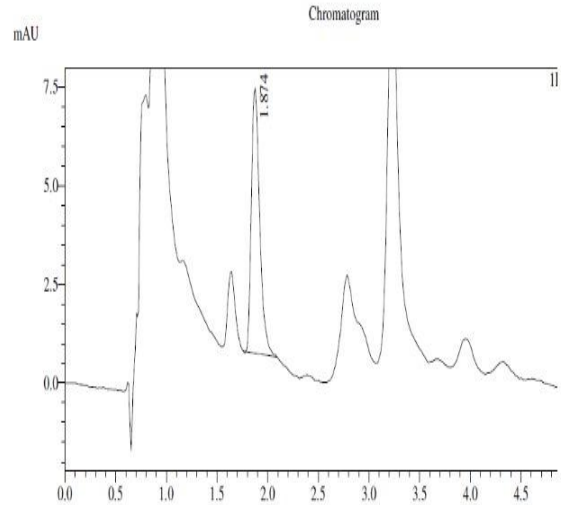


<Peak Table>

PDA Ch1 221nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	1.876	177156	29454	0.000		M
Total		177156	29454			

<Chromatogram>



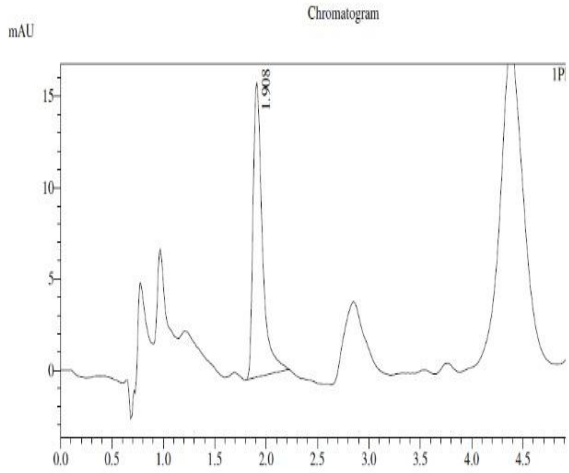
<Peak Table>

PDA Ch1 221nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	1.874	38336	6710	0.000		M
Total		38336	6710			

Fig: UPLC Chromatograms for 10, 20, 30 and 40 days when A. migulans KTPP was treated with Napthalene (200ppm)

<Chromatogram>

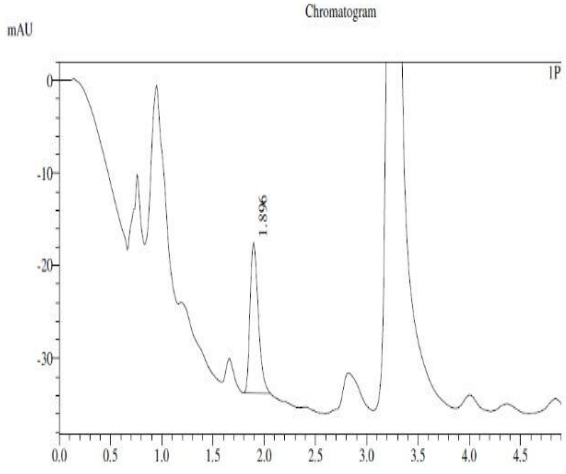


<Peak Table>

PDA Ch1 221nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	1.908	102458	16145	0.000		M
Total		102458	16145			

<Chromatogram>

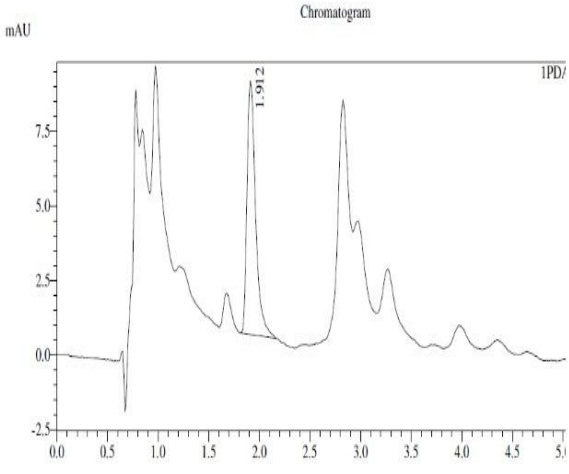


<Peak Table>

PDA Ch1 221nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	1.896	90755	16225	0.000		M
Total		90755	16225			

<Chromatogram>

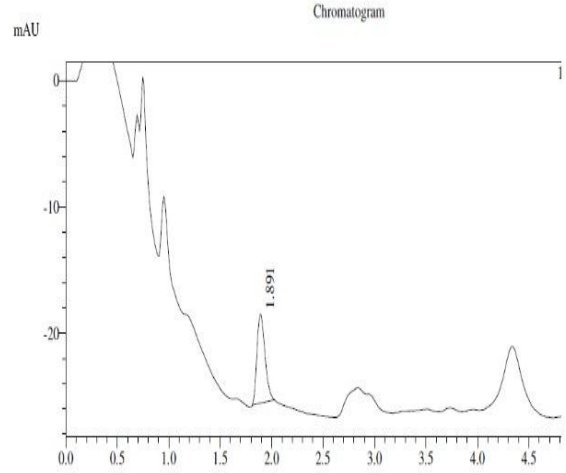


<Peak Table>

PDA Ch1 221nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	N
1	1.912	50046	8508	0.000		M	
Total		50046	8508				

<Chromatogram>

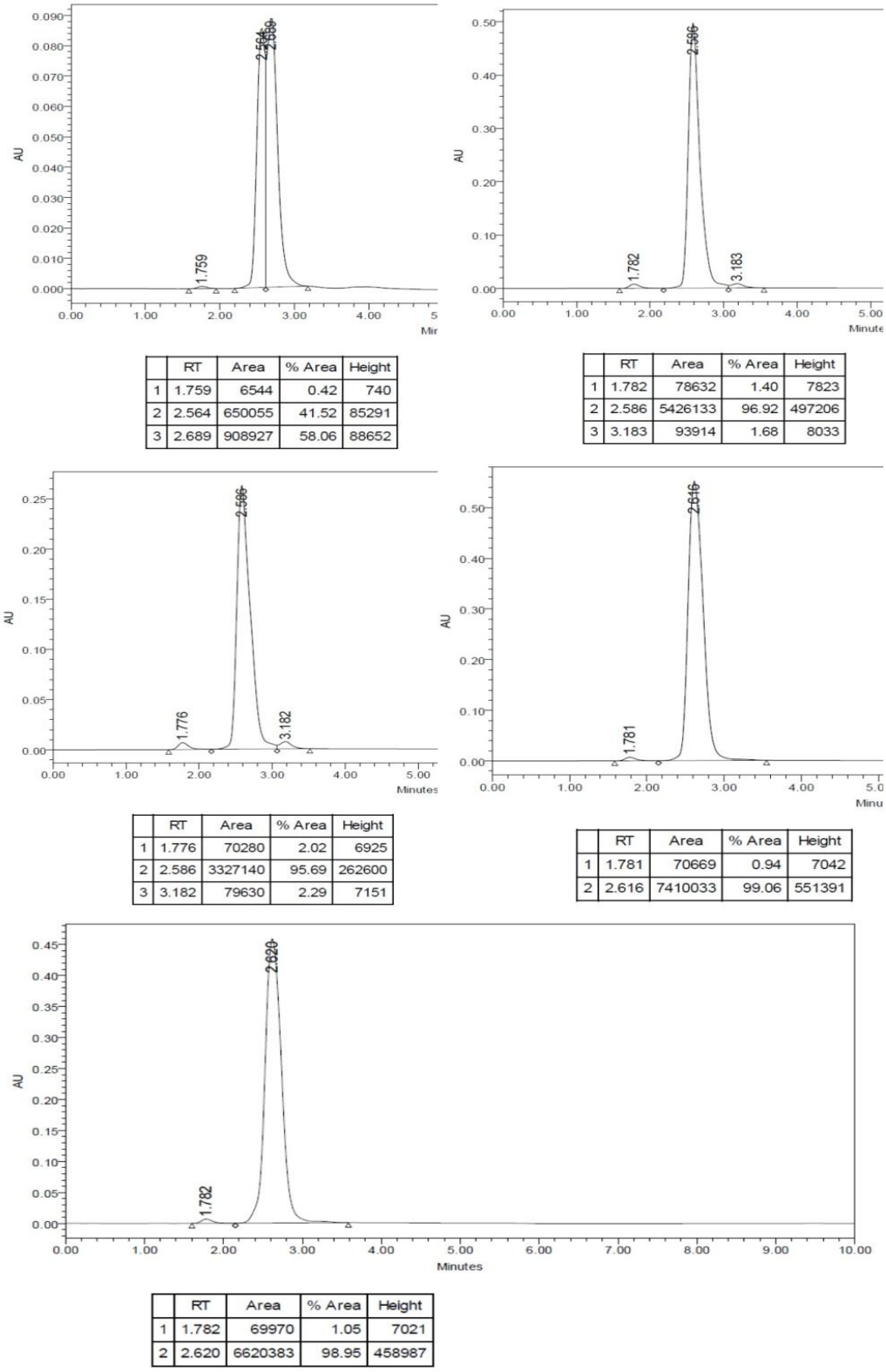


<Peak Table>

PDA Ch1 221nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	1.891	38455	7056	0.000		M
Total		38455	7056			

Fig: UPLC Chromatograms for 10, 20, 30 and 40 days when A. aneurinilyticus RSP was treated with Napthalene (200ppm)



	RT	Area	% Area	Height
1	1.759	6544	0.42	740
2	2.564	650055	41.52	85291
3	2.689	908927	58.06	88652

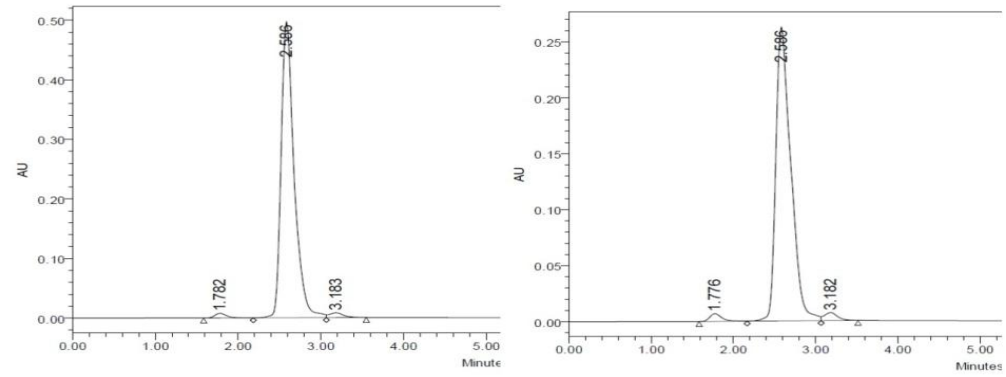
	RT	Area	% Area	Height
1	1.782	78632	1.40	7823
2	2.586	5426133	96.92	497206
3	3.183	93914	1.68	8033

	RT	Area	% Area	Height
1	1.776	70280	2.02	6925
2	2.586	3327140	95.69	262600
3	3.182	79630	2.29	7151

	RT	Area	% Area	Height
1	1.781	70669	0.94	7042
2	2.616	7410033	99.06	551391

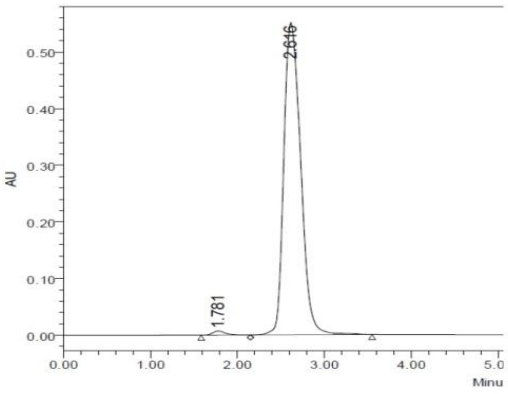
	RT	Area	% Area	Height
1	1.782	69970	1.05	7021
2	2.620	6620383	98.95	458987

Fig: HPLC chromatograms for 10 days when all the five bacterial species were treated with 0.1 % benzene

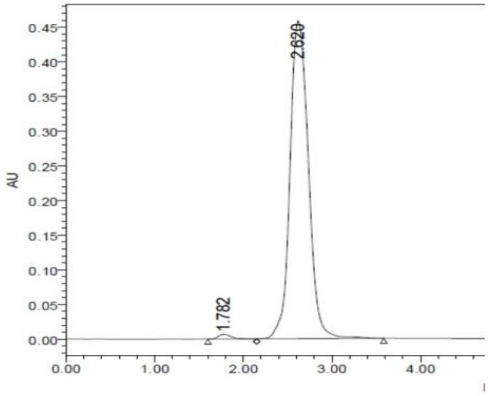


	RT	Area	% Area	Height
1	1.782	78632	1.40	7823
2	2.586	5426133	96.92	497206
3	3.183	93914	1.68	8033

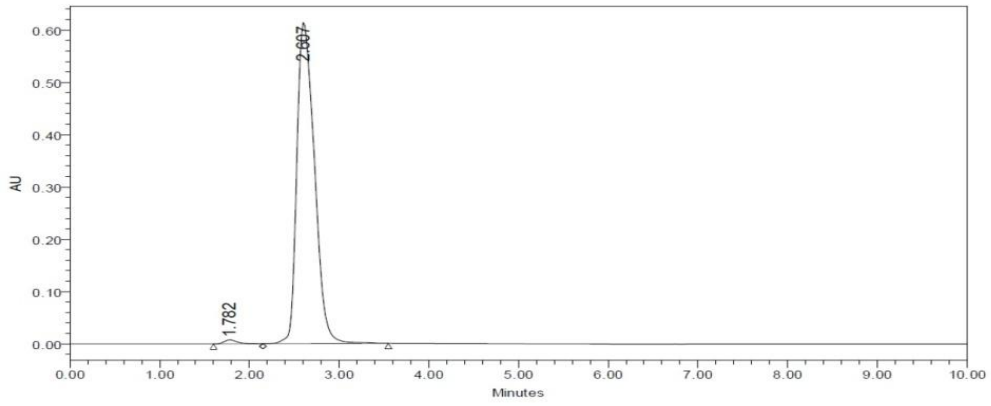
	RT	Area	% Area	Height
1	1.776	70280	2.02	6925
2	2.586	3327140	95.69	262600
3	3.182	79630	2.29	7151



	RT	Area	% Area	Height
1	1.781	70669	0.94	7042
2	2.616	7410033	99.06	551391

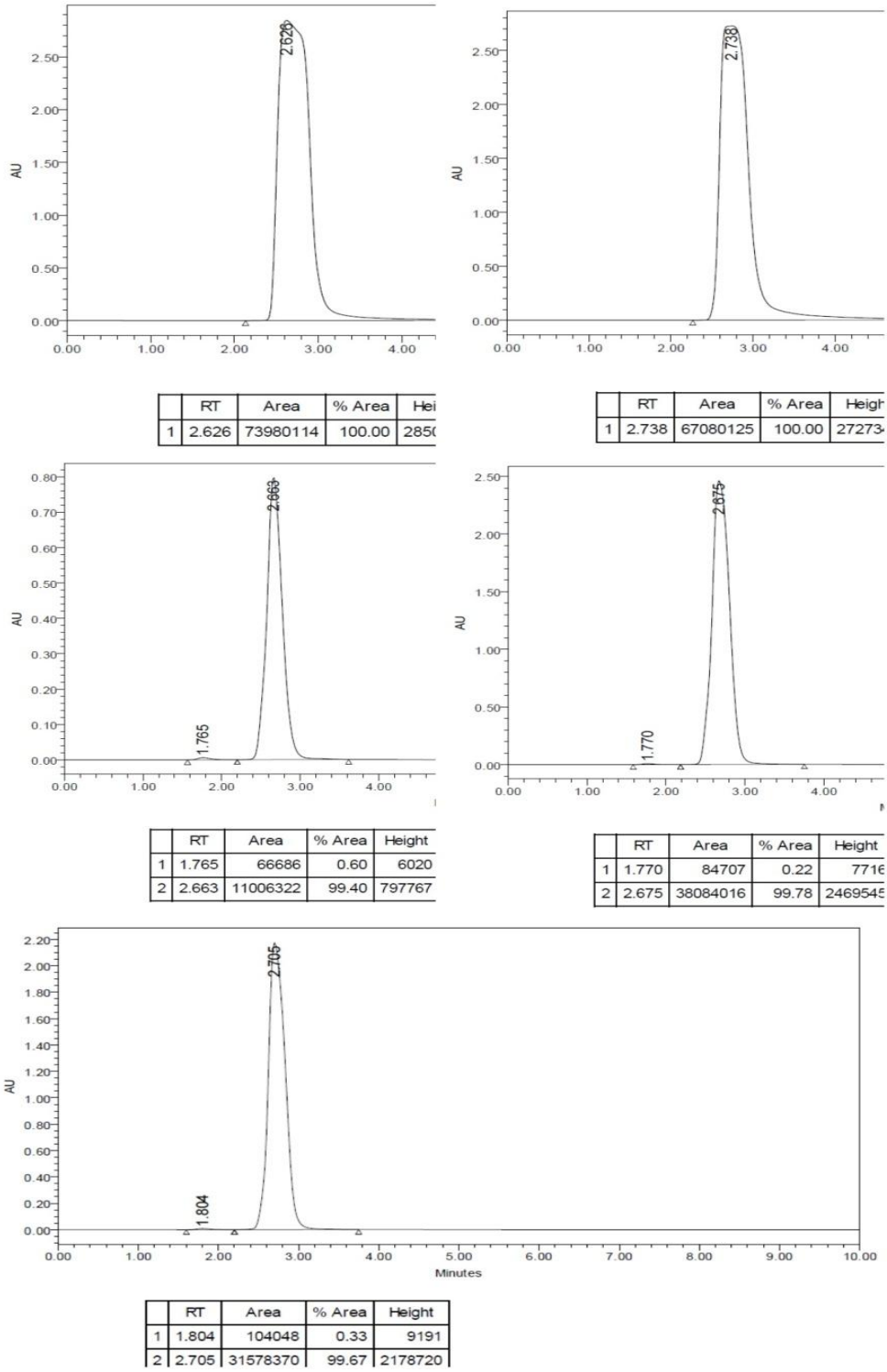


	RT	Area	% Area	Height
1	1.782	69970	1.05	7021
2	2.620	6620383	98.95	458987



	RT	Area	% Area	Height
1	1.782	75410	0.89	7428
2	2.607	8365363	99.11	615662

Fig: HPLC chromatograms for 20 days when all the five bacterial species were treated with 0.1 % benzene



	RT	Area	% Area	Hei
1	2.626	73980114	100.00	285(

	RT	Area	% Area	Heigh
1	2.738	67080125	100.00	27273

	RT	Area	% Area	Height
1	1.765	66686	0.60	6020
2	2.663	11006322	99.40	797767

	RT	Area	% Area	Height
1	1.770	84707	0.22	7716
2	2.675	38084016	99.78	2469545

	RT	Area	% Area	Height
1	1.804	104048	0.33	9191
2	2.705	31578370	99.67	2178720

Fig: HPLC chromatograms for 10 days when all the five bacterial species were treated with 0.1 % toluene

Appendix 2

List of Publications

S.No.	Title of the paper	Name of Journal
1	Antibiotic and Heavy Metal Tolerance of Some Indigenous Bacteria Isolated From Petroleum Contaminated Soil Sediments with A Study of Their Aromatic Hydrocarbon Degradation Potential	Int. J. Curr. Microbiol. App. Sci. (Accepted)
2	Xenobiotic Compounds Present in Soil and Water: A Review on Remediation Strategies	J. Of Environ. and Analytical Toxicology
3	Hydrocarbon Bioremediation Efficiency by five Indigenous Bacterial Strains isolated from Contaminated Soils.	Int. J. Curr. Microbiol. App. Sci.
4	Biodegradation of One Ring Hydrocarbons (Benzene and Toluene) and Two Ring Hydrocarbons (Acenapthene and Napthalene) by Bacterial Isolates of Hydrocarbon Contaminated Sites Located in Chhattisgarh: A Preliminary Study.	J. Pet. Environ. Biotechnol.
5	Growth Potential Assessment of Actinomycetes Isolated from Petroleum Contaminated Soil.	J Bioremed. Biodeg.
6	Advances in Molecular Biology Approaches to Guage Microbial Communities and Bioremediation at Contaminated Sites.	Int. J. Bioreme. Biodeg.
7	Biodegradation of keratin from chicken feathers by fungal species as a means of sustainable development.	J Bioremed. Biodeg.