"Degradation and Characterization of HMW polycyclic aromatic compounds (Anthracene, Coronene and Nepthacene) by indigenous bacterial strains from hydrocarbon contaminated (Oil Refineries) sites"

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DOCTOR OF PHILOSOPHY
IN
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Under the guidance of

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2017

Dedication

To
The Spirit of my
Parent

DEPARTMENT OF BIOTECHNOLOGY
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CERTIFICATE

This is to certify that the research work, reported in this thesis entitled ***"Degradation and Characterization of HMW polycyclic aromatic compounds (Anthracene, Coronene and Nepthacene) by indigenous bacterial strains from hydrocarbon contaminated (Oil Refineries) sites"*** is an original work, unless otherwise stated, carried out by **Mr. Sudhir K Shekhar** under my constant supervision in the Department of Biotechnology, Babasaheb BhimraoAmbedkar University, Lucknow – 226025, Uttar Pradesh, India.

It is further certified that Mr. Sudhir K Shekhar has fulfilled all requirements laid down by the BBA University, Lucknow for the submission of thesis for the award of the degree of Doctor of Philosophy in accordance with UGC rules and regulations - 2009.

Place: Lucknow

Dinesh Raj Modi
(Supervisor)

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CANDIDATE'S DECLARATION

I Sudhir K Shekhar, Ph D Scholar, Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow hereby declare that my research work incorporated in the dissertation entitled ***"Degradation and Characterization of HMW polycyclic aromatic compounds (Anthracene, Coronene and Nepthacene) by indigenous bacterial strains from hydrocarbon contaminated (Oil Refineries) sites"*** is an authentic research work carried by me in the Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow under the direct guidance and supervision of Dr. D. R. Modi, Associate Professor, Department of Biotechnology, BBAU, Lucknow. The research work is original and no part of this work has been submitted for any other degree or diploma. All the given information is true to best of my knowledge.

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Certificate from the Supervisior

This is to certify that above statement made by the candidate is correct to the best of my knowledge.

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Sudhir K Shekhar

ABBREVIATIONS

ABTS: 2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
ATP: Adenosine 5’triphosphate
ANT: Anthracene
bp: basepair
CFU: Colony Forming Unit
CHR: Chrysene
COD: Chemical oxidation process
DMAB: 3-Dimethylaminobenzoic acid
DNA: deoxyribonucleic acid
Fig: figure
FLT: Fluoranthene
HMW: High molecular weight PAH
HPLC: High performance liquid chromatography
kb: kilobase(s)
Km: Michaelis constant
LMW PAH: Low molecular weight PAH
LSD: least significant data difference
mRNA: messenger RNA
min: minutes
NOM: Natural Organic matter
OP: Oxidation process
OD: optical density
PAH: Polycyclic aromatic hydrocarbons
PHE: Phenanthrene
PCR: polymerase chain reaction
PYR: Pyrene
RNA: ribonucleic acid
SOM: Soil Organic matter
SDS: sodium dodecyl sulphate
SDW: sterile distilled water
WRF: white rot fungi
WHC: water holding capacity
BTEX Benzene, Toluene, Ethyl-benzene, Xylene
COD: Chemical Oxygen Demand
DGGE: Denaturing Gradient Gel Electrophoresis
GC: Gas Chromatography
HC: Hydrocarbon
NRC: National Research Council
OC: Oxygen Consumption
SO: Sediment Oil
ANOVA: ANalysis Of Variance
API: American Petroleum Institute
BS: Biosurfactants
CMC: Critical Micelle Concentration
CTAB: Cetyltrimethylammonium Bromide
DO: Dissolved Oxygen
FTIR: Fourier Transform Infrared spectroscopy
HPCL: Hindustan Petroleum Corporation Limited
MSM: Minimal Salts Media
SEM: Scanning Electron Microscope
ST: Surface Tension
TPH: Total Petroleum Hydrocarbon
%: Percent
BaP: Benzo[a]pyrene
BSM: Basal Salts Medium
^{o}C: Degree Celsius
cfu: Colony Forming Unit
DCM: Dichloromethane
dNTP: Deoxynucleotide triphosphate
EDTA: Ethylenediaminetetra-acetic Acid
EPA: Environment Protection Authority (Australia)
FID: Flame Ionisation Detector
g: Grams
HgCl2: Mercuric Chloride
kg: Kilogram
l: Litre
LB: Luria-Bertani
MW: Molecular Weight
Mg: Milligram
mM: Millimolar
ml: Millilitre
MPN: Most Probable Number
NA: Nutrient Agar
NB: Nutrient Broth
nm: Nanometre
pH: Hydrogen Ion Concentration
rpm: Revolutions per Minute
Tris: Tris-(hydroxymethyl) aminoethane
USEPA: United States Environment Protection Authority
v/v: volume per volume
w/v: weight per volume
Ace: Acenaphthene.
Anth: Anthracene.
B-aAnth: Benzo-a-anthracene.
HDB: Hydrocarbon Degrading Bacteria.
UPLC: Ultra Performance Liquid Chromatography.
Mg/L: Milligram per liter.
Naph: Naphthalene.
NCBI: National Center for Biotechnology Information.
Phen: Phenanthrene.
Pyr:Pyrene

SUMMARY

Soil polluted with petroleum oil from Mathura and Baruni Oil Refinery, India were used for isolation of indigenous microbial communities. These microbial communities were grown on three polycyclic aromatic hydrocarbon compounds were identified by 16S-rRNA, morphology by SEM and biochemically. Five identified isolates *VIZ* (*Bacillus licheniformis* strain MBOR, *BacteriumCT* BORL, *Bacillus pumilus* BORS, *Paenibacillus lautus* MORL and *Bacillus acidovirans* MORS) having accession no. KX376312, KX376311, KX376310 KX376309 and KX376308 were submitted to NCBI. All five screened isolates were capable to grow on different concentrations of three PAHs (Anthracene 50mg/l, Coronene20mg/l and Napthacene50mg/l) at temperature 30^0C-35^0C and pH 6.5-7.5. The best degraders' bacterial isolates *Bacillus licheniformis* MBOR and *Paenibacillus lautus* MORL were shown maximum degradation at concentration 50mg/l of anthracene, napthacene and 20mg/l of coronene. The most promising bacterial strain were enhance the degradation by suplimentation of surfactant/emulsifier (tween 20, tween 80, and triton x100) to enhance its qualities of degradation.

Bacterial isolates from soils contaminated with a large range of petroleum organic pollutants of oil refinery Mathura and Baruni were tested for their ability to metabolise, mineralized and degrada the selected recalcitrant PAHs (anthracene , coronene and napthacene) as their sole energy and carbon source. Plating of soil microorganisms isolated from 50 petroleum-contaminated soils on solid medium (MSM), supplemented with the PAH (50 mg/l anthracene, 20mg/l coronene and 50mg/l napthacene) as sole carbon source, yielded 58 PAH degrading bacterial strains, of which most potent isolates was selected and were further characterized. By different parameters, identified 5 of the 58 colonies aspotentially competent in HMW PAH degradation (anthracene, coronene and napthacene). The 5 bacterial isolates were further screened by UPLC for their capacity to mineralize three HMW PAHs serving as their sole carbon and energy source. Under such conditions, bacterial isolate of five strains cumulatively mineralized 98% of anthracene, 95% of napthacene, and 97% of coronene after 30-days incubation period period, but could not degrade coronene (24%) as much compaire to napthacene and coronene.

Three different characterisation approaches were applied to five isolate (*Bacillus licheniformis strain* MBOR, *BacteriumCT* BORL, *Bacillus pumilus* BORS *Paenibacillus lautus* MORL and *Bacillus acidovirans* MORS in Genbank): (i) micro/macromorphological stydy byu phase-contrast microscopy showed five isolates to be a Gram variable rodcoccus; produced creamy yellow, whitish and circular colonies that were catalase-positive, except *Paenibacillus lautus* all bacteria was gram positive (ii) SEM for morphological and structral analysis (iii) molecular species confirmed by others‘ 16S rRNA gene sequence analysis. Among five strains *Bacterium CT* BORL mineralization of PAH also showed it to be new and unique PAH-degrading bacteria.

This multidisciplinary study highlights the fact that five strains shows a great potential to be useful in biodegradation of high molecular weight PAHs., and strongly supports the general concept of *Bacillus licheniformis* MBOR, *BacteriumCT* BORL, *Bacillus pumilus* BORS *Paenibacillus lautus* MORL and *Bacillus acidovirans* MORS having a significant role in PAHs-biodegradation, bioremediation, and biodetoxification in contaminated envirnoment, Petroleum refinery and hazardous compounds effluents of industry

Keywords: Polycyclic- SEM, UPLC, FTIR, Aromatic- Hydrocarbon (antracene, napthacene and coronene) – Biodegradation-Pollution.

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Chapter- 1

Introduction

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are global pollutants in the environment and are a serious matter of concern**(Yucheng *et al.,* 2008; Driscoll *et al.,* 2010).**PAHs are hydrophobic aromatic compounds containing two or more fused phenyl and/or pentacyclic rings in linear, angular or in cluster arrangements and are ubiquitous, semi-volatile pollutants**(Cerniglia, 1992).** They are known to be toxic, carcinogenic, mutagenic, genotoxic as well as endocrine disrupting substances **(Haritash and Kaushik, 2009; Beyer *et al.,* 2010).**They are persistent organic pollutant affecting both aquatic and terrestrial organisms **(Phillips and Grover, 1994; Cachot *et al.,* 2006; Yucheng *et al.,* 2008; Amezcua-Allieri *et al.,* 2012).** As a result of geogenic and anthropogenic activities.They have the potential to cause adverse impacts on ecosystem and human health **(Juhasz and Naidu, 2000; Rostami and Juhasz, 2011).**

Soil contaminating PAHs are produced in many processes including burning of fossil fuels, incomplete combustion of organic matter, manufacture of gas and coal tar, wood processing, automobile gasoline exhausts, fuel-burning kitchen stove, incineration of wastes, petroleum products, oil spillage and also partly from natural calamitieslike forest fires and volcanic eruptions **(Cai *et al.,* 2007; Das *et al.,* 2008; Motelay-Massei *et al.,* 2007; Wang *et al.,* 2009).**Due to their hydrophobicity and chemical stability, these compounds accumulate progressively in soils, where they persist for several years. PAHs are contaminants of agricultural soil and may contribute to food chain and therefore represents a human health risk due to the consumption of contaminated meat or vegetables **(Wild and Jones, 1992; Fismes *et al.,* 2002; Grova *et al.,* 2002; Martorell *et al.,* 2010).** Moreover, PAHs contaminants of agricultural soil also alter soil microbial functioning which are themajor playersof soil ecosystem services. Soil contaminations resulting from chronic atmospheric PAHsdepositions are characterized by a multiple contamination including several PAHs **(Motelay-Massei *et al.,* 2004, 2007).** Their Removal from the environment is a necessity for ensuring human health and ecosystems **(Yucheng *et al.,* 2008; Haritash and Kaushik, 2009).** Many strategies have been developed to remove PAHs from the environment, including volatilization, photo-oxidation, chemical oxidation, adsorptionand biodegradation**(Haritash and Kaushik, 2009; Lin *et al.,* 2010; Run *et al.,* 2010).**Among them,biodegradation techniques have received considerable attention, particularly in the context of cleaning up contaminated sites, due to their efficacy, environmental safety and comparatively lower cost **(Yang *et al.,* 2009; Lin *et al.,* 2010; Run *et al.,* 2010).** A large number of bacteria that metabolize PAHs have been isolated and characterized.

Most of these microorganisms belonged to genera*viz.Pseudomonas*, *Alcaligenes*, *Mycobacterium*, *Rhodococcus*, *Neptunomonas*, *Stenotrophomonas*, *Sphingomonas*, *Cycloclasticus*, *Staphylococcus*, *Burkholderia*, *Acinetobacter*, *Ochrobactrum*, *Agmenellum*, *Aeromonas*, *Bacillus*, *Beijerinckia*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Moraxella*, *Nocardioides*, *Lutibacterium*, *Streptomyces*, *Vibrio*, *Paenibacillus***(Zhang *et al.,* 2006; Labana *et al.,* 2007; Doyle *et al.,* 2008; Zhao *et al.,* 2008; Lin *et al.,* 2010; Ferhat *et al.,* 2011; Yessicaa *et al.,* 2013).**

Degradation of PAHs-based pollutants can be enhanced by the provision of nutrients to stimulate the activity of indigenous microorganisms **(Seklemova *et al.,* 2001; Ueno *et al.,* 2006).** However, soil characteristics, such as moisture content, aeration , nutrients, co-substrates, the redox potential, dry-wetting sunlight, soil organic matter, pH, Na^+, Cl^-, CO_3^{2-}, and SO_4^{2-}content, particle and pore size distribution, bioavailability, bioaccessibility and toxicity are all known to affect the removal of PAHs from soil **(Gan *et al.,* 2009).**Polycyclic aromatic hydrocarbons of high molecular weight (HMW-PAHs) are organic compounds that can be biodegraded by a native microbiota that is capable of synthesizing and releasing enzymatic material that is able to break down the molecular links of these compounds. When such enzymatic material is not available, HMW-PAHs can be transformed by co-metabolism by providing very specific microorganisms. Some of these microorganisms are native of highly polluted soils **(Mancera-López *et al.,* 2008).** A promising means to enhance the bioavailability of PAHs is the application of surfactants. Addition of surfactant increased the solubilization and biodegradation of PAHs **(Frutos *et al.,* 2012).** Biosurfactants from *Acinetobacter sp*. increases the apparent solubility and biodegradation of PAHs **(Haritash *et al.,* 2009).** They are biodegradable, less toxic and cheap in comparison to chemical surfactants **(Zang *et al.,* 1997; Barkay *et al.,* 1997; Rosenberg *et al.,* 1997 and Zhao *et al.,* 2009).** Anotherbioemulsifier-producing bacterial strain capable of degrading some alkanes and PAHs compounds was isolated from petroleum-contaminated soil and identified as *Rhodococcus ruberem***(Li *et al.,* 2003; Li *et al.,* 2004 and Huang *et al.,* 2007).**It is well known that the low biodegradability of high molecular weight PAHs (HMW-

PAHs) with 4 or more rings is due to their low aqueous solubility and high sorption to soil particles **(Couto *et al.,* 2010; Mohan, 2006).**

Anthracene (ANT), a tricyclic PAH, is found in high concentrations in PAHs-contaminated sediments, surfaces soils and waste yards**(Wilson and Jones, 1993; Choi and Oris, 2003).** In addition, ANT is considered as a benchmark PAHs and serves as a signature compound to detect PAHs contamination, since its chemical structure is found in carcinogenic PAHs, such as benz[a] anthracene and benzo[a] pyrene. PAHs strongly adsorbes and accumulates in humus layer of soil and reveale as a sink of PAHs in atmosphere. Soil system seem to be an important long-term repository for PAH and are considered to be a steady indicator of the environmental pollution state. PAHs accumulates in the bottom of aquatic bodies; a further pathway of PAHs dissipation from the environment may be volatilization, irreversible sorption, leaching, accumulation by plants and microbial biodegradation **(Park *et al.,* 1990).** Loading of PAHs in surface soils is a function of total atmospheric deposition, excluding losses due to volatilization and biodegradation which may cause direct or indirect PAHs exposure to humans. Accumulation of PAHs in soils and sediments may further lead to contamination of vegetables and the food chain **(Kipopoulou *et al.,* 1999; Mueller and Shann, 2006).** Soil contaminated with PAHs (10–100 ppm) shows phytotoxicity for vegetation. It was also observed that phytotoxicity increases with increasing in number of aromatic rings.Bioconcentration factors (BCFs) of PAH in aquatic organisms frequently range between 100–200 and increases with increasing molecular weight.Metabolic PAHs intermediates bind covalently with cellular DNA and shows carcinogenesis (Malik *et al.,* 2004). Oil refineries can be a major source of PAHs and heavy metal pollution due to poor management, faulty equipment and lack of environmental controls and may have a significant impact on the surrounding environment. Some countries including Netherlands, Finland, Denmark, United Kingdom, Moldavia, Russia, and Ukraine have established the critical limits for soil selected PAHs. Populations living in the vicinity of an oil refinery may be at greater risk of potential exposure to PAH through inhalation, ingestion and direct contact with contaminated media. Identification and quantification of PAHs and heavy metals (iron, lead, copper, nickel, cobalt, and chromium (total)) surrounding an oil refinery soils are extremely important in terms of pollution control and risk management.PAHsin suspended particulate matter (SPM) near oil refinery is 12% to 45%, while in urban areas, it varies from 0.45% to 0.65%. Percentage of PAHs in relative suspended particulate matter (RSPM) near an oil refinery varied from 6% to 20%, while in urban areas it varies from 0.5% to 1.45%. The oil refinery contributed 11.5%–44% PAHs tototal ambient suspended particle (TSP) and 5.5% to 18.5% PAH to ambient RSPM **(Padma *et al.*, 2007).**Petroleum industry generates a huge amount of oily and saline waste water (oily brines, productionwaters) after separation of crude oil from reservoir water, which has to be disposed. Petroleum oil (a complex mixture of organic liquid called crude oil and natural gas, which occurs naturally in the ground, formed millions of years ago) consists of five types of components, saturated non-cyclic hydrocarbons(paraffins), cyclic hydrocarbons (cycloalkanes), olefinic hydrocarbons (alkenes), aromatics, sulphur compounds, nitrogen-oxygen compounds, and heavy metals **(Cote, 1976).**The main contaminants in this production water are aromatic and polycyclic aromatic hydrocarbons (PAHs) **(Borgne *et al.*, 2008; Zhuang *et al.*, 2010).**

Chapter- 2

Review of Litrature

1. REVIEW OF LITERATURE

Biodegradation, A process that involves microorganisms are used to break down and there by detoxify hazardous chemicals into the environment. Detoxification and elimination of hazardouspollutants from the environment can be carried out through severalremediation processes that involvebacterial applicationvia: microbial degradation, photo catalytic oxidation, chemical oxidation, volatilization and sedimentation **(Gao*et al.*, 1998).** It has been observed that industrial and agricultural processes release various polluting compounds in the environment **(Gainfreda*et al.*, 2005).**Aforesaid polluting compounds are responsible for deleterious effects on environmental quality and human health. It is therefore advisible to control the release of pollutants and understand their fate of action once they enter the soil. Biodegradation is an inexpensive and an effective approach to degrade and remove pollutants from contaminated soils. The microbial community has been suggested to play an important role in the decomposition of pollutants **(Beulke*et al.*, 2005).**PAHs composed of more than three ringsare the Centre of choice for the researchersfor the bacterial biodegradation during the last couple of decades.

Chemical pollutants can be classified into inorganic and organic pollutants as described below:

2.1. Organic pollutants In recent years, many organic compounds are used in day to day life. These compounds are produced for different uses such as petroleum refineries pesticides, plasticizers, lubricants, refrigerant, fuels, solvents and preservatives **(Liu, 2010).** Some of these organic compounds are biologically harmful even in minute concentrations while some are relatively inert and harmless. Some of these pollutants that enter the soil may inhibit or kill soil microorganisms, thereby perturbing the balance of the soil community.

Crude oil is a complex mixture of varying molecular weight and other organic compounds found beneath the earth's surface. It is a dark sticky fluid naturallyoccurring in certain rock formations. Crude oil are rich sources of carbon and hydrogen, with or without nonmetallic elements such as oxygen and sulfur. It is highly flammable and generates energy. Its derivative i.e. natural gas is an excellent fuel. The term "Petroleum" has been used as a synonym to crude oil. This term was first used in the treatise "De NaturaFossilium" published in 1546 by the German mineralogist Georg Bauer **(BauerGeorg *et al.*, 1955).**However, some may also be transported from the soil to air, water or vegetation where they may come into physical contact, could be inhaled or ingested by number of organisms **(Beulke*et al.*, 2005).**Of these, the major types of organic pollutants are aliphatic, alicyclic and aromatic hydrocarbons.

2.1.1. Aliphatic hydrocarbons

Aliphatic hydrocarbon consists of alkanes, alkenes, and alkynes. The alkanes saturated hydrocarbons (i.e. methane) are fairly inert and generally inactive in atmospheric photochemical reactions **(Leahy & Colwell, 1990).**

2.1.1(a) Saturated hydrocarbons

All hydrogen atoms attached to carbon bonds are together linkedin a chain, molecule is said to be saturated. For example: H3C-CH3 (ethane) **(Krafft& Crooks, 1988).**

2.1.1(b) Unsaturated hydrocarbons*:*

If two adjacent carbon atoms each lose a hydrogen atom, a double bond forms between them. Such a molecule is said to be unsaturated **(Krafft& Crooks, 1988).** For example: H2C=CH2 (ethylene, ethene).Aliphatic and aromatic hydrocarbons are differentiated as they provide a useful method for categorizing these compounds. For example, although anaerobic biodegradation of aromatic hydrocarbons has been reported, it is uncommon and relatively slow compared to aerobic biodegradation **(Leahy & Colwell, 1990).**

2.1.2. Alicyclic hydrocarbons

Alicyclic hydrocarbons are made up as cyclic saturated carbon chains. Most common alicyclic hydrocarbons occur naturally. For example, alicyclic hydrocarbons are a major component of crude oil, comprising 20 to 67% by volume. Examples of complex, naturally occurring alicyclic hydrocarbons include camphor, which is plant oil; cyclohexyl fatty acids, which are components of microbial lipids. **Rios-Hernandez *et al.*, (2003)** studied "Biodegradation of an alicyclic hydrocarbon by a sulfate-reducing enrichment from a gas condensate-d contaminated aquifer." Ethylcyclopentane (ECP) which is an alicyclic hydrocarbon was used in research conducted to study its metabolism by sulfate-reducing bacterial enrichment. Moreover, the research suggests that (ECP)-alicyclic hydrocarbons are anaerobically activated by addition of fumarate. Alkylsuccinate derivatives are obtained as by-products under sulfate- reducing condition.

2.1.3. Aromatic hydrocarbons

Aromatic hydrocarbons are found in petroleum components and its refined products. Naturally occurring aromatic hydrocarbons consists of benzene and its derivative derivatives e. Benzene is an aromatic compound exhibiting similar chemical behaviour and is one of the simplest forms of petrochemicals **(Krafft& Crooks, 1988).** Naphthalene is considered as one of the simplest representative of polycyclic aromatic hydrocarbon and benzene, toluene and ethyl benzene are amongst the other important aromatic petroleum hydrocarbons **(Wrenn*et al.*, 1998).** Benzene exhibits important properties and is a naturally occuring of aromatic hydrocarbons. The elemental composition of benzene is organic compound with molecular composition of C_6H_6 with six-member ring and with three carbon-carbon double bonds. Also, due to delocalised nature of bonding, benzene is represented with ring inside hexagonal arrangements of carbon atoms **(Wilson & Jones, 1993).** It is structurally similar to cyclic alkenes and is cyclic in nature. It is a colourless and highly flammable. Benzene is considered as an aromatic hydrocarbon and is naturally occurring in crude oil. It is, however, unusually stable and does not readily participate in reactions that are characteristic of alkenes **(Wrenn*et al.,* 1998).** Many chemical compounds are originated from benzene by substituting one or more of its hydrogen atoms with some other functional group. The resulting effect on the reactivity of these molecules that distinguishes aromatic hydrocarbons from unsaturated aliphatic hydrocarbons depends on the relative stability of benzene and its derivatives **(Rios-Hernandez *et al.,* 2003).**

2.2. Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are a class of stable organic molecules which consist of hydrogen and carbon molecules. As PAHs are commonly found widespread contaminants, these are of environmental concern **(Uyttebroek*et al.,* 2007).** The structure of PAHs compounds appears flat and consists of carbon and hydrogen atoms **(Cutright, 2006).** However, other atoms like sulphur, nitrogen and oxygen get readily substituted in the benzene ring and get converted to heterocyclic aromatic compounds. These heterocyclic aromatic compounds are commonly grouped together with PAHs **(Wilson & Jones, 1993).** Also, unsubstituted PAHs are non-polar, neutral and hydrophobic compounds that are randomly scattered during energy conversion and industries dealing with petroleum **(Juhasz*et al.,* 2000).** PAHs possess different chemical and physical properties due to their chemical structure. The examples of PAHs and their molecular formulae have been shown in **Table 2.1 (Johnsen*et al.,* 2005).**

Table 2.1: PAHs and their molecular formulae.

S.No.	PAH/PAH Name	Molecular formula
1.	Pen/Pentalene	C8H6
2	Ind/Indene	C9H8
3.	Nap/Naphthalene	C10H8
4.	Azu/Azulene	C10H8
5.	Hep/Heptalene	C12H10
6.	Bip/Biphenylene	C12H8
7.	aIn/asIndacene	C12H8
8.	sIn/sIndacene	C12H8
9.	Can/Acenaphthylene	C12H8
10.	Flu/Fluorene	C13H10
11.	Phe/Phenalene	C13H10
12.	Phr/Phenanthrene	C14H10
13.	Ant/Anthracene	C14H10
14.	Flt/Fluoranthene	C16H10
15.	Acp/Acephenanthrylene	C16H10
16.	Aca/Aceanthrylene	C16H10
17.	Tpl/Triphenylene	C18H12
18.	Pyr/Pyrene	C16H10
19.	Chr/Chrysene	C24H16
20.	Npc/Naphthacene	C18H12
21.	Ple/Pleiadene	C18H12
22.	Per/Perylene	C20H12
23.	Pic/Picene	C22H14
24.	Pen/Pentaphene	C22H14
25.	Pec/Pentacene	C22H14
26.	Tpl/Tetraphenylene	C24H16
27.	Hep/Hexaphene	C26H16
28.	Hex/Hexacene	C26H16
29.	Rub/Rubicene	C26H14
30.	Cor/Coronene	C24H12
31.	Trp/Trinaphthylene	C30H18
32.	Hep/Heptaphene	C30H18
33.	Hec/Heptacene	C30H18
34.	Pya/Pyranthrene	C30H16
35.	Ova/Ovalene	C32H14

The PAH molecules exhibits biochemical persistence due to the presence of dense cloud of π electrons on both sides of ring structures. Hence, PAHs compounds are more resistant to nucleophilic attack **(Haritash&Kaushik, 2009).** PAHs compounds exhibit various noxious and hazardous properties. Thus, PAH compounds are toxic, potential mutagens and carcinogens that results in the fate of PAHs in the environment contributing towards environmental concerns **(Martens, 1995).**

PAHs are formed due to thermal decomposition of various organic molecules in the environment. PAHs are produced as a result of incomplete combustion of hydrocarbons at high temperature (500-800^{0}C) or for long durations. PAHs appear to be colorless in nature with white/pale yellow solids. They have low solubility in water with low vapour pressure and with high melting and boiling points **(Johnsen*et al.*, 2005).**

PAHs in the environment are found commonly from natural sources or from artificial sources(anthropogenic):-

Natural sources include forest and rangeland fires, oil seeps and volcanic eruptions. Anthropogenic sources include exudates from coal tar, wood, garbage, refuse, used lubricating oil, oil refineries and oil filters discharge. Soil, air and water act as the ultimate depository of PAHs. PAHs are found to be major pollutants of air and soil **(Haritash & Kaushik, 2009).**

2.3. Formation of PAHs

PAHs are formed due to incomplete combustion of organic compounds **(Keith &Telliard, 1979).**Different types of combustion (domestic, industrial and agricultural) contribute to their emissions. Waste combustion is (due to the heterogeneous mix) a potential source for different types of PAHs. The chemical and physical formation of PAHs is very complex and many decades of scientific study have been dedicated to this. The different compounds involved during formation of PAHs include unburned fuel and lubricating oil, fuel that has undergone pyrolysis but has not passed through a flame zone as well as combustion in flame **(Longwell, 2007).**

Hydrophobicity and stability of PAHs results in recalcitrance of high molecular weight (HMW) PAHs in the environment **(Kanaly & Harayama, 2000).** Thus, presence of dense clouds of π electrons on each side of aromatic rings restricts the PAHs to limited nucleophilic attack leading to biochemical stability **(Johnsen *et al.,* 2005).** Solubility in water or polar solvents is prevented by the hydrophobic nature of PAHs and results in less bioavailability **(Boocham *et al.,* 2000).** However, to enhance the biodegradation process it is necessary to increase the bioavailability of PAHs and therefore, solubility in water and polar solvents have to be increased **(Field *et al.,* 1995).**

2.4. Sources of PAHs

Industrial and daily human activities such as coal processing, combustion of wood, crude oil, natural gas for heating, vehicle transportation, cooking and smoking; even natural processes such as carbonization and oil refineriesplay an important role for the incomplete combustion of organic materials. Incomplete combustion of organic material further produces PAHs through pyrolytic processes **(Hati *et al.,* 2009).** Among the several PAHs; benzoic[a]pyrene (Bap), anthracene, fluoranthene, phenanthrene and naphthalene are the main PAHs that are produced in the environment. Apart from these, some other PAHs such as heterocyclic aromatic compounds (e.g. carbazole and acridine), and nitro-PAHs (for example: pyrene) are also generated through incomplete combustion **(Neff, 1985).**

2.5. Toxicity of PAHs

PAHs exert deleterious effects on human health. According to US Environmental Protection Agency PAHs Benzo[a]pyrene is recognized as the most dangerous pollutant due to its extreme carcinogenic potential. It is also a major component of smoke released from the cigarettes. PAHs have very high lipid solubility and hence are quickly absorbed into the gastrointestinal tract of humans **(Gibson & Subramanian, 1984).** Moreover, PAHs have been investigated to be carcinogenic, mutagenic and teratogenic along with potential endocrine- disrupting properties. The mutagenic effects of PAHs are responsible for tumor initiation **(Lee & Hosomi, 2001).** Foods may contain PAHs in varying quantities especially when some food is exposed to high temperatures. Studies have suggested that PAHs are found in oil, fats and cereals. Furthermore, they are also obtained from cooked meat and vegetables **(Eriksson *et al.*, 2003).** PAHs are distributed in soil and harm human health as they may pass into the food chain, therefore, PAHs contaminantssoil andare important source of great concern with respect to human health **(Tao *et al.*, 2006).**

Skin is another major route of absorption of PAHs which accounts for 75% of the total PAHs (specifically pyrene) absorbed. Absorption rates of PAHs are fast due to high potential for biomagnifications in the food chain. In general, **Cerniglia, (1992)** states, “the greater the number of benzene rings, the greater the toxicity of the PAH.” The relative acute toxicity of PAHs can be measured using LD50 values (the lethal dose in 50% of the population tested). PAHs are also suspected carcinogens however, they are not considered to be genotoxic until mammalian enzymes are ‘activated’ from reactive epoxide and quinones. The Cytochrome P450 monoxygenase enzyme is responsible for degradation and is mostly found in fungi which oxidises the aromatic ring to form epoxide and diol-epoxide reactive intermediates. Due to the oxidation by cytochrome P450 enzymes the genotoxic effect comes into consideration and the aromatic ring forms epoxide and diol–epoxide reactive intermediates **(Harvey, 1996).** During oxidation processes these intermediates combine with DNA, or attack DNA and undergo oxidation or hydrolysis. Further intermediates combine and attack DNA and form covalent adducts with DNA causing mutation which may further lead to tumor formation **(Bamforth & Singleton, 2005b).**

2.6. Chemical and physical properties of PAHs

PAHs are a group of compounds containing carbon and hydrogen. Most PAHs consist of a “bay-region”, a “K-region” and an “L-region” **(Figure 2.1).**

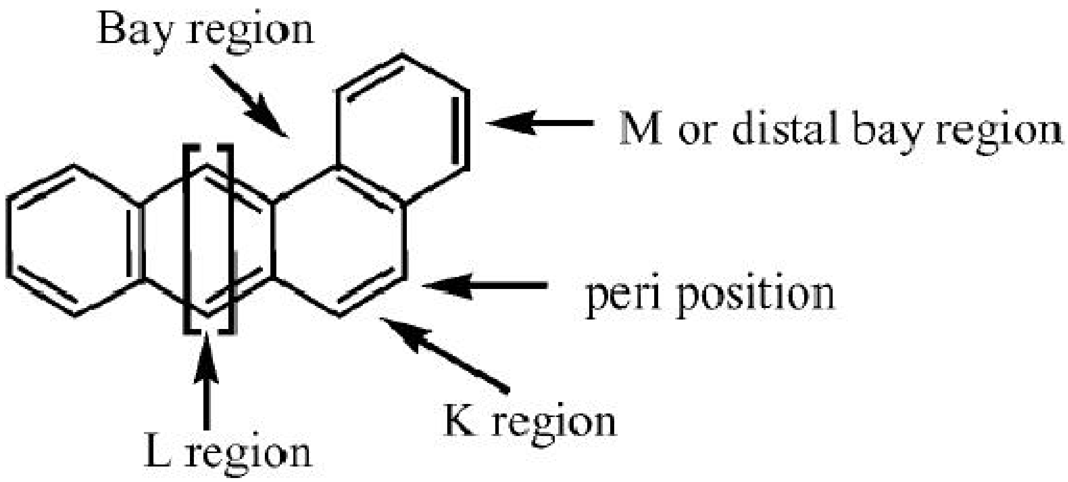


Figure 2.1: Biological activity of PAHs in Different regions.

The bay- region is an internal open inner corner for an example, open inner corner of phenanthrene structure. The K-region is an external closed corner and L-region represents the pair of opposed anthracenic point atoms **(Aramandla*et al.,* 2004).** These bay- and K-region epoxides are chemically reactive. Thus, they are developed metabolically and biologically. Phenanthrene is the simplest aromatic hydrocarbon composed of these regions. The bay-region of phenanthrene is a sterically hindered area between carbon atoms 4 and 5 and the K-region is the 9, 10 double bond, which is the most oleinic aromatic double bond with high electron density **(Figure 2.1) (Sikkema*et al.,* 1995).** Low-molecular weight (LMW) PAHs is relatively volatile, soluble and degrades more quickly. Whereas, high molecular weight (HMW) PAHs are more resistant to microbial degradation due to their strong anchoring to soils. Due to the solid state of PAHs compounds, high molecular weight PAHs compounds are hydrophobic and these PAHs are very toxic to whole cells **(Cerniglia, 1992).** The chemical properties, of a PAH molecule depends on the molecular size including the number of aromatic rings, and molecule topology or the pattern of ring linkage. Ring linkage patterns in PAHs may occur in a way such that the tertiary carbon atoms are centers of two or three interlinked rings for example the case of linear kata-annelated PAH anthracene or the peri-condensed PAH pyrene(**Kanaly & Harayama, 2000).** Some properties of selected PAHs are presented in **Table 2.2.**

Table 2.2: Chemical and physical properties of selected PAHs (adopted from Neff, 1979; CCREM, 1987; NRCC, 1983; USPHS, 1990).

PAH	**Mol.Wt (g)**	**Solubility at 25°C (µg/L)**	**Vapor Pressurea t 25 °C (mm Hg)**	**Log Kow (Log Koc)**	**Carcinogenicity**	**Benzene (and total) rings**
Naphthalene	128.2	12500 to 34000	1.8x 10^{-2}	3.37	NC	2
Acenaphthylene	152.2	3420	10^{-3}- 10^{-4}	4.07 (3.40)	NC	2
Acenaphthene	154.2			3.98 (3.66)	NC	2
Fluorene	166.2	800		4.18 (3.86)	NC	2 (3)
Anthracene	178.2	59	2.4x 10^{-4}	4.5 (4.15)	NC	3
Phenanthrene	178.2	435	6.8x 10^{-4}	4.46 (4.15)	NC	3
Acridine	179.2			(4.48)	NC	3
2-Methylanthracene	192.3	21.3		4.77	NC	3
9-Methylphenanthrene	192.3	261		4.77	NC	3
1-Methylphenanthrene	192.3	269		4.77	NC	3
Fluoranthene	202.3	260		4.90 (4.58)	NC	3 (4)
9,10-Dimethylanthracene	206.3	56		5.13	NC	3
Benzo[a]fluorine	216.3	45		5.34	NC	3 (4)
Benzo[b]fluorine	216.3	29.6		5.34	NC	3 (4)
Pyrene	202.1	133	6.9x 10^{-7}	4.88 (4.58)	NC	4
Benz[a]anthracene	228.3	11.0	1.1x 10^{-7}	5.63 (5.30)	C	4
Naphthacene	228.3	1.0		5.65	NC	4
Chrysene	228.3	1.9		5.63 (5.30)	WC	4
Triphenylene	228.3	43		5.63		4
Benzo[b]fluoranthene	252.3	2.4		6.04 (5.74)	C	4 (5)
Benzo[j]fluoranthene	252.3	2.4		6.21	C	4 (5)
Cholanthrene	254.3	2.0		6.28	C	4 (5)

7,12-Dimethylbenz[a] Anthracene	256.3	1.5		6.36	SC	4
Dibenzo[a,h]fluorene	266.3	0.8		6.57	WC	4 (5)
Dibenzo[a,g]fluorene	266.3	0.8		6.57	C	4 (5)
Dibenzo[a,c]fluorene	266.3	0.8		6.57	WC	4 (5)
3-Methylcholanthrene	267.3	0.7		6.64	SC	4 (5)
Benzo[ghi]fluoranthene	214.2	0.5		6.78	NC	4 (5)
Benzo[a]pyrene	252.3	3.8	5.5x 10^{-9}	6.06 (5.74)	SC	5
Benzo[e]pyrene	252.3	2.4	5.5x 10^{-9}	6.21	NC	5
Perylene	252.3	2.4		6.21	NC	5
Indeno(1,2,3-cd)pyrene	276.3	-		6.58 (6.20)	C	5(6)
Dibenz[a,h]anthracene	278.3	0.4		6.86 (6.52)	C	5
Benzo[ghi]perylene	276.4	0.3	1.0x 10^{-10}	6.78 (6.20)	NC	6
Coronene	300.3	0.14	1.5x 10^{-11}	7.36	NC	7

NC= non-carcinogenic; WC=weakly carcinogenic; C=carcinogenic; SC=strongly carcinogenic; Kow=Octanol/water partition coefficient; Koc= partitioning coefficient for organic carbon

2.7. Persistence of PAHs inthe environment

The persistence of PAHs in the environment is determined by various factors. These factors include chemical structure, the concentration, and dispersion of PAH, as well as the bioavailability of the co-existing contaminants. Apart from these factors environmental factors such as soil type and texture, pH and temperature are other important factors that control the persistence of PAHs in the environment **(Boyle *et al.*, 1998).**

Thus, the long environmental persistence of higher molecular weight PAH molecules are due to higher hydrophobicity and toxicity. The persistence of PAHs is also influenced with the ‘age’ of the co-existing contaminants in the soil matrix. If the age of coexisting contaminants is higher the persistence of PAHs is longer. However, PAHs in the environment can be removed through a natural techniques using microbial degradation or using physical or chemical processes as shown in **Figure 2.2 (Hatzinger*et al.*, 1995).**

The association of PAHs with co-pollutants such as hydrocarbons and heavy metals is another factor that can prolong their residence time in the environment. Aliphatic hydrocarbons and BTEX compounds are readily biodegradable by the *in situ* microbial community relative to the more complex chemical structures of the PAHs. This results in the depletion of available oxygen in the surrounding environment and the onset of anaerobicity. Though recent work has shown that there is a real potential for the biodegradation of PAHs in the absence of molecular oxygen (see 'Anaerobic metabolism of PAHs'), details regarding the efficiency and scale of PAH degradation in anaerobic environments is still limited, with rates of anaerobic organic matter oxidation up to an order of magnitude less than those under aerobic conditions. In addition, it is possible that the presence of heavy metals in soil could inhibit microbial growth and hence limit the metabolism of contaminants under anaerobic conditions.

Phenanthrene has shown reduced biodegradability due to depletion of oxygen and increase of anaerobicity in the environment. However, work has shown that biodegradation of PAHs can occur even in anaerobic conditions in the absence of molecular oxygen **(Rockne & Strand, 1998)** but the PAH degradation under anaerobic conditions has limited efficiency **(Bamforth & Singleton, 2005a).**

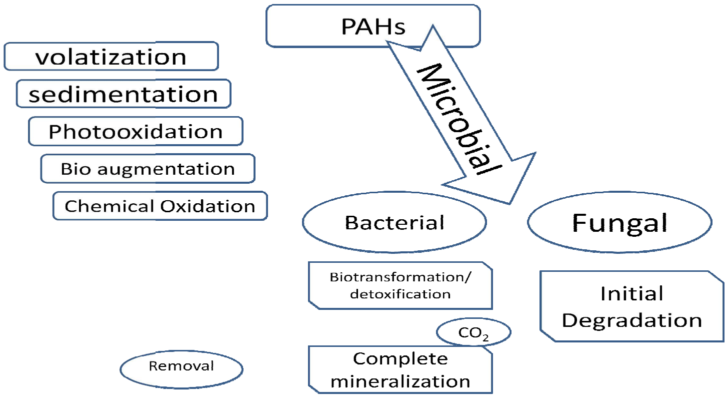


Figure 2.2: Schematic representation of the environmental fate of polycyclic aromatic hydrocarbons.

In the environment the fate of PAHs is dependent upon the extent of loss of contaminants due to mechanisms of biodegradation **(Rockne *et al.,* 2000).** PAH biodegradation processes can be considered as a removal of anthropogenic pollutants as well as a normal process of carbon cycles from the environment. The fate of organic contaminants in the environment is associated with both abiotic and biotic factors including chemical oxidation, volatilization, and photo-oxidation **(Pathak*et al.,* 2009).**

2.7.1. Chemical oxidation

In situ chemical oxidation is a process where oxidants (ozone, hydrogen peroxide, hypochlorite, chlorine and chlorine dioxide) are injected into the contaminated soil to convert PAHs to more stable and less mobile forms **(Figure 2.3)** and/or to provide an oxygen source for microbes (coupled with bioremediation). It is important to consider soil properties and injection method when choosing the appropriate oxidant. There are several drawbacks to this process including, but not limited to:

- Oxidant introduction can negatively impact subsurface soils
- Decreased soil permeability (colloid formation)
- Release of previously sorbed metals to groundwater resources
- Toxic byproduct production
- Heat and gas production
- Logistics of handling and storing oxidizing chemicals

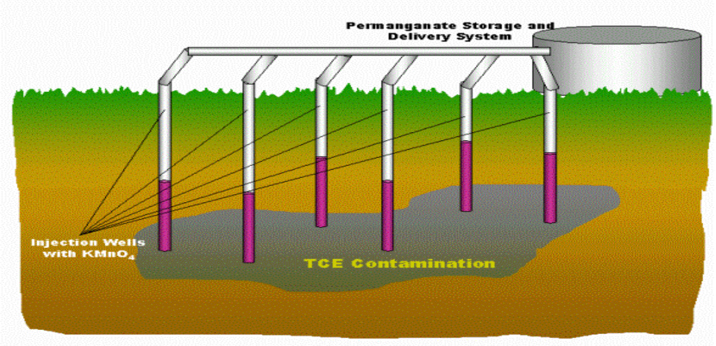


Figure 2.3: In situ chemical oxidation where oxidants are injected into the contaminated soil to convert PAHs to more stable and less mobile forms.

Bioremediation processes are efficient and economically feasible remediation strategies but tend to be slow, and have shown limited capacity to degrade HMW PAHs **(Wilson and Jones, 1993; Lundstedt *et al.*, 2003).** The introduction of a strong chemical oxidant into the soil can overcome some of the limitations encountered with biodegradation, and may result in a faster and more efficient degradation of HMW compounds **(Kawahara *et al.*, 1995; Nam *et al.*, 2001; Watts *et al.*, 2002).** Combining chemical oxidation processes with biodegradation may enhance PAH removal since chemical oxidation byproducts of PAHs are more water-soluble and available to biodegradation **(Nam *et al.*, 2001; Matscheko *et al.*, 2002).** Some researchers have suggested that biodegradation procedures be used as a polishing step after the bulk of contaminants is removed by other means **(Lundstedt *et al.*, 2006).**

Chemical oxidation procedures have some other advantages over biodegradation in that the processes can be better controlled since the conditions can be modified. They are also relatively insensitive to external environmental disturbances **(Rivas, 2006).** Conversely, there is always a possibility of wastage of chemical reagent added, especially when the sediments/soils have high organic matter content, which will also be oxidized. High organic matter content may increase the cost of treatment but can also increase PAH availability since sorbed PAHs will be released from organic matter during oxidation **(Rivas, 2006).**

2.7.2. Volatilization

The process of conversion of a chemical substance from a liquid or solid state to a gaseous or vapor state by the application of heat, by reducing pressure, or by a combination of both is called volatilization. However most of the compounds are degraded slowly and thus tend to accumulate in the environment **(Urgun-Demirtas*et al.*, 2006).** Organic contaminants are present in soil and their transportation to air is one of the important processes in volatilization. Therefore, it affects and controls the transport and fate of organic pollutants present in soil **(Liu *et al.*, 2010).**He also investigated that volatilization of PAHs can be inhibited with respect to solublization of PAHs by micelles. However, sorbed surfactant formation drastically inhibited solid-vapor volatilization of PAHs.

2.7.3. Photo-oxidation

Photo-oxidation is potentially one of the important and prevalent ways for PAH modification along with bioremediation in the environment. Photo-catalysis is a process that uses a catalyst, for example titanium oxide (TiO2) which facilitates a photoreaction to degrade the toxic compounds. TiO2 induced-photo catalyzed degradation of a variety of all organic substrates is gaining attention due to its potential to degrade PAHs from the environment **(Wen *et al.,* 2002).** Apart from microbial degradation of PAHs, photo-catalysis is another efficient process, and can be used for the elimination of PAHs. Upon absorbing sunlight, a PAH can be rapidly transformed to a variety of compounds, most of which are oxidation products **(Mallakin*et al.,* 2000).** The principle of photo-catalytic oxidation is shown in **Figure 2.4.**

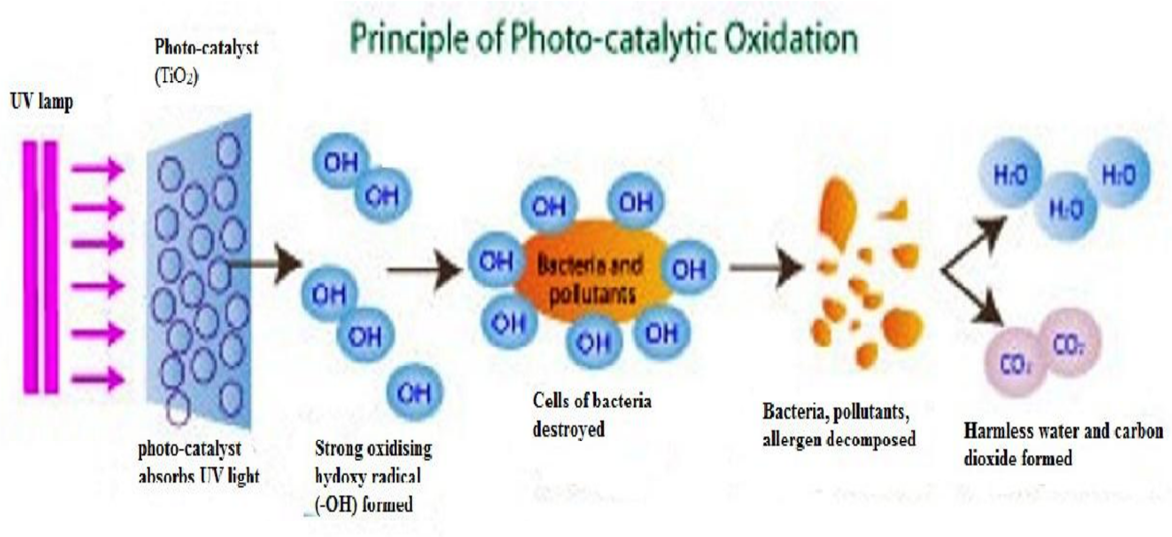


Figure 2.4: Principle of photo-catalytic oxidation (www.peakpureair.com).

It has been found that when aromatic compounds are exposed to UV light, partially oxidized intermediates of aromatic compounds are produced which more susceptible to degradation than their parent ones. Because of this property of aromatic compounds, photo-degradation has been recommended as an early stage strategy for biodegradation **(Mueller *et al.,* 1997).** Photo-degradation of PAHs in the presence of catalytic solution is considered as an oxidative process which is further augmented in the presence of photo-inducers. The polarity of the solvent is directly proportional to the rate of the degradation process. Thus, higher the polarity of the solvent faster is the degradation process. It has been suggested that reactive oxygen species and hydroxyl radicals do not play an important role in PAH photolysis. PAHs photo-decomposition initiated by photo-ionization results in the production of PAH radical cations and hydrated electron which further destroys PAH in the presence of water **(Zeep & Schlotzhauer, 1979; Zepp, 1982).**

2.8. Bioremediation

The main purpose of bioremediation is to detoxify the toxic compounds by microbial degradation in the environment. The degradation particularly includes organic and can be defined as the use of microorganisms to remove environmental pollutants of soils, waters, and sediments **(Gogoi*et al.,* 2003).** The bioremediation processes may be enhanced/facilitated by various applications to remove pollutants; to treat polluted environment is one of the most efficient practices toward a healthier environment **(Gogoi *et al.,* 2003; Van Gestel *et al.,* 2003).** In the bioremediation process, microorganisms are used to degrade the toxic compounds. However, biodegradation rates of hydrocarbons in soil can be defined by many factors, for example: microorganism type, nutrients, pH, temperature, moisture, oxygen, soil properties and contaminant concentration **(Semple *et al.,* 2001; Ghazali *et al.,* 2004).**

2.9. Types of bioremediation

2.9.1. *In-situ* bioremediation

In-situ bioremediation technologies are used to enhance the mechanisms that degrade PAH as well as various eminent polluting compounds in contaminated soil and groundwater. Generally, *in-situ* bioremediation technologies employ engineered systems to enhance the effects of naturally occurring degradation mechanisms **(Fiedler, 2000).** *In-situ* bioremediation includes:

2.9.1. a. Biostimulation

It Provide nutrition and suitable physiological conditions for the growth of the indigenous microbial populations. Thus, during the degradation of contaminants metabolic activities are enhanced.

2.9.1. b. Bioaugmentation

Introduction of specific blends of laboratory cultivated microorganisms into a contaminated environment or into a bioreactor to initiate the bioremediation process **(Fahnestock *et al.,* 1998).** Degradation carried out by microbial populations use carbon, nitrogen and phosphorous as sole source of nutrient. However, phosphate and nitrogen are two of the nutrients in the soil with limiting concentration **(USEPA, 1994).**

2.9.2. Types of *In-situ* Bioremediation

2.9.3. Intrinsic Bioremediation

In order to degrade contaminants from the environment, bioremediation process depends on different intrinsic factors. Intrinsic bioremediation is defined as the degradation of contaminants without alteration or amendment, to achieve *in-situ* bioremediation. In monitored natural attenuation (MNA) sites, intrinsic bioremediation may play a role that helps broaden the term defined by the US National research Council (NRC) and EPA as "biodegradation, dispersion, chemical or biological stabilization, transformation or destruction of contaminant" **(Sturman *et al.*, 1995).**

2.9.4. Engineered in-situ Bioremediation

In-situ bioremediation techniques include many selection procedures at contaminated sites. During remediation of a contaminated site the identification of degradation mechanisms provides enhancement of technologies that are more beneficial for use at the site. The steps in selection and implementation of *in-situ* bioremediation are:

- Evaluation of site characteristics
- Identification of site conditions and engineering solutions
- Identification of primary reactants
- Perform test based on treatability (bench-scale)
- Perform design system, field test, and implementation **(Fiedler, 2000).**

2.10. Ex-situ Bioremediation

The main metabolic activities and genetic features of microbial degradation need to be evaluated for a successful bioremediation approach. The microbial degradation at contaminated site can be evaluated considering the design and implementation suitable for microbial detoxifying strategies. It thus helps in monitoring the effectiveness of the bioremediation and understanding the metabolic and genetic features **(Andreoni & Gianfreda, 2007).** There are some aspects that help for easier *ex-situ* bioremediation and the most concerning aspects are: (a) Pollution of the environment by two classes of aromatic pollutants namely, polycyclic aromatic hydrocarbons (PAHs) and volatile aromatics are collectively indicated as BTEX (benzene, toluene, ethylbenzene, and xylene) (b) The main metabolic pathways and the genetic basis of their microbial degradation (c) The biological strategies to reduce or to eliminate their contamination, and (d) More advanced monitoring techniques to evaluate the effectiveness of a bioremediation process **(Andreoni & Gianfreda, 2007).**

2.11. The contaminated soil matrix

Air, water and soil are major natural resources. Soil is a complex mixture of air (25%), water (25%), minerals that come from rocks below or nearby surfaces (45%), and organic matter (5%) **(Figure 2.5).**The organic matter present in soil is due to remains of plants and animals that use the soil and living organism that reside in soil **(Bollag & Liu, 1990).** The type of soil depends on the clay, sand and silt present in it. However, other factors like climate, vegetation, time, the surrounding terrain, and even human activities (e.g. farming, grazing, gardening, etc.), are also important in influencing the soil that is formed and the types of soil that occur in a particular landscape **(Pritchard & Bourquin, 1985).**Thus, soil quality depends in part on its natural composition, and also on the changes caused by human use and management **(Larson & Pierce, 1991).**

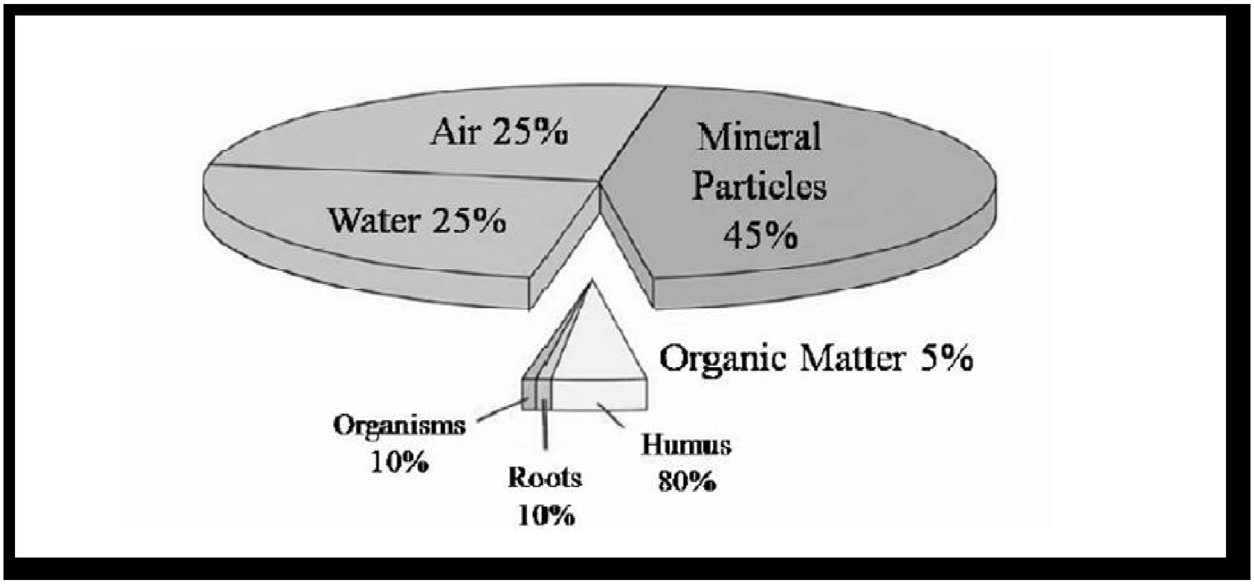


Figure 2.5: Soil contains four basic components: mineral particles, water, air, and organic matter. Organic matter can be further sub-divided into humus, roots and living organisms (http://www.physicalgeography.net).

2.12. Fate of organic contaminants in the soil environment

An organic contaminant (OC) in the soil environment may be lost by both biological and physical-chemical pathways in soil. Living organisms carry out biological degradation. The abiotic processes include leaching or volatilization, accumulation within the soil biota or sequestration within the soil mineral and organic matter fractions as shown in **Figure 2.6 (Lifongo&Nfon, 2009; Bollag& Liu, 1990).**

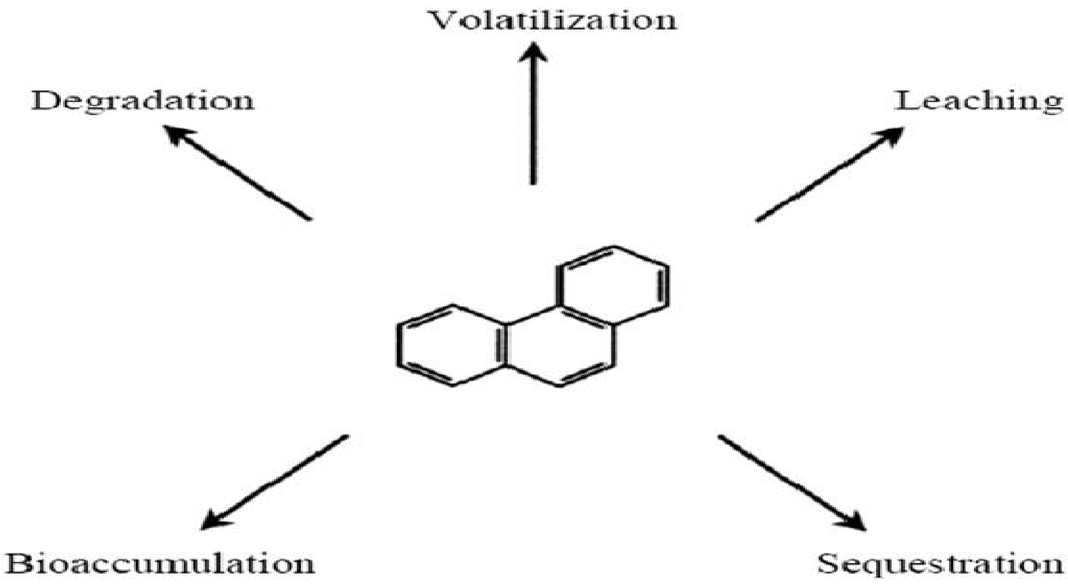


Figure 2.6: Assumed fate of behavior of a model organic contaminant (phenanthrene) in soil (Semple *et al.,* 2003).

2.13. Remediation of PAHs in soil systems

Biodegradation is one of the most common mechanisms harnessed in order to treat organic contaminants. Soil bioremediation is dependent upon desorption from the solid to the liquid phase of contaminant. Limited oxygen or nutrients, lack of bioavailability however, limit the bioremediation process. Under denitrifying conditions, anaerobic degradation of some PAHs occurs in the presence of excess nitrate **(Mihelcic & Luthy,**

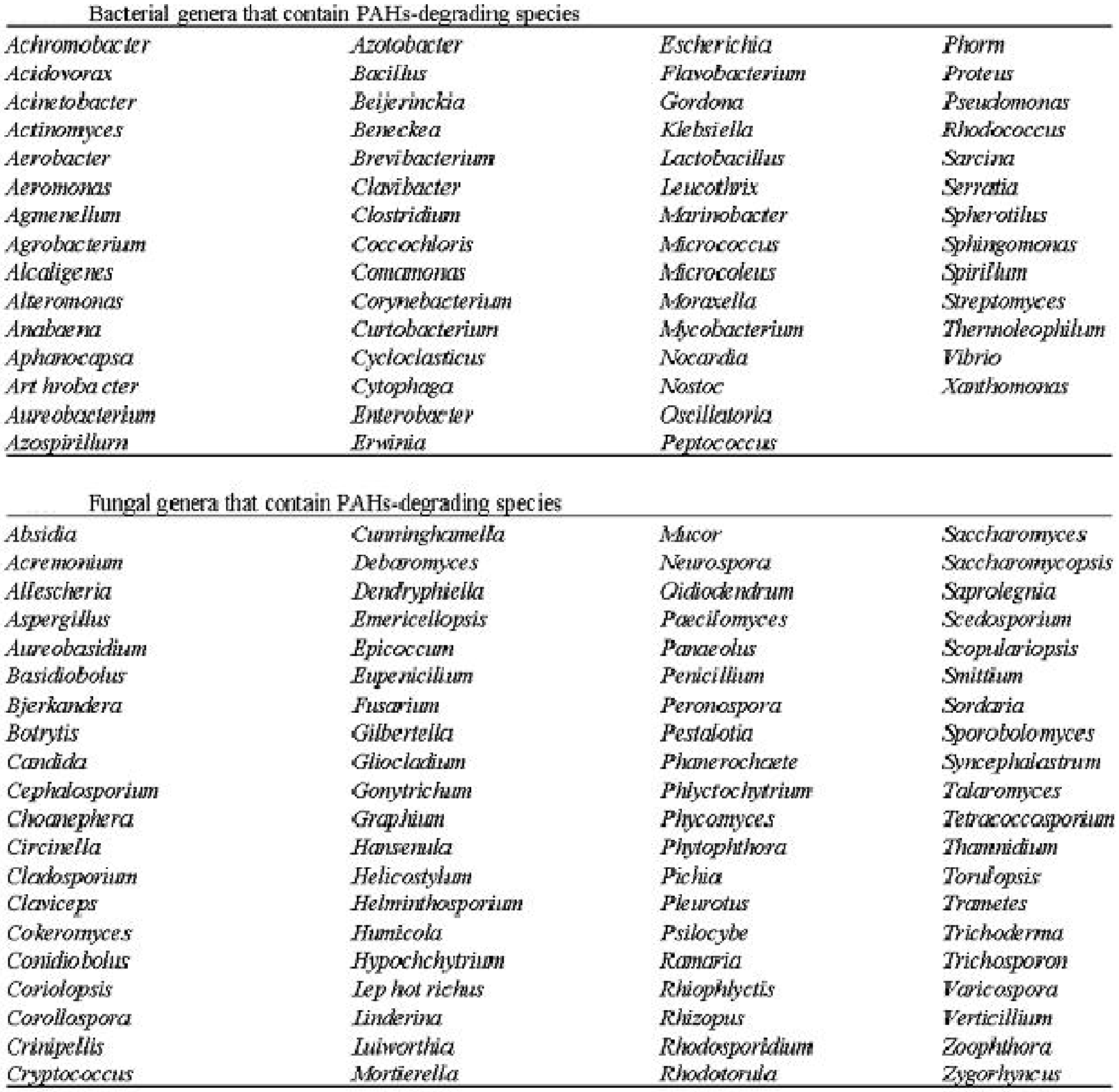
1988). The hydrophobicity of these compounds constitutes the main factor that determines their persistence in the environment; in particular they tend to be strongly absorbed by soil particles with low bioavailability and possibly accumulate in the food chain as well **(Mollea *et al.,* 2005).** In the soil matrix, as the contaminants are released, they bind to the surface and become sequestrated into the soil matrix. Due to sequestrating of contaminants, sorption strength increases over time. Further, sorption strength reduces the susceptibility of the contaminants to remediation. Soil organic matter (SOM) is not homogenous; it consists of varying proportions of combustible residues, non-aqueous phase liquids, and natural organic matter (NOM), all of which vary in their affinity for contaminants **(Jonsson *et al.,* 2007).** Mass transfer rates of the PAH molecule is an independent factor. Degradation rates often depend on the mass transfer rates of the PAH from the solid phase to the water phase. Enhanced mass transfer can be achieved by the consumption of minor substrate molecules, thereby altering the surface area and resulting in a higher dissolution rate **(Tiehm, 1994).**

2.14. Metabolism of PAHs by microbes

Prokaryotes are considered as major decomposers of organic compounds in an ecosystem **(Campbell & Reece, 2005).** Enzymes present in bacteria, digest these organic compounds and make them available for absorption. Higher molecular weight PAHs are often oxidized in variety of organic compounds by white rot fungi providing more advantages compared to bacteria **(Pointing, 2001).** PAH degradation is carried out by white rot fungi under aerobic conditions however, in bacteria it occurs under aerobic and anaerobic conditions. Bacteria and white rot fungi have different biochemistry for the catabolism of organo-pollutants **(Pointing, 2001).** Organo-pollutants are degraded by bacteria as their cells use a source of carbon and nitrogen as nutritional benefits, while PAHs and toxic pollutants are co-metabolically oxidized/ transformed with no energy supply to their cells. As a result white rot fungi require additional nutrients to manage their cellular activities **(Pointing, 2001; Boochan *et al.,* 2000).**

Microorganisms that degrade PAHs are universally distributed in the natural environment, such as in soils (bacteria and non-ligninolytic fungi) and woody materials (ligninolytic fungi). A comprehensive listing of microorganism genera capable of PAHs degradation is tabulated in **Table 2.3.**

Table 2.3: Bacterial and fungal genera that contain PAHs- degrading species (Al-Turki, 2009).



Bacterial genera that contain PAHs-degrading species

Achromobacter
Acidovorax
Acinetobacter
Actinomyces
Aerobacter
Aeromonas
Agmenellum
Agrobacterium
Alcaligenes
Alteromonas
Anabaena
Aphanocapsa
Arthrobacter
Aureobacterium
Azospirillum
Azotobacter
Bacillus
Beijerinckia
Beneckea
Brevibacterium
Clavibacter
Clostridium
Coccochloris
Comamonas
Corynebacterium
Curtobacterium
Cycloclasticus
Cytophaga
Enterobacter
Erwinia
Escherichia
Flavobacterium
Gordona
Klebsiella
Lactobacillus
Leucothrix
Marinobacter
Micrococcus
Microcoleus
Moraxella
Mycobacterium
Nocardia
Nostoc
Oscillatoria
Peptococcus
Phorm
Proteus
Pseudomonas
Rhodococcus
Sarcina
Serratia
Spherotilus
Sphingomonas
Spirillum
Streptomyces
Thermoleophilum
Vibrio
Xanthomonas

Fungal genera that contain PAHs-degrading species

Absidia
Acremonium
Allescheria
Aspergillus
Aureobasidium
Basidiobolus
Bjerkandera
Botrytis
Candida
Cephalosporium
Choanephera
Circinella
Cladosporium
Claviceps
Cokeromyces
Conidiobolus
Coriolopsis
Corollospora
Crinipellis
Cryptococcus
Cunninghamella
Debaromyces
Dendryphiella
Emericellopsis
Epicoccum
Eupenicilium
Fusarium
Gilbertella
Gliocladium
Gonytrichum
Graphium
Hansenula
Helicostylum
Helminthosporium
Humicola
Hypochchytrium
Lephotrichus
Linderina
Luiworthia
Mortierella
Mucor
Neurospora
Oidiodendrum
Paecilomyces
Panaeolus
Penicillium
Peronospora
Pestalotia
Phanerochaete
Phlyctochytrium
Phycomyces
Phytophthora
Pichia
Pleurotus
Psilocybe
Ramaria
Rhiophlyctis
Rhizopus
Rhodosporidium
Rhodotorula
Saccharomyces
Saccharomycopsis
Saprolegnia
Scedosporium
Scopulariopsis
Smittium
Sordaria
Sporobolomyces
Syncephalastrum
Talaromyces
Tetracoccosporium
Thamnidium
Torulopsis
Trametes
Trichoderma
Trichosporon
Varicospora
Verticillium
Zoophthora
Zygorhyncus

Large molecular weight (LMW) PAHs in contaminated soil can be effectively treated by bioremediation. In the bioremediation processes, the native microbes are of a great interest as these organisms may be expected to be adapted to the soil environment. These microorganisms when used in particular soil environments and with optimized abiotic conditions are more likely to out-compete the introduced microorganisms **(Silva *et al.,* 2009).** Microbial degradation of PAHs by aerobic mechanisms is expressed by three fundamental mechanisms. The specific details of degradation metabolism by bacterial and fungal are shown in **Figure 2.7.** Several environmental factors influence the rate of PAHs degradation. Low oxygen concentrations limit PAHs degradation that requires oxygenase activity. Some PAHs metabolites, particularly epoxide, dihydrodiols and quinones, affect the survival and viability of microorganisms, since they are cytotoxic and genotoxic. It has been found that the pH of culture media affects the levels of two constitutive PAHs o-quinonereductases particularly a pyrene-degrading enzymes **(Kim *et al.,* 2005).**

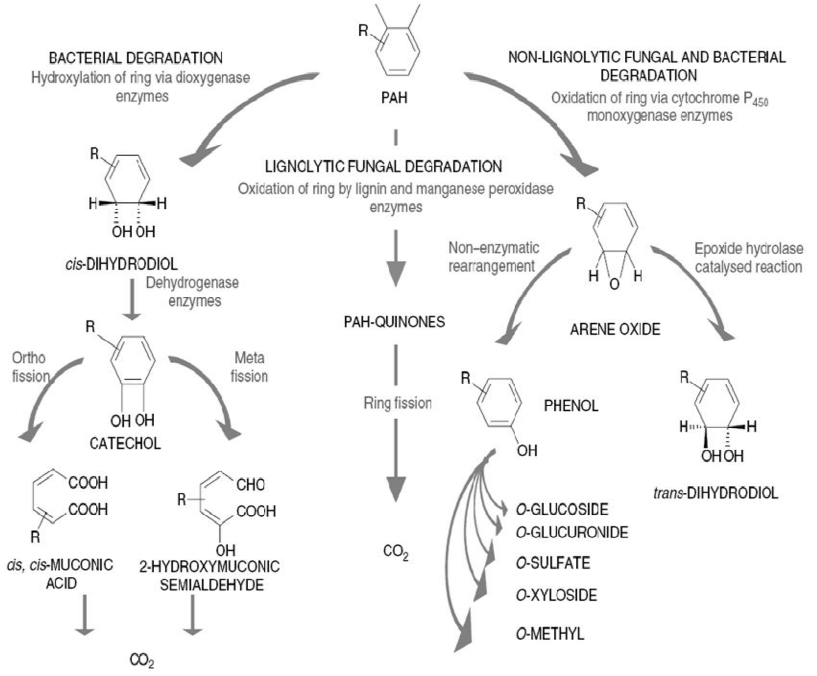


Figure 2.7: Three main pathways for polycyclic aromatic hydrocarbon degradation by fungi and bacteria (Bamforth and Singleton, 2005b).

2.15. Bacterial metabolism of PAHs

Active degradation of organic pollutants from the contaminated site in the environment is performed by bacteria. Natural processes of degradation via bacteria can however be carried out more rapidly by altering abiotic factors at these contaminated site **(Haritash & Kaushik, 2009).** The principal mechanism for the aerobic bacterial metabolism of PAHs is illustrated in **Figure 2.7.** The initial step involves oxidation of the benzene ring by the action of dioxygenase enzymes to form cis-dihydrodiols **(Parales *et al.,* 1998; Kanaly & Harayama, 2000).** Further, re-aromatization occurs and these cis-dihydrodiols are dehydrogenated to form dihydroxylated intermediates. These dihydroxylated intermediates are exposed to cleavage by dioxygenase *via* ortho or meta cleavage resulting in production of carboxylic acid cycle **(Parales *et al.,* 1998)** which can then be further metabolized *via* catechols to carbon dioxide and water **(Figure 2.7).** There is a large diversity of bacteria that are able to oxidize PAHs using dioxygenase enzymes, including organisms from the genera *Pseudomonas* and *Rhodococcus*. A few bacteria such as *Mycobacterium* species are also capable of oxidizing PAHs by the action of the cytochrome P450 monoxygenase enzyme to form trans-dihydrodiols **(Kelley *et al.,* 1990).**

2.16. Degradation of anthracene

Anthracene is a tricyclic aromatic hydrocarbon, found widely in the environment. It has been a model substrate for various studies on degradation of PAHs **(Moody *et al.,* 2001).**Anthracene is oxidised in the positions 1, 2 and gets converted to cis-1, 2-dihydroxy-1, 2-dihydroanthracene. It is further converted to 1, 2-dihydroxyanthracene which uses NAD+ dependent dihydrodiol dehydrogenase. Moreover, oxidation of 1, 2-dihydroxyanthracene undergoes ring fission to form a cis-4-(2-dihydroxynaphth-3-yl)-2-oxobut-enoic acid as a product. This product gets converted to 2-hydroxynaphthoic acid. Further, the fission helps the metabolic product to form salicylate and catechol through 2, 3-dihdroxynapthalene **(Cerniglia, 1984; Evans *et al.*, 1965).** The schematic proposed pathway for anthracene degradation is exhibited in **Figure 2.8.**

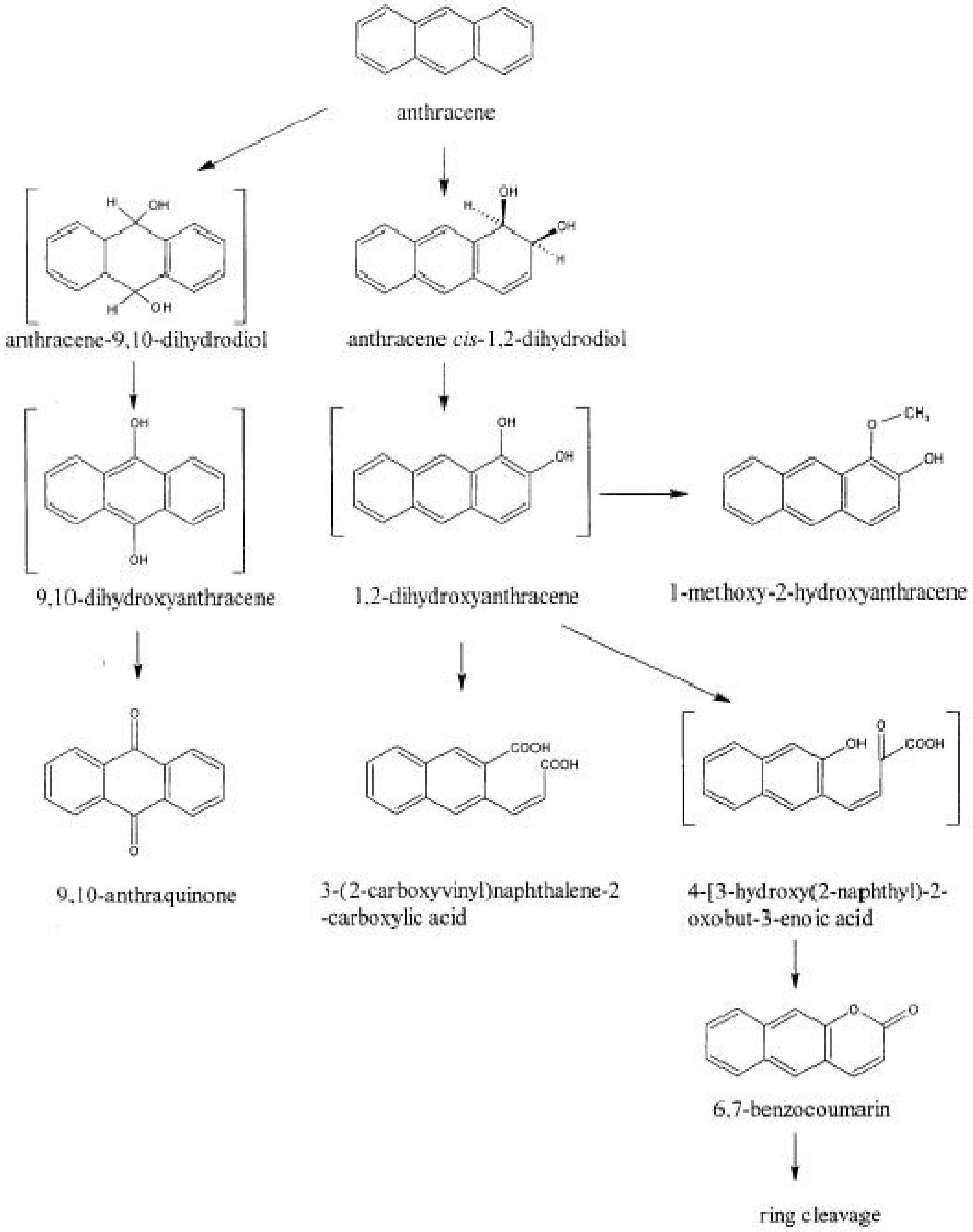


Figure 2.8: Anthracene degradation pathway (Bamforth and Singleton, 2005b).

2.17. Microbial biomass and activity

Microbial metabolic activity in soil can be evaluated using CO2 evolution as an indicator of microbial respiration. Moreover, the relation between respiration and biomass (qCO2) is a parameter considered to be a good indicator of the amount of pollution and the efficiency of bioremediation in contaminated soils **(Silva *et al.*, 2009)** further, leading to lower biomass yield found in most of contaminated soils **(Sirguey *et al*, 2008).** Arginine ammonification is one of the methods to measure the microbial activity which serves as a convenient test for microbial activity in soil samples. Although a wide range of methods are available to determine the microbial activity, soil ATP measurements, arginine ammonification may be used as a convenient, inexpensive and relatively fast method for routine estimation of microbial activity potentials **(Alef & Kleiner, 1987).**

2.18. Factors affecting the bioremediation of PAHs

Stacking and composting of soil from contaminated site is one of the classic examples for successful remediation. The efficiency of bioremediation on a bench scale and under ideal laboratory conditions have been investigated in many studies, often at specified neutral pH and mesophilic temperatures. However, the environmental factors vary from site to site (such as bioavailability of contaminant, nutrient availability, temperature and soil pH) and regulate the process of bioremediations **(Bamforth & Singleton, 2005b).**

2.18.1. Temperature

The effect of temperature is considered as one of the factors influences during degradation of PAHs. As temperature increases, the solubility of PAH molecule increases. During different seasons of the year, temperature at most contaminated sites is not helpful for bioremediation **(Margesin & Schinner, 2001).** However, **Erickson *et al.*, (2003)** suggests that oxygen solubility decreases with increase in temperature. This results in decreased metabolic activity of aerobic microorganisms. Different populations of hydrocarbon-utilizing microorganisms which are adapted to ambient temperatures are selected and expected in different climates and seasons. In some Arctic and temperate regions, soil temperature is below 10°C all around the year, and availability of oxygen in wet conditions is limited. The, cost of increasing the temperature may be prohibitive, so low temperatures need to be optimized **(Eriksson *et al.*, 2003).**

2.18.2. Oxygen (O_2)

Organic contaminants can be metabolized to achieve remediation both under aerobic and anaerobic conditions. Most work has concentrated upon the dynamics of aerobic metabolism of PAHs **(Gibson *et al.*, 1968).** Oxygen is necessary for the action of microbial mono- and dioxygenase enzymes in the initial process as carried out during aerobic PAHs metabolism. There is still a debate and issues have been raised as to whether the benefits of anaerobic bioremediation are outweighed by the negatives. This has been carried out using hydrogen peroxide, sodium nitrate and perchlorate **(Coates *et al.*, 1999).** However, it has also been reported that rates of anaerobic PAH degradation under denitrifying conditions were comparable to those under aerobic conditions **(Mcnally *et al.*, 1998).** This suggests that the dominant *in situ* microbial community consist of PAH-degrading microorganisms and that bioremediation was not limited by low numbers of PAH-degrading microorganisms rather than adverse environmental conditions. Oxidation of the substrate by oxygenase is involved due to catabolism of alicyclic and aromatic hydrocarbons by bacteria and fungi. Therefore, the route of microbial oxidation of hydrocarbons in the environment makes it necessary for aerobic conditions. The rates of microbial oxygen consumption and, type of soil, are fairly dependent on the availability of oxygen. The oxygen availability when soil is waterlogged is low and the high availability of utilizable substrates can also lead to oxygen depletion **(Genthner *et al.*, 19997).**

2.18.3. Nutrient availability

For cellular metabolism during degradation of PAHs microorganisms require mineral nutrients for successful growth. Different mineral nutrients such as nitrogen, phosphate and potassium (N, P and K) are required for growth and so help to readily degrade carbon source. Therefore, contaminated land may need to be supplemented with various nutrients, generally nitrogen and phosphates to stimulate *in situ* microbial activity and further enhance bioremediation strategies **(Brandli *et al.,* 2008).** The ratio of C:N:P in microbial biomass has been estimated for optimal growth and hence for the bioremediation process **(Bamforth & Singleton, 2005a).** However, a recent study due to nature of the pollutants in contaminated sites, where organic carbon levels are often high, available nutrients can rapidly deplete during microbial metabolism. The failure in the bioremediation of high molecular weight PAHs due to the temporary increased long duration inhibition of functionally important organisms **(Bamforth & Singleton, 2005a).**

2.18.4. Bioavailability

PAH compounds have low bioavailability because they are hydrophobic **(Genthner *et al.,* 1997).** Bioavailability depends on the rate and extent of biodegradation and is the most important factor in bioremediation **(Muller *et al.,* 1997).** In addition, in the presence of different soil matrices rapid sorption to mineral surfaces such as clays and organic matter such as humic and fulvic acids are carried out by PAHs. As long as PAHs compounds are in direct contact with soil, sorption becomes stronger and chemical and biological extractability of contaminants is lowered. This phenomenon is called ‘ageing’ of the contaminant. Therefore the bioavailability of a pollutant is linked to its persistence in an environment **(Vandyke *et al.,* 1991).** PAHs are chemicals with low water solubility that are resistant to biological, chemical and photolytic breakdown. The higher the molecular weight of the PAH, the lower its solubility, and due to lower solubility, the accessibility of PAHs compounds during the metabolism by the microbial biota is lower **(Fewson, 1988).** Various surface-active agents for example, surfactants or detergents, help in release of PAHs from the surface of minerals and organic matter. These can be monitored as microbial respiration rates (mineralization) of 14C-labelled contaminants, the bioluminescence of microorganisms. The surface active compounds contain both a hydrophobic and hydrophilic moiety. A 'bridge' between the hydrophobic PAHs molecule and the hydrophilic microbial cell is formed due to these hydrophobic and hydrophilic moieties and this maintains the bioavailability **(Bamforth & Singleton, 2005a).**

2.18.4. Salinity

The mineralization of phenanthrene and naphthalene in fine soil sediments is positively correlated with salinity and the rate of mineralization. It has been reported in general that correlation decreases with the increase in salinity. In a study of soil containing salt in high quantities, it has been shown that rates of hydrocarbon metabolism decreased with increase in salinity in the range of 3.3 to 28.4% and the results are attributed to a general reduction in microbial metabolic rates **(Leahy & Colwell, 1990).** Thus, there are few published studies which deal with effects of salinity on the microbial degradation of hydrocarbons.

2.18.5. Water activity (aW)

The water activity or water potential of soils can range from 30% to 45% water saturation. The hydrocarbon biodegradation in an acidic soil may therefore be limited by the available water for microbial growth and metabolism.

2.18.6. pH

For bioremediation, most of PAHs contaminated sites are not at the optimal pH. Leaching processes will however, increase the pH of the soil and result in limited favourable conditions for microbial metabolism. On the other hand the oxidation and leaching of coal will create an acidic environment by the release and oxidation of sulphides **(Kastner**

***et al.*, 1998).** As the pollutants are frequently linked with the pH of contaminated sites, the indigenous microorganisms may not be able to transform PAHs under the prevalent acidic or alkaline conditions. Therefore, it is common practice to adjust the pH at these sites, for example by the addition of lime **(Alexander, 1995).** Many pollutants determine the fate of soil pH however soil acidity is also affected by microbial activity, where fungi are found to be more active in acidic pH while bacteria tend to be active in neutral/alkaline pH **(Brady & Weil, 2002).** Few investigations show phenanthrene degradation, although bacterial and fungal growth is significant, since phenanthrene removal was only 40% at pH 5.5 after 16 days, whereas at neutral pH values, phenanthrene removal was 80%. If an acid pH is considered as an important abiotic factor that requires lime, suggests that consortium of both fungi and bacteria are necessary to accomplish removal **(Kastner *et al.*, 1998).** These findings show that future research would benefit from the isolation and characterization of PAHs degrading microorganisms from both acidic and alkaline environments. The degradation of PAHs can be enhanced and carried out faster by optimizing the pH conditions. The various findings related to pH of soil at various contaminated sites indicate that *in situ* microorganisms are tolerant of the site conditions. These may also have the potential to metabolize PAHs in sub-optimal conditions **(Bamforth & Singleton, 2005a).** Most heterotrophic bacteria and fungi favor a pH near neutrality, with fungi being more tolerant of acidic conditions. Extremes in pH, as can be observed in some soils, would therefore be expected to have a negative influence on the ability of microbial populations to degrade hydrocarbons **(Leahy & Colwell, 1990).** Low water solubility and dissolution rates of hydrophobic substrates are always limiting factors during the process of biodegradation. The rate of mass transfer can be enhanced in order to increase the degradation rate. However, the mass transfer can be achieved by the use of small substrate particles, thereby increasing the surface area and resulting in a higher dissolution rates **(Tiehm, 1994).**

AIM AND STRUCTRE OF THE WORK

The overall objective of this study is to degrade the serious hazardous , low and high molecular weight (LMW and HMW) polycyclic aromatic hydrocarbons (PAHs) which are carcinogenic and mutagenic by ecofriendly manner via indigenous bacterial communities, and getting the most potent strains able to degrade different PAHs isolated from the indigenous bacterial communities of petroleum oil contaminated samples.

This main objective can be divided to subobjectives:

1. Isolation, Screening and Enrichment culture of PAHs degrading bacteria from contaminated soil sediment.
2. Molecular charecterization and Phylogentic analysis of PAHs degrading bacterial strains.
3. FTIR based analysis of PAHs contaminated soil for presence of HMW PAHs.
4. Optimization of physico-chemical condition favoring maximum degradation of HMW PAHs.
5. Plasmid mediated degradation and Extraction, analysis of bisurfactant from PAHs degrading bacteria.
6. Chromatographic identification of degradation product of HMW PAHs by GCMS/UPLC.

Chapter- 3

Materials and Methods

3. MATERIALS AND METHODS

3.1. Collection of sample

Sampling

Samples were collected from Mathura and Barauni Oil Refinery, India and transported to the laboratory in sterile plastic containers and stored at 4 °C until they were used. Microorganisms used in the degradation study were isolated from sand and soil samples collected from the petroleum contaminated (hydrocarbon) contaminated sites.

3.2 Chemicals

Pure and analytical grade chemicals used in all experiments including media preparation for microbial culture growth and culture maintenance. Nutrients used for microbial culture and the testing kits were supplied by M/s Hi Media chemicals, India. Petroleum crude oil was collected from Mathura oil refinery UP and Baruni oil refinery. Other chemicals and nutrient salts procured from Merck Chemical, India and Sigma Aldrich.

3.3 Glassware and Instruments

All glassware's (Conical flasks, Measuring cylinders, Beakers, Petriplates and Test tubes etc.) purchased from Borosil. The instruments and apparatus used throughout the experiment listed in Table

Instrument	**Make**	**Function**	**Operation conditions or specification**
Analytical balance	Sartorius (BS223S)	Weight measurement	1mg - 100g
pH meter	Systronics (361)	Measurement of pH	pH 1 to 12
Vertical autoclave	Test master	Sterilization of nutrient media	121^oC temperature, 15 psi pressure, 15 min.
Laminar airflow chamber	Zhichen (ZhJH-1109C)	Aseptic Environment	Air filter: HEPA Filter efficiency: FS209E, Class 100. Air Velocity: 0.3-0.6m/s Visible Light source:

			Fluorescent Lamp UV light source: 8watt, mercury lamp (254nm)
BOD incubator	Vikram scientific instruments	Incubation of cultures	Temperature range: 5 - 70 ºC.
Incubator shaker	Environmental orbital Shaker	Shaking of culture flasks used in degradation study	Speed range: 50 - 200 rpm. Temperature: 5 - 70 ºC.
Spectrophotometer (UV/Vis)	Jasco (V-530)	Estimation of biomass and analysis of organic compounds	Bacterial Biomass: 600 nm TPH analysis: 228 nm Anthrone reagent: 620 nm Biuret reagent: 540 nm
Bioreactor IIC	Industrial Corporation	Biosurfactant production	2 liters capacity
Scanning Electron Microscope JEOL	(JSM-6480 LV)	To study the clear morphology about the structure and shape of the microbial cells with dimensions.	Magnification: up to 500k. Resolution : 3 nm Detector: Everhardt Thornley secondary electron detector and Solid state backscattered detector. X-Ray Analysis: Oxford Instruments ISIS 310 system with "windowless" detector. Light element analysis: silicon detector with ATW.
Optical microscope .	Hund (H-600)	Examining the morphology of microbial cells	100x, 250x, 400x and 1000x Magnification
Surface tensiometer	Data physics (Dcat-11)	Measuring the surface tension of the solution at	Method: Wilhelmy Plate. Plate material:

		air/liquid interface	Platinum. Temperature: Ambient
Fourier Transform Infrared Spectroscopy (FTIR)	Perkin Elmer (3500)	Analyze the organic functional groups present in the Biosurfactant sample	Measured range: wave number 600-4000 cm^{-1}. Resolution: 4 cm
Micro Centrifuge	Remi (RM12C)	Separation of biomass from the culture broth	8000 RPM for 10 min
Digital colony counter	EI Products (D12)	Measure the number of microbial colonies	Dimension: L 274 x B 320 x 167 mm Magnification :110 mm
UPLC			
NMR			

3.4 Media

Three different media were used in this study:

Bushnell Haas broth consisting of

0.2 g MgSO4, 0.02 g CaCl2, 1.0 g KHPO4, 1.0 g K2HPO4, 1.0 g NH4NO3, 0.05 g FeCl3 per litre of distilled water, pH 7.0;

Mineral salts medium consisting of

5.0 g NaCl, 5.0 g KH2PO4, 1.0 g K2HPO4, 1.0 g (NH4)2SO4, 0.25 g MgSO4·7H2O, 2.0 g NaNO3, 0.02 g FeCl2·4H2O, 0.02 g CaCl2 per litre of distilled water, pH 7.2;

Nutrient Agar

Ingredients Gms / Litre

5.0 g Peptone , 5.0 g Nacl, 1.5 g Beef extract, and 15 g Agar in one litre distilled water, pH 7.5.

Luria Bertani Agar

Ingredients Gms / Litre

10.0 g Casein enzymic hydrolysate 5.0g Yeast extract, 10.0 g Sodium chloride and 15 g Agar in one litre distilled water, pH 7.5.

3.3.3 Enrichment culture, isolation and screening of bacteria from PAH contaminated soil sediment

PAH degrading bacteria was isolated from petroleum contaminated soil sediment by conventional enrichment method. For this experiment, soil samples that were contaminated with petroleum oil were collected from 25 different of each site from surroundings of an oil refinery Mathura and Bauruni India. These were mixed thoroughly, sieved through 2mm mesh, placed in polyethylene bags, which were closed tightly and were stored at 4°C in the laboratory.

In 250 ml flask, 10 g of petroleum contaminated soil, collected from Mathura and Barauni Oil Refinery, India was added to 100 ml minimal salt medium (MSM) and incubated for 15 days in an orbital shaker set at 35°C and 150 rpm. After 15 days of incubation, 2 ml inoculum of bacterial culture was transferred to the flasks having fresh sterilized MSM supplemented with petroleum crude oil (2% w/v) and incubated in an orbit shaker set at 35°C and 150 rpm for 7 days the enrichment of petroleum hydrocarbon degrading bacterial strains. After repeating this procedure for 3 times, the active inoculum was used for the isolation of TPH (Total petroleum hydrocarbon) degrading bacteria, following serial dilution method and spreading over the nutrient agar plate and contained PAH compound as carbon source (**Liu *et al.*, 2010, 2014**).

2.5. Isolation of pure microbial cultures:

Microbial isolation experiments performed in solid Minimal salts media (MSM) containing 1.5% agar and 2% of the crude oil as a sole carbon source. The medium was autoclaved before inoculation. Broth culture samples from microbial enriched jar were serially diluted and 1ml aliquot of 10^{-4} dilution samples was added to sterile petri plate by spread plate technique. Agar plates were incubated for four days at room temperature (28 - 30ºC) at a pH of 7.4 ± 0.2 **(Ilyina *et al.*, 2003).** Randomly selected colonies from the plate were restreaked on new plates.

2.6. Hydrocarbon utilization efficiency and Screening of PAH degrading bacteria:

The isolated bacterial pure culture species were tested for screening based on the ability of the bacterial species to degrade PAH. The screening studies were performed in Mineral Salt Medium (MSM) that contains PAH as the sole source of carbon.

The isolated fifty eight bacterial strains species from different sites were streaked in Mineral Salt Medium agar paltes amended with 2%v/v Napthacene. Anthracene., Coronene.

Three replicates were used for each strain on each compound. The plates were incubated at 30°C. The growth was shaked every day for 15 days. The growths of the isolated bacterial species were also monitored in broth culture at regular intervals by measuring the optical density at absorbance 600 nm. Based on the growth of bacterial species on PAH (Anthracene, Coronene and Napthacene), the best degraders were selected for further experiment.

3.7. Sub culturing of pure cultures and inoculum preparation:

Isolated pure cultures were sub-cultured at an interval of every 15 days at nutrient agar medium and the inoculum used in the degradation study was acclimatization using PAH.

3.8. Identification and characterisation of PAH degrading bacterial isolates

3.8.1. Morphological characterisation

Pure colonies of PAH degrading bacteria were identified and characterised based on the results of their gram reaction tests and their morphological features when compared to *Bergey's manual* **(Garrity *et al.,* 2005; Cerqueira *et al.,* 2011).** The morphological properties of the cell were examined under compound microscope through which we can get the information about Gram staining, Motility and citrate utilization test were done to characterize the isolates.

3.8.2. Colony morphology

The shape, size, elevation, margin and colour of the colony were observed in the culture plates with MSM, NA, and Luria Bertani Agar used as the nutrient medium. The observations were noted down. Colony morphology: Shape, size, colour, elevation and margin of colony and appearance are observed in overnight plate culture on Nutrient agar media and noted down.

3.8.3. Cultural characteristics: It is important to describe the color or pigment of the colony. It also includes descriptive terms for any other relevant optical characteristics such as: opaque, cloudy, translucent, and iridescent. Bacterial colonies are frequently shiny and smooth in appearance.

Cell shape: The form refers to the shape of the colony. These forms represent the most common colony shapes you are likely to encounter. These are circular, irregular, filamentous, rhizoid.

Cell size: The size of the colony can be a useful characteristic for identification. The diameter of representative colony may be measured.

Gram staining:

Gram staining is an important method of differentiating bacterial species into two large groups such as Gram-positive and Gram-negative, based on the chemical, chiefly the presence of high levels of peptidoglycan in its cell wall, and physical properties. The Gram staining is almost always the first step in the identification of a bacterial organism, it is mainly performed to know whether the culture is pure or not. While Gram staining is a valuable diagnostic tool in both clinical and research backgrounds, not all bacteria can be classified by this type of process, thus producing Gram-variable and Gram-indeterminate groups as well. A Gram positive reaction results in a purple/blue color while a Gram negative reaction results in a pink/red color.

Staining mechanism:

Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50- 90% of cell wall), which are absorb purple colour stain by crystal violet, whereas those are Gram-negative bacteria have a thinner layer (10% of cell wall), which are stained pink by the counter stain,safranin. There are four basic steps of the Gram staining: A clean grease free slide was taken and a smear of the bacterial culture was made on it with a sterile loop. The smear was air-dried and then heat fixed. Then it was subjected to the following staining reagents:

Applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture for 2 minutes. The addition of a trapping agent (Gram's iodine), Rapid decolorization with alcohol and Counterstaining with safranin.

Cell morphology The Gram stained cells were observed under the light microscope under 100x using oil immersion. The shape, colour and morphological characteristics of the cells were determined.

Motility test: It is used to check the ability of bacteria to migrate away from the stabbing line which is the line of inoculation. The bacterium was inoculated by a needle into motility media, i.e, mannitol agar by stabbing the culture in a straight line and was observed after 24-48 hours incubation. If the test organism migrates away from the line of inoculation, the bacteria are motile.

Biochemcial characteristics

Following biochemical tests were done:

Indole test

The test was performed to find out the ability of organism to oxidize tryptophan to indole, pyruvic acid and ammonia. The isolated organism was inoculated in tryptone broth (Appendix). The inoculated and uninnoculated (control) tube were incubated at 28˚C for 4 to 7 days. After incubation, Kovac's reagent (Appendix) was added to inoculated and control tubes. Development of cherry red colour at the top layer in the form of ring indicated positive test while absence of ring formation indicated negative test.

Methyl-Red test

0.1 ml of inoculum was added in test tube containing MR-VP (Appendix) broth. Uninoculated tubes served as control. The tube was incubated at 28˚C for 4 to 7 days. After incubation 5 drops of methyl red indicater (Appendix) was added to the tube. Appearance of red colour was assigned as methyl red positive where as colour change to yellow was assigned as negative test.

Catalase test

Catalase test was performed to determine the ability of organism to produce oxygen gas during aerobic respiration. Microorganism produces hydrogen peroxide which is lethal to the cell. Catalase test was performed by adding hydrogen peroxide solution (Appendix) to the culture on a clean glass slide. The enzyme catalase present in the microorganisms breakdown hydrogenperoxide to water and oxygen. Release of free oxygen bubbles was considered as positive catalase test.

Carbohydrate fermentation test

Fermentative degradation of various carbohydrates such as Glucose, Sucrose, Mannitol, Xylose and Arabinose by microorganism under aerobic condition was carried out in a fermentation tube.

Culture tube that contained an inverted Durham's tube for the detection of gas production as an end product of metabolism. The fermentation broth contained ingredient of basal medium (Appendix), a specific carbon (glucose, mannitol, xylose, sucrose and arabinose) and pH indicator (methyl red) (Appendix) which was red at neutral pH and turned yellow at pH below 6.8 due to production an organic acid was considered to show positive result.

Starch Hydrolysis test

Starch agar plate (Appendix 1.10) was streaked with the isolated organism under aseptic condition. After that the plate was incubated at 28˚C for 7 days. in an inverted position. After incubation, the surface of the plate was flooded with iodine solution with the help of dropper for 30 seconds. The plates were examined for the colour change of the medium. A clear zone surrounding the microbial colonies indicated a typical positive starch hydrolysis while a negative reaction was showen as dark blue colouration of the medium.Uninoculated plate served as control.

Casein Hydrolysis test

The casein agar plates (Appendix) were streaked with organism under aseptic condition and uninoculated plate was used as control. After that, the inoculated and uninocilated plates were incubated at 28˚C for 4 to 7 days in an inverted position. A clear zone surrounding the bacterial growth indicated a positive reaction for extra cellular caseinase secretion while absence of clear area indicated negative reaction.

Gelatin Liquefaction test

Stab inoculation of the agar column from top to bottom and withdrawal of the needle through the same path was done in the gelatin agar (Appendix). Uninoculated tube was used as a control. Inoculated and uninoculated tubes were incubated at 15°C for 4-7 days. After incubation, tubes were placed in refrigerator at 4°C for 24 h. The tubes were examined to check whether the medium was solid or liquid. The tubes that remained liquefied produced gilatinase and showed positive test for gelatin hydrolysis and the tubes that remained solid demonstrated negative reaction for gelatin hydrolysis.

Nitrate reduction test

The test was used to identify the ability of organism to reduce nitrate into nitrites and further into ammonia or molecular nitrogen. The isolated microorganism was inoculated into the nitrate broth (Appendix) that contained an inverted Durham's tube for detection gas. An uninoculated (control) tube was used as control all tubes were incubated at 28 ±0.2°C for 4 - 7 days. After incubation the organism’s ability to reduce nitrate to nitrite and finally to nitrogen gas was determined by the addition of two reagents, solution A followed by solution B (Appendix). Development of cherry red colour indicated positive test for nitrate reduction where as no change in colouration indicated further two possibility *i.e.* negative nitrate reduction or direct nitrogen gas formation that was detected using a pinch of zinc dust to all tubes whose colour did not change. The tubes that changed its colour to cherry red after addition of zinc dust was assigned as negative nitrate reduction test while no colour change with evolution of gas bubbles in Durhams tube indicated nitrogen gas formation.

Characterization of PAH degrading bacteria by Scanning Electron Microscopy (SEM)

The freshly cultured strains were centrifuged at 8000 g, 4°C for 5 min and the cells were washed with 0.1 mM phosphate buffer saline (PBS) for 3 times. Then the cells were fixed by adding 2 % glutaraldehyde prepared in 0.1 M PBS and incubate at room temperature overnight for fixation. Then next day the cells were washed thrice with PBS. Then these cells were centrifuged at 8000 g, 4°C for 5 min. Then de-hydrolysis was done of each sample by different ethanol concentrations like 30 %, 50 %, 70 %, 90 %, 100 % and incubate for 18 hours. Then these were incubated for 1 h with ethanol (100 %). Then these slides were air dried and observed at various resolutions under SEM.

3.9. Molecular characterization: Sequence analysis of the 16S rRNA gene

Molecular identification and Phylogentic analysis of isolates

3.9.1. Genomic DNA isolation

Genomic DNA of bacterial isolates was isolated on the basis of versatile quick preparation method **(Masayaki *et al.*, 1993).**Cells were grown in Nutrient broth for 7-8 days keeping on rotatory shaker. The cultures were centrifuged at 8000rpm for 8mins at 20°C. Pellet was collected and rinsed with the TE and was repeated twice. Pellet was resuspended in 0.5ml SET buffer. 25µl of lysozyme was added and incubated for 3 hrs at 37°C. 0.1 volumes of 10% SDS and 20µl of proteinase K was added and incubated at 55°C for 2 hours. The mixture was centrifuged at 10000rpm for 8 minutes at 20°C and aqueous phase was removed with the blunt end tip.1 volume of 3M sodium acetate and 1 volume of propanol was added and incubated at room temperature for 30 minutes. The mixture was centrifuged at 10000 rpm for 5 minutes and washed with 70% ethanol. It was centrifuged at 10000 rpm for 5 minutes and dried for1 hour in the laminar air flow chamber. Finally DNA pellet was dissolved in 40µl TE buffer.

3.9.2. PCR programme and methods

Amplification of 16S rDNA was carried out by polymerase chain reaction using a thermal cycler (boired). 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. Amplification of 16S rDNA was carried out by polymerase chain reaction using a thermal cycler (Boired). The cyclic condition for the amplification reaction was as follows:

I. 96°C for 2 min. (Initial denaturation).

II. 96°C for 45 sec. (30 cycles).

III. 56°C for 30 sec. (annealing).

IV. 72°C for 2 min. (extension).

V. 72°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 55v and visualized under UV light **(Cook and Meyers, 2003).**

3.9.3. PCR product purification:

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

3.9.4. Sequencing and phylogenetic analysis:

Miniprep kit (Qiagen) and sequenced by using Big Dyeterminator with an automated capillary sequencer (Applied Biosystems). Inserts were sequenced using vector-specific and a suite of 16S rRNA-specific primers to generate an overlapping set of sequences that were assembled into one contiguous sequence. The sequences obtained were compared with those from the EZTaxon database; aligned using the Clustal W software and phylogenetic trees inferred using the neighbor-joining method in the MEGA 7 program. The identified sequences were submitted to NCBI database.

3.10. FTIR based analysis of PAHs contaminated soil for presence of HMW PAHs.

Fourier Transform Infrared Spectrometry (FTIR) analysis was conducted to identify potential functional group on the surface of petroleum contaminated sediment at refinery sites. The first step, an approximately 2 g of contaminated soil samples were extracted by carbontretrachloride CCl_4 (50 ml). The extraction was performed in a Soxhlet extractor (Soxtec System HT Tecator 1043 Extraction Unit). The final extract was collected in anhydrous sodium sulfate and then filtered in a column containing florisil 60-100 mesh in order to remove polar compounds.

For the analysis, the samples were grounded with KBr in the ratio of 100:1 (100 mg KBr and 1 mg sample) in an agate motor to make the pellets by using a Hydraullic press (CAP.- 15T) at a pressure of ten tons. Spectra were obtained with a total of 32 scans against a KBr background using FTIR (Fourier-Transform Infrared Spectrometer), Nicolet model- 6700, which works in covering frequency IR range of 4000-400 cm^{-1}. This standard allowed the determination of Total Petroleum Hydrocarbon (TPH) extractable by carbon tetrachloride in acidic medium.

3.11. Optimization of physico growth parameter favoring maximum degradation of HMW PAHs.

Effect of Temprature and pH on PAH degradation

The temperatures considered for optimization study were 15ºC, 25ºC, 35ºC, 55ºC and 55ºC. The cultures are prepared in five different flasks; each was maintained at different temperature. In this study pH of 7.4 ± 0.2 was maintained, since isolation of microbial strains was conducted at this pH. Experiment was carried out in 100 ml conical flasks containing 50 ml MSM broth with 2% PAH compound used as a carbon source. The flasks were kept in shaker incubator at 120 rpm, for 15 days. Similarly, the pH optimization study was considered by varying the pH 5.5, 6.5, 7.5 and 8.5 and 9.5. The culture flasks were kept in shaker incubator at 35ºC and 120 rpm, for 15 days. Growth of the five isolate in the presence of three PAH at varying concentrations, viz., 20mg/l for coronene, 50 mg/l for anthracene and coronene was observed by measuring the optical density of the culture broth at 600nm at 15 days h intervals using UV–visible spectrophotometer.

3.12. Plasmid mediated degradation of PAHs degrading bacteria.

Plasmid Curing and Isolation: Plasmid Curing was carried out on the isolates using the methods of **Winkler *et al.,* (1979)** and **Lexmi*et al.,* (1987).** The curing agent used was acridine orange. Physical evidence for the presence or loss of plasmid(s) in isolates was obtained by plasmid band. The plasmids were characterized using agarose gel electrophoresis **(Sambrook *et al.,* 1989).** The molecular weights of plasmids were visualized using UV transilluminator (Alpha Imager TM 2200) at 302-365nm. The role of plasmid in PAH degradation was confirmed by curing. Add 50µl of Acridine orange (0.10 mg/ml) to 5ml of LB broth, Keep it for incubation (24 hrs) in a shaker incubator. After incubation, this culture is swabbed in to the MHA Plates, Plates are incubated for 24 hrs at 35°C. The plasmid cured strains were screened for PAH degradation, uncured strarains used as control.

For Qulitative

For Qulitative degradation the cultures are prepared in 15 different flasks with respect to 3 PAH and 5 isolates; each was maintained at 30ºC, temperature. In this study pH of 7.4 ± 0.2 was maintained, since isolation of microbial strains was conducted at this pH. Experiment was carried out in 100 ml conical flasks containing 50 ml MSM broth with PAH compound (20 mg coronene, 50 mg anthracene and napthacene) for degradation. The flasks were kept in shaker incubator at 120 rpm, for 15 days. PAH degradation was measured by bacterial growth calculated at absorbance 600nm.

For Quantitative

Experiment was carried out in 100 ml conical flasks containing 50 ml MSM broth of five bacterial isolates with PAH compound (20 mg/l coronene/l, 50 mg/l of anthracene and napthacene) for degradation. The flasks were kept in shaker incubator at 120 rpm, for 15 days. Incubations were performed in triplicate for each analysis type at 35°C, shaking at150 rev min^{-1} in the dark. Triplicate individual cultures were removed and extracted with ethyl acetate for chemical, analysis at 15 days.

3.13. Biosurfactant extraction from PAH degrading bacteria and FTIR based analytical analysis.

Screening of biosurfactant

The isolated colonies were obtained in pure cultures and tested for their biosurfactant production by the following methods.

Five bacterial strains were screened for biosurfactant production on different carbon sources anthracene, napthacene and coronene on the basis of its ability to reduce the surface tension. For biosurfactant production, 2 ml of inoculum was added to 100 ml of sterile basal medium containing PAH (2 %, v/v) used as the carbon source. Flask cultures were incubated at 35 °C, on a rotary shaker at 150 rpm for 6days. Surface tension of cell-free supernatants was measured by a Gibertini tensiometer (TSD 132389, Milan, Italy), according to the method described previously **(Mnif *et al.*, 2011).** Values given are the mean of three replicates± standard deviation.

Surface tension Measurement

50 ml of the crude biosurfactant of 5 strains were taken for surface tension measurement with respect to distilled water. By digital tensiometer the surface tensions of each strain were plotted. Hence the surface tensions with respect to distilled water were determined **(ABU-Ruwaida *et al.*, 1991).**

Oil spreading technique

In oil spreading assay (Morikawa *et al.,* 1993), 10 µl of crude oil was added to the surface of 40 ml of distilled water in a petri dish to form a thin oil layer. Then, 10 µl of culture or culture supernatant were gently placed on the centre of the oil layer. The presence of biosurfactant would displace the oil and a clear zone would form. The diameter of the clearing zone on the oil surface would be visualized under visible light and measured after 30 seconds, which correlates to the surfactant activity, also known as oil displacement activity.

Drop-collapse test

The bacterial strains were inoculated in mineral salts medium with 1% crude oil and incubated for 48 hours. Drop collapse test was performed to screen the biosurfactant production (Jain *et al.,* 1991). 2µl of crude oil i.e., crude oil was applied to the well regions delimited on the covers of 96-well microplates and these were left to equilibrate for 24 hours. The 48 hour culture was centrifuged at 12,000g for 15mins at 25°C to remove the cells. 5 µl of the supernatant was transferred to the oil-coated well regions and drop size was observed after 1 min with the help of a magnifying glass. The result was considered to be positive when the diameter of the drop was increased by 1mm from that which was produced by distilled water which was taken as the negative control (Youssef *et al.,* 2004).

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3.13.1. Extraction of biosurfactants

The cultures were inoculated in 50 ml Mineral salt broth to which 1ml of 2% PAH solvent was added. The cultures were incubated at 35°C for 7 days with shaking conditions. After incubation, the cultures were centrifuged at 5000rpm, 4°C for 30 minutes to remove the bacterial cells. To the supernatant thus obtained, 1M HCL was added to adjust the pH at 2. Equal volumes of chloroform: methanol was added in the ratio of 2:1. These mixtures were shaken well to ensure proper mixing and were left overnight for evaporation. White coloured precipitate if seen at the interface between the two liquids proved the presence of biosurfactant.

3.13.2. Purification of biosurfactants

The biosurfactant formed was carefully taken out with the help of micropipette and kept in eppendorf tubes. 1ml distilled was added to the eppendorf containing biosurfactant and was thoroughly vortexed to ensure uniform mixing. These were centrifuged at 7000rpm, 4°C for 30 minutes. The supernatant was discarded and the pellet was all owed to dry for 24 hours. The dry pellet thus obtained was the crude extract of biosurfactant.

3.13.3. Characterization of biosurfactant

Structural characterization: The chemical structures of the components in the crude biosurfactant sample were preliminarily determined by using Fourier transform infrared (FT-IR) spectroscopy. Moreover and nuclear magnetic resonance (NMR) analysis were employed to identify the chemical structures of the components in the crude biosurfactant.

2.13.4 Fourier Transform Infrared analysis (FTIR)

FTIR spectroscopy was carried out using crude biosurfactant extract obtained from the acid precipitation of the cell free culture supernatant. IR Prestige- 21Fourier Transform Infrared spectrophotometer (Nicolet model- 6700) was used to measure the absorption spectra, solvent extracted biosurfactant sample was dropped on the Potassium bromide (KBr) crystal at a resolution of 4 cm^{-1} and measurement wave length range from 600 to 4000 cm^{-1} **(Saher *et al.,* 2011)** and determine the chemical nature of the bisurfactant by the KBr pellet method **(Das *et al.,* 2008a, b; Mukherjee *et al*., 2009).**

The organic functional groups present in the biosurfactant sample were determined using FTIR analysis. The analysis was carried out using FTIR- Nicolet model- 6700. To measure the absorption spectra, solvent extracted biosurfactant sample was dropped on the Potassium bromide (KBr) crystal at a resolution of 4 cm-1 and measurement wave length range from 600 to 4000 cm^{-1} **(Saher *et al.,* 2011).**

3.14. Degradation of PAH (Antracene, Coronene and Nepthacene)

3.14.1. Qulitative degradation by bacterial growth (OD measurment)

For Qulitative degradation the cultures are prepared in 15 different flasks with respect to 3 PAH and 5 isolates; each was maintained at 35ºC, temperature. In this study pH of 7.4 ± 0.2 was maintained, since isolation of microbial strains was conducted at this pH. Experiment was carried out in 100 ml conical flasks containing 50 ml MSM broth with PAH compound (20 mg coronene, 50 mg anthracene and napthacene) for degradation. The flasks were kept in shaker incubator at 120 rpm, for 10, 20 and 30 days. PAH degradation was measured by bacterial growth calculated at absorbance 600nm.

First bacterial cultures were inoculated in Luria Bertani broth for 2 days. Next day 100 µl of these bacterial cultures were sub cultured in 50 ml Mineral salt agar medium with 50 mg/l of anthracene, napthacene and 20mg/l coronene for 7 days for enrichment culture. At 7th day the pellets of each bacterial culture was collected by centrifuging at 6000 rpm, 10 min at 4°C. Then these pellets were re-suspened in Mineral salt broth (2 ml). Then the O.D. of each bacterial pellet (300 µl) was taken at 595 nm in ELISA Plate Reader. As O.D. of each strain was found less than 1, then 50 µl of enriched pellet of each strain was transferred to 5 ml of Mineral salt agar medium with Anthracene, Napthacene (50 mg/l), (Coronene-20 mg/l) and kept in shaker incubator (in dark) at 180 rpm, 35°C. Then at Day-10, Day-20 and 30 days extraction was done by adding the equal volume of n-hexane. After adding n- Hexane, it was vortexed for 5 min and then centrifuged at 6000 rpm for 10 minutes at 4°C to collect the Hexane layer. Then O.D. of the Hexane extract was taken at 254 nm and also scanned from 200 nm to 400 nm **(Tao *et al.*, 2007).**

3.14.2. Quantitavive degradation by UPLC

Analysis of percentage degradation of HMW PAHs

Media and stock solutions

A Mineral salts medium (MSM) was used for PAH degradation studies **(Juhasz *et al.*, 2000).** Stock solutions of each PAH were prepared in ethyl acetate and 0.1ml added to 9.9 ml of MSM to achieve final PAH concentrations of 50mgl $^{-1}$.When a mixture of PAHs was used (Antracene and nepthacene) PAHs were added to achieve a final concentration of 50mgl^{-1} for each PAH, except for coronene which was supplied at 20mgl^{-1}.

Degradation of PAHs by five bacterial strains

Inocula for PAH degradation studies were prepared as described previously **(Juhasz *et al.*, 2000).** Cell suspensions (10ml; 7.5X10^{-7} cells ml^{-1}) were inoculated into sterile 30ml serum bottles to evaluate the degradation of single PAHs or the PAH mixture. Incubations were performed in triplicate for each analysis type at 35°C, shaking at150 rev min^{-1} in the dark. Triplicate individual cultures were removed for chemical, microbiological, toxicological and mutagenic analysis at 10, 20, 30 and 40d. Controls consisted of inoculated cultures which were killed by the addition of mercuric chloride (2%w/v). Percentage degradation and remaining PAH concentration was analyzed by UPLC.

Calculations

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

Residual concentration (in ppm)

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

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

3.15. Effect of surfactant/ Emulsifier to enhance PAH degradation

Effect of biodegradation experiments were performed in 3 replicates. 1.5 ml of the culture were inoculated in 150 ml MSM medium containing 1% w/w of the corresponding surfactant. The resulting initial concentration for each surfactant was 15.9 mM for Tweens20, 17.1 mM for Triton X-100 and 8.1 mM for Tween-80. PAH was added to a concentration of 50 mg/l for anthracene and napthacene, 20mg/l for coronene. Erlenmeyer flasks were incubated at 200 rpm and 35^{0}C during 30 days. Samples were withdrawn at different time intervals to monitor PAHs depletion and cell density. Control cultures without the addition of any surfactant were conducted for all bacterial strains. In addition, an abiotic control without bacterial inoculum was performed in order to evaluate PAH depletion due to physicochemical processes. Cell density was monitored by measuring the absorbance at 600 nm using a spectrophotometer.

3.16. Statistical analysis

Three replicates were used throughout the experiments, and the mean values with standard deviation were calculated using Microsoft Excel 2013, Prism (GraphPad 6.0) and SPSS ver. 16.

The experimental data are presented in terms of arithmetic averages of at least three replicates and the standard deviations are indicated by the error bars. The analyses were done using SigmaPlot software, version 8.02 (SPSS Inc., UK).

Chapter- 4

Results

4. RESULTS

4.1.Collection of sample

Soil samples were collected from the surface layer (0–10 cm) from Mathura oil refinery (Northern) - Latitude - $27^0$26′ N, Longitude - 77043′ E and Barauni refinery (Eastern) - Latitude - 25028′ N Longititude - $85^0$59′ E which had been contaminated for more than 30 years. Soil had the following physic-chemico characteristics: pH, organic carbon, nitrogen, phosphorus, potassium, soil moisture, texture, pH and temperature were shown in following table. Soil samples were air dried in the dark, passed through 2mm sieve, and stored at 4 ◦C until use.

Most of the microbes species present in the environment genetically have an efficiency to utilize the hydrocarbons as a carbon source. This ability comes to expression only when they are grown in the hydrocarbon environment (Atlas, 1981). Sample collection from petroleum oil's contaminated sites will facilitate to isolate microbial strains with hydrocarbon degradation efficiency **(Li *et al.,* 2000; Length, 2010).** So, samples have been collected from ten diffrent sites from each refinery.

Sample site	**No. of sample**
Mathura oil refinery	**25**
Barauni oil refinery	**25**

Table 4.1: Incidence of PAH degrading bacteria from petroleum refinery sites.

Total sample	Total Isolates	Individual Incidence				
		Bacillus Licheniformis	Bacterium	Bacillus pumilus	Paenibacillus	Bacillus acidovirans
Barauni refinery(25)	26	8	2	5	6	5
Mathura refinery (25)	32	10	4	5	9	4
Total -50	58	18	6	10	15	9

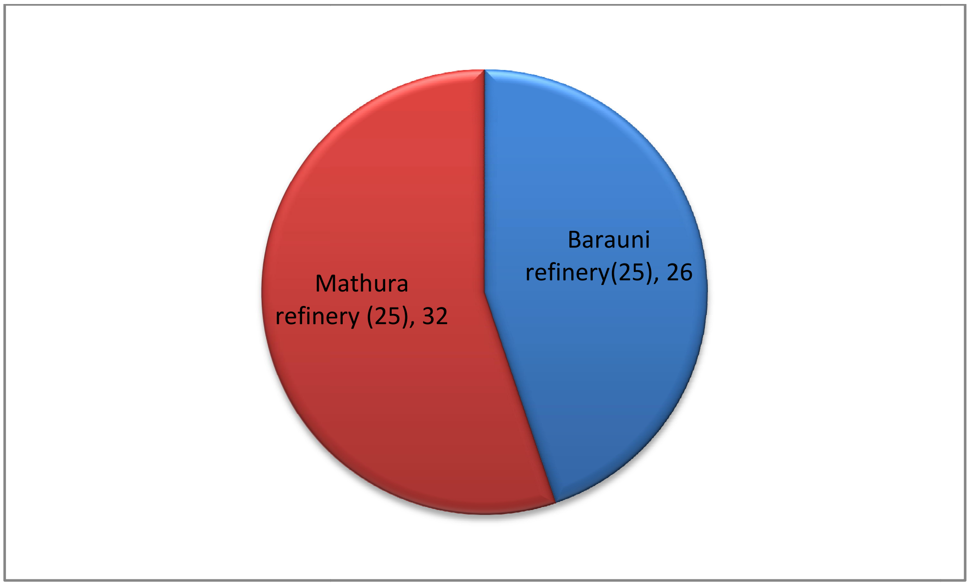


Figure 4.1: Isolates distribution of samples from refinery sites.

Table 4.2: Incidence and percentage distribution of bacterial strains from two site i.e. Barauni and Mathura refinery sites.

Total sample	Total Isolates	Individual Incidence				
		Bacillus Licheniformis	Bacterium	Bacillus pumilus	Paenibacillus	Bacillus acidovirans
Barauni refinery(25)	44.8%	13.79%	3.44%	8.62%	10.34%	8.65%
Mathura refinery (25)	55.17%	17.24%	6.89%	8.62%	15.51%	6.89%
Total -50	58=100 %	31.03%	10.34%	17.24%	25.86%	15.51%

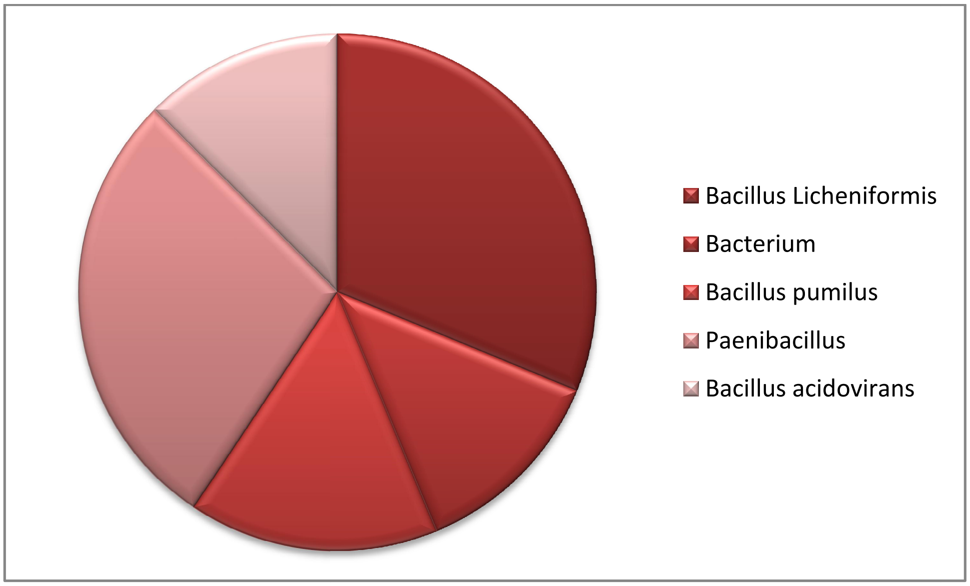


Figure 4.2: Incidence and percentage distribution of bacterial strains from two sites.

4.2.Physico Chemico Properties of soil sample

Physico-chemicoproperties of was succeed by sigma Aldrich kit for analysis of total nitrogen, organic carbon, phosphorous and potassium.

Table 4.3: Physico-Chemical properties of Mathura oil refinery.

Physico-Chemical properties of Mathura oil refinery (Northern) - Latitude - 27^{0}26′ N Longitude - 77^{0}43′ E									
Sample	Color	Soil Moisture	Soil texture	Temperature	pH	Total Nitrogen (%)	Organic carbon (%)	Extractible Phosphorous (ppm)	Exchangeable Potassium (ppm)
S1	Dark Black	35.57 ± 5.12	Cl=06.67 ± 03.47 Si=21.87 ± 09.78 Sa=71.54± 00.86	18^{0}C	5.50 ±0780	0.12 ± 1.005	8.04 ± 0.90	4.57 ± 1.70	38.83 ± 2.45
S2	Black	32.67 ± 5.22	Cl=06.67 ± 03.47 Si=21.87 ± 09.78 Sa=71.54± 00.86	19^{0}C	5.90 ±0.80	0.18 ± 1.003	8.05 ± 0.99	4.47 ± 1.40	39.64 ± 2.75
S3	Dark Black	31.67 ± 3.12	Cl=06.67 ± 03.47 Si=21.87 ± 09.78 Sa=71.54± 00.86	14^{0}C	6.00 ±0.80	0.19 ± 1.005	7.95 ± 0.99	4.67 ± 1.30	39.63 ± 2.95
S4	Black	25.67 ± 4.12	Cl=06.67 ± 03.47 Si=21.87 ± 09.78 Sa=71.54± 00.86	14^{0}C	5.40 ±0.86	0.12 ± 1.001	8.05 ± 0.99	4.07 ± 1.10	39.43 ± 2.42
S5	Dark B	36.67 ± 6.12	Cl=06.67 ± 03.47 Si=21.87 ± 09.78 Sa=71.54± 00.86	16^{0}C	5.90 ±0.60	0.13 ± 1.006	8.05 ± 0.99	4.47 ± 1.50	39.84 ± 2.45
Physico-Chemical properties of Barauni refinery (Eastern) - Latitude - 25^{0}28′ N Longititude - 85^{0}59′ E									
B1	Dark Black	39.67 ± 6.18	Cl=06.67 ± 03.47 Si=21.87 ± 09.78 Sa=71.54± 00.86	18^{0}C	6.40 ±0.80	0.15 ± 1.006	8.55 ± 0.27	4.17 ± 1720	39.63 ± 2.45
B2	Dark	37.66 ± 5.22	Cl=06.67 ± 03.47 Si=21.87 ± 09.78 Sa=71.54± 00.86	19^{0}C	5.80 ±0.80	0.18 ± 1.005	8.25 ± 0.49	4.47 ± 1.20	39.63 ± 2.45
B3	Dark	36.67 ± 4.12	Cl=06.67 ± 03.47 Si=21.87 ± 09.78 Sa=71.54± 00.86	15^{0}C	5.90 ±0980	0.14 ± 1.004	8.85 ± 0.99	4.67 ± 1.20	39.63 ± 2.45
B4	Black	35.67 ± 5.12	Cl=06.67 ± 03.47 Si=21.87 ± 09.78 Sa=71.54± 00.86	18^{0}C	7.80 ±0.70	0.12 ± 1.004	8.00 ± 0.99	4.97 ± 1.00	39.63 ± 2.45
B5	Black	32.67 ± 3.12	Cl=06.67 ± 03.47 Si=21.87 ± 09.78 Sa=71.54± 00.86	17^{0}C	5.90 ±0.50	0.12 ± 1.005	8.00 ± 0.89	4.07 ± 110	39.63 ± 2.45

4.3.Enrichment culture, isolation and screening of bacteria from PAH contaminated soil sediment

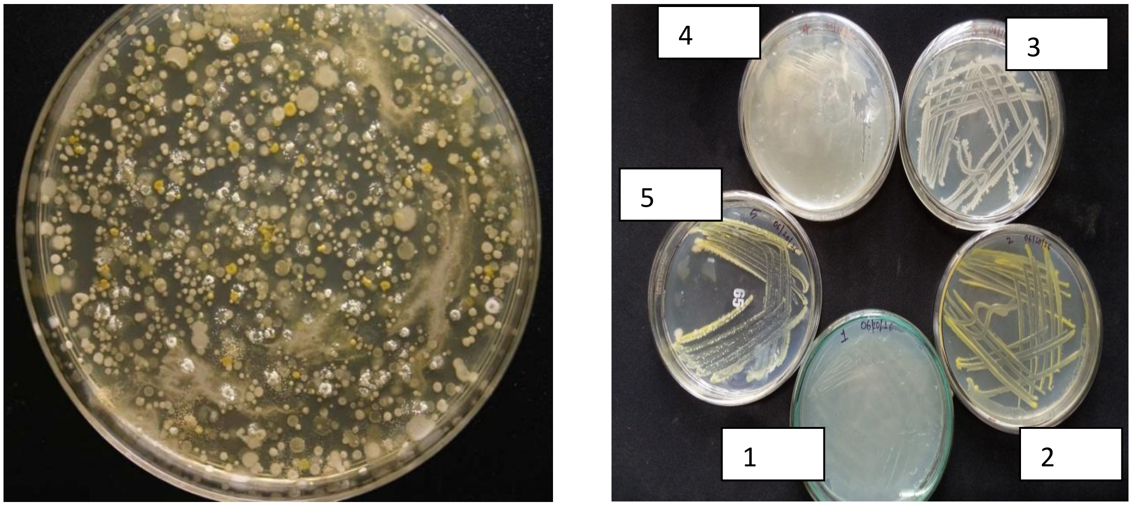
To enrichment and isolate the efficient PAH degrading microorganisms was proceed by enrichment culture method **(Figure 4.3)**. 10 g of petroleum contaminated soil, collected from Mathura and Barauni Oil Refinery, India was added to 100 ml minimal salt medium (MSM) and incubated for 15 days in an orbital shaker set at 35°C and 150 rpm. After 15 days of incubation, 2 ml inoculum of bacterial culture was transferred to the flasks having fresh sterilized MSM supplemented with petroleum oil (2% w/v) and incubated in an orbit shaker set at 37°C and 150 rpm for the enrichment of petroleum hydrocarbon degrading bacterial strains. After repeating this procedure for 3 times, the active inoculum was used for the isolation of TPH (Total petroleum hydrocarbon) degrading bacteria, following serial dilution method and spreading over the nutrient agar. 500µl of 10^{-4}serial diluted microbial enriched water sample from the refinery soil inoculated liquid MSM, Culture plates were incubated for 2 days at temperature 35ºC. After two days of incubation microbial colonies were observed on the petri dishes.

Figure 4.3: Enrichment culture, isolation and screening of bacteria from PAH contaminated soil sediment

Microbial colonies grown on crude oil supplemented petri plates were further tested to examine the growth on higher hydrocarbon fractions for screening of best degraders to select for next experiment. MSM with 2% PAH (antracene, coronene and napthacene) was used as a carbon source and the culture plates were incubated at 35ºC temperature for 7 days. After incubation period growth of microbial colonies were observed on the petri dishes. Following table were shown the incidence of isolates at each site and also represented the individual bacterial incidence with respect to site and species.

4.4.Identification of PAH degrading bacteria

From two refinery soil sampling sites, fifty eight bacteria were isolated after screening of PAH utilization test on mineral salt agar plates containing antracene, coronene and napthacene respectively. Among fifty eight isolates five different type of bacterial were selected after primary identification procedure were carried by morphological characteristics, cultural characteristics and biochemical test. According to the Berge's manual of systematic bacteriology bacterial strains are characterized. **(Table 4.3).** All isolates were gram positive rod shaped bacillus cells except Penibacillus lautus which was gram negative **(Figure 4.4).** Colony color was off white, yellow, creamy white, creamy and light yellow.



Mix culture **Pure culture**

Figure 4.4: Morphological and cultural characteristics of PAH degrading bacteria

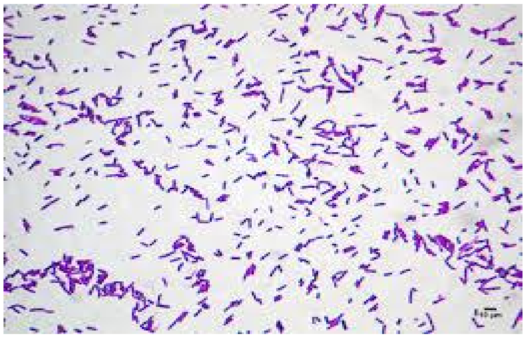
1,2,34, and 5 mentioned as *licheniformis*, *Bacterium CT, Bacillus Pumilus Bacillus Paenibacillus lautus* MORL, *Bacillus acidovirans* .

Table 4.3: Identifying characteristics of bacteria from petroleum contaminated soil

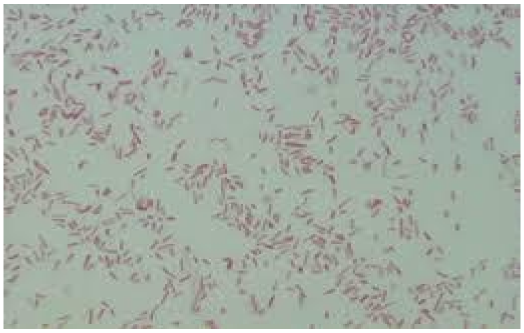
Characteristics		***Bacillus licheniformis***	***Bacterium C-T***	***Bacillus pumilus***	***Paenibacilllus Lautus***	***Bacillus acidovorans***
Colony morphology	Color	white	yellow	Creamy white	creamy	Light yellow
	Margin	Undulate	Irregular	Irregular	Entire	Wavy
	Elevation	Raised	Raised	Flate	Concave	Convex
	Shape of colony	irregular	Circular	Undulate	Filamentous	Filamentous
	Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
	Pigmentation	Creamy	No	white	No	No
	Areal hyphae	-	-	-	+	-
Cell morphology	Spore chain	Cylindricall	spiral	spiral	spiral	-
	Gram stain reaction	+	+	+	-	+
	Acid-fast	-	-	-	+	+
	Cell shape	Rod in chain	Rod	Rod	Rod	Rod
Biochemical characteristics	Casein hydrolysis test	+	+	+	-	-
	Gelatin Liquefaction	+	-	+	-	-
	Starch hydrolysis test	+	+	+	+	+
	M.R	_	-	-	-	+
	VP	+	+	+		
	Indole test	-	-	+	-	-
	Nitrate Reduction	+	+	+	-	+
	Catalase test	+	+	+	+	+
Utilization of Carbon source	D-Glu.	$A^+ G^+$	A^-G^-	$A^+ G^+$	A^-G^-	A^-G^-
	Sucr.	$A^+ G^+$	$A^+ G^+$	$A^+ G^+$	$A^+ G^+$	$A^+ G^+$
	D-Man.	$A^+ G^-$	$A^+ G^-$	$A^+ G^-$	$A^+ G^-$	$A^+ G^-$
	D-Xyl.	$A^- G^-$	$A^- G^-$	A^+G^+	$A^- G^-$	$A^- G^-$
	L- Ara.	$A^+ G^+$	$A^+ G^+$	$A^+ G^+$	$A^+ G^+$	$A^+ G^+$

4.5. Gram staining microscopic structure:-

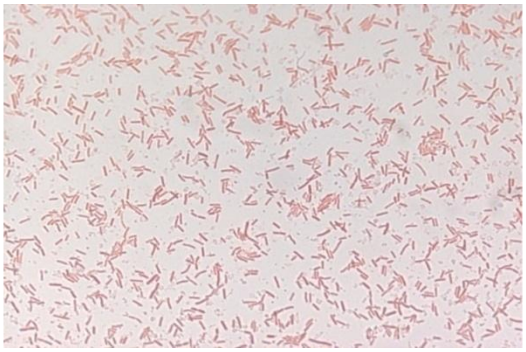
The staining studies revealed the Gram +ve nature and rod shape of the isolates



Bacillus licheniformis

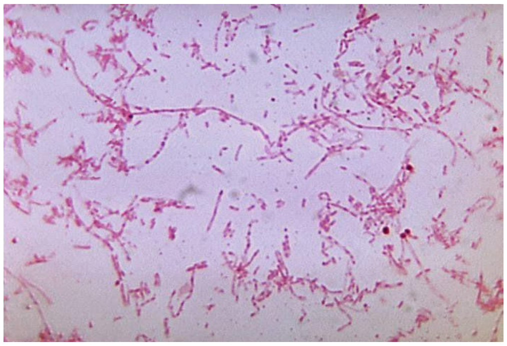


Bacillus pimulus



Paenibacillus lautus

Bacillus acidovorans

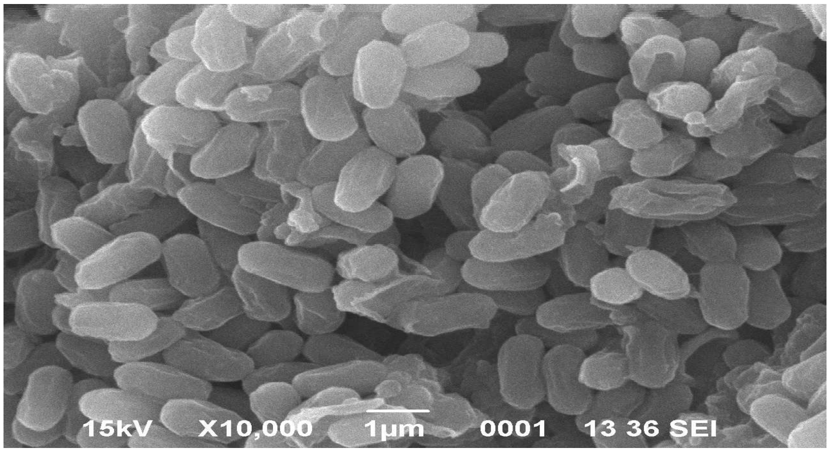


Bacterium C-T j 29

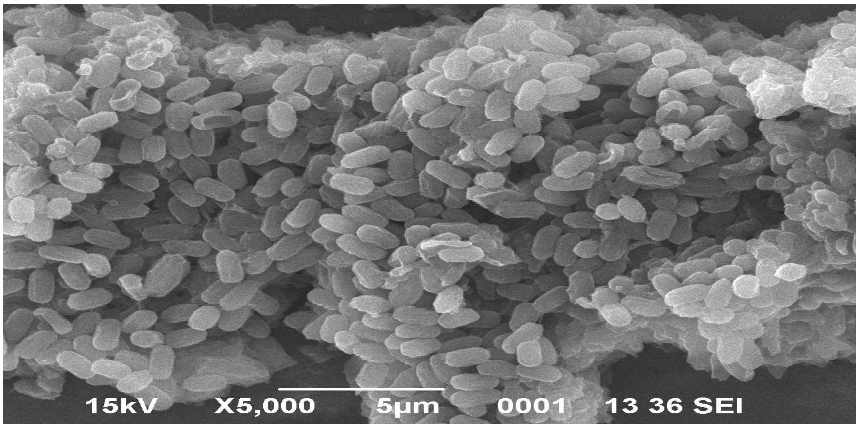
Sucr. = Sucrose, L- Ara. = L- Arabinose, D-Glu. = D-Glucose, D-Man. =D-Mannitol, D-Xyl. =D-Xylose, +ve = Acid positive;

A^+ = Acid production, A^- = No acid production, G^+ = Gas production, G^- = No gas production.

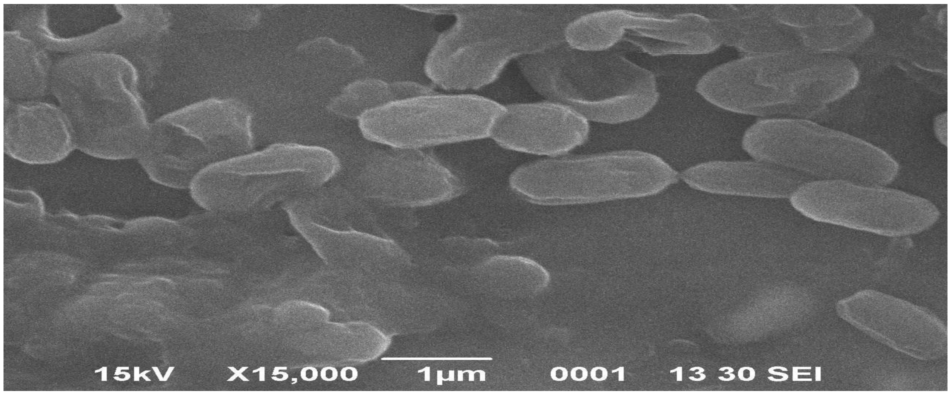
the short rod nature of the isolate belongs to *Bacillus licheniformis*. SEM is a type of electron microscope that divulges the image of a sample by scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interrelate with the atoms that make up the sample, producing signals that contain information about the sample's surface structure. Morphology of isolates *Bacterium CT, Bcillus pimulus* and *Bcillus acidovorans* shown smooth surface. All bacterial morphology after observation was found rod shape.



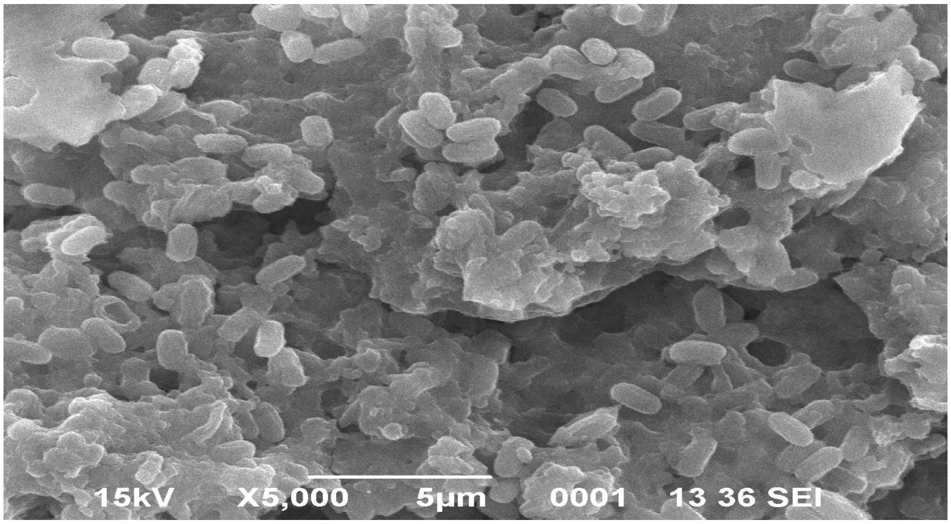
***Bacillus licheniformis*.**



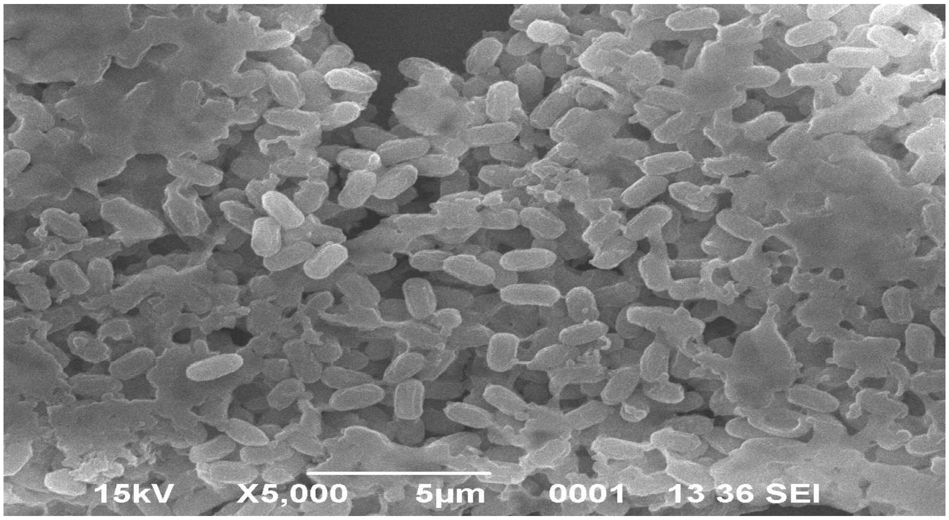
Bacterium C-T j 29



Bacillus pimulus



Paenibacillus lautus



Bacillus acidovorans

4.7.Molecular characteristics of PAH degrading bacteria

4.7.1. 16s rRNA gene Sequencing and phylogenetic analysis:

Miniprep kit (Qiagen) and sequenced by using Big Dyeterminator with an automated capillary sequencer (Applied Biosystems). Inserts were sequenced using vector-specific and a suite of 16S rRNA-specific primers to generate an overlapping set of sequences that were assembled into one contiguous sequence. The sequences obtained were compared with those from the EZTaxon database; aligned using the Clustal W software and phylogenetic trees inferred using the neighbor-joining method in the MEGA 7 program. The identified sequences were submitted to NCBI database.

Figure 4.5: DNA Band Isolated from bacterial isolates

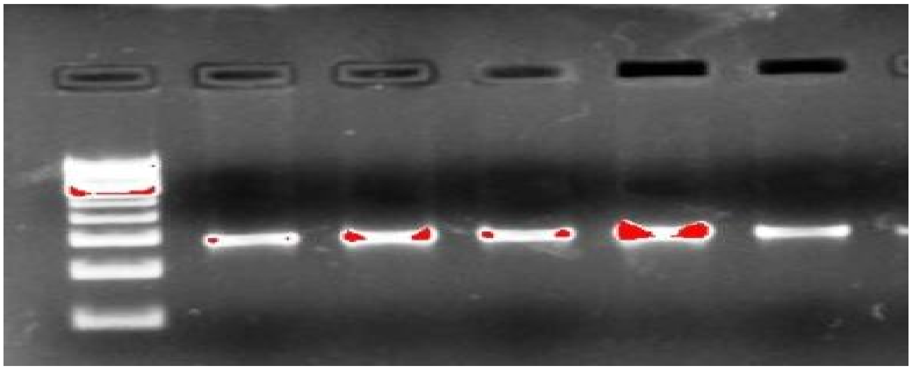


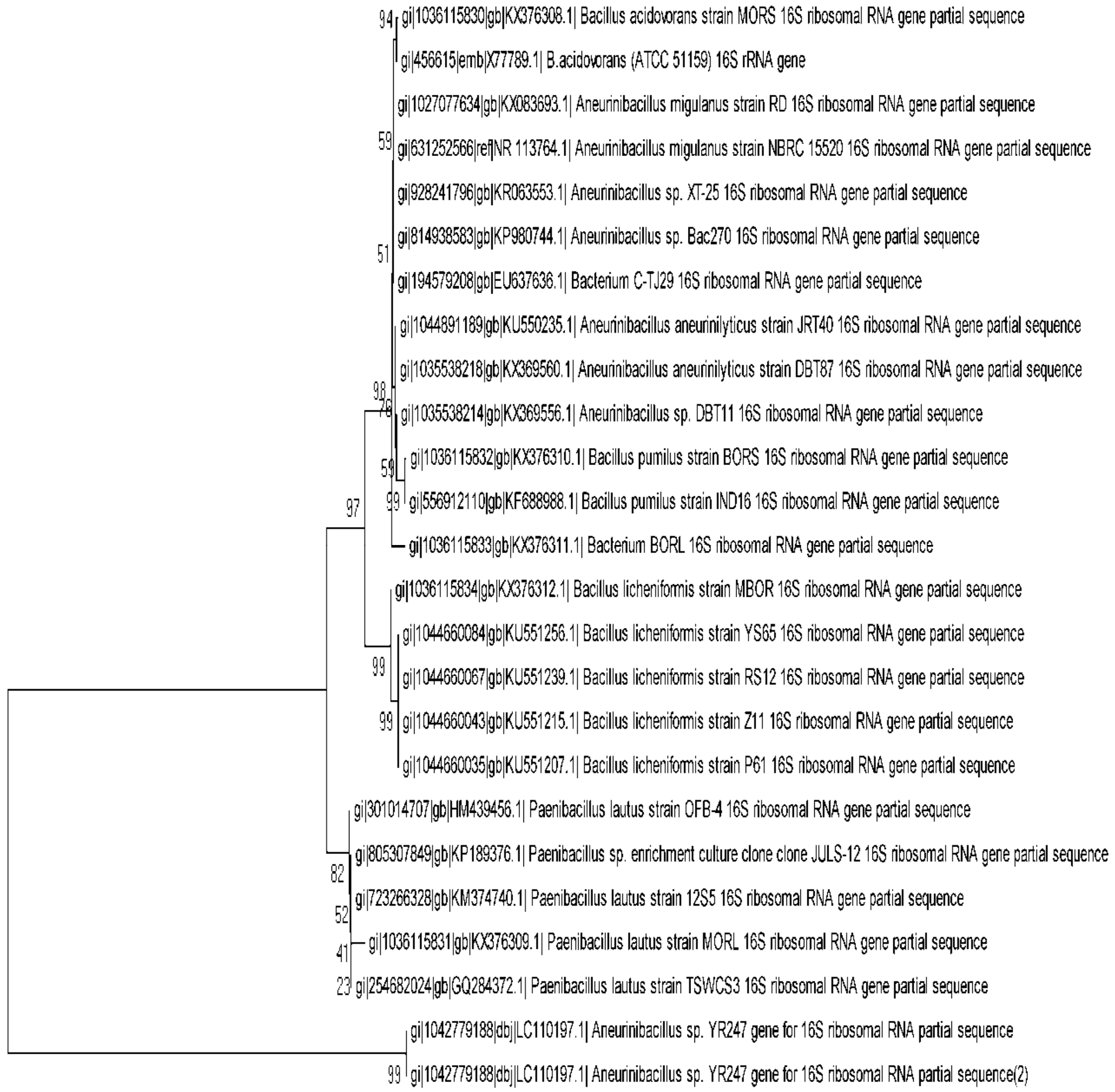
Figure 4.6: DNA Band after PCR product

4.8. Isolated sequence submitted in National Centre of Biotechnology Information (NCBI) after identification/purification and sequence analysis.

Following sequences with accession number have been submitted in NCBI:

Identified Isolates		**Accession no at NCBI**
1	*Bacillus licheniformis* strain MBOR	**KX376312**
2	*Bacterium* BORL	**KX376311**
3	*Bacillus pumilus* strain BORS	**KX376310**
4	*Paenibacillus lautus* strain MORL	**KX376309**
5	*Bacillus acidovirans* strain MORS	**KX376308**

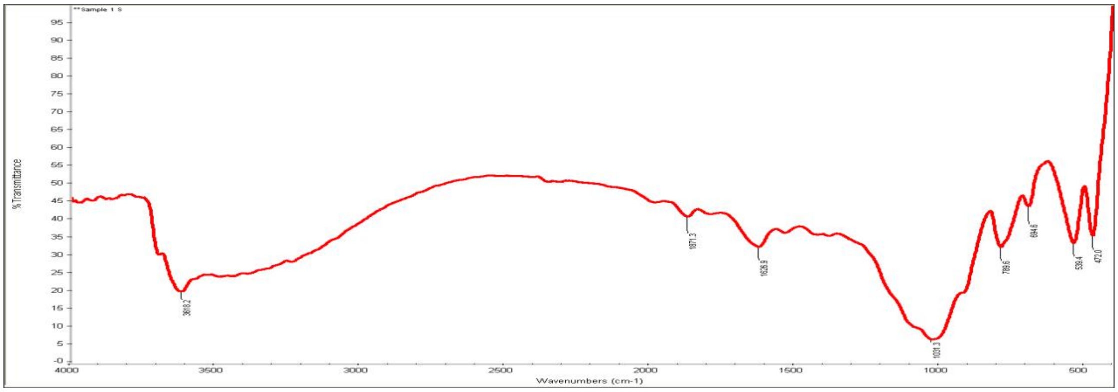
4.9.Phylogentic tree of PAH degrading Five Isolates



4.10. FTIR based analysis of PAHs components extracted from contaminated soil for presence of HMW PAHs.

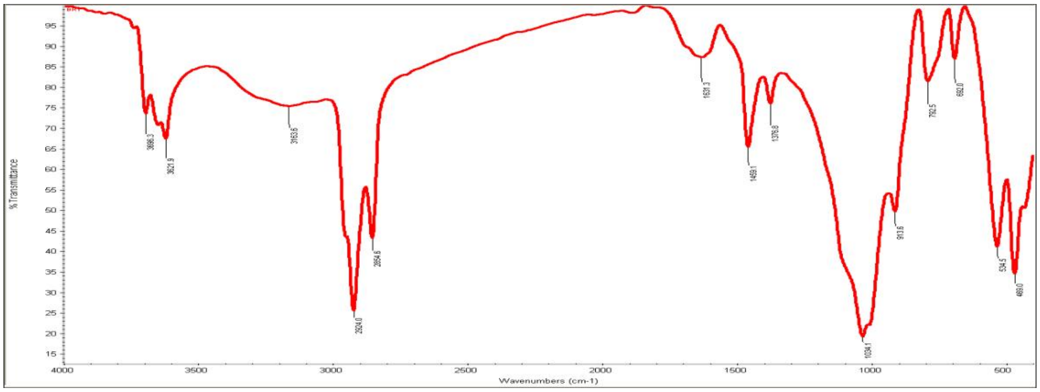
Result observation was shown that four sample of Mathura and Baruani refinery, two samples from each site had heavily contaminated with hydrocarbon and PAHs compounds. All analysis was succeeding by comparative study with control and sample at particular absorbance of IR. The different range of wave number shown the different hydrocarbon and aromatic compound present in samples, 3100–3000 (s) C–H stretch aromatics, 3100–3000 (m) =C–H stretch alkenes, 3000–2850 (m) C–H stretch alkanes, C– stretch alkynes, 1750–1735 (s) C=O saturated aliphatic, 1740–1720 (s) C=O saturated aliphatic, 1730–1715 (s) C=O stretch–unsaturated esters, 1715 (s) C=O stretch, saturated aliphatic, 1680–1640 (m) –C=C– stretch alkenes, 1600–1585 (m) C–C stretch (in–ring) aromatics, 1500–1400 (m) C–C stretch (in–ring) aromatics, 1470–1450 (m) C–H bend alkanes, 1370–1350 (m) C–H rock alkanes, 1000–650 (s) =C–H bend alkenes, 900–675 (s) C–H "oop" aromatics, 725–720 (m) C–H rock alkanes, C–H: C–H bend alkynes.

Control for all soil sample



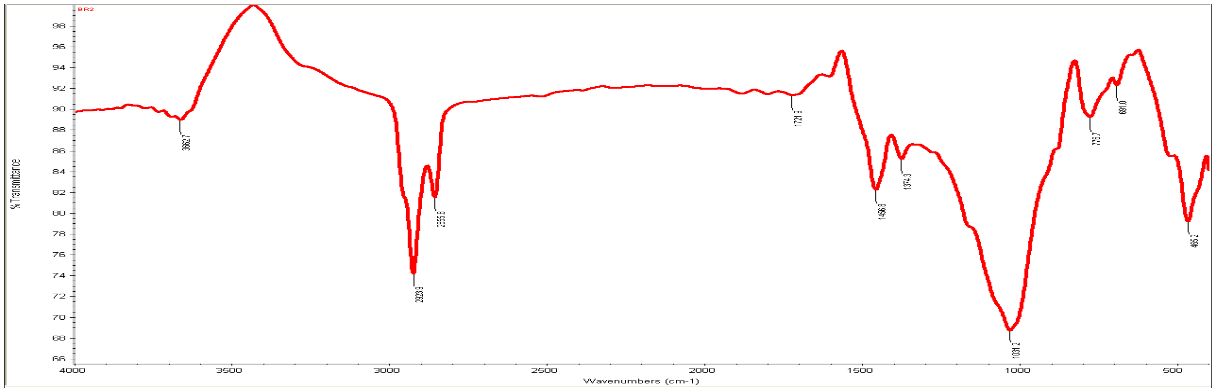
Frequency, cm^{-1}	Bond	Functional group
1031.3	C-O stretch, C-N strech	Alcohal, Corboxylic ester, ether, aliphatic amines
789.6	C-H bend N-H wag C-H "opp"	Alkanes $1^0 2^0$ Amineslkyl Alkyl halides
694.6	-C-H- Rocks C-H "opps" -C=C-H: C-H	Alkanes Aromatic Alkynes
539.4	C-Br	Alkyl halide

Sample 1: BR_1 (Baruani refinery)



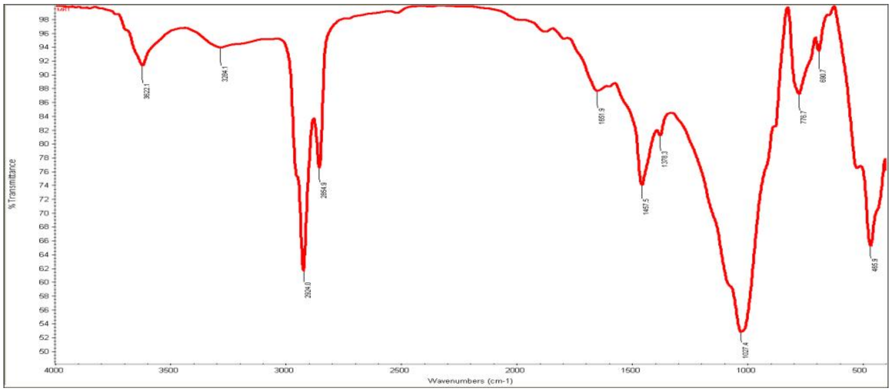
Frequency, cm^{-1}	Bond	Functional group
2924.0, 2854.6, 1459.1	C-H bend	Alkanes
1459.1	C-C Strech in ring	Aromatic
913.6, 792.5, 692.0	=C-H Bend	Alkene
792.5, 692.0	C-H “opps”	Aromatic

Sample 2: BR_2 (Baruani refinery)



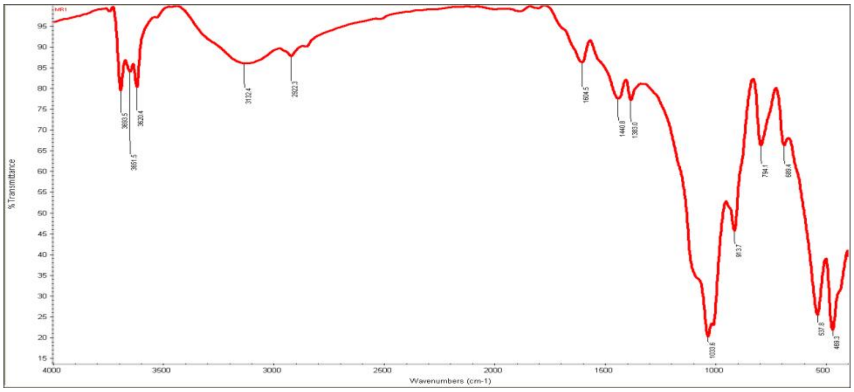
Frequency, cm^{-1}	Bond	Functional group
2923.4, 2855.8, 1456.8	C-H bend	Alkanes
1456.8	C-C Strech in ring	Aromatic
776.7, 691.0	=C-H Bend	Alkene
776.7	C-H “opps”	Aromatic

Sample 3: MR$_1$(Mathura oilrefinery)



Frequency, cm^{-1}	Bond	Functional group
2922.4, 2854.9, 1457.5	C-H bend	Alkanes
1651.9	-C=C-	Alkenes
1457.5	C-C Strech in ring	Aromatic
776.7,690.7	=C-H Bend	Alken
776.7, 690.7	C-H “opps”	Aromatic

Sample 4: MR$_2$



Frequency, cm^{-1}	Bond	Functional group
2922.4, 913.7	C-H bend	Alkanes
1440.8	C-C Strech in ring	Aromatic
794.1, 689.4	-C-H- Rocks	Alkanes

	C-H “opps”	Aromatic

4.11. Optimization of physico growth parameter favoring maximum degradation of HMW PAHs.

Samples withdrawn at scheduled temperature intervals 15, 25, 35, 45 and 55^{0}C for seven days. The optimized concentration of PAH was 50 mg/L Napthacene., 50 mg/L Anthracene., 20 mg/L Coronene, supplementation in MSM medium. For pH optimization same processes was followed at pH 4.5, 5.5, 6.5, 7.5, and 8.5 and incubates at 35^{0}C for 15 days .The growth of isolates was calculated based on the absorption displayed in UV–visible spectral analysis at 600 nm. Result was shown that maximum growth was observed at pH 6.5 and temperature 35^{0}C.

4.11.1. Effect of Temprature and pH on PAH degradation

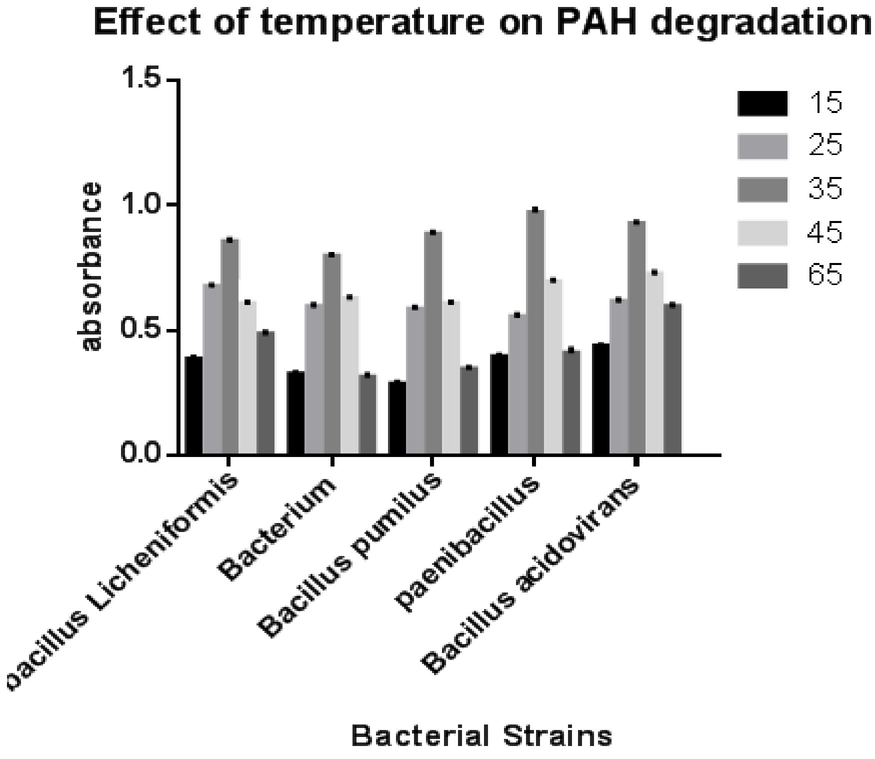
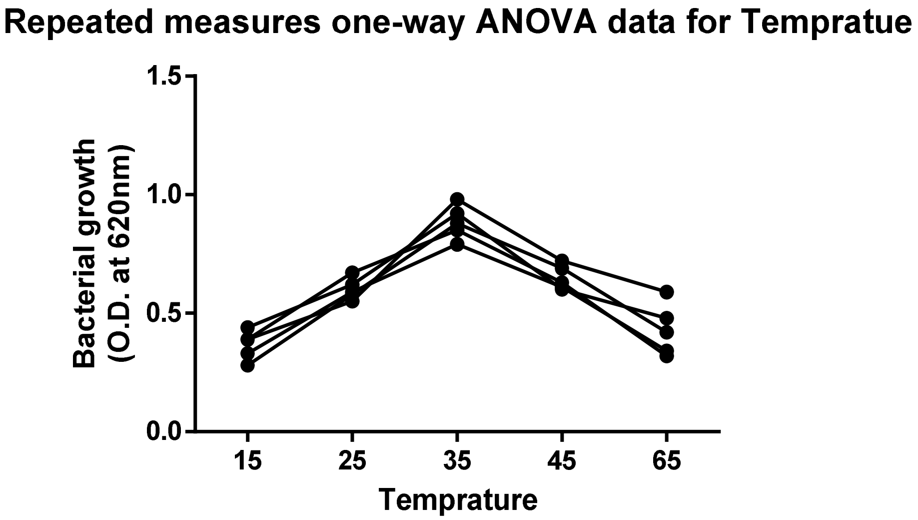


Figure 4.7. Effect of Temprature and temperature on PAH degradation



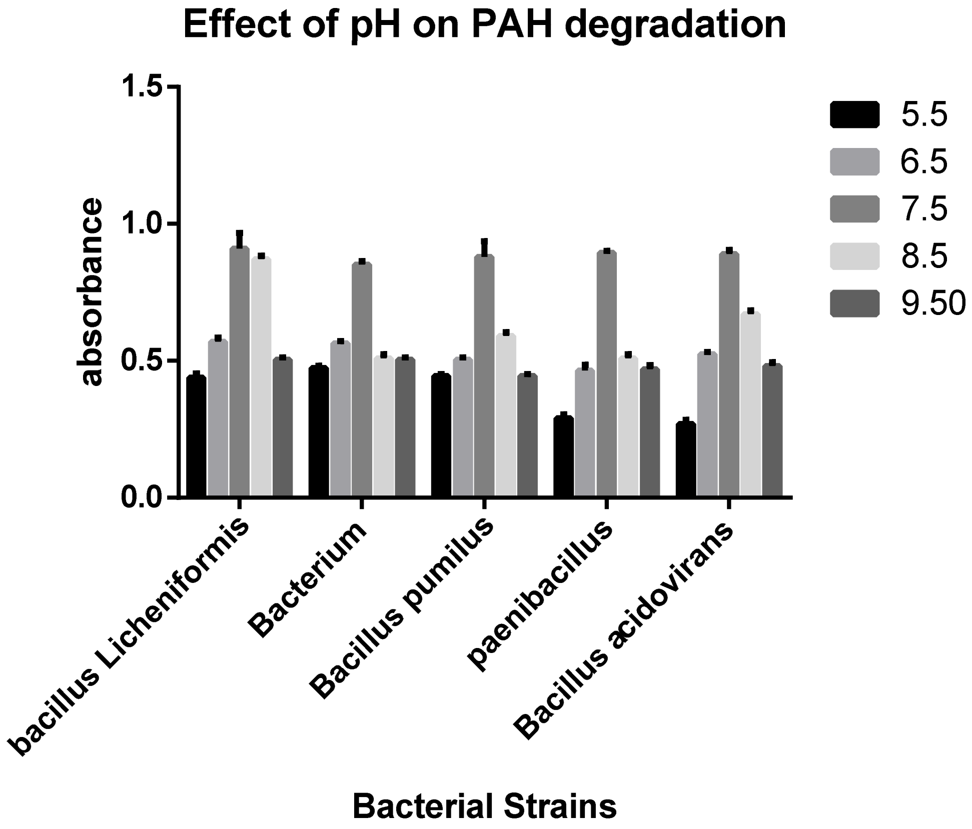


Figure 4.8. Effect of pH and temperature on PAH degradation.

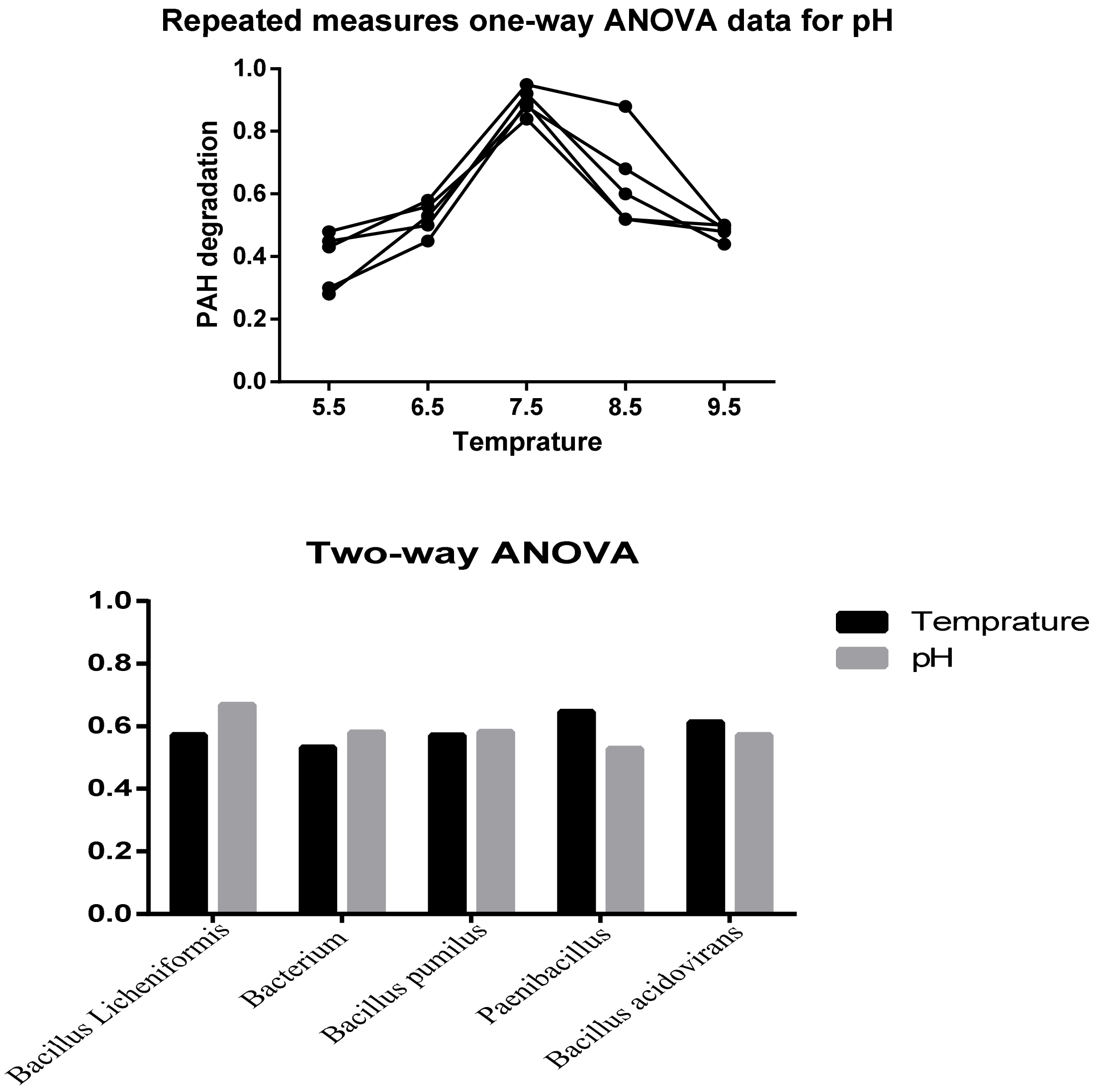


Figure 4.9. Comparative two-way ANOVA for pH and Temprature.

4.12. Plasmid mediated degradation and

Plasmid mediated degradation was succeed by plasmid curing. In this study plasmid DNA was observed and check the molecular weights of plasmids were visualized using UV transilluminator (AlphaImager TM 2200) at 302-365nm. Observation shown the loss of plasmid DNA after curing processes. The plasmid cured strains were screened for PAH degradation and uncured strarains used as control. After screening processes cured isolates were loss the degrading activity. Following plasmid DNA band loss was shown in for band.

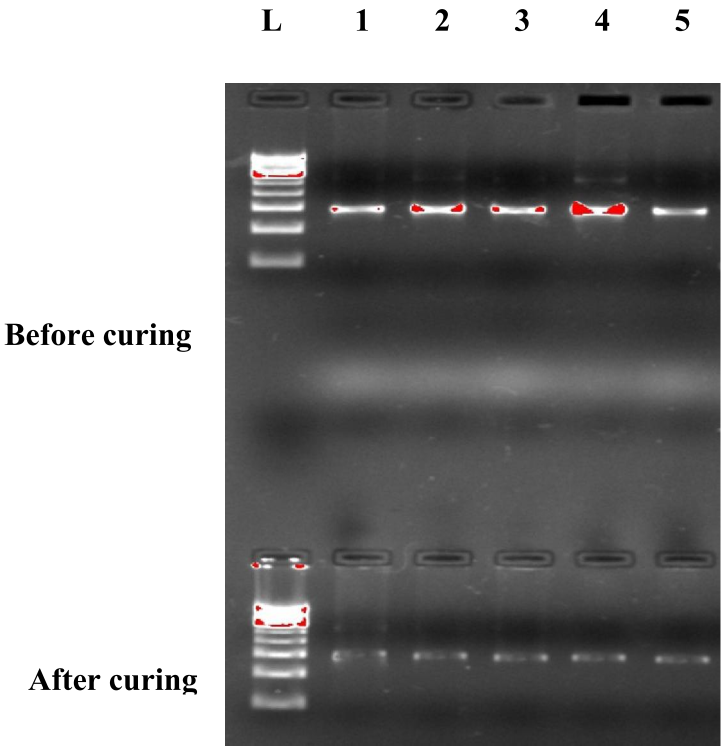
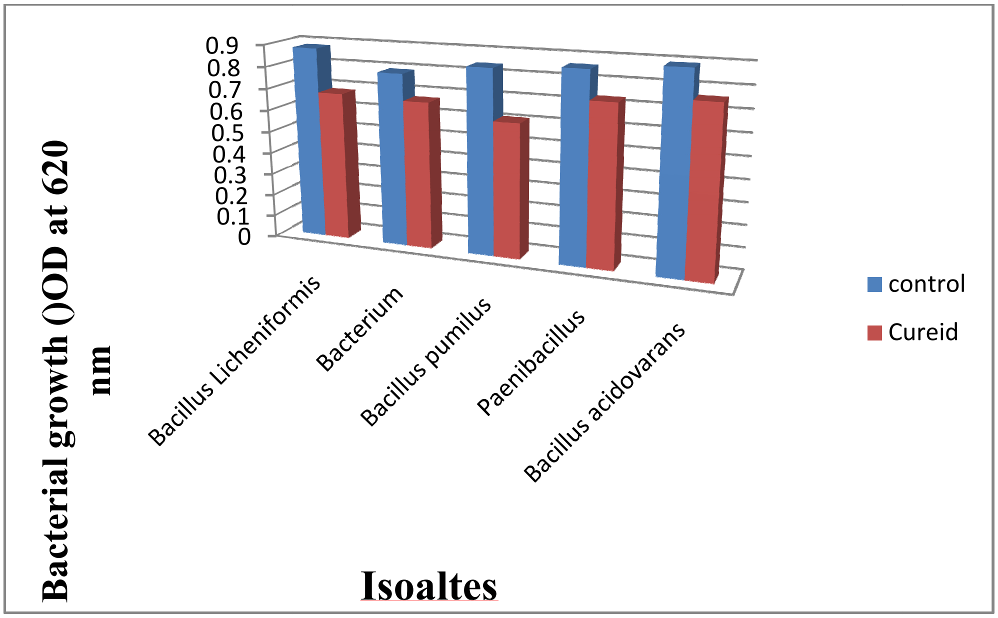


Figure 4.10. Plasmid curing of PAH degrading bacteria: Isolation of plasmid DNA after and before curing. L: 300 bp Ladder, 1: B.licheniformis, 2: Bacterium, 3: Bacillus pumilus, 4: Paenibacillus, 5: B.acidovirans

4.13. Effect of plasmid curing of isolate on PAH degradation

Following result were illustrated that after curing bacteria had loss the plasmid DNA and degrading ability could reduced.

Isolates	Isolates				
	Bacillus Licheniformis	Bacterium	Bacillus Pumilus	Paenibacillus lautus	Bacillus acidovarans
Curied	**0.68**	**0.67**	**0.61**	**073**	**0.76**
Control	**0.88**	**0.79**	**0.84**	**0.86**	**0.89**



Biosurfactant extraction from PAH degrading bacteria and FTIR based analytical analysis.

4.14. Extraction, analysis of bisurfactant from PAHs degrading bacteria.

Screening of biosurfactant

Oil spreading technique and drop collapse method were used for screening of bacterial biosurfactant for PAH degradation. All five bacterial isolates shown the positive result they were shown bellow. The supernatant of each strain were added to the plates containing that contained distilled water and cride oil. The zone of displacement were shown by strains had ability to displaced the oil. The organisms which produce biosurfactant can only displace the oil. The results obtained were noted down as follows:

Strains name	**Result**
Bacillus Licheniformis	Positive
Bacterium CT	Positive
Bacillus pumilus	Positive
Paenibacillus lautus	Positive
Bacillus acidovorans	Positive

Drop collapse test:

Drop collapse test evaluated the ability of bacterial biosurfactants to collapse or destabilized the crude oil or hydrocarbon liquid droplets. Therefore, cell suspension or of culture supernatant drops were placed on an oil coated, solid surface. If the liquid bacterial suspension contains surfactants, the drops were spread or even collapsed because the force or interfacial tension between the liquid drop and the hydrophobic surface was reduced. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension. All the strains tested gave positive results for drop collapse test.

Strains name	**Result**
Bacillus Licheniformis	Positive
Bacterium CT	Positive
Bacillus pumilus	Positive
Paenibacillus lautus	Positive
Bacillus acidovorans	Positive

4.15. Extraction and FTIR based characterization of biosurfactants.

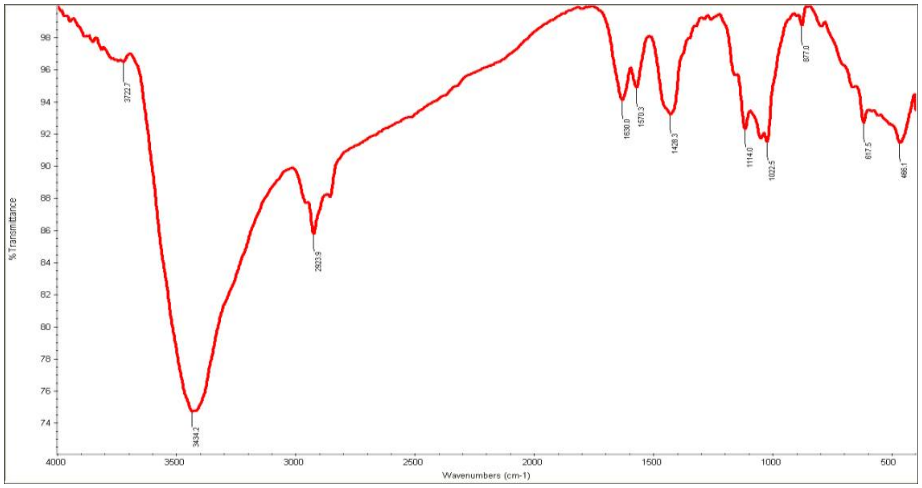
Biosurfactant extraction was carried out by the five bacterial isolates were inoculated in 50 ml Mineral salt broth to which 1ml of 2% PAH(anthracene, coronene and napthacene) was added. After biosurfactant analysis of five bacterial strains *Bacillus licheniformis, Bcterium CT, Bacillus pumilus, Paenibacillus and Bacillus acidovorans* respectively, for three PAH compound *viz.* anthracene, coronene and napthacene. FTIR based analysis of biosurfactant at absorbance of particular wave number was state that various functional groups and component of biosrfactant were presented, *Bacillus licheniformis* produces lipopeptide, polypeptide, plipastatin and surfactatin, *Bcterium CT*-lipopeptide, *Bacillus pumilus,*-polipeptide, lipopeptide, *Paenibacillus*-lipopeptide, polypeptide, *and Bacillus acidovorans*-lipoprotiens.

4.16. FTIR characterization of biosurfactant of *Bcillus licheniformis* to degrade antracene, coronene and napthacene

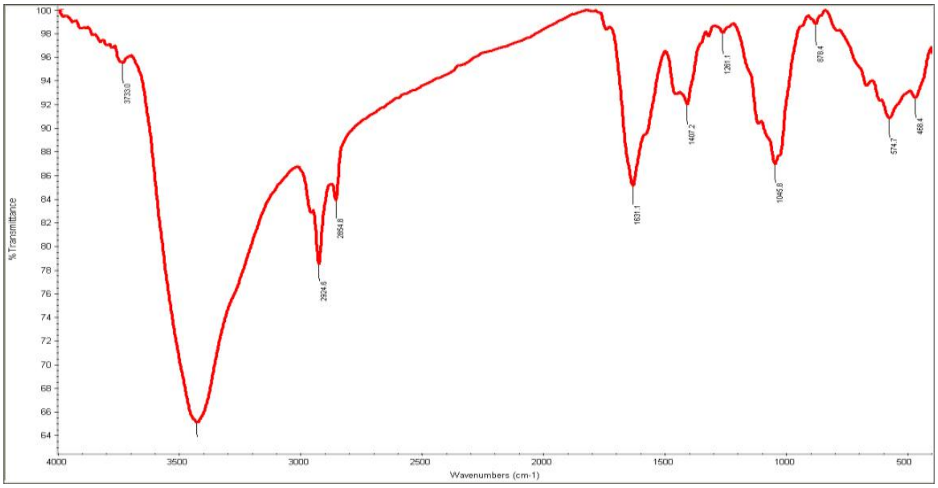
Abosorption of IR at particular wave number was shown the various functional groups presented which indicate these functional groups were seemes to be biosurfactant. Presence of groups OH, C-O, C=O, Alkynes, corboxyl ester and CH_2 indicate the presence of Plipastatien .The group NH bend, CO streches, OH. COOH, Ester, CN, and alkynes are present in biosurfactant shown lipopeptide and poly peptide. Following table was represented the three graph for Bacillus licheniformis produces biosurfactant to degrade anthracene, napthacene and coronene.

Wave number	**Functional group**
3430	**-NH-**
1655	**-CO-**
3000-2800	**-CN-**
3400	**Aliphatic**
2850-2950	**OH-**
1740	**-CH-**
1270	**-C=O**

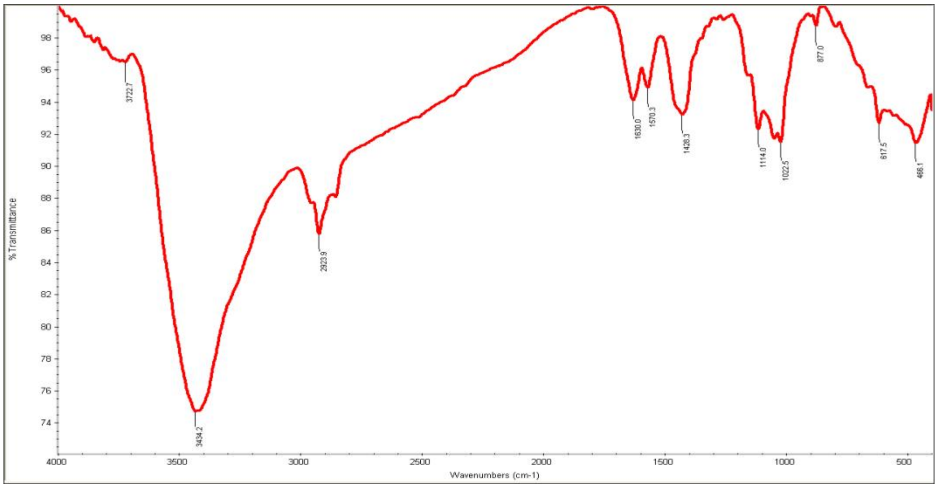
Biosurfactant for Anthracene



Coronene



Napthacene

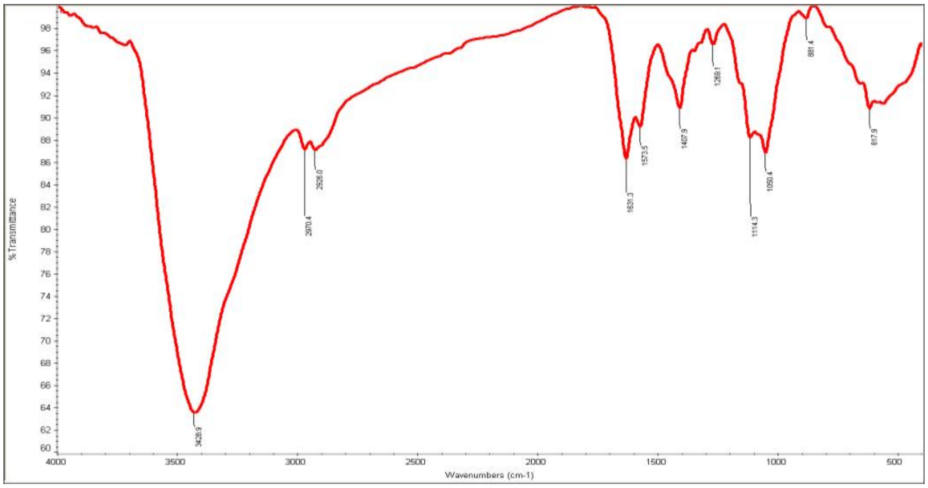


4.17. FTIR characterization of biosurfactant by *Bacterium CT* to degrade anthracene, coronene and napthacene

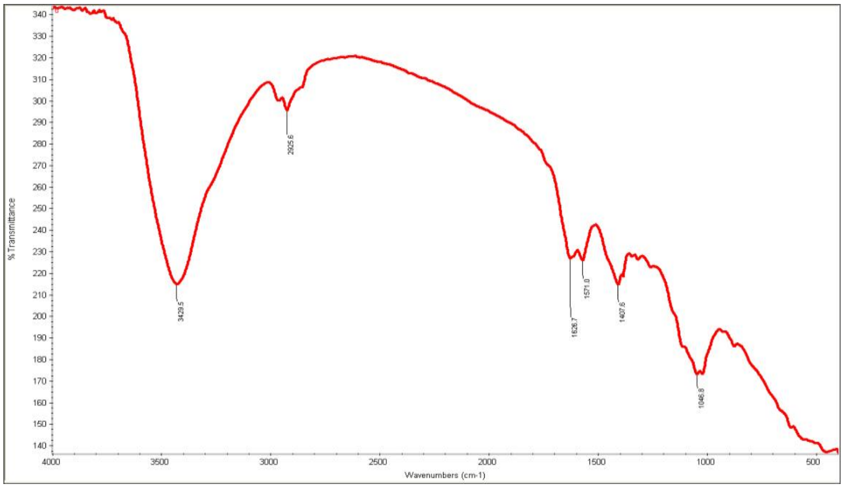
The result was found that group NH bend, CO streches, OH. COOH, Ester, CN, and alkynes was present in biosurfactant shown lipopeptide produced from *Bacterium CT* to degrade anthracene napthacene and coronene which was shown in following graph.

Wave number	Functional group
3433	-OH-, 1^0Amine
1655	-CO-
3000-2800	-CN-
3400,1111	Aliphatic
2850-2950	OH-
1740	-CH-
1270	-C=O
2925.1	COOH
1630	-NH- 1^0Amines
617.6	Alkenes

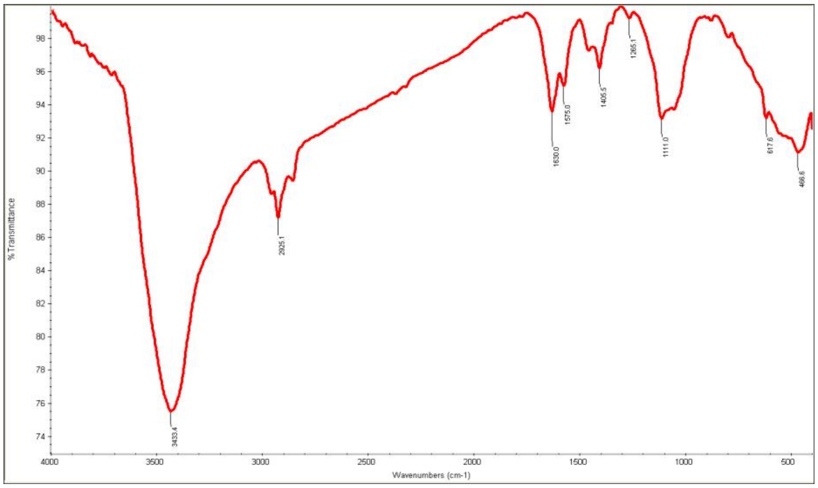
Biosurfactant for Anthracene degradation



coronene



Napthacene

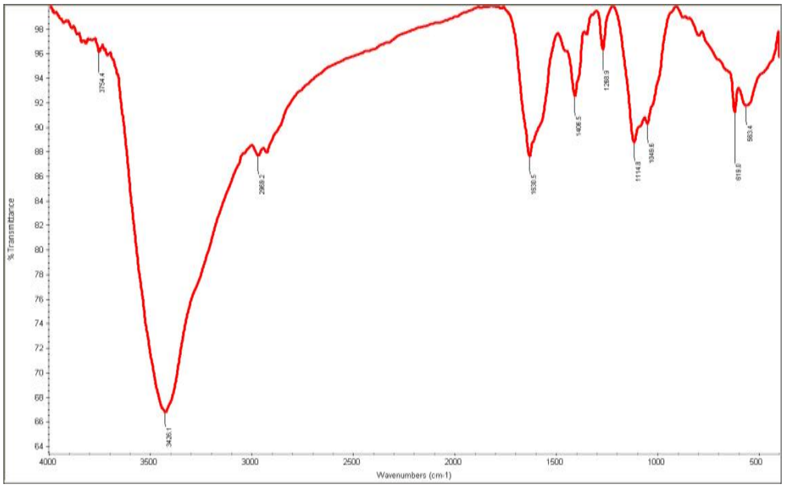


4.18. FTIR characterization of biosurfactant by *Bacillus pumilus* to degrade anthracene, coronene and napthacene

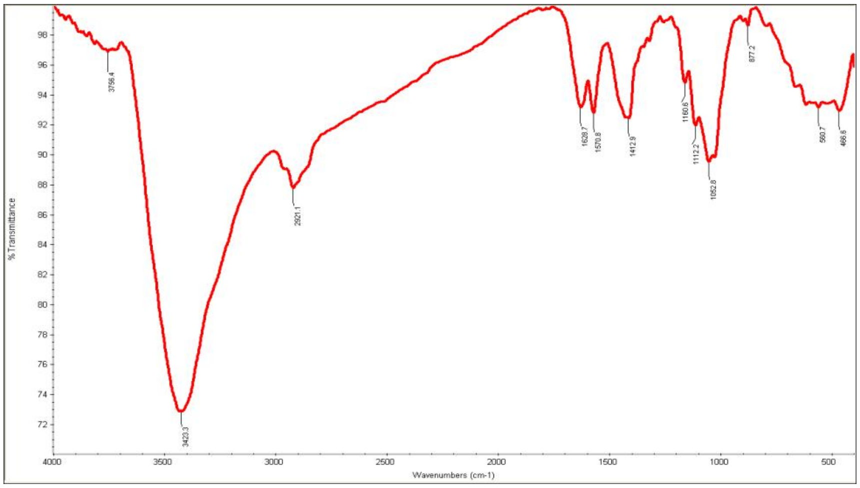
The group aliphatic amine, NH bends, CO streches, OH. COOH, Ester, CN, 1°2° amine and alkynes were presented in biosurfactant shown lipopeptide. Following functional group in graph represented *Bacillus pumilus* produces lipopeptide, peptide, glycolipid and surfactatin to degrade anthracene, napthacene and coronene.

Wave number	**Functional group**
3433	**-OH-, 1^0Amine**
1408	**-COOH-, Ester, Aliphatic amines**
2923.9	**OH, COOH**
3400,1111	**Aliphatic**
2850-2950	**OH-**
868	**1020 Amines, NH Bend**
1270	**-C=O**
2923.9	**OH, COOH**
1628	**-NH- 1^0Amines**
1083	**CH Bend, Alkenes**

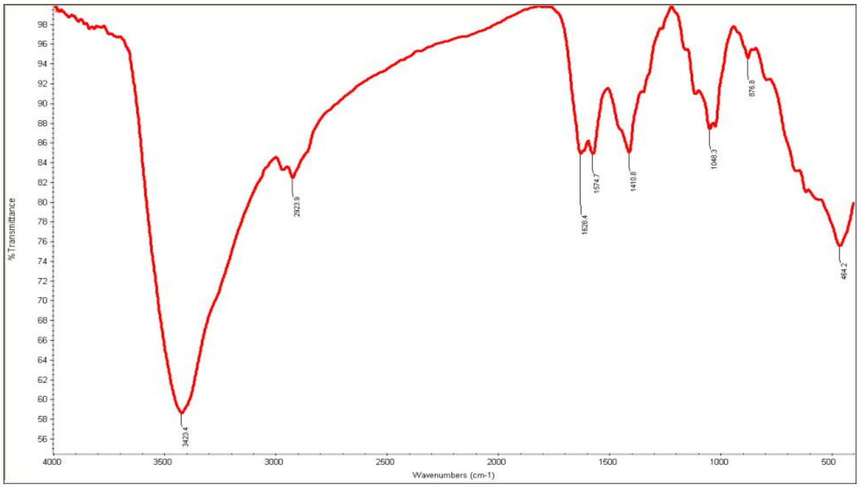
Biosurfactant to degrade anthracene



Coronene



Napthacene

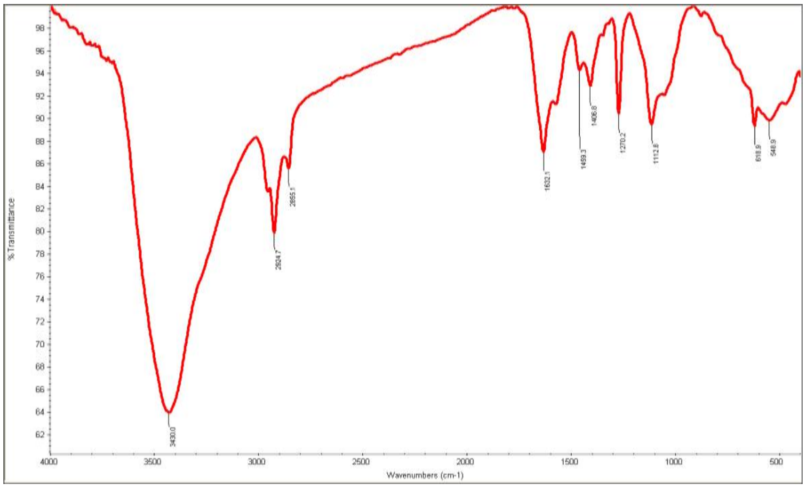


4.19. FTIR characterization of biosurfactant by *Peanibacillus leutus* to degrade anthracene, coronene and napthacene

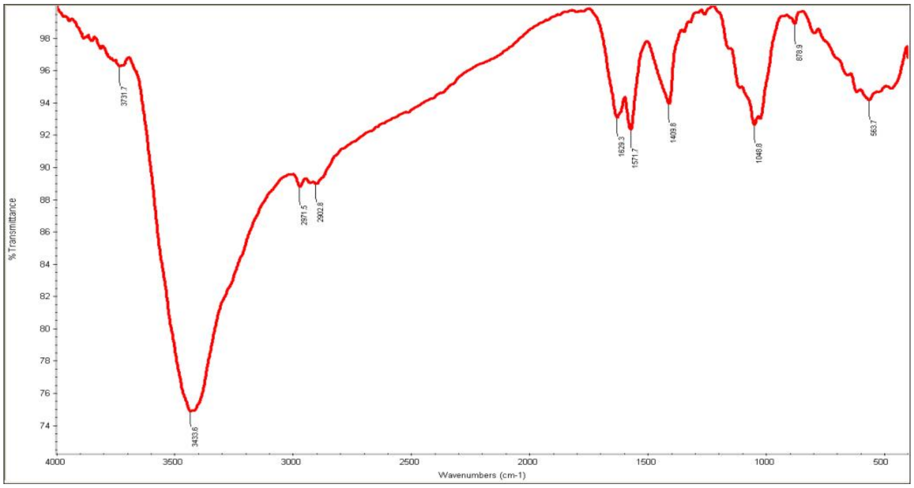
The streches at absorbance 618, 1286, 1022 cm^{-1}shown the functional group Aliphatic amine, NH bend, CO streches, OH. COOH, Ester, CN, 1°2° amine and alkynes are present in biosurfactant shown lipopeptide. Following graph and table were shown that *Peanibacillus leutus* produces lipopeptide and peptide as biosurfactant to degrade following PAH compound which was shown in graph.

Wave number	Functional group
618	-C=C-H- Alkyne, Methyle
1286	-COOH-, CN
1022	Alkune, Methyle, -C-H-bend
3400,1111	Aliphatic
2850-2950	OH-
868	1020 Amines, NH Bend
1270	-C=O
2923.9	OH, COOH
1628	-NH- 1^0Amines
1083	CH Bend, Alkenes

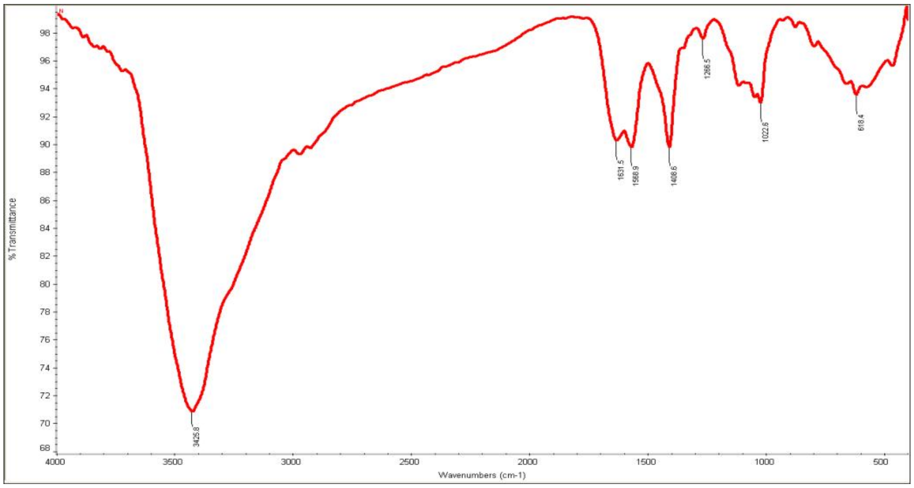
Biosurfactant to degrade anthracene



Coronene



Napthacene

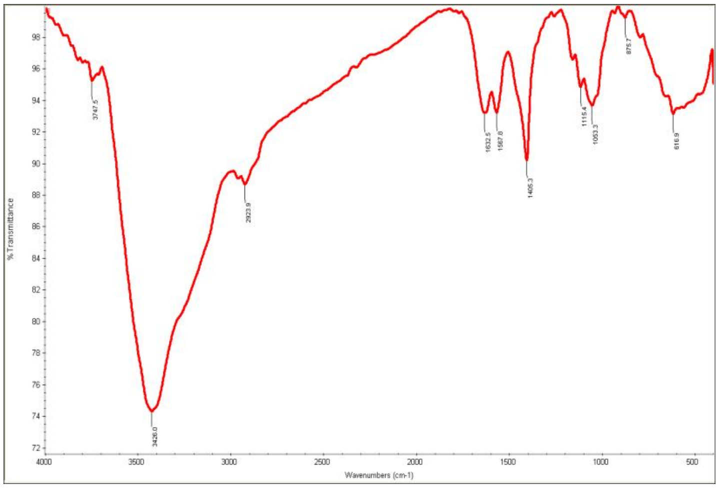


4.20. FTIR characterization of biosurfactant by *Bacillus acidovirans* to degrade anthracene, coronene and napthacene

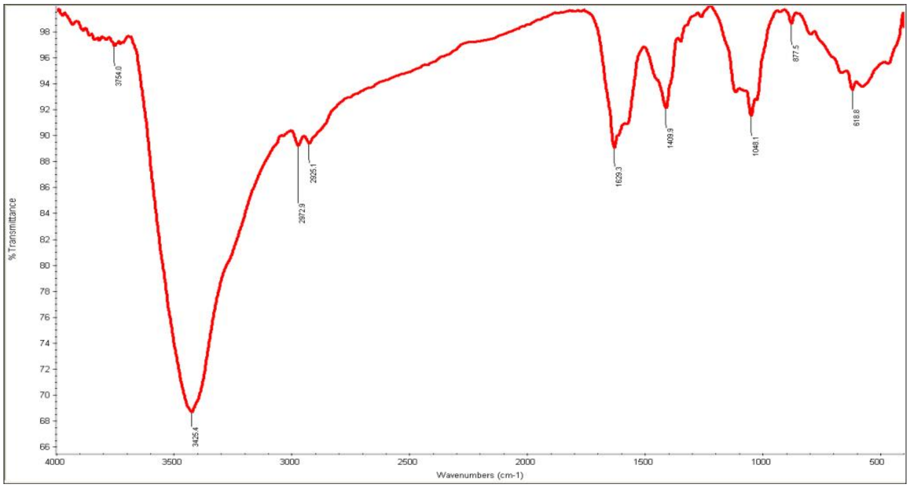
The absorbance at wave length 618, 1286, 1022 cm^{-1}shown the functional group Aliphatic amine, NH bend, CO streches, OH. COOH , Ester, CN, 1°2° amine and alkynes are present in biosurfactant shown lipopeptide.

Wave number	**Functional group**
618	**-C=C-H- Alkyne, Methyle**
1286	**-COOH-, CN**
1022	**Alkune, Methyle, -C-H-bend**
3400,1111	**Aliphatic**
2850-2950	**OH-**
868	**1020 Amines, NH Bend**
1270	**-C=O**
2923.9	**OH, COOH**
1628	**-NH- 1^0Amines**
1083	**CH Bend, Alkenes**

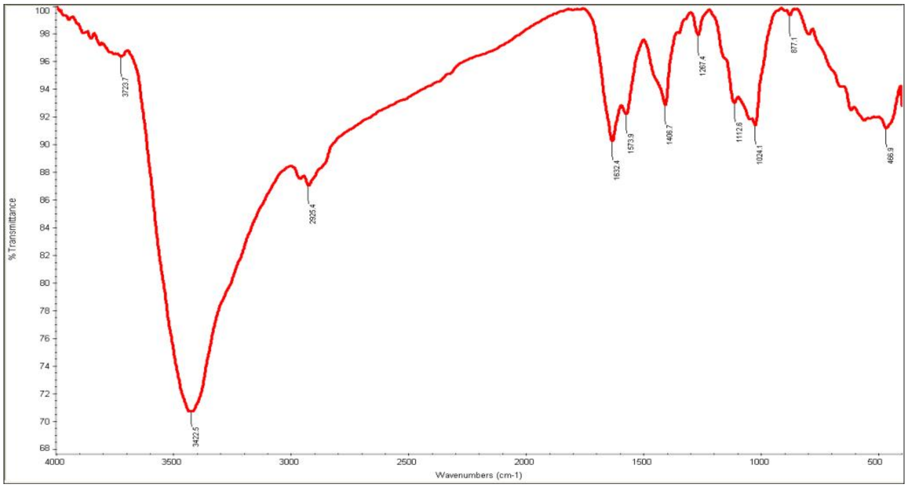
Biosurfactant to degrade Anthracene



Coronene

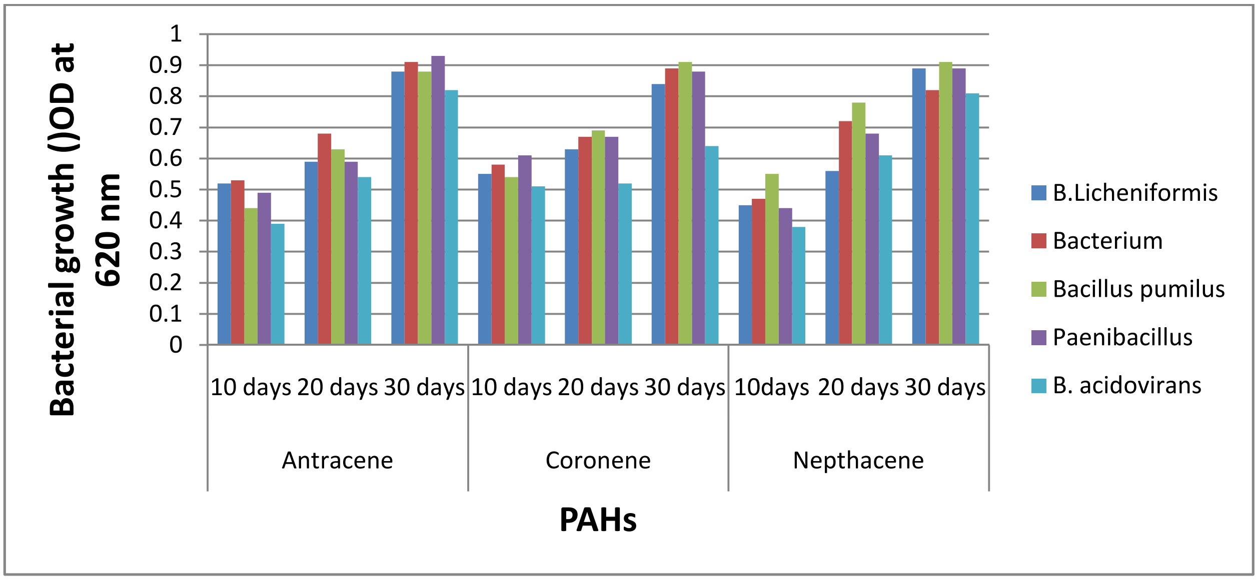


Naptahcene



Degradation of PAH (Antracene, Coronene and Nepthacene), Qulitative degradation by bacterial growth (OD measurment).

After experiment observation was made for qualitative degradation of five isolates with three PAH compound by experimental setup prepared in 15 different flasks with respect to 3 PAH and 5 isolates; each was maintained at 35ºC, temperature. In this study pH of 7.4 ± 0.2 was maintained, since isolation of microbial strains was conducted at this pH. Experiment was carried out in 100 ml conical flasks containing 50 ml MSM broth with PAH compound (20 mg coronene, 50 mg anthracene and napthacene) for degradation. The flasks were kept in shaker incubator at 120 rpm, for 15 days. PAH degradation was measured by bacterial growth calculated at absorbance 600nm.



4.21. Quantitavive degradation by UPLC

Analysis of percentage degradation of HMW PAHs

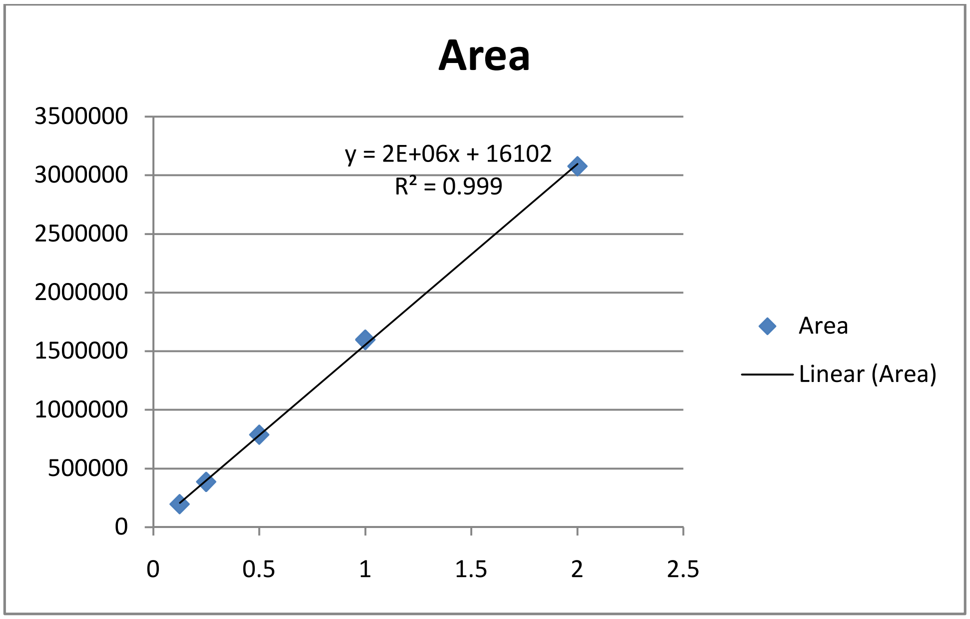
Observation was made after complete incubations were performed in triplicate for each analysis type at 37°C, shaking at150 rev min^{-1} in the dark. Triplicate individual cultures were removed for chemical, microbiological, toxicological and mutagenic analysis at 10, 20, 30 and 40d. Controls consisted of inoculated cultures which were killed by the addition of mercuric chloride (2%w/v). follwing table and graph shown the degradation of three PAH by five isolates after compaire with standard calliberation curve.

Degradation of PAHs by five bacterial strains

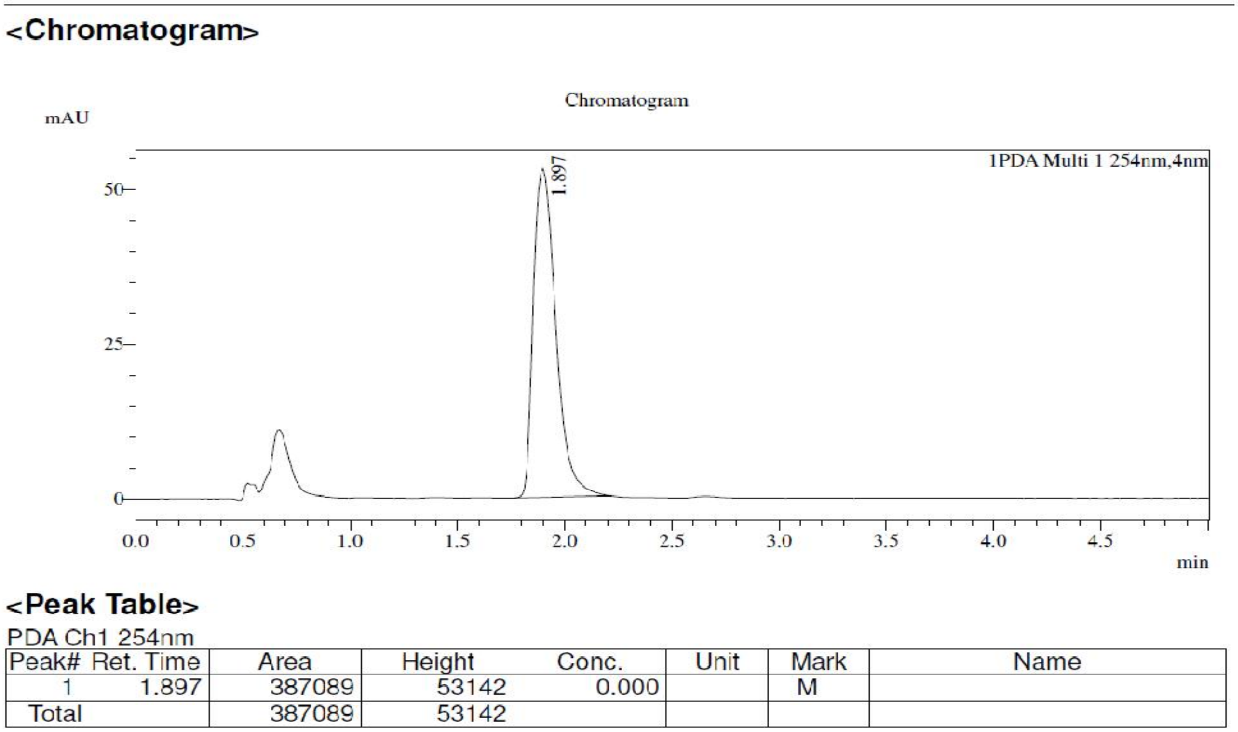
Anthracene degradation by UPLC method

Result was evaluated that degradation of Anthracene was calculated with help of anthracene standard peak area, retention time and colliberation curve to find out the remaining concentration of anthracene compound. There was following colliberation curve for concentration 0.125, 0.25,0.5, 1 and 2 mg.

Calliberation curve of anthracene standard



Chromatogram for anthracene standard at 0.25 mg used for calculation.



<Chromatogram>

<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	1.897	387089	53142	0.000		M	
Total		387089	53142				

Retation time and peak area for 5,10 and 15 days incubation time

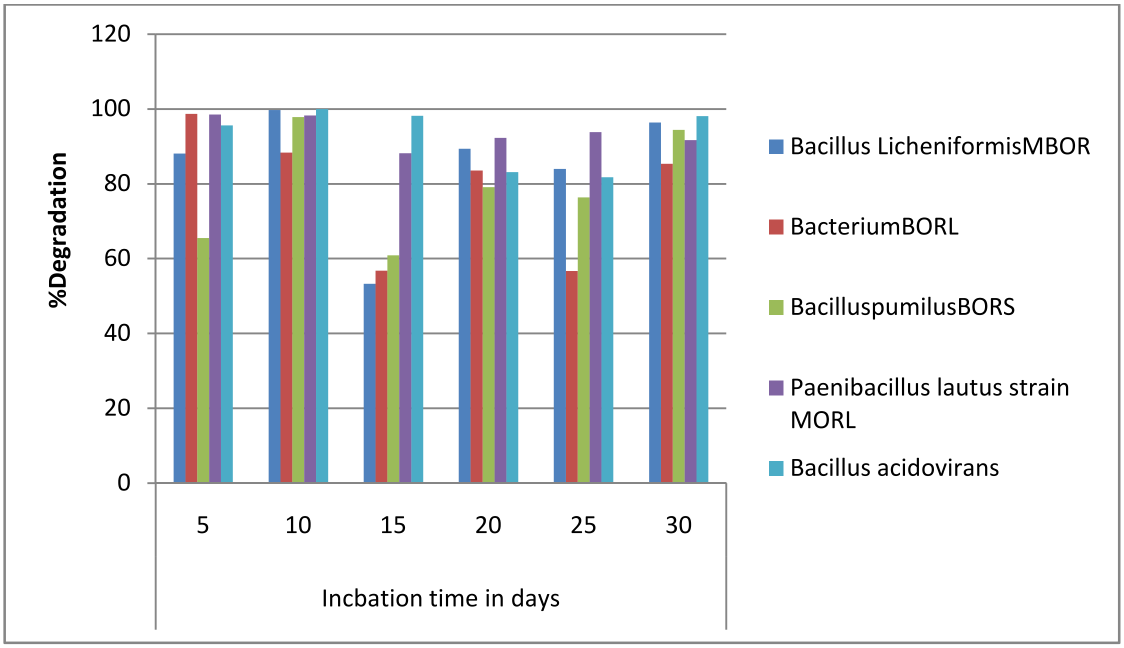
Isoaltes	**Incubation time**					
	5 days		**10day**		**15days**	
	Ret. Time	**Peak area**	**Ret. Time**	**Peak area**	**Ret. Time**	**Peak area**
*Bacillus Licheniformis*MBOR	1.928	2211360	2.676	46478	1.935	8677601
Bacterium CT BORL	1.930	233672	2.680	2167331	1.931	8023267
*Bacillus pumilus*BORS	1.929	6411516	2.680	398507	1.926	7272065
Paenibacillus lautus MORL	1.928	266626	2.681	320512	1.931	2200915
Bacillus acidovirans	1.918	809191	2.671	41341	1.920	340423

Retation time and peak area for 15,20 and 30 days incubation time

Isoaltes	**Incubation time**					
	20 days		**25day**		**30days**	
	Ret. Time	**Peak area**	**Ret. Time**	**Peak area**	**Ret. Time**	**Peak area**
*B.licheniformis*MBOR	1.934	1964424	1.935	2977860	1.942	5212841
Bacterium CT BORL	1.952	3056983	1.948	8044848	1.929	6585351
*Bacillus pumilus*BORS	1.947	3878389	1.941	4387995	1.930	483900
Paenibacillus lautus MORL	1.952	1429341	1.936	1147818	1.932	5593126
Bacillus acidovirans	1.950	3126115	1.946	3380328	1.928	1875494

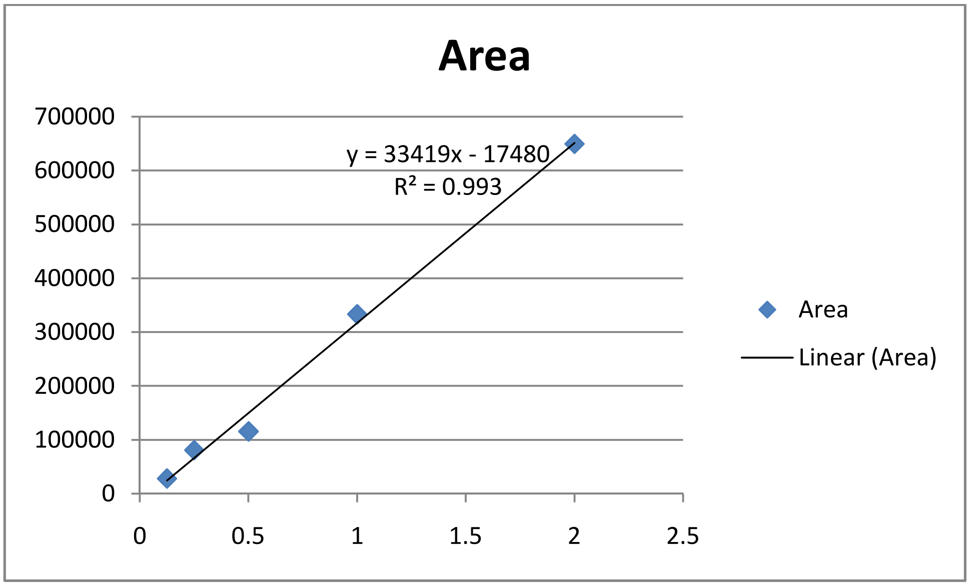
Percentage degradation of Anthracene

After analysis of degradation of anthracene, all isolates were shown highly degradation activity during incubation period 10 days. During 15 days incubation period *Bacillus licheniformis*, *Bacterium CT* and *Bacillus Pumilus* were shown minimum degradation but in next exeperimental setup for anthracene degradation were increases regularly during incubation period 20, 25 and 30 dayes for isolates *Bacillus licheniformis*, *Bacterium CT* *Paenibacillus lautus* MORL, *Bacillus acidovirans* and *Bacillus Pumilus* respectively. These finding were revealed that 10, 20, 25 and 30 dayes incubation period presented highest degradation for all isolates at anthracene concentration 50 mg/l. *Bacillus licheniformis*, *and* *Paenibacillus lautus* MORL was more significat in anthracene degradation for incubating periods.



Coronene degradation by UPLC method

Calliberation curve of coronene standard



Chromatogram for coronene standard at 0.25 mg used for calculation.

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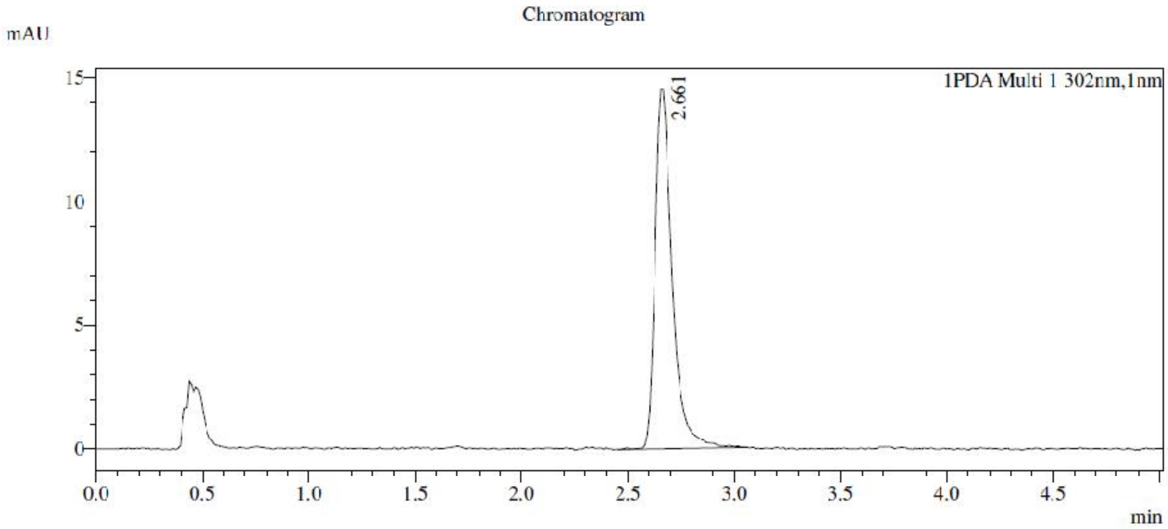


<Peak Table>

PDA Ch1 302nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.661	81099	14564	0.000		S	
Total		81099	14564				

Retation time and peak area for 5,10 and 15 days incubation time

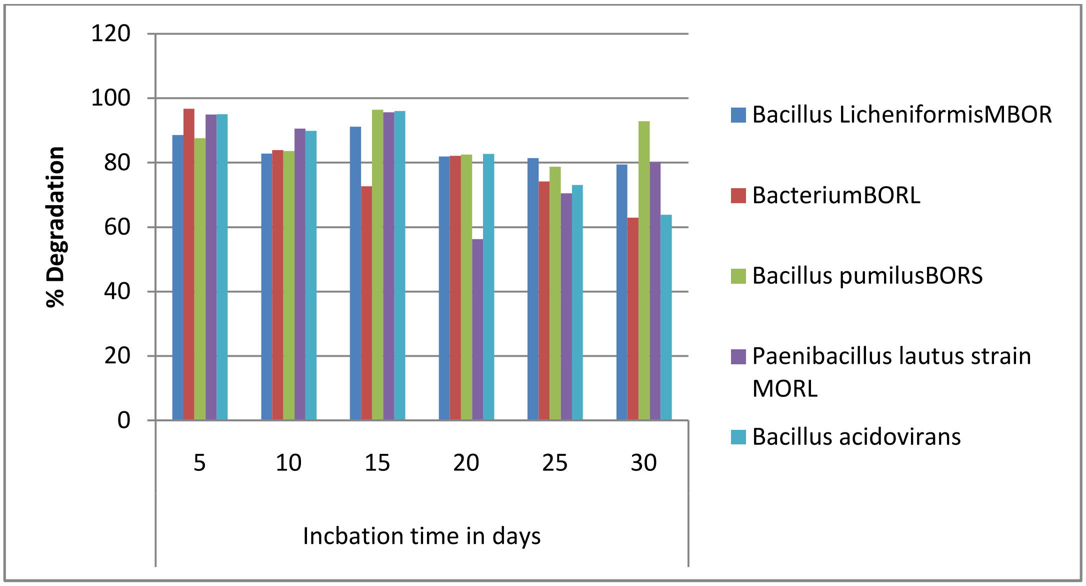
Isoaltes	Incubation time					
	5 days		10day		15 days	
	Ret. Time	Peak area	Ret. Time	Peak area	Ret. Time	Peak area
*B. Licheniformis*MBOR	2.658	295331	2.676	445939	2.725	229543
Bacterium CT BORL	2.646	85137	2.680	417307	2.688	709197
*B.pumilus*BORS	2.661	322807	2.680	424084	2.685	91809
Paenibacillus lautus MORL	2.656	129874	2.681	245659	2.689	112821
Bacillus acidovirans	2.663	128054	2.671	263524	2.690	101769

Retation time and peak area for 15,20 and 30 days incubation time

Isoaltes	**Incubation time**					
	20 days		**25 day**		**30 days**	
	Ret. Time	**Peak area**	**Ret. Time**	**Peak area**	**Ret. Time**	**Peak area**
*Bacillus Licheniformis*MBOR	2.664	469453	2.660	481558	2.651	533894
Bacterium CT BORL	2.658	463402	2.241	669477	2.663	960978
*Bacillus pumilus*BORS	2.660	454292	2.252	550265	2.654	185276
Paenibacillus lautus MORL	2.658	1133265	2.246	766634	2.659	515744
Bacillus acidovirans	2.660	449371	2.249	699603	2.653	939426

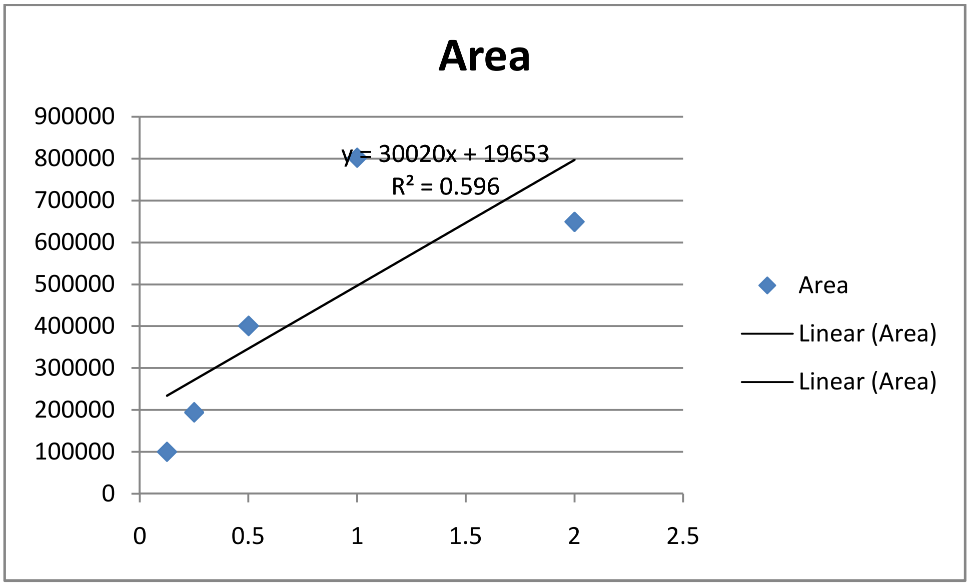
Percentage degradation of Coronene

Result evaluated during 5,10 and 15 dayes of incubation of degradation setup for all isolates had shown maximum activity at concentration 20mg/l of coronene in *Bacillus Pumilus Bacillus licheniformis*, *Bacterium CT, Paenibacillus lautus* MORL, *Bacillus acidovirans* and *Bacillus Pumilus* MSM . Degradation potential of isolates was gradually decreases after 15 days of incubation , 30 days had shown minimum degradation activity.



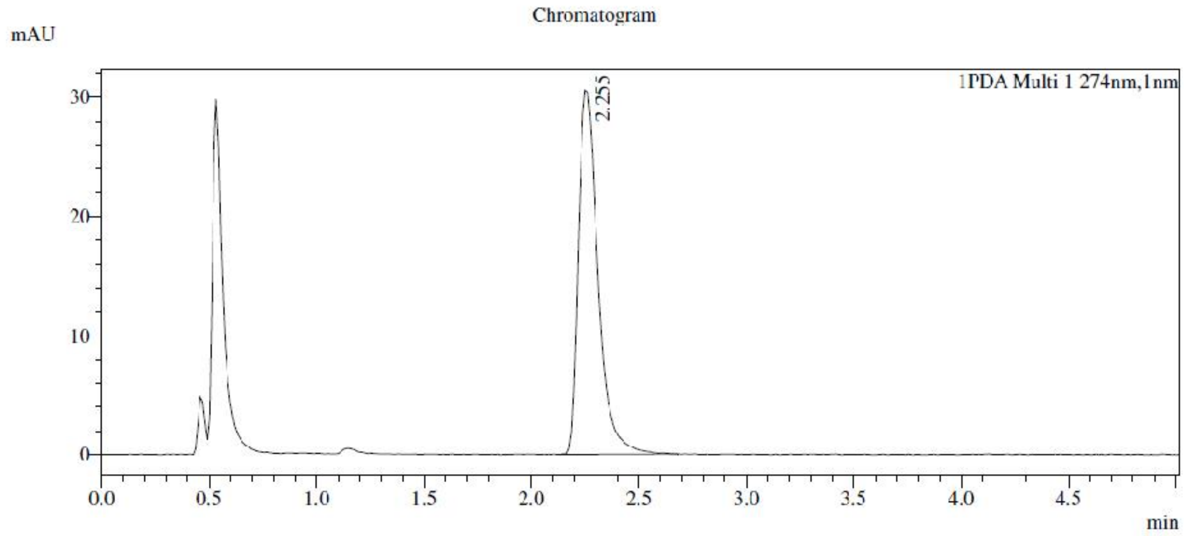
Napthacene degradation by UPLC method

Calliberation curve of napthacene standard



Chromatogram for naphthacene standard at 0.25 mg used for calculation.

<Chromatogram>



<Peak Table>

PDA Ch1 274nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.255	194334	30555	100.000			
Total		194334	30555				

Retation time and peak area for 5,10 and 15 days incubation time

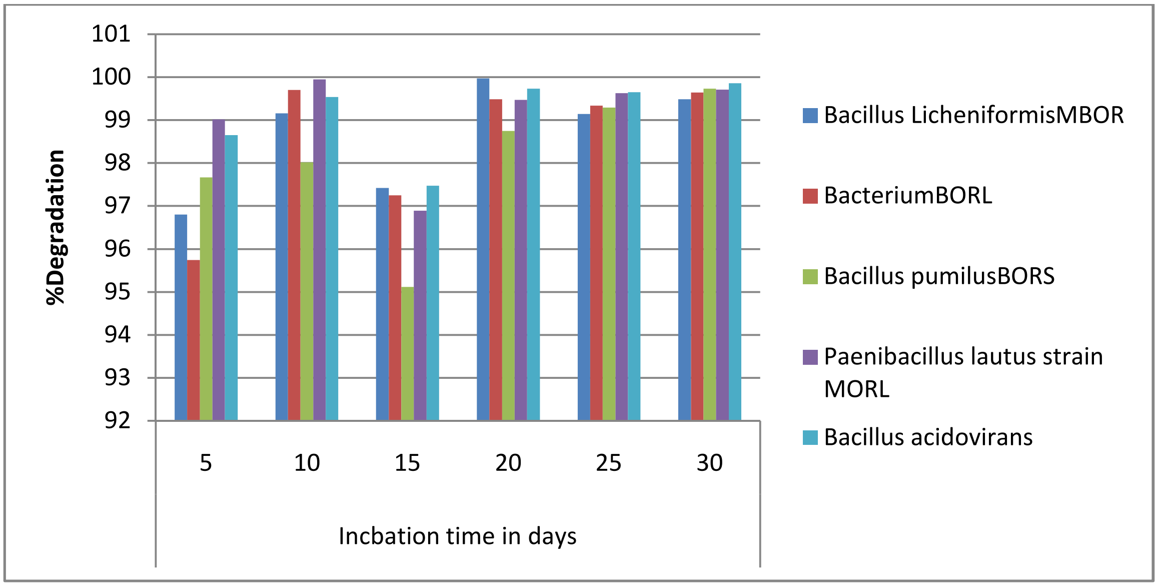
Isoaltes	Incubation time					
	5 days		10day		15 days	
	Ret. Time	Peak area	Ret. Time	Peak area	Ret. Time	Peak area
*B.Licheniformis*MBOR	2.243	297878	2.255	77681	2.248	240543
Bacterium CT BORL	2.239	397028	2.249	27349	2.271	255957
*B. pumilus*BORS	2.252	216979	2.264	184591	2.275	454301
Paenibacillus lautus	2.281	91901	2.259	4638	2.265	289538
B.acidovirans	2.245	125579	2.259	42297	2.257	235244

Retation time and peak area for 15,20 and 30 days incubation time

Isoaltes	Incubation time					
	20 days		25 day		30 days	
	Ret. Time	Peak area	Ret. Time	Peak area	Ret. Time	Peak area
*B.Licheniformis*MBOR	2.257	1896	2.241	79710	2.239	47177
Bacterium CT BORL	2.234	46683	2.252	61212	2.255	32772
*B. pumilus*BORS	2.184	116310	2.246	66006	2.178	24867
Paenibacillus lautus MORL	2.211	49371	2.249	33744	2.243	26891
*B. acidovirans*MORS	2.213	24578	2.245	32337	2.239	12878

Percentage degradation of Napthacene

Experimental observation was state that degradation activity of all isolates respectively *Bacillus Pumilus Bacillus licheniformis*, *Bacterium CT, Paenibacillus lautus* MORL, *Bacillus acidovirans* and *Bacillus Pumilus* against 50 mg/l napthacene, gradually increases during incubation period 5,10,15,20,25,and 30 days, 15 days were shown maximum degradation. Among all isolates *Paenibacillus lautus* MORL, and *Bacillus acidovirans* were shown maximum degradation potential. During antracene degradation Bacterium CT shown minimum activity.



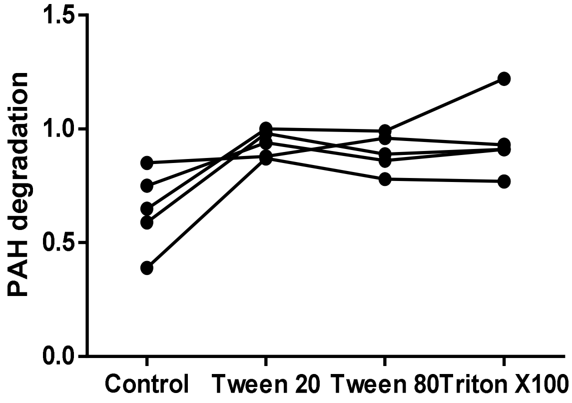
Effect of surfactant/ Emulsifier to enhance PAH degradation

Following results were shown that degradation of anthracene, coronene and napthacene was enhanced by addition of synthetic surfactant known as Tweens 20, Tween 80 and Triton X 100 during degradation experiment. Tween 20 and Triton X 100 was shown more effective than another surfactant in degradation of PAHs.

Antracene

Surfactant/ Bioemulsifiers	Isolates				
	Bacillus Licheniformis	Bacterium	Bacillus Pumilus	Paenibacillus lautus	Bacillus acidovarans
Tween 20	0.88	0.87	0.98	1.00	0.94
Tween 80	0.96	0.78	0.89	0.99	0.86
Triton X 100	0.93	0.77	0.91	1.22	0.91
Control	0.85	0.39	0.59	0.65	0.75

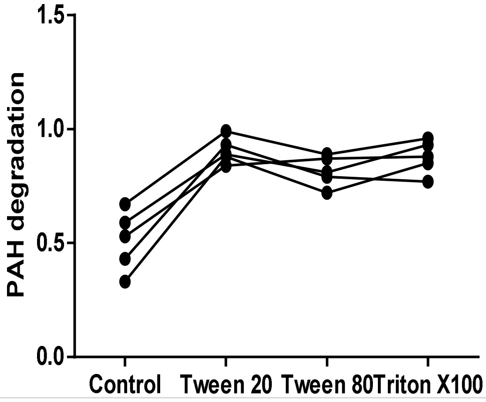
Repeated measures one-way ANOVA data surfactant / Bioemulsifiers on PAH degradation (Antracene)



Coronene

Surfactant/ Bioemulsifiers	Isolates				
	Bacillus Licheniformis	Bacterium	Bacillus Pumilus	Paenibacillus lautus	Bacillus acidovarans
Tween 20	0.88	0.93	0.84	0.99	0.89
Tween 80	0.72	0.79	0.87	0.89	0.81
Triton X 100	0.85	0.77	0.88	0.96	0.93
Control	0.33	0.43	0.53	0.67	0.59

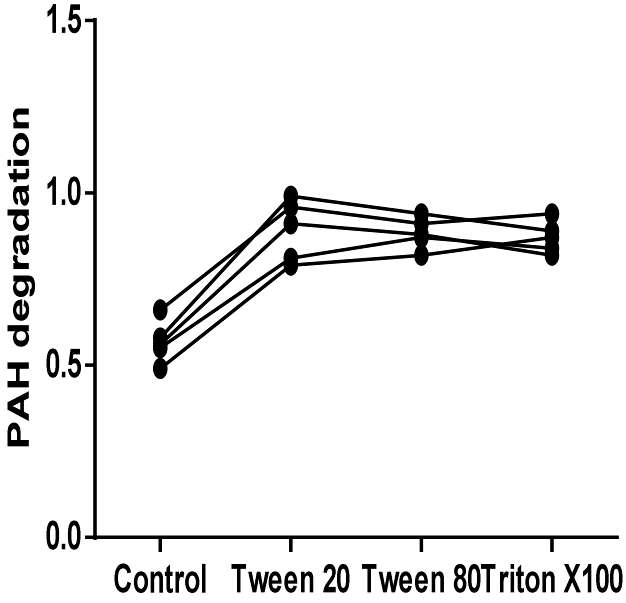
Repeated measures one-way ANOVA data surfactant / Bioemulsifiers on PAH degradation (Coronene)



Nepthacene /Tetracene

Surfactant/ Bioemulsifiers	Isolates				
	Bacillus Licheniformis	Bacterium	Bacillus Pumilus	Paenibacillus lautus	Bacillus acidovarans
Tween 20	0.99	0.79	0.91	0.96	0.81
Tween 80	0.94	0.82	0.88	0.91	0.87
Triton X 100	0.89	0.87	0.82	0.94	0.84
Control	0.58	0.49	0.56	0.66	0.55

Repeated measures one-way ANOVA data surfactant / Bioemulsifiers on PAH degradation (Nepthacene /Tetracene)



Chapter- 5
Discussion

4. DISCUSSION

The present study focused on the isolation, identification, screening and biosurfactant production of bacteria to degradae Anthracene, Napthacene and Coronene from the petroleum contaminated soil sediment of Mathura and Baurauni oil refinery. According to the finding results, both sites were highly contaminated with various PAH compound. Among these sites, physicochemical properties of some soil sample showen correlation relationships with the contamination in the oil refinery and around landfarming site, the PAH degradation in forming fields was higher than the unforming soil areas. However, the degrading microorganism counts had significant differences between forming, unforming and petroleum contaminated field. The petroleum contamination level of refinery sites had played no more roles for correlation between the degrder incidance. Soil environments of Mathura and Barauni refinery were had slight variable in their physicochemical properties which was important to favour for microbial growth and bioavailability of contaminants. Quite limitetaion was observed in aerated soils subsurface, where aerated soils shown, much degradation by microorganisms than those unaerated surface soils. The dry matter of contaminated soil content reflects the moisture condition which is mainly determined by the soil texture. Some studies performed in artificial condition in which moisture content microcosms had a significant effect on bioremediation of hydrocarbon **(Gray *et al.,* 2000; Yerushalmi *et al.,* 2003; Ramírez *et al.,* 2009).** It was reported that aerobic PAH biodegradation was the optimal moisture content 50-80 % of holding capacity soil. However, the clayed soils had experienced more difficulties for hydrocarbon biodegradation because the pollutants of organic compounds was sequestrated by the organic compounds. **Ramírez *et al.,* (2009)** According to their experimental results, the highest degradation rate was obseroved in the treatments with water content corresponding to better water holding capacity. Studied shown that best experimental condition for hydrocarbon degradation in clayed and weathered polluted soil.

According to experimental result 35^0C temperature and pH 6.5 favour good potential effect to degrade PAH compounds as well known, bioremediation requires an optimal pH range to be successful. In return, the metabolites accumulated during degradation and released CO_2 by microbial respiration can change the soil pH value. Oxidation of oil during biodegradation leads to an increase of acidity **(Head *et al.,* 2003). Roberts (1998)** believed that a pH range of 6.5 to 8.5 is optimum for hydrocarbon degradation. Besides, **Dibble and Bartha (1979)** proposed an optimal pH range of 5.0 to 7.8 for oily sludge mineralization in soils. Due to my study, all the samples were in such a pH range which indicates that both contaminated sites had a good acidity-alkalinity condition for bioremediation. It was also indicated that farming field, unforming field and oil contaminated soil , pol sludge waste and another treated soil had no significant alteration of soil pH in terms of hydrocarbon biodegradation **(Kastner *et al.*, 1998).**

Study of biosurfactant extraction was carried out by the five bacterial isolates viz: *Bacillus licheniformis, Bcterium CT, Bacillus pumilus, Paenibacillus and Bacillus acidovorans* respectively for PAH compound (anthracene, coronene and napthacene) degradation . After biosurfactant analysis of five bacterial strains, for three PAH compound *viz.* anthracene, coronene and napthacene. FTIR based analysis of biosurfactant at absorbance of particular wave number was state that various functional groups and component of biosrfactant were presented, *Bacillus licheniformis* produces lipopeptide, polypeptide, plipastatin and surfactatin, *Bcterium CT*-lipopeptide, *Bacillus pumilus,*-polipeptide, lipopeptide, *Paenibacillus*-lipopeptide, polypeptide, *and Bacillus acidovorans*-lipoprotiens. Similar study was carried for biosurfactant analysis by FTIR method, this method harnesses the spectra of the stretching and bending vibration associated with a molecule when it absorbs energy in the IR region of the electromagnetic spectrum for property elucidation (Weisman et al 1998). In the electromagnetic spectrum, spectra of hydrocarbon derivatives originate mainly from combinations or overtones of C-H stretching modes of saturated CH_2 and terminal $–CH_3$ or aromatic C-H functional groups **(Aske, et al 2001).** In the IR region, these occur within the wave number range of 3000 to 2900 cm-1 (~3333 to 3448 nm) or at the specific wavenumber of 2930 cm-1 (~3413 nm) (Brassington et al 2010). Usually, as-received samples are first extracted with an eluting solvent containg no C-H bonds and the eluate is contacted with silica gel to remove biogenic polar components before being subjected to IR spectrometry. The absorbance of the eluate is then measured at the specified wavenumber and compared against the calibration curve developed for the instrument. The instrument calibration standard usually is a petroleum hydrocarbon of known TPH concentration **(Weisman *et al* 1998).** The primary advantage of the IR-based methods is that they are quick, simple and inexpensive with commonly detection limits of approximately 10mg/kg in soil **(Weisman *et al* 1998, Lambert *et al* 2001).**

Fifty eight bacterial strains were isolated from Mathura and and Baurauni oil Refinery petroleum contaminated soil sediment by enrichment bacterial culture on mineral salt medium. Further enrichment bacterial culture processes repeated three- four times with supplementation of 1% PAH concentration as corbon sole source for bacterial growth in medium to make the most potent bacterial isolate to degrade the HMW PAHs (anthracene, napthacene and coronene). Growth was observed by absorbance at 600nm after that streaked the bacterial isolate at nutrient agar and mineral salt agar plates for further used and maintained at pure culture. These bacterial strains were screened to check the PAH degrading and biosurfactant produceing activity.

In oil displacement test, all five strains were showing the positive results which were selected after screening processed. In drop collapse method, all five screened strains were showing positive results. From these biosurfactant screening assays, we have seen that five strains named *Bacillus licheniformis, Bcterium CT, Bacillus pumilus, Paenibacillus and Bacillus acidovorans* were showing positive results of producing biosurfactant. So for further study these five strains were taken. The characterizations of these five strains were done in SEM (Scanning Electron Microscopy) and it was observed that all five were rod shaped having smooth surface. From biochemical tests, motility test, nitrate reduction test and indole motility tests, it was observed that *Bacillus licheniformis, Bcterium CT, Bacillus pumilus, Paenibacillus and Bacillus acidovorans*, were showing sugar fermentation and also motile in nature. Others two were showing negative results in sugar fermentation and also motile. Like this also in nitrate reduction test, *Bacillus licheniformis, Bcterium CT, Bacillus pumilus, Paenibacillus and Bacillus acidovorans* showing in reduction of nitrate to nitrite and others were showing negative results.

The growth pattern of the biosurfactant producing isolates was usually between 6-10 days having good biosurfactant production during this time period. By using FTIR, chemical characterization of the produced biosurfactant were showed that that the peak obtained through FTIR analysis usually represented to primary and secondary amines functional groups with having carboxylic acid stretch, alkane stretch and aromatic groups present. Similar study was described the carbohydrate estimation using Phenol-sulphuric acid test and protein estimation using Bradford assay showed that there was considerable carbohydrate and protein content in the extracted biosurfactant. Different aliphatic and aromatic carbon sources were used as substrate for the growth of biosurfactant producing bacteria to have an optimization study of which sources are widely utilized **(Chukwudi *et al.*, 2012).** It had been shown that all five strains could easily PAHs compound that represented the growth on different aromatic compounds like naphthalene, pyrene, phenanthrene, biphenyl, kerosene showing that the bacteria could easily utilize naphthalene and phenanthrene more readily, and then comes biphenyl by observed data. The rationale behind biosurfactant production on hydrocarbon utilization should stimulate itself by enhancing the substrate availability. In some literatures it was mentioned that biosurfactant production in presence of hydrocarbons showed better production of biosurfactants **(Kumar *et al.,* 2006).** Here the result was same; the bacterial strains were showing better production of biosurfactant by utilizing the PAHs as carbon source. Biosurfactants usually lowers the tensioactive force between the two phases indicating its powerful surface tension-reducing property.

Biosurfactant utilised in bioremediation has been harnessed relentlessly for biotechnological purposes. we have isolated and identified five potent isolates having high surface tension reducing property. Among *Bacillus licheniformis, Bcterium CT, Bacillus pumilus, Paenibacillus and Bacillus acidovorans*, for degradation of Anthracene , napthacene and coronene, *Bacillus licheniformis, Paenibacillus and Bacillus acidovorans*, Shown maximum production of biosurfactant properties.

Results were shown that degradation of anthracene, coronene and napthacene was enhanced by addition of synthetic surfactant known as Tweens 20, Tween 80 and Triton X 100 during degradation experiment. Tween 20 and Triton X 100 were shown more effective than another surfactant in degradation of PAHs. Data showed that the supplimentation of surfactants a accelerate positively for PAHs biodegradation, increasing the solubility and reduces surface tension to dissolved the molecules PAHs and enhance their progressive biodegradation up to concentration levels lower than the initial values. This result was clearly observed considering the low solubility and biodegradation degree of PAHs, in which surfactant was not supplemented. The chemical degradation of naphthalene in presence of 1% w/w Tween-80, was not observed for both PAHs compound napthacene and coronene , when we used bacterial surfactant with suynthetic surfactant had shown better degradation. However, in presence of PAHs-degrding strains, anthracene, coronene and naphthalene degradation was significantly faster than that in the abiotic control degradation represented that degradation of anthracene,naphthalene and coronene was mainly due to biological processes.

Of the three surfactants studied, Tween-20, Triton X100 was the one that caused the higher level of PAHs degradation. Moreover, the progressive decrease in toxicity and the larger bacterial growth seem to favour a significant reduction in naphthalene, phenanthrene and anthracene. For Triton X-100 the results obtained were favourable and similar in both cases. However, the degree and rates of biodegradation were lower for results using Tween-20.

The higher bacterial growth and PAHs-biodegradation rate observed in presence of Tween-80 during PAHs-biodegradation experiments could be due to the fact that this surfactant was biodegradable and can be used as an additional carbon source for the microorganisms **(Franzetti *et al.,* 2006; Boonchan *et al.,* 1998; Yoshimura, 1986; Swisher, 1987),** although the surfactant biodegradation could compete for the substrates **(Tiehm, 1994).** The growth of Pseudomonas sp., Enterobacter sp. and Stenotrophomonas sp. strains with PAHs as carbon source indicates that these microorganisms are potential PAHs' biodegraders. The results showed that in most cases growth rate of Enterobacter sp. and its PAHs-biodegradation rate were higher than for the other bacteria. Furthermore, the rates of degradation and growth observed for Pseudomonas sp. and Stenotrophomonas sp. were also significant. Many authors have studied the genes involved in the PAHs-degradation metabolism for naphthalene **(Dunn and Gunsalus, 1973; Kuhm *et al.,* 1991; White *et al.,* 1997; Filonov *et al.,* 2006),** phenanthrene **(Balashova *et al.,* 1999; Puntus *et al.,* 2008) and anthracene (Jacques *et al.,* 2005)** or other aromatic hydrocarbon with higher molecularweight **(Boonchan *et al.,* 1998; Juhasz *et al.,* 2000).** Anyway, the highest levels of growth and degradation of PAHs were obtained when the three bacteriaworked together. Probably this fact is due to the existence of a microbial succession during the incubation.

Result from degradation of PAHs : anthracene, ccoronene and napthacene was evaluated that degradation of Anthracene was calculated with help of anthracene standard peak area, retention time and colliberation curve to find out the remaining concentration of anthracene compound. There was following colliberation curve for concentration 0.125, 0.25,0. , 1 and 2 mg. After analysis of degradation of anthracene, all isolates were shown highly degradation activity during incubation period 10 days. During 15 days incubation period *Bacillus licheniformis*, *Bacterium CT* and *Bacillus Pumilus* were shown minimum degradation but in next exeperimental setup for anthracene degradation were increases regularly during incubation period 20, 25 and 30 dayes for isolates *Bacillus licheniformis*, *Bacterium CT Paenibacillus lautus* MORL, *Bacillus acidovirans* and *Bacillus Pumilus* respectively. These finding were revealed that 10, 20, 25 and 30 dayes incubation period presented highest degradation for all isolates at anthracene concentration 50 mg/l. *Bacillus licheniformis*, *and Paenibacillus lautus* MORL was more significat in anthracene degradation for incubating periods. Coronene degradation during 5,10 and 15 dayes of incubation of degradation setup for all isolates had shown maximum activity at concentration 20mg/l of coronene in *Bacillus Pumilus Bacillus licheniformis*, *Bacterium CT, Paenibacillus lautus* MORL, *Bacillus acidovirans* and *Bacillus Pumilus* MSM . Degradation potential of isolates was gradually decreases after 15 days of incubation, 30 days had shown minimum degradation activity. For napthacene experimental observation was state that degradation activity of all isolates respectively *Bacillus Pumilus Bacillus licheniformis*, *Bacterium CT, Paenibacillus lautus* MORL, *Bacillus acidovirans* and *Bacillus Pumilus* against 50 mg/l napthacene, gradually increases during incubation period 5,10,15,20,25,and 30 days, 15 days were shown maximum degradation. Among all isolates *Paenibacillus lautus* MORL, and *Bacillus acidovirans* were shown maximum degradation potential. During antracene degradation Bacterium CT shown minimum activity.

Similar study was compared that represented the PAHs-degrading efficiency depends on the molecular weight and the number of aromatic rings **(Warmer and Peters, 2005),** since, the solubility in water, volatility and persistence of PAHs decrease when molecular weight and the number of aromatic rings decrease **(Juhasz and Naidu, 2000; Kanaly and Harayama, 2000; Dimitriou- Christidis *et al.*, 2007).** The results showed that the evolution of the PAHs during the biodegradation process followed the order of solubility of these pollutants: naphthalene>phenanthrene>anthracene. The depletion of both three-ring hydrocarbons was not associated to any abiotic processes since depletion of phenanthrene and anthracene was not observed from the abiotic control experiments. In the case of naphthalene, the depletion measured in this abiotic control was likely due to volatilization because of its higher vapour pressure (11.6 Pa at 298 K) than phenanthrene and anthracene (0.13 Pa and 0.08 Pa at 298 K, respectively). However, the overall rate of naphthalene depletion was higher than that observed in control experiments, so that the biological contribution was significant. The large increase in the degradation rate of each PAH in presence of surfactant was not only due to the large increase in PAHs solubility, but to a concomitant increase in the value of the kinetic constant. The significant interactions between type of microorganism and type of surfactant showed by the ANOVA performed for each PAH proved that the choice of the best surfactant to increase the biodegradation efficiency depends on the microorganism or consortium used as well as the nature of the hydrocarbon to be removed.

The small decrease in TOC (total organic carbon) confirmed the PAHs biodegradation. The partial mineralization of PAHs and intermediate products to CO_2 could explain this trend. Nevertheless the results showed two increases in TOC levels during the biodegradation process. The first one, from the beginning of the experiment, could be caused by the formation of intermediate degradation products and the dissolution of PAHs precipitated in the media along the exponential growth phase. In the case of the biodegradation experiments using Tween-80 as surfactant that effect was even larger since Tween-80 was biodegraded by all three strains used. At the end of the growth phase a second increase in TOC took place, most likely due to the release of intracellular matter during the death phase. In absence of surfactant, the low solubility of PAHs produced very low values of TOC in aqueous solution.

The biodegradation of low molecular weight (two- and threering) PAHs occurred much more rapidly and extensively than that of the high molecular weight (four-, five- and six-ring) hydrocarbons **(Nam *et al.*, 2001; Taylor *et al.*, 2001).** However, high degradation of the latter would have been obtained if some pretreatments had been done. In this study, the degradation of three-ring PAHs was similar to that of five-ring PAHs, andwas lower than that of fourring PAHs. This indicated that, besides chemical pre-oxidation, the particular microbial consortia screening using typical rings contaminants might be one of the other pretreatment methods **Kulik *et al.*,**

CONCLUSION

Based on the findings of the present study, it may be concluded that the isolated bacterial shown maximum degradation at pH 7.5 and temperature 35°C. It is capable of utilizing low and high molecular weight PAHs such as anthracene, coronene and napthacene as sole carbon source under aerobic condition. The isolated bacteria produces biosurfactant lipopeptide,glycopeptide, surfactatin and palipastatin is also capable of degrading another PAHs present in crude oil contaminated soil . Hence it may be concluded that the isolated bacteria could be a potential candidate in environmental and industrial application concerning crude oil contamination.

Fifty eight microbial stains were isolated from various sampling sites namely Mathura and Ba ruani oil refinery soil sediment, that showed existence of these species on petroleum containing PAH compound. Among the Fifty eight isolates Five bacterial species had shown very good growth on PAH (anthracene, coronene and coronene). The morphological, physiological and biochemical tests, 16S-rRNA sequencing and morphology study by SEM was confermed that bacterial strain were *Bacillus licheniformis* MBOR, *Bacterium* CT BORL, *Bacillus pumilus* BORS, *Paenibacillus lautus* MORL and *Bacillus acidovirans* MORS. Optimization of process parameters like temperature and pH source were conducted by one. *BacteriumCT* BORL, *Bacillus pumilus* BORS, *Paenibacillus lautus* MORL and *Bacillus acidovirans* MORS) having accession no. KX376312, KX376311, KX376310 KX376309 and KX376308 were submitted to NCBI. All five screened isolates were capable to grow on different concentrations of three PAHs (Anthracene 50mg/l, Coronene20mg/l and Napthacene50mg/l) at temperature 30^0C-35^0C and pH 6.5-7.5. The best degraders' bacterial isolates *Bacillus licheniformis* MBOR and *Paenibacillus lautus* MORL were shown maximum degradation at concentration 50mg/l of anthracene, napthacene and 20mg/l of coronene. The most promising bacterial strain were enhance the degradation by suplimentation of surfactant/emulsifier (tween 20, tween 80, and triton x100) to enhance its qualities of degradation.

Bacterial isolates from soils contaminated with a large range of petroleum organic pollutants of oil refinery Mathura and Baruni were tested for their ability to metabolise, mineralized and degrada the selected recalcitrant PAHs (anthracene , coronene and napthacene) as their sole energy and carbon source. Plating of soil microorganisms isolated from 50 petroleum-contaminated soils on solid medium (MSM), supplemented with the PAH (50 mg/l anthracene, 20mg/l coronene and 50mg/l napthacene) as sole carbon source, yielded 58 PAH degrading bacterial strains, of which most potent isolates was selected and were further characterized. By different parameters, identified 5 of the 58 colonies aspotentially competent in HMW PAH degradation (anthracene, coronene and napthacene). The 5 bacterial isolates were further screened by UPLC for their capacity to mineralize three HMW PAHs serving as their sole carbon and energy source. Under such conditions, bacterial isolate of five strains cumulatively mineralized 98% of anthracene, 95% of napthacene, and 97% of coronene after 30-days incubation period period, but could not degrade coronene (24%) as much compaire to napthacene and coronene.

Three different characterisation approaches were applied to five isolate (*Bacillus licheniformis strain* MBOR, *BacteriumCT* BORL, *Bacillus pumilus* BORS *Paenibacillus lautus* MORL and *Bacillus acidovirans* MORS in Genbank): (i) micro/macromorphological stydy byu phase-contrast microscopy showed five isolates to be a Gram variable rodcoccus; produced creamy yellow, whitish and circular colonies that were catalase-positive, except *Paenibacillus lautus* all bacteria was gram positive (ii) SEM for morphological and structral analysis (iii) molecular species confirmed by others‘ 16S rRNA gene sequence analysis. Among five strains *Bacterium CT* BORL mineralization of PAH also showed it to be new and unique PAH-degrading bacteria. This multidisciplinary study highlights the fact that five strains shows a great potential to be useful in biodegradation of high molecular weight PAHs., and strongly supports the general concept of *Bacillus licheniformis* MBOR, *BacteriumCT* BORL, *Bacillus pumilus* BORS *Paenibacillus lautus* MORL and *Bacillus acidovirans* MORS having a significant role in PAHs-biodegradation, bioremediation, and biodetoxification in contaminated envirnoment, Petroleum refinery and hazardous compounds effluents of industry. Field trials for biodegradation of hydrocarbons in aqueous environment by the microbial isolates were to be friendly processes. While applying the isolated microorganisms and produced biosurfactants in large quantity to degrade PAHs. Degradation studies of petroleum hydrocarbons present in soil by the two isolates namely*Bacillus licheniformis strain* and *Paenibacillus lautus* were had more potential for HMW PAH degradation done. Impact of environmental conditions like temperature, pH and moisture and oxygen on action of Lipopeptide, lipoprotein, glycolipid and glycoprotiens biosurfactants were examined.

Chapter- 6

Conclusion

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Chapter- 7
References

APPENDIX

The media used and their compositions are given below

Luria Bertani Media

Components	Quantity (g/l)
Tryptone	2.00
NaCl	1.00
Yeast Extract	0.5%

pH (at 25 °C) 7.0

Bushnell Haas Broth (BHB)

Components Quantity	(g/l)
Magnesium sulphate	0.20
Calcium chloride	0.02
Monopotassium phosphate	1.00
Dipotassium phosphate	1.00
Ammonium nitrate	1.00
Ferric chloride	0.05

pH (at 25 ° C) 7.0 ±0.2

Muller Hinton Broth (MHB)

Components Quantity	(g/l)
Beef infusion solids	4.0
Starch	1.5
Casein hydrolysate	17.5

pH (at 37 °C) 7.4± 0.2

Polycyclic aromatic Hydrocarbons

Used polycyclic aromatic hydrocarbons are: - Anthracene, Coronene, Napthacene

These are taken in the concentration of 50mg/l for Anthracene, Napthacene and 20 mg/l for Coronene,

Basal medium

Tryptone	10.0 g
NaCl	5.0g
Bromocresol purple	10 ml
Distilled water	1000ml
pH	7.0±0.2

Autoclaved at 15 lbs/in^2 for 15 min. 10 % sugar solution (mannitol, glucose, lactose, maltose, sucrose, and xylose) was made and 0.5 ml were added to the test tube containing 5 ml Basal medium.

Casein Starch Agar Medium

Soluble Starch	10g
Casein	0.3g

Potassium Nitrate	2g
Sodium Chloride	2g
Dipotassium Phosphate	2g
Magnesium Phosphate	0.05g
Calcium Carbonate	0.02g
Ferrous Sulphate	0.01g
Distilled Water	1000g
pH	7.0±0.5

All the ingredients were dissolved, heated and autoclaved at 15 lbs/in^2 for 15 min.

Gelatin Agar Medium

Peptone	5.0 g
Beef extract	3.0 g
Gelatin	120.0 g
Distilled water	1000 ml
pH	6.8±0.2

Dissolved all the ingredients in water by heating. Adjusted pH and Sterilized by autoclaving at 15 lbs/in^2 for 15 min.

Methyl Red – Voges Proskauer Broth

Peptone	7.0 g
Potassium phosphate	5.0 g
Dextrose	5.0 g
Distilled water	1000 ml
pH	6.9±0.2

All the ingredients were dissolved, heated and autoclaved at 15 lbs/in^2 for 15 min.

Mineral Salt Agar Mdium

Casein	0.3g
Potassium Nitrate	2g
Sodium Chloride	2g
Dipotassium Phosphate	2g
Magnesium Phosphate	0.05g
Calcium Carbonate	0.02g
Ferrous Sulphate	0.01g
Agar	18g
Distilled Water	1000g
pH	7.0±0.2

All ingredients were dissolved in distilled water and pH was adjusted to 7.3±0.2 followed by sterilization using autoclave at 15lbs/in^2 for 15 min.

Mineral Salt Broth

Casein	0.3g
Potassium Nitrate	2g
Sodium Chloride	2g
Dipotassium Phosphate	2g
Magnesium Phosphate	0.05g
Calcium Carbonate	0.02g

Ferrous Sulphate	0.01g
Distilled Water	1000g

All the ingredients were dissolved, heated and autoclaved at 15 lbs/in^2 for 15 min.

Nitrate Reductase Media

Peptone	5.0 g
Beef extract	3.0 g
Potassium nitrate	5.0 g
Distilled water	1000 ml
pH	7.2±0.2

All the ingredients were dissolved, heated and autoclaved at 15 lbs for 15 min.

Ringer Solution

Potassium chloride	0.42g
Sodium chloride	9.0g
Sodium bicarbonate	0.2g
Anhydrous Calcium Chloride	10.0g
Distilled water	1000ml
pH	7.3±0.2

All ingredients were dissolved in distilled water and pH was adjusted to 7.3±0.2 followed by sterilization using autoclave at 15lbs/in^2 for 15 min.

Skimmed Milk Medium (pH 6.8)

Skimmed milk powder	100.0 g
Distilled water	1000.0 ml

Skimmed milk was dissolved in distilled water and autoclaved at 10 lbs/inch^2 for 10 minutes.

Starch Agar

Starch	20.0 g
Sodium Chloride	5.0 g
Agar	18.0 g
Distilled Water	1000 g
pH	7.0±0.2

Dissolved all the ingredients in water by heating. Adjusted pH and Sterilized by autoclaving at 15 lbs/in^2 for 15 min.

Tryptone broth

Peptone	10.0 g
Distilled water	1000 ml
pH	7.0±0.

Autoclaved at 15 lbs for 15 min.

Reagent and Stains

Alpha naphthol solution

α-naphthol	5.0 g

Ethyl alcohol (absolute)	95.0 ml

Gram's stains

I Crystal violet

a) Solution A

Crystal violet (90% dye content)	2.0 g
Ethyl alcohol (95%)	20.0 ml

b) Solution B

Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Crystal violet was dissolved in Ethyl alcohol and the Ammonium oxalate in distilled water. Then solution A and B was mixed.

II Gram's Iodine

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300 ml

III Ethyl alcohol (95 %)

Ethyl alcohol (100 %)	95.0 ml
Distilled water	5.0 ml

IV Safranin

Safranin (2.5% sol. + 95% ethyl alcohol)	10.0 ml
Distilled water	100 ml

Kovac's Reagent

p-dimethyl aminobenzaldehyde	5.0 g
Amyl alcohol	75.0 ml
Conc. HCl	25.0 ml

Aldehyde was added to a flask containing alcohol and dissolved by warming at 55 ^{0}C, cooled and then HCl was added.

Methyl Red solution

Methyl Red	0.04 g
Ethyl alcohol (absolute)	40.0 ml
Distilled water	60.0 ml

Dissolved Methyl Red in ethyl alcohol and volume was made to 60 ml .
using distilled water.

Nitrate Test Solution

a) Solution A

Sulfanilic acid	8.0 g
Acetic acid (5N)	1000 ml

b) Solution B

α-Naphthylamine	5.0 g

Acetic acid (5N)	1000 ml

Phenol Red indicator

Phenol Red	0.2 g
Ethyl alcohol (95 %)	50.0 ml
Distilled water	50.0 ml

Potassium hydroxide (40 %)

Potassium hydroxide (KOH)	40g
Distilled water	100ml

3% Hydrogen peroxide Solution :

Hydrogen Peroxide	3.0 ml
Distilled water	97.0 ml

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Research Article Open Access

Biodegradation of Keratin from Chicken Feathers by Fungal Species as a Means of Sustainable Development

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Abstract

Keratinolytic microorganisms have a great importance in feather waste degradation and its use for improvement of livestock feed and production of protein hydrolysates. Annually several thousand tons of feather wastes are discharged into the surrounding environment as a by-product of commercial poultry processing. Microorganisms could minimize regulatory problems of uncontrolled accumulation of waste feathers. This residue is almost pure keratin, which is not easily degradable by common proteolytic enzymes.

The present study deals with identification of fungi that play a significant role in the degradation of chicken feather and keratin degradation ability of the isolated fungi. Feathers of broiler chicken were collected from Jaggi poultry farm, Mandir Hasaud, Raipur. Fungi were isolated by feather baiting technique. Feathers were inoculated in Sobouraud Dextrose Agar (SDA) medium and their pure culture was prepared. Fungus were identified by Lacto phenol cotton blue staining method as *Trichoderma*, *Gliocladium*, *Fusarium*, *Syncephalastrum*, *Mucor*, *Aspergillus Flavus*. The pure culture were grown in mineral media with 500 mg of feathers as a sole source of Nitrogen and Carbon and incubated for the period of 25 days. At 5 days intervals, the biochemical changes associated with biodegradation was evaluated by analyzing the culture filtrate. The release of Nitrate, Cystine, Cysteine and methionine components during the process of biodegradation was studied which proved the efficient degradation of keratin. There was also a change in pH of the medium towards alkalinity. *Mucor* and *Aspergillus Flavus* were the most powerful bio remedial fungus in the current study. With an increasing world-wide concern for the environment it is possible to use these six fungus for the degradation of enormous quantity of waste feathers. Biodegradation leads to recycle the wastes and thus maintaining the environmental quantity of the biosphere.

Keywords: Fungus; Bioremediation; Poultry processing; Keratin; Feather baiting; Lacto phenol cotton blue staining

Introduction

Feather is generated in bulk quantities as a by-product in the poultry industry globally. It is a very rich source of protein with β-keratin constituting 91% of feather protein. The presence of keratin makes feather recalcitrant to most common proteases like trypsin, pepsin, papain, and so forth, thus slowing down its degradation process in nature [1]. Typically, each bird has up to 125 gm of feather and with more than 400 million chickens being processed every week worldwide, the daily accumulation of feather waste reaches five million tons [2]. The bulk of feather waste is poorly recycled in nature and has limited utility due to the chemically unreactive nature of keratin. Conventionally, this waste has been converted into feed supplement, resulting in feed of poor quality which is nonviable economically [3]. Thus, recycling of this by-product is neither profitable nor environmentally friendly. The disposal of this waste is a global environmental issue leading to pollution of both air and underground water resources [4]. In recent years, feather treated with microbial keratinase is attracting wide attention with several applications. Keratinase-treated feather is increasingly considered as a viable source of dietary protein in food and feed supplements, as the enzyme-treated end product retained high nutritive value. Keratinases are projected to generate a potential worldwide market similar to other proteases. Diverse groups of microorganisms are reported to produce keratinase like fungi (*Doratomyces microsporus, Alternaria radicina, Trichurus spiralis, Aspergillus sp., Rhizomucor sp., Absidia sp., Stachybotrys alba,* etc.), *actinomycetes* (*Streptomycespactum, S. alvs, S. thermoviolaceus, S. fradiae, Thermoactinomyces candidus* etc.), and several bacterial species (*Fervidobacteriumislandicum, Pseudomonas aeruginosa, Microbacterium sp.*, and many species of *Bacillus* including *Bacilluslicheni formis* and *B. pumilus*) earlier [2,5-8]. However, the full commercial potential of keratinases is yet to be realized. Major component of feather is keratin which is insoluble fibrous protein. Keratin is highly resistance to hydrolysis by week acids, alkalies, ethanol or salt solution [9] and also to enzymatic digestion [10]. The durability of Keratin is due to cross binding of closely packed polypeptide chain in which cystine molecules are held together by disulphide bonds(S-S). However, the keratinophilic fungi have been frequently isolated from soil, where they colonize various keratinous substrates, degrade them and add the mineral content to the soil [11].

Feathers of birds are most suitable substrate for the survival of much fungus in nature [12]. Ramesh [13] isolated a number of pathogenic and non-pathogenic fungi from keratin substrates and studied their intensity and the type of hair degradation. However, there is no detailed investigation on the degradation of feathers. Therefore, the present investigation was envisaged to study the biodegradation of feathers. Currently, almost all the habitats of the world have been surveyed for the presence of keratinophilic fungi [14]. Most of these fungi belong to families Arthrodermataceae and Onygenaceae, order Onygenales in Ascomycetes [15].

Methodology

Method of sample collection and isolation of potential fungal colonies

Feathers of broiler chicken were collected from Jaggi poultry farm, Mandir Hasaud, Raipur. Fungi were isolated by feather baiting technique. Feathers of hens were cut and finely powdered and sterilised by using 70% sodium hypochlorite solution for 5 minutes. These sterilised feathers were then inoculated on Sabouraud Dextrose Agar (SDA) media to obtain culture of fungal colonies. Six different colonies obtained were inoculated in 50 ml mineral media (Sodium Nitrate, 3 g; Dipotassium Hydrogen phosphate, 1 g; Potassium Chloride, 0.5 g; Magnesium Sulphate 0.5 g, Ferrous Sulphate 0.01 g) along with 500 mg of feathers as a sole source of Nitrogen and Carbon [16] in 250 ml flasks and incubated for observation.

Identification of isolates

Fungus was identified by Lacto phenol cotton blue staining method. A drop of Lacto phenol cotton blue was taken on a slide. Fungal hyphae was picked from plate with the help of a needle and placed on the slide containing Lacto phenol. Then the Slide was covered with a cover slip and observed under the microscope (Figures 1-6).

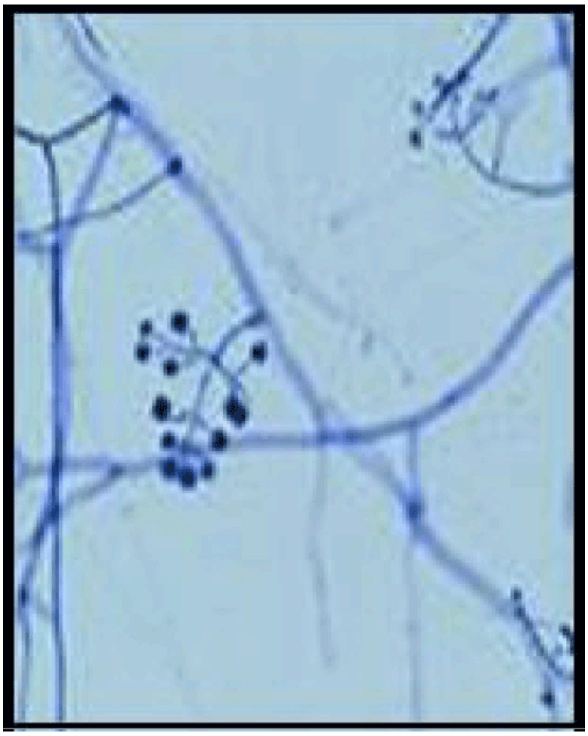

Figure 1: Trichoderma

Figure 2: Gliocladium

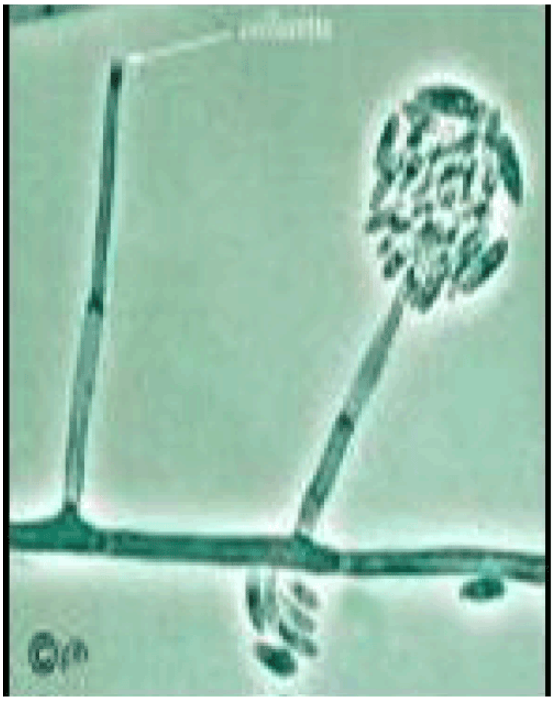

Figure 3: Fusarium

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Figure 4: Syncephalastrum

Figure 5: Aspergilus

Estimation of keratin degradation

Filtrates were collected in an interval of 5 days starting from the 10th day of incubation. Filtrate was collected by filtering the incubated media by arranging 2-3 Wattman filter paper on one above another. This filtrate does not contain any feathers, fungal culture and mineral media (Table 1). Change in pH was measured by using digital pH meter with a glass electrode. Determination of nitrate release (NO_3) was done by the method of Goldsmith [17]. Determination of cysteine and cystine was done by the method of Ramakrishna [18]. Determination of methionine was done by the method of Timothy [19].

Figure 6: Mucor

Filtrate	Microorganism
Filtrate 1	*Trichoderma*
Filtrate 2	*Gliocladium*
Filtrate 3	*Fusarium*
Filtrate 4	*Syncephalastrum*
Filtrate 5	*Aspergillus*
Filtrate 6	*Mucor*

Table 1: No. of filtrates

Result and Discussion

Increase in pH

During the process of biodegradation there was a gradual increase of pH in to the alkaline phase for feathers till 25 day incubation. However, the pH increases from 10th day, 20th day for 1st to 4th culture and increases continuously from 10th to 25th day in case of 5th and 6th culture (Figure 7). Such an alkalinisation of the medium may be due to excretion of excess nitrogen via deamination and ammonium excretion. Keratin degradation involves rupturing the disulphide linkage between the peptide chain of keratin molecules by some extra and intra cellular enzymes collectively called keratinise.

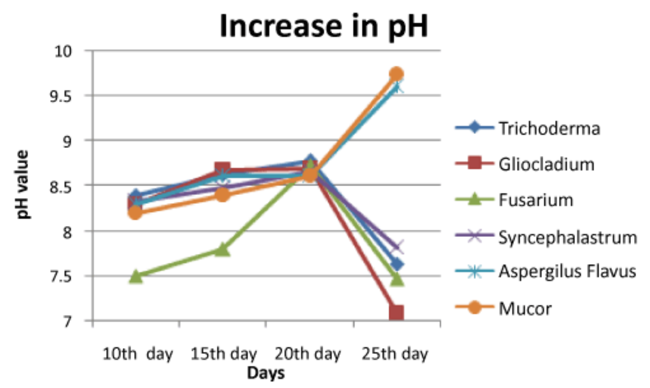

Figure 7: Increase in pH

Increase in Nitrate

The liberation of nitrogenous compound gradually increases from 10th day to 20th day for 1st to 4th culture and increases continuously from 10th day to 25th day in case of 5th and 6th culture (Figure 8).

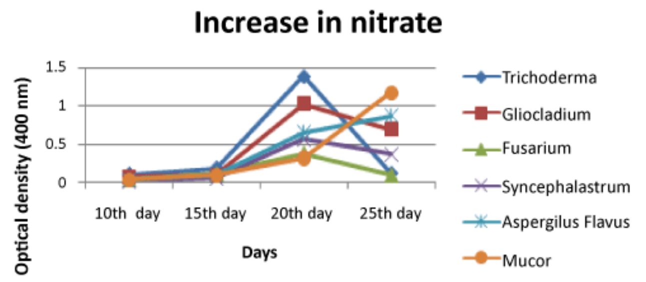

Figure 8: Increase in Nitrate

Increase in Methionine

Methionine release in the filtrate increases from 10th to 20th day in 1st to 5th culture and increases continuously from 10th to 25th day way in case of 6th culture (Figure 9).

Increase in Cystine

Accumulation of cystine may be direct reduction of disulphide bridges of keratin [20]. Chicken feathers, a type of eukeratin which connected histidine, lysine, and arginine in a definite proportion of 1:4:12 and 3-5% of sulphur nearly all of which is in the form of cystine [21]. In filtrate 1, 2, 3 and 6 cystine release increases from 10th day to 25th day whereas increases continuously in 4th and 5th culture (Figure 10).

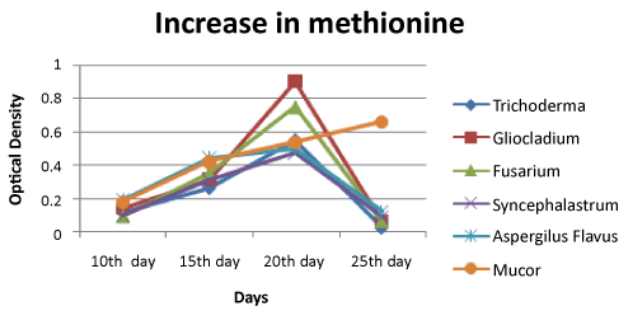

Figure 9: Increase in Methionine

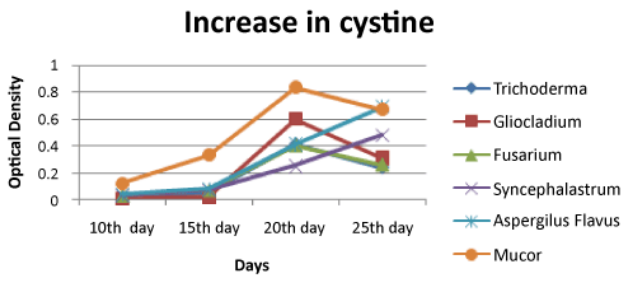

Figure 10: Increase in cystine

Increase in Cysteine

The release of sulphydryl compounds namely, cysteine release increases from 10th day to 20th day and decreases in 25th day in all the culture except in 5th culture in which cysteine release increases continuously (Figure 11). During the process of degradation the -S-sulfo groups (-S.SO3H) and Sulfhydryl groups were formed by Sulfitolysis [22].

The fungus degrades this highly resistant Keratin of feather. With an increasing world-wide concern for the environment it is possible to use these 6 fungi for the degradation of enormous quantity of waste feathers. Biodegradation leads to recycle the wastes and thus maintaining the environmental quantity of the biosphere.

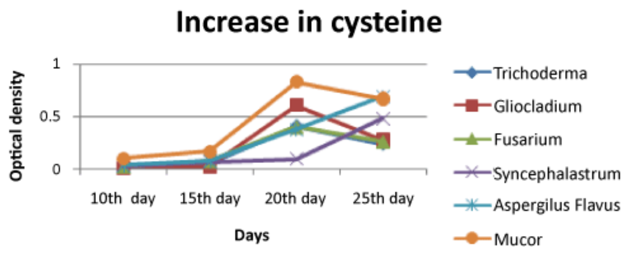

Figure 11: Increase in cysteine

Current and future use

Wastage of protein-rich reserve is ultimately converted into feather meal using keratinolytic fungi [23,24]. The addition of microbial digested feather meal to the animal feed improves digestibility and bolstered growth of poultry. Nutritional enhancement can also be achieved by hydrolysis of raw feathers using these keratinolytic fungi. Microbial-digested feather meal is also used as slow nitrogen releasing fertilizer. Keratinophilic fungi are used for the production of biodegradable films, coatings and glue from keratinous waste. Keratinases of these fungi are utilized in enzyme-based detergents which are used in the removal of keratinous soils, common in the laundry, on collars of shirts, etc. These enzymes are also used for cleaning up of drains clogged with keratin waste. These keratinases are also employed in the leather industry in hair saving dehairing in place of chemical based dehairing. Recently, these keratinases have been found to degrade prion protein leading to the prevention/cure of mad cow disease [25]. Further, keratinases are applied in the modification of silk and wool fibers, for acne or psoriasis, for making vaccines of dermatophytosis and has addictives in skin-lightening agents. In addition to the keratinases, these fungi have the potential to generate natural gas for fuel from poultry-waste degradation.

Waste material, hen feather, a bio sorbent, was successfully utilized in removing a water-soluble hazardous triphenyl methane dye, Brilliant Blue FCF from wastewater. Chicken feathers could help save trees by taking the place of wood pulp in air filters, paper products, and other uses, according to chemist Walter Schmidt of the U.S. Agricultural Research Service. Replacing half the wood-pulp content of composite paper with chicken feathers means only half as many trees. Chicken feathers can also to be use for the production of fuel.

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Research Article Open Access

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

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Abstract

Aromatic hydrocarbons are common groundwater and soil contaminants associated with petroleum product releases. They are hydrophobic and can readily bio-accumulate in the environment. Some PAHs (Polyaromatic Hydrocarbons) are harmful and known to be carcinogenic, mutagenic or genotoxic. Soil samples from hydrocarbon contaminated sites of Chhattisgarh were taken aseptically and their physicochemical properties studied. Soil samples were cultured in BBH media from which a total of eight positive isolates were studied for utilization of four aromatic hydrocarbons viz. Benzene, Toluene, Acenapthene and Napthalene. After biochemical analysis the genera identified were *Alacaligens, Arthobacter, Burkholderia, Psuedomonas, Ralstonia, Enterobacter, Micrococcus, Kluyvera*. All the isolates except strains *Burkholderia* and *Ralstonia* were able to use all the aromatic hydrocarbons studied as sole source of carbon indicated by the color change of resazurine indicator. Strains *Pseudomonas* and *Kluyvera* were considered as best degraders since they degraded the broadest spectrum of tested aromatic hydrocarbons and they showed the highest microbial activity in the presence of hydrocarbons tested indicated by the rapidity of the color change of resazurine. Furthermore, strains *Enterobacter* and *Micrococcus* exhibited moderate microbial activity against all of the tested hydrocarbons. Isolate Ralstonia was able to utilize benezene, toluene and naphthalene but couldn't utilize acenapthene. Isolate *Burkholderia* was able to utilize benzene, acenapthene and naphthalene, but their proliferation was not supported by toluene.

Keywords: Bioremediation; Aromatic hydrocarbons; BBH media; Resazurin

Introduction

Microorganisms, owing to their biodiversity and vast catabolic potential, have been enormously harnessed for the biodegradation of toxic pollutants for a long time [1]. Remediation work on a contaminated site, besides being an instrument for environmental and human health protection, is an important vector for social and economic development as it encourages the transformation of entire areas from nonproductive zones to zones of environmental, urban and economic redevelopment. The suitability of different remedial technologies must be assessed on the basis of a series of parameters connected with the pollutant type and its relative concentration, the characteristics of the contaminated matrix and the site typology, the specific environmental matrix, the chemical-physical characteristics of the pollutants, the age of the pollution and, finally, with the matrix microbial activity [2,3]. Production, refinery, transportation or storage of crude oil and its derivatives, through accidental leakages may lead to contamination of soils with BTEX compounds (benzene, toluene, ethyl-benzene and xylenes), polycyclic aromatic hydrocarbons (PAHs), as well as aliphatic hydrocarbons. Oil wells, petroleum plants, distribution and storage devices, transportation equipments are the main sources of the contaminations [4]. Polycyclic aromatic hydrocarbons (PAHs), produced by incomplete combustion of fossil fuels, and also manufactured for use in the pesticide, pharmaceutical and dye making industry are ubiquitous and persistent in the environment [5,6]. They are hydrophobic and can readily bio-accumulate in the environment. Some PAHs are harmful and known to be carcinogenic, mutagenic or genotoxic [7-9]. Cases of lung, intestinal, liver, pancreatic and skin cancer, have been reported [10]. PAH exposure occurs by inhalation, ingestion and dermal contact and, as they are highly lipid-soluble, they are quickly absorbed through the gastrointestinal tract in mammals [11]. Most of the PAHs are used to conduct research. However, some of the PAHs are used to make dyes; plastics; detergent; fungicides and pesticides. Some are even used in medicines. One of the most common ways PAHs can enter the body is through breathing contaminated air. The PAHs get into the human lungs when they breathe them. In addition if the human eat or drink food and water that are contaminated with PAHs, they could be exposed. Exposure to PAHs can also occur if the skin touches PAH contaminated soil or products like heavy oils, coal tar, roofing tar or creosote [10].

A large number of microorganisms (bacteria, fungi and some algae) that are capable of using petroleum hydrocarbons as the sole source of carbon and energy have been described [12]. Since PAH are hydrophobic compounds with low solubility in water, they have a tendency to bind with organic matter or soil, limiting their availability to microorganisms. Despite these properties, many bacterial strains have been isolated for their ability to transform, degrade and utilize

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PAH as a source of carbon and energy [13]. The isolation of bacteria from mixed contaminated soils which display versatile catabolic activity to tolerate toxic metals and degrade PAHs could be a potential cost effective remediation strategy [14]. Significant bacterial communities with ability to degrade PAH in soil play a critical role in biodegradation in spite of their low bioavailability. Microorganisms inoculated into PAH-contaminated soil environments must find and mobilize PAH before degradation and hence motility and chemotaxis are thought to be desired properties [15].

Materials and Methods

Site description and soil sampling

Raipur (20.91° N, 82.00° E), Bilaspur (22.09° N, 82.15° E) and Bacheli (18.70° N, 81.25° E) are located in Chhattisgarh state. Soil was moderately acidic with sandy-loam texture, increased humus, nitrogen and phosphorus content (Table 1). Hydrocarbon-contaminated soil samples were taken in vicinity of aboveground, as well as underground diesel-oil storage units. Samples serving as negative controls were taken from adjacent non-contaminated areas, from the same soil types. Sample labels are summarized in Table 2. The top 10 cm of soil was collected using sterile spatula into sterile glass bottles closed with cotton plug for microbiological analysis and with rubber septa for chemical analysis. Samples were stored at 4°C until further processing (within 24 h). Soil pH of samples was determined by following the SR ISO 10390-1999 standard (Muntean and Rusu, 2011). Humus content of soils (expressed in %) was determined according to Damian et al. [16] while the amount of mobile forms of phosphorous and potassium in accordance with Lacatusu and Lacatusu [17] using spectrophotometry, respectively flame spectrometry in acetatelactate ammonium solution at pH 3.7. Nitrogen index was calculated on basis of total humus content and soil base saturation according to Budoi et al. [18].

Enrichment, purification and culturing of hydrocarbon degrading bacteria

Diesel-oil degrading bacteria were isolated using enrichment containing: 99 ml BBH mineral broth medium supplemented with 1% (v/v) diesel-oil and inoculated with 1 g of contaminated soil. After 2 weeks of incubation at 23°C, 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).

Soil (0-20 cm)	pH	H_T (%)	N_i	P_m (ppm)	K_m (ppm)
Raipur	7.12	0.35	1.19	5.5	34
Bilaspur	6.9	0.89	1.49	7.9	48
Bacheli	6.6	1.50	1.25	9.0	23

H_T–total humus; N_i–nitrogen index; P_m–Phosphorous mobile; K_m–potassium mobile

Table 1: Soil characteristics within the studied areas.

S.No.	Serial Dilution Taken	Contaminated Site
1	10^{-3}	RNCS (Raipur Non Contaminated Site)
2	10^{-4}	RNCS
3	10^{-5}	BICS (Bilaspur Contaminated Site)
4	10^{-7}	BICS
5	10^{-4}	BCS (Bacheli Contaminated Site)
6	10^{-5}	BCS
7	10^{-3}	RCS (Raipur contaminated Site)
8	10^{-6}	RCS

Table 2: Designation of Investigated Sample.

Grouping and identification of strains

A total of eight different plates Gram staining and biochemical analysis was done for identifying different strains. Biochemical tests were performed (Gram stain, Starch hydrolysis test, Urease test, Indole test, Methyl Red test, Voges Proskauer test, Citrate test, Catalase test, H_2S test, Nitrate Reduction test, Cellulase test, Triple Sugar Iron, Caesinase, Gelatinase and Oxidase test). The genera of microorganisms were identified according to the data generated by abisonline.com.

Hydrocarbon degradation potential of isolates

Identified microorganisms were tested to degrade different hydrocarbons: benzene, toluene, acenapthene and naphthalene (Analytical Reagents-HiMedia). Test media contained 50 ml BBH mineral broth, supplemented with one of the filter sterilized (0.2 mm) hydrocarbons and resazurine (10 mg/L) as a redox indicator. In order to select strains that possess an increased hydrocarbon degradation potential the concentration of applied carbon sources was set to 0.5 g/L. Test solutions were inoculated with 250 ml strain culture solutions (OD_{600}=0.5). In the case of hydrocarbon degradation the initial blue color of test solution changed to colorless via pink [19]. The test runs were incubated for a week in a rotary shaker at 145 rpm and 28°C. Samples with no degradation activity (blue color) were marked "-", minimum microbial activity (bluish pink color) "+", the medium activity pink samples by "++", while samples showing increased hydrocarbon degradation activity (colorless) were marked "+++".

Results

Grouping and identification of strains

Mixed culture colonies were obtained after inoculation of enriched cultures in nutrient agar (Figure 1). The morphological and biochemical results (Table 3) were used to identify bacterial genus through online method (www.abisonline.com) were *Alacaligens, Arthobacter, Burkholderia, Psuedomonas, Ralstonia, Enterobacter, Micrococcus, Kluyvera* (Figures 2-9).

Hydrocarbon degradation potential of isolates

All the isolates except strains *Burkholderia* and *Ralstonia* were able to use all the aromatic hydrocarbons studied as sole source of carbon (Table 4) indicated by the color change of resazurine indicator (Figures 10-12). Strains *Pseudomonas* and *Kluyvera* were considered as best degraders since they degraded the broadest spectrum of tested aromatic hydrocarbons and they showed the highest microbial activity in the presence of hydrocarbons tested indicated by the rapidity of the color change of resazurine. Furthermore, strains *Enterobacter* and *Micrococcus* exhibited moderate microbial activity against all of the tested hydrocarbons. Isolate *Ralstonia* was able to utilize benezene, toluene and naphthalene but couldn't utilize acenapthene. Isolate *Burkholderia* was able to utilize benzene, acenapthene and naphthalene,

Figure 1: Mixed Colony plates.

Properties	**RNCS 10^{-4}**	**RNCS 10^{-3}**	**BICS 10^{-5}**	**BICS 10^{-7}**	**BCS 10^{-4}**	**BCS 10^{-5}**	**RCS 10^{-3}**	**RCS 10^{-6}**
Gram stain	+	-	+	-	-	+	+	-
Color	White	White	White	White	White	White	Yellow	White
Shape	Cocci	Cocci	Rod	Rod	Rod	Cocci	Cocci	Cocci
Starch hydrolysis	-	+	+	+	-	+	-	+
Urease	-	-	-	-	-	-	+	-
Indole	-	-	+	-	+	-	+	+
Methyl red	-	+	-	-	-	+	-	-
Voges Proskaeur	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Hydrogen Sulphide	-	-	-	-	-	-	-	-
Nitrate Reduction	-	-	-	+	+	-	+	-
Cellulase	-	+	+	+	+	+	+	+
Gelatinase	-	+	+	+	+	+	+	+
Glucose	-	+	+	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-
Lactose	-	-	-	+	-	-	-	-
Caseinase	-	-	-	-	-	-	-	-
Oxidase	+	-	-	+	+	-	-	+
Possible Genera →	*Alcaligens*	*Arthobaccter*	*Burkholderia*	*Enterobacter*	*Psuedomonas*	*Ralstonia*	*Micrococcus*	*Kluyvera*

Table 3: Morphological and Biochemical analysis chart of isolates.

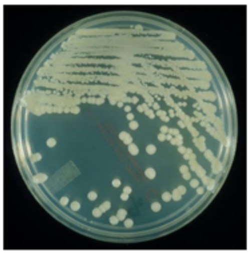

Figure 2: *Alcaligens.*

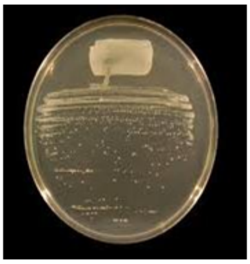

Figure 3: *Arthobacter.*

Figure 4: *Burkholderia.*

Figure 5: *Acinetobacter.*

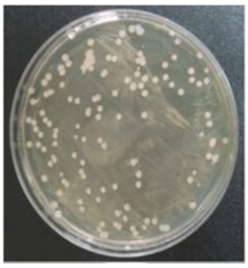

Figure 6: *Kluyvera.*

Figure 7: *Ralstonia.*

S. No	**Serial Dilution**	**Sample site/Bacterial Sp.**	**Benzene**	**Toulene**	**Acenapthene**	**Napthalene**
1	10^{-3}	RNCS (Raipur Non Contaminated Site)/*Arthobacter*	+	+	++	++
2	10^{-4}	RNCS/Alcaligens	++	+	++	++
3	10^{-5}	BICS (Bilaspur Contaminated Site)/*Burkholderia*	-	+	-	-
4	10^{-7}	BICS/Enterobacter	+	+++	++	++
5	10^{-4}	BCS (Bacheli Contaminated Site)/*Psuedomonas*	++	+++	+++	+++
6	10^{-5}	BCS/Ralstonia	+	+	-	+
7	10^{-3}	RCS (Raipur contaminated Site)/*Micrococcus*	++	++	++	++
8	10^{-6}	RCS/*Kluvera*	+++	+++	++	++

Table 4: Aromatic hydrocarbon degradation potential.

Figure 8: *Enterobacter.*

Figure 9: *Micrococcus.*

Figure 10: Dye color before degradation.

Figure 11: Dye color after degradation.

but their proliferation was not supported by toluene.

Discussion

Identification results of isolated aerobic heterotrophic hydrocarbon-degrading strains suggested the prevalence of the representatives of gamma-proteobacteria and beta-proteobacteria in the contaminated samples. This phenomenon is in accordance with results of several studies which established the dominance of Proteobacteria division in hydrocarbon polluted soils [20]. The dominant cultivable species in all samples were members of the genus *Pseudomonas*, these well known and widely investigated petroleum hydrocarbon-degrading organisms [21,22]. Genus *Burkholderia* was able to utilize Toluene only because of more complex nature of other hydrocarbons whereas Genus *Ralstonia* was not able to utilize Acenapthene because of its higher aromaticity. We have to mention however, that our results at least are influenced by the applied enrichment techniques, using BBH broth and diesel-oil as supplements. The quantitative analysis of hydrocarbon degradation will be carried out through GC-MS.

Figure 12: Dye color after complete degradation.

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Advances in Molecular Biology Approaches to Guage Microbial Communities and Bioremediation at Contaminated Sites

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Abstract Over the past 40 years, research on the microbial degradation of polycyclic aromatic hydrocarbons (PAHs) has resulted in the isolation of numerous genera of bacteria, fungi and algae capable of degrading PAHs. With the development of biology, molecular techniques such as PCR, fingerprinting technique (mainly DGGE/TGGE), ARDRA, TRFLP, FISH, RISA and gene reporter technique have been intensively applied to gain further insight into the mechanism of PAHs degradation. Further recent developments in moleclar microbial ecology like genotypic profiling, ultrafast genome pyrosequencing, metagenomics, metatranscriptomics, metaproteomics and metabolomics along with bioinformatics tools offer new tools that facilitates molecular analyses of microbial populations at contaminated and bioremediated sites. Information provided by such analyses aids in the evaluation of the effectiveness of bioremediation and the formulation of strategies that might accelerate bioremediation. The potential for the use of molecular methods in toxicological risk assessment and in developing bioremediation strategies is expanding rapidly as new methodologies become available. In this paper we present an overview of some molecular methods we feel have the most potential for use in assessment and monitoring in the field.

Keywords: *bioremediation, DGGE, TGGE, ARDRA, TRFLP, FISH, RISA PAH*

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1. Introduction

PAHs, a large and diverse group of organic molecules, are known for their adverse and cumulative effect in ubiquitous environment [1]. The non-environmentally friendly nature of PAHs makes difficult its treatment and depletion and thereby PAHs in environment impose sinful effect on the whole ecosystem. Modern molecular techniques provide an exciting opportunity to overcome the requirement for culturing and have greatly increased our understanding of bacterial diversity and functionality during bioremediation of oil-polluted soils. Unfortunately, only a fraction of the microorganisms involved in the biodegradation of pollutants in different ecosystems can currently be cultured using standard laboratory agars and conditions [2-11]. Molecular tools are especially useful in bioaugmentation, in which exogenous microorganisms that are introduced to accelerate pollutant biodegradation need to be monitored. To elucidate how microbial communities change due to the effects of environmental disturbances like pollution, investigations need to rely on rapid methods that can characterize cellular constituents such as nucleic acids, proteins (enzymes) and other taxa-specific compounds [12,13,14,15]. These molecules can be extracted directly from the soil without the need for culturing and thus can be used to elucidate the microbial community composition in such polluted environments during bioremediation [16]. To attain expected research goal, molecular techniques were indispensable to compensate the restriction of traditional applications. For example, polymerase chain reaction (PCR) and fingerprinting techniques based on PCR such as 16S rRNA gene library, denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), terminal restriction fragment length polymorphism (T-RFLP), rRNA intergenic spacer analysis (RISA) were selectively employed in microbial flora and ecology research. Besides above mentioned techniques, other molecular approaches including DNA hybridization such as fluorescence in situ hybridization (FISH) and DNA microarray, gene reporters and biosensors were also frequently used. However, as for PAHs biodegradation investigation, the techniques extensively employed were PCR, fingerprinting technique (mainly DGGE), DNA hybridization technique and gene reporters. The major aim of this article is to review these techniques. Genomics has been instrumental in providing complete genome sequence data of Pseudomonas, Shewanella, Deinococcus, Dehalococcoides and other micro-organisms pertinent to bioremediation. Genomics based recognition of various promoters, genes and degradative pathways have influenced the construction of more efficient contaminant degrading strains for pollution abatement. Cultivation-independent analyses of the overall microbial community structures at contaminated sites using molecular profiling techniques have been instrumental in our understanding of the community dynamics, relative abundance and distribution of micro-organisms actively involved in bioremediation. Metagenomics refers to collective analyses of the overall microbial community genomes of a given environment. Metagenomic analyses have enabled researchers to explore the previously uncultivable microorganisms and exploit their genetic potential in pollutant bioremediation [17]. Recent technological breakthroughs in de novo sequencing of microbial metagenomes based on pyrosequencing have provided rapid and relatively inexpensive methods to generate microbial community profiles simultaneously from different environmental samples [18]. Recently, development of microbial ecological DNA microarrays has enabled researchers to simultaneously analyze thousands of phylogenetic or functional genes in order to characterize microbial communities involved in bioremediation [19]. Metaproteomics approaches utilizing two-dimensional electrophoresis (2-DE), mass spectroscopy (MS) have aided in global analysis of catabolic enzymes involved in microbial biodegradation pathways [20,21]. One of the upcoming ‘‘-omics” technologies known as metabolomics refers to the colossal analyses of primary and secondary proteinaceous metabolites produced by microbial cells under defined physiological conditions [22]. This review highlights advances in the application of these technologies for studying microbial communities and their functional roles in environmental bioremediation.

2. Polymerase Chain Reaction (PCR)

The screening and cultivation of PAHs degrading microorganisms usually proceed the other steps in studies of PAHs microbial degradation. Once such microorganisms were obtained, identification and categorization of related microorganisms is a frequently confused technical problem. Categorization and identification of newly isolated microorganisms traditionally depend on phenotypic characteristics such as colony and cell morphology as well as biochemical and serological characteristics such as protein and fatty acid pattern profiles. However, it is often time-consuming and need expertise. With the rapid development of molecular biology, modern taxonomy prefer sequencing technologies of molecular markers such as 16S rRNA or 18S rRNA. These technologies allow the identification of colonies isolated from microbial consortia and the establishment of phylogenetic relationships between them [23]. In addition to taxonomy, PCR combined with other approaches could also be used to estimate in situ, how pollution affects the bacterial community structure and composition of sediments. Several PCR techniques such as random amplified polymorphic DNA (RAPD-PCR), arbitrarily primed-PCR (AP-PCR), repetitive extragenic palindromic-PCR (REP-PCR) and enterobacterial intragenic consensus sequence-PCR (ERIC-PCR) could be used to identify species. Besides, bacterial 16S rRNA has become the most commonly used molecular index for its evolutionary distinctive sequence. The 16S rRNA gene is essential as it encodes the small subunit of the prokaryotic ribosome and is therefore present in all prokaryotes. In bacteria, the rRNA genes are transcribed from the ribosomal operon as 30S rRNA precursor molecules and then cleaved by RNase III into 16S, 23S and 5S rRNA molecules [24]. The ribosomal operon size, nucleotide sequences, and secondary structures of the three rRNA genes are conserved within a bacterial species [25]. Since 16S rRNA is the most conserved of these three rRNAs, it has been proposed as an ‘‘evolutionary clock’’, which has led to the reconstruction of the tree of life [26].

Real-time quantitative PCR (also referred to as qPCR) has emerged as a promising tool for rapid, reproducible and accurate estimations of microbial community dynamics or monitoring their catabolic activity during active bioremediation processes [27]. The principle of qPCR assay is based on real-time detection of a reporter molecule whose fluorescence increases as the PCR product accumulates during each amplification cycle. The fluorescence chemistry in the qPCR reactions are either based on hybridization probes (TaqMan-molecular beacons with (FRET) fluorescence resonance energy transfer) or utilize double stranded DNA intercalating dyes SYBR Green along with carboxy-X-rhodamine (ROX) as a passive reference dye. However, to design probes and primers, the ‘‘signature sequences” unique to a particular mirco-organism or a catabolic gene need to be determined by comparison with database sequences using alignment tools. In each qPCR assay a known concentration of standard DNA (usually a linearized plasmid or genomic DNA) is used to prepare standard curves for quantification of unknown target microbial genes. The initial amount of target DNA is inversely proportional to the cycle threshold (CT) value which can be defined as the amplification cycle when the signal of fluorescence in the assay is statistically significant above the baseline level of fluorescence. Based on cycle threshold (CT) values, the relative abundance of specific group of microorganisms in the total microbial community DNA can be quantified by targeting either taxon/species/phylum specific rRNA genes or any other catabolite biomarker genes. A disadvantage of qPCR is optimization of amplification efficiencies and PCR biases in each run for accurate quantification. Nyyssonen et al. 2006 [27] validated a sensitive real-time PCR assay for quantification of naphthalene hydroxylating dioxygenase (nahAc) genes of naphthalene-degrading Proteobacteria within soil samples recovered from large-scale remediation processes. These authors also investigated naphthalene biodegradation using qPCR assay for enumeration of naphthalene dioxygenase genes in soil slurry microcosms. Furthermore, their study reports that qPCR assay is more sensitive than hybridization based analysis in monitoring bioremediation processes. Recently, [28] determined abundance of active bacterial populations of an enriched bacterial consortium-AIE2 during the steady-state condition within continuous bioreactors treating Cr(VI) and azo dye mixtures by calculating 16S rRNA gene copy numbers using qPCR assays. Cébron et al. 2008 [29] developed qPCR assays to determine copy numbers of a functional gene that encodes the alpha subunit of the PAH-ring hydroxylating dioxygenases (PAH-RHDa) within bacterial populations capable of degrading PAHs by aerobic metabolism in soil and sediment samples.

3. Denaturing Gradient Gel Electrophoresis (DGGE)/ Temperature Gradient Gel Electrophoresis (TGGE)

A number of PCR-based genotypic fingerprinting techniques are available for monitoring microbial communities and efficacy of bioremediation processes. Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are based on the principle of amplifying rRNA or functional gene PCR products from community DNA using primers containing a 50 bp GC-clamp and their separation on polyacrylamide gels having chemical or temperature based denaturing gradients [30]. Many studies have reported use of DGGE in monitoring microbial communities and their functional genes at sites contaminated with anthropogenic pollutants [31], such as microbial community shifts were detected in poly-metal contaminated soils [32] and abundances of dsrB (dissimilatory sulfite reductase b-subunit)-genes were assessed at an in situ metal precipitation site using DGGE fingerprinting technique [33]. However, these commonly used techniques (DGGE or TGGE) have inherent limitations, since they are labor-intensive and often less reproducible in terms of band pattern and intensity detection obtained after electrophoretic separation. These limitations can be addressed by designing improved group-specific primers [34] or by using a variant technique known as denaturing high performance liquid chromatography (dHPLC) that utilizes chromatographic separation instead of electrophoresis [35]. Muhling et al. 2008 [34] designed phylum- and class-specific PCR primers and tested their application in denaturing gradient gel electrophoresis (DGGE) analysis of complex bacterial communities. Wagner et al. 2009 [35] reported application of denaturing high performance liquid chromatography (dHPLC) in analyses of different microbial ecosystems within fermentor sludge, compost and soil samples using an ordinary HPLC system. For environmental or contaminated source samples where microbial diversity is largely unknown [36], DGGE/TGGE technique provides the opportunity for the identification of the microbial population through the excision of selected bands followed by their reamplification, cloning and sequencing that can lead to the phylogenetic affiliation of the ribotypes [37,38]. DGGE in particular has been widely used for the assessment of microbial community structure in contaminated soil and water in a number of studies [39-44].

4. Amplified Ribosomal DNA Restriction Analysis (ARDRA)

In amplified ribosomal DNA restriction analysis, PCR-amplified 16S rRNA fragments are digested or ut at specific sites with restriction enzymes and the resulting digest separated by gel electrophoresis. Different DNA sequences will be cut in different locations and will result in a fingerprint unique to the community being analyzed [45,46]. Divergence of the community rRNA restriction pattern on a gel is highly influenced by the type of restriction enzyme used [47]. Banding patterns in ARDRA can be used to screen clones or be used to measure bacterial community structure. ARDRA is simple, rapid and cost-effective, and as a result has been used in microbial identification [48,49,50] and microbial community studies [51,52,53]. In recent studies, ARDRA has been combined with other molecular techniques such as T-RFLP and DGGE to characterize microbial communities from contaminated sources [54,55,56]. A major challenge in using ARDRA lies in the interpretation of the fingerprints obtained from complex microbial communities.

5. Terminal-restriction Fragment Length Polymorphism (T-RFLP)

Terminal-restriction fragment length polymorphism is a major modification and improvement of the ARDRA method. The main advancement of T-RFLP over ARDRA lies in the fact that per organism detected, only the terminal restriction fragments (T-RFs) will be detected. Terminal-restriction fragment length polymorphism analysis (T-RFLP) is yet another most popular technique in studying microbial communities, since the last decade of its introduction to microbial ecological research. Main advantage of this profiling technique is its simplicity, automation and provision for accurate analysis of in silico data. T-RFLP analysis involves amplification of small subunit (SSU) rRNA genes from the total microbial community DNA using one or two primers tagged with different fluorescent labels. The resultant mixture of community rRNA amplicons is then digested with one or more four-base cutter restriction enzymes to generate T-RFs that are separated through capillary electrophoresis. Every single T-RF represents community fingerprints of a particular length unique to a particular phylogenetic microbial lineage or operational taxonomic unit (OTU). Based on the polymorphisms present in the SSU rRNA genes, different size of T-RF patterns of the whole microbial community are obtained. The size and relative abundances of these florescent T-RFs can be detected using an automated DNA sequencing instrument. Patterns of T-RF peaks on the output electropherogram can be identified using online database comparison tools. Many online automated fragment length assignment tools such as (taxonomic assignment pipeline) TAP-TRFLP (http://rdp.cme.msu.edu), torast (http://www.torast.de), and MiCA (http://mica.ibest.uidaho.edu), have been developed to perform in silico T-RFLP analysis of 16S rRNA gene sequences available in the databases. Similarly, an online tool known as TRF-CUT (www.arb-home.de) has been developed to predict in silico T-RFs from ARB database using aligned small-subunit rRNA gene or functional gene sequences (e.g. pmoA, nirK, and nifH) [57]. Unlike the above mentioned softwares, another online program TRiFle is available that can simulate and create T-RF datasets using arbitrary sets of DNA sequences from specific targets (e.g. genes involved in any metabolic pathways) or from unpublished sequences [58]. These online predictive tools are useful in choosing appropriate combinations of primers and restriction endonucleases to achieve best resolution up to taxonomic level in T-RFLP analysis. Several web based tools such as phylogenetic assignment tool (PAT), TRUFFLER, APLAUS are available to determine microbial community composition by comparision with T-RFs predicted from an in silico analysis of rRNA database sequences. Notwithstanding few of the limitations of TRFLP, such as overestimation of diversity by ‘pseudo T-RFs’ and non-specific action of restriction enzymes, it has become a valuable profiling technique for rapid and sensitive estimation of temporal and spatial variations in microbial communities [30]. Vázquez et al. 2009 [59] utilized T-RFLP to interpret temporal microbial community dynamics during bioremediation of diesel oil-contaminated antarctic soil. These authors performed a mesocosm study which suggested that nutrient addition (biostimulation) was a main factor in restoration of chronically contaminated Antartic soils, which significantly increased the detection of catechol degradation genes (nahH and catA) at treated plots. One of the advances in T-RFLP analysis is the demonstration of multiplex T-RFLP (M-TRFLP) which is useful in simultaneous profiling of multiple taxonomic groups of micro-organisms (two or four different taxa) within an ecosystem [60]. M-TRFLP analysis was validated using multiple primer sets targeted to bacteria, archaea and fungi in the same PCR reaction to study different microbial taxa in an ecosystem. This multiplex molecular profiling is useful in identifying bio-indicators of pollution, environmental health and to study how differently various microbial taxa respond to environmental stress [60].

The PCR primers used in T-RFLP analysis are fluorescently labelled at the 5′-terminus and the resultant PCR products are visualised and quantified [61] T-RFLP relies on variations in the positions of restriction sites among sequences and the determination of the length of fluorescently labelled terminal restriction fragments by high-resolution gel electrophoresis on an automated DNA sequencer. The electropherogram represents the profile of a microbial community as a series of peaks varying in migration distance. The use of fluorescently tagged primers limits the analysis to only the terminal fragments of the digestion [62]. This simplifies the banding pattern, hence enabling the analysis of complex communities as well as providing information on diversity as each visible band represents a single operational taxonomic unit or ribotype [63].

6. Fluorescent *in Situ* Hybridization (FISH)

The FISH technique is based on selective hybridization of rRNA targeted fluorescent dye-labeled oligonucleotide probes to the ribosomes of permeabilized microbial cells prefixed on membrane filters or glass slides. The resultant microbial cells stained by the complementary rRNA-targeted probes can be visualized or counted using epifluorescence microscopy, confocal laser scanning microscopy (CLSM), or flow cytometry techniques [64]. Multiple group-specific rRNA probes targeting prokaryotic and eukaryotic microbial taxa can be used in a FISH experiment for simultaneous phylogenetic classification as well as quantification of physiologically active microbial populations in an environmental sample. In the FISH approach it is assumed that actively growing microbes have many ribosomes and should theoretically yield brighter fluorescence signals due to higher rRNA-targeted probehybridizations. However, this assumption does hold true for many microbial cells that are smaller in size, slow growing or starving or containing low cellular rRNA content for example Dehalococcoides [65]. Hence, to over-come these limitations and improve the sensitivity of conventional FISH techniques, two new combinative approaches have been developed, namely (CARDFISH) catalyzed reporter deposition-fluorescence in situ hybridization [65] and (FISH-MAR) fluorescence in situ hybridization-microautoradiography [64]. Microautoradiography (MAR) is a process that relies on uptake of radioactive substrates by growing cells; the radioactivity incorporated into these cells is then visualized using radiation-sensitive photographic emulsions and microscopy. Therefore, coupling of FISH with microautoradiography (FISH-MAR) facilitates both phylogenetic as well as functional identification of substrate-active cells within complex microbial communities. The FISH-MAR technique involves short incubation of the environmental sample with radio-actively labeled substrate, followed by identification of microbial populations using FISH and in-parallel processing of identified microbial cells using radiation-sensitive photographic silver emulsions. Consequently, the silver particles deposited around the actively growing cells are visualized under transmission electron microscopy (TEM) to determine whether the microbe identified using rRNA-targeted probes was functionally or metabolically active in consuming the radio-labeled substrate offered at the time of incubation [64]. FISH-MAR technique has been most commonly used to identify key biodegradative microbial phylotypes within activated sludge systems owing to easy availability of sludge biomass for fixation, staining and hybridization experiments. Recently, [66] investigated the functional Bacteria and Archaea community structures responsible for decomposition process in a full-scale anaerobic sludge digester using FISH-MAR technique. In this study, it was observed that [14C] glucose-degrading microbial communities were dominant in terms of abundance and diversity as compared to fatty acids-[14C] propionate-[14C] butyrate-utilizing microbial communities. Moreover, despite the dominance of Betaproteobacteria in the community structures, members of Chloroflexi, Smithella, Syntrophomonas and Methanosaeta groups were more capable of utilizing radio-labeled sugars and fattyacids [66].

7. DNA Microarray Technologies

Microarrays (‘chips’) containing nucleic acids as probes represent a major advancement in molecular detection technology. They are ideal for the high-throughput study of the sequence diversity of 16S rRNA genes as well as of functional genes in environmental samples. DNA microarray technology is a very powerful taxonomic and functional tool that is widely used to study biological processes, including mixed microbial communities involved in pollutant degradation. This technique is similar to FISH, but provides a means for simultaneous analysis of many genes [67]. DNA microarrays are glass chips fabricated with different types of probes (pre-synthesized PCR products, cDNA, oligonucleotides, and known genomic fragments). These probes are deposited or spotted on the glass surface using metal pins (contact printing) or by ink-jets (non-contact printing). In addition, high-density oligonucleotide microarrays are generated by synthesizing probes at discrete locations using photodeprotection by photomasks/digital mirrors/chemical deprotection and ink-jet printing. Oligonucleotide microarrays are commercially available from several companies (Affymetrix, MWG, Agilent Technologies, GE Healthcare- Amersham Biosciences etc.). The probes ranging from sizes of approximately 25–1000 bps can be used to generate homogeneous microarrays (probes from a single genomic source) or heterogeneous microarrays (probes from different genomic sources) [68]. In principle, DNA microarray technique is based on hybridization of the target DNA molecules (single cell genomes or community genomes) to the array probes detected by measuring change in fluorescence signals (probe or target DNA can be tagged with several fluorescent dyes such as Cy3 or Cy5). The fluorescence signals from each of the probe-target hybridization spots are designated for quantification using mean signal intensity of each signal spot relative to its local background by signal-to-noise ratio (SNR) and measured using commercially available image analysis softwares. DNA Microarrays are amenable for rapid, sensitive and quantitative as well as simultaneous monitoring of several microbial populations within complex ecosystems. Based on the probes utilized in the fabrication of an array or depending on their applications, microbial ecological microarrays can be classified into several different types [68]. Of these different types of ecological microarrays; phylogenetic oligonucleotide arrays (POA), functional gene arrays (FGA) and whole-genome arrays (WGA) are most frequently employed in bioremediation studies. Phylogenetic oligonucleotide arrays (POA) or PhyloChips are constructed using short stretches of known oligonucleotide sequences based on rRNA genes from different microbial phyla and are amongst the most commonly used microarrays to decipher microbial community structures in environmental samples. Due to huge numbers of rRNA gene sequences available from public databases (such as RDP-II, ARB, NCBI, EMBL and DDBJ) along with easy in silico accessibility of online rRNA-targeted probe design and probe match tools, designing probes for a POA experiment becomes very convenient and user friendly. However, shorter stretches of oligonucleotide probes are less effective in resolving species-level phylogeny for some bacterial lineages [68]. Loy et al. 2005 [69] used a 16S rRNA gene-targeted oligonucleotide microarray (RHC-PhyloChip) consisting of 79 probes for diversity analysis of the betaproteobacterial order ‘‘Rhodocyclales” in activated sludge samples from an industrial wastewater treatment plant. RHC-PhyloChip was successful in detection of uncultured Zoogloea, Ferri bacterium/Dechloromonas and Sterolibacterium related bacterial lineages from the activated sludge samples that play an important role in waste water bioremediation process. An advanced version of PhyloChips is the Isotope Array, which is based on incorporation of radioactivity into rRNA of microbes incubated with radioactively labeled substrates [70]. Isotope array approach of hybridization of the radioactive rRNA of growing cells to PhyloChips enables microbial community diversity and activity to be assessed simultaneously using fluorescence and radioactivity detection modules.

8. Ribosomal Intergenic Spacer Analysis (RISA)

The RISA method makes use of the length and sequence heterogeneities that are present in the intergenic spacer (IGS) region between the small (SSU) and the large subunit (LSU) rRNA genes in the rRNA operon. It is a PCR-based technique that amplifies the region between the 16S and 23S rRNA genes. The IGS region, depending on the species, has both sequence and length (50–1500 bp) variability [71] and this unique feature facilitates taxonomic identification of organisms [72]. RISA has been used to distinguish between different strains and closely related species of *Staphylococcus* [73,74], *Bacillus* [75,76], *Vibrio* [77,78], and other medically important microorganisms. In environmental studies, RISA has been used to detect microbial populations involved in the degradation of PAH at low temperature under aerobic and nitrate-reducing enriched soil conditions [79]. RISA has also been used to define microbial diversity and community composition in freshwater environments [80]. RISA is a very rapid and simple fingerprinting method but its application in microbial community analysis from contaminated sources is limited partly due to the limited database for ribosomal intergenic spacer sequences is not as large or as comprehensive as the 16S sequence database. Kostka et al. [81] used ARISA to determine the diversity of hydrocarbon degrading bacteria and bacterial community response in beach sands impacted by the Deepwater Horizon oil spill in the Gulf of Mexico. Their findings indicated that oil contamination from the Deepwater Horizon had a profound impact on the abundance and community composition of autochthonous bacteria in the beach sands. Also members of the *γ-proteobacteria* (*Alcanivorax*, *Marinobacter*) and *α-proteobacteria* (*Rhodobacteraceae*) were identified as the key players in crude oil degradation.

9. Nucleic Acids Based Stable Isotope Probing (SIP)

Nucleic acids-based stable isotope probing (SIP) is a novel approach which directly links the microbial community structure with its function without the need for cultivation of individual micro-organisms. In principle, SIP technique consists of providing heavy isotope-labeled substrates (e.g. 13C-labeled substrates) to microbial communities and separation of the total cellular pool of nucleic acids within these microbial communities by isopycnic density gradient ultra-centrifugation [82]. The total extracted nucleic acids will form two different centrifugal zones, one with 13C-labeled (high buoyant density) and the other with C12-containing nucleic acid fragments (low buoyant density). Functional microbial communities that utilized the heavy isotope-labeled substrates can be identified from the resultant 13C-labeled nucleic acid fragments using molecular techniques discussed above. Natural C12-containing nucleic acid fragments are usually used as negative control in the SIP experiments to differentiate between active (13C-labeled) and inactive (C12-containing) microbial populations. SIP technique has been applied using wide variety of xenobiotic compounds to delineate the active microbial populations that utilize these compounds as substrates in cellular metabolism. Singleton et al. [83] supplied 13C-napthalene, 13C-salicylate and 13C-phenanthrene into PAH contaminated soils of a bioreactor to identify enriched bacterial degraders of PAHs within the bioreactor community DNA. In this DNA-SIP experiment, analysis of distinct 16S rDNA-based taxa within 13C-labeled community nucleic acids using DGGE fingerprinting technique identified Pseudomonas sp., Ralstonia sp. as degraders of naphthalene and salicylate and Acidovorax sp. as degraders of phenanthrene hydrocarbons. The SIP-approach in complementation with molecular fingerprinting or sequencing method can identify potential degraders of xenobiotics; however, the actual biodegradative pathways cannot be identified using this technique.

10. Molecular Biosensors / Bioreporters

In bioremediation of PAHs, in situ aerobic biodegradation of PAHs has been shown to be one of the most effective remediation strategies for detoxification of PAHs contaminated sites. In this sense, better understanding of the ecological behaviour of indigenous PAHs-degrading microorganisms and potential inoculants strains is required to optimise their potential use in bioremediation processes. However, it is impossible nowadays to reveal the whole veridical information of the ecological behaviour of microorganisms present in PAHs-contaminated environment. One of the feasible approaches to date is to mark a target strain before mixing with other microorganisms and then detect its activities to discover the role of this microorganism. With the increase of such microorganisms, theoretically, it is possible to elucidate the relationship between each microorganism in degrading of PAHs. Nevertheless, up to now, such research was preferable rare.

Environmental biosensors have made significant advancement towards monitoring of pollutants at contaminated sites as they have the unique ability to measure the interaction of specific compounds (pollutants) with biological systems through highly sensitive bio-recognition processes (signals). A biosensor is composed of a biosensing component interfaced with a transducing element that produces a measurable signal. Biosensing systems cover a wide array of integrated devices that utilize enzymes, antibodies, and sections of organs or tissues depending upon their applications [84]. A molecular biosensor has a recombinant plasmid as the biological component. It has a specific promoter, whose expression is sensitive to a target molecule and uses the reporter system to generate the signal. The promoters can be turned on or off with specific molecules, hence they provide the required specificity in signal generation. The generation of signals is directly proportional to the expression of the promoter. A biomarker, or marker gene, is defined as a DNA sequence, introduced into an organism, which provides a distinct genotype or phenotype to facilitate monitoring in a given environment [85]. Bundy et al. [86] showed use of single species biosensor responses to monitor recovery of oil polluted soil. Majority of bioreporters used in the study of microbial ecology are genetically engineered organisms in which responsive promoters are fused with suitable reporter genes, including lacZ coding for bgalactosidase, lux genes for the luciferase system, gfp for green fluorescent protein (GFP) and inaZ for ice nucleation protein [87]. The gene of green fluorescent protein (gfp) from the jellyfish Aequoria victoria, possess several advantages over other visual marker genes. The introduction of gfp into microorganisms is a useful tool with which to study microbial distribution, gene expression and protein interactions. The combination of transposon tagging with fluorescent reporter genes like gfp may lead to the development of internal markers for in situ detection of PAHs-degrading mycobacteria. Bastos et al. [88] chromosomally labeled an Alcaligenes faecalis strain isolated from an Amazonian soil sample and after PCR and Southern blot analyses confirmed that the gfp gene was integrated into the chromosome and the addition of the gfp marker did not affect phenol degradation ability compared with the wild-type. Environmental mycobacteria are considered promising for the biological clean-up of PAHs-contaminated environment for their peculiar physiological and structural properties. However, the molecular study of PAHs-degrading mycobacteria is hampered by the lack of adequate tools and methods. Wattiau et al. [89] employed suitable vectors carrying the pAL5000-based plasmids containing the green fluorescent protein (gfp) gene to investigate PAHs-degrading mycobacteria. Although they demonstrate the suitability of the pAL5000 replicon for the development of recombinant DNAbased studies in PAHs-degrading Mycobacterium spp., difficulties were encountered during their study in terms of electroporation efficiency and stability of plasmid constructs. Based on the above mentioned researches, Dandie et al. modified the transformation systems, especially transposon-based mycobacterial vectors to enhance electroporation efficiency. In their studies, the vectors pEM32 and pEM42 were employed to be electroporated into Mycobacterium sp. strain [90]. The electroporation condition was not satisfying similar to the results obtained by Wattiau et al. which demonstrated standard methods were inadequate for electroporation of environmental mycobacterial isolates and a systematic investigation of electroporation conditions and growth/preparation of electrocompetent cells is required to fully optimise transformation conditions for this strain [89].

11. Metagenomic Libraries and Pyrosequencing

Metagenomic libraries are constructed by direct cloning of DNA fragments extracted from an environmental sample in a suitable vector (e.g. plasmid, phage, fosmid, cosmid or bacterial artificial chromosomes BAC), which is then transformed into a suitable host strain. Cloned

DNA fragments are then analyzed using either sequence based or function-based screening procedures. Many studies have reported construction and screening of metagenomic libraries to identify genes involved in bioremediation. Martin et al. [91] constructed metagenomic libraries to decipher ecological and metabolic functions of microbial communities involved in enhanced biological phosphate removal (EPBR) systems. This remarkable metagenomic study addressed the 30 year-old mystery of phosphate removal in EPBR systems by determining the complete genome and the associated phosphate accumulation genes within an uncultured, yet dominant poly-phosphate accumulating micro-organism (POA) known as Candidatus Accumulibacter phosphatis. In a similar study, Suenaga et al. [92] constructed fosmid libraries from metagenomic DNA fragements recovered from sludge samples. The resulting library was screened for extradiol dioxygenases (EDOs) using catechol as a substrate, which yielded 91 EDO-positive clones. Sequencing of the inserts within these positive clones depicted new EDO gene subfamilies involved in degradation of aromatic compounds. Metagenomics has now become a widespread approach to discover novel biocatalysts or gene products involved in biodegradation of anthropogenic compounds. However, the scope of constructing metagenomic libraries from environments having lower microbial abundance is very limited. These limitations have been over-come by application of whole community genome amplification (WCGA) based on principle of multiple displacement amplification (MDA), improving the accessibility and efficacy of metagenomic gene discoveries from lowbiomass environments. In this technique, all the metagenomic DNA is evenly amplified which ensures the representativeness of community microbial genomes [93]. Initial WGA (whole genome amplification) reactions utilized PCR-based techniques such as degenerate oligonucleotide primed PCR and primer extension PCR. However, these were limited by non-specific artifacts of amplification, strong bias and short amplification products. Majority of researchers now utilize the multiple displacement amplification (MDA) for whole genome amplification, this procedure involves an isothermal (30^0C) strand displacement synthesis in which the highly processive phi29 DNA polymerase repeatedly extends random primers on the template as it concomitantly displaces previously synthesized copies [94]. MDA has been applied in many metagenomic studies, for example Gonzalez et al. [93] used MDA as a pre-PCR enrichment step to alleviate the problem of co-extraction of humic acids and exopolysaccharides associated with low concentration of biomass in samples from cave, meadow soil and wastewater treatment systems. In this study, MDA from a defined mixture of pure-culture DNA accurately reflected the microbial community composition using denaturing gradient gel electrophoresis fingerprints. A yet another innovative technical breakthrough in the field of metagenomics is the massively parallel pyrosequencing also known as metagenomic pyrosequencing. Many pyrosequencing based chemistries and instruments are now commercially available, such as the Genome Sequencers from Roche/454 Life Sciences [GS-20 or GS-FLX;], the 1G Analyzer from Illumina/Solexa and the SOLiD System from Applied Biosystems (solid.appliedbiosystems.com). 454 Life Sciences has scaled up this technology enabling massively parallel sequencing of more than 300,000 sequences at once. In addition to the massive parallelization, the 454 technology does not require cloning from metagenomic DNA, thus eliminating many of the problems that are associated with this step of metagenomics. The only limitation of pyrosequencing is the short read lengths of approximately 250–400 bp that provide poor phylogenetic information as compared to full length 16S rRNA gene sequences (~1500 bp). However, these limitations can be over-come by using error-correcting barcoded primers [94] or by accurate taxonomic assignments of 16S rRNA sequence reads obtained from massively parallel pyrosequencers [96]. Hamady et al. [95] constructed error-correcting DNA barcodes that allow massively parallel pyrosequencing in a single run; this approach was successful in characterization of 16S rRNA gene sequences representing microbial communities in 286 environmental samples.

12. Transcriptomics and Metatranscriptomics in Bioremediation

Transcriptomic or metatranscriptomics tools are used to gain functional in-sights into the activities of environmental microbial communities by studying their mRNA transcriptional profiles [97]. Transcriptomic analyses entail the following basic steps; (1) extraction and enrichment of the total mRNA, (2) cDNA synthesis, (3) followed by either microarray hybridization of cDNA or sequencing of the complete cDNA transcriptome. Jennings et al. [98] performed transcriptomics analysis on a cisdichloroethene (cDCE)-assimilating Polaromonas sp. JS666 strain in order to identify the genes upregulated by cDCE using DNA microarrays. In this study, whole-genome expression arrays for Polaromonas sp. strain JS666 were synthesized and hybridized with the cDNA isolated from the strain JS666 during its growth on cDCE. The genes upregulated by cDCE were determined to be antioxidant proteins, ABC transporters and sodium/solute symporters. Moreover, it was hypothesized that a major degradation pathway involving carbon-chloride cleavage and a minor degradation pathway involving monooxygenase-catalyzed epoxidation were responsible for biodegradation of cDCE by Polaromonas sp. JS666 strain. In a similar study, Holmes et al. [99] deciphered the transcriptome of Geobacter uraniireducens strain growing in uranium-contaminated subsurface sediments. In this study, a whole-genome microarray analysis comparing sediment-grown G. uraniireducens with cells grown in defined culture medium indicated that there were 1084 genes that had higher transcript levels during growth in uranium contaminated sediments. It was observed that thirty four c-type cytochrome genes involved in Fe(III) and U(VI) reduction were upregulated in the sediment grown cells of G. uraniireducens. The above mentioned studies demonstrate that it is feasible to monitor gene expression of micro-organisms growing in presence of anthropogenic contaminants. Recent advances in direct extraction of mRNA from archaeal, bacterial and eukaryotic microbial cells have enabled researchers to obtain the gene expression profile of the entire microbial community also known as ‘‘metatranscriptome”. Extraction of this metatranscriptomes coupled with pyrosequencing or construction of cDNA microarrays provides a useful tool to monitor transcriptional activities of entire microbial communities (Urich et al., 2008). Urich et al. [100] employed a metatranscriptomic approach to simultaneously obtain information on both structure and function of soil microbial communities. In this study, the total community RNA was extracted and randomly reverse transcribed into cDNA, this resultant cDNA was subjected to pyrosequencing in order to produce 193, 219 rRNA-tags with valid taxonomic information, together with 21, 133 mRNA-tags exhibiting functional capabilities of the soil microbial communities. Transcriptomics approaches discussed above have a wide-applicability in linking structure and function of environmental microbial communities by performing a single experiment.

13. Pahbase, a Freely Available Functional Database of Polycyclic Aromatic Hydrocarbons (Pahs) Degrading Bacteria

Microbial population is a highly diverse and a ubiquitous group among the living world. One of the novel features of the microbes relates to their versatility in utilizing a large numbers of natural and manmade compounds. This property proves highly valuable in bioremediation for the complete destruction and removal of pollutants [101]. Contamination of soils and sediments by Polycyclic Aromatic Hydrocarbons (PAHs) is widespread, which raises enormous environmental concerns. It has been observed that PAH degradation in soil is dominated by bacterial strains belonging to a very limited number of taxonomic groups such as *Sphingomonads, Burkholderia, Pseudomonas, Bacillus, Micrococcus and Mycobacterium* [102-109] Members of these genera are specialized in the degradation of aromatic chemicals [110,111]. As such, bioremediation may provide relatively low-cost and less intensified technology with high public acceptance. Bioinformatics based analysis and prediction is playing a pivotal role in understanding and capturing the in-depth knowledge of biological molecules particularly with reference to proteomics and genomics. Although with this advancement, there have been only limited efforts on the collection of all relevant information for a specific field of interest. With this realization, present study focuses on the wide spread data and information related to the occurrence and potential of PAH degrading bacteria. The information and detailed account on these bacteria are quite limited and scattered in scientific journals. Therefore, details from the research papers were extracted, analyzed and presented in form of a precise informative database: PAHbase reflecting the diversity and functional analysis of PAHs degrading bacteria.

PAHbase is a freely available functional database of Polycyclic Aromatic Hydrocarbons (PAHs) degrading bacteria. The database consists of relevant information obtained from scientific literature and databases. The database provides a comprehensible representation of PAH degrading bacteria with reference to its occurrence, phylogeny, and stress adaptation, potential to withstand extreme conditions, biodegradative ability, metabolic pathways and genetic basis of the degradation. The narrow search and limit options of the constructed database provide comparable information from the relevant PAH degrading candidates. The user friendly approach of PHP front end facilitates to add sequences of reported entries leading to scientific information for the specific purpose. The functional PAH database available freely on internet under URL: www.pahbase.in.

14. Conclusions

As mentioned above, PAHs as one kind of the most serious marine pollutants now has become a worldwide environmental problem and posed harmful threaten to humanbeings. Studies about PAHs biodegradation by microbes is a most focused branch in marine environment research field and research emphasis has been changed from finding PAHs-degrading microorganisms into metabolic pathways of microbes, genetic regulation and construction of high efficiency engineering microorganisms. Techniques introduced in this article has become powerful tool in biology and should be applied more intensively in studying of PAHs biodegradation and therefore provide prospective achievements in future. At the same time, as the rapid development of science, new molecular techniques such as meta-genomics sequencing (meta sequencing), DNA stable-isotope probing (DNA-SIP), function- driven screening method were also introduced into microbial ecological research and manifested powerful prospect. However, the microflora in PAHs-contaminated environment is pretty complex and therefore it is difficult to discover the nature of the biodegradation process by using solely one kind of methods. In this sense, only the integrated utilization of molecular techniques and other approaches could the nature of PAHs biodegradation be discovered.

15. Future Prospects

Developing efficient and consistent bioremediation strategies requires in depth understanding of the parameters governing the community structures and metabolic functioning of innate microbial communities. In this review, we described the recent advances in molecular and -omics technologies that have become an integral tool to manage and monitor bioremediation processes. Emergence of specialized techniques for monitoring the microbial genome, transcriptome, proteome, metabolome and fluxome in this ‘‘integrative- omics” era have paved way towards development and successful execution of efficient bioremediation strategies. However, the interpretation and management of the massive data generated by these ‘‘-omics” approaches still requires development of efficient statistical algorithms and bioinformatics tools. The progress made by recent molecular and ‘‘omics” explorations in microbial biodegradation and/or biotransformation have an enormous impact on our efforts to sustain industrialized societies with a cleaner environment.

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Growth Potential Assessment of Actinomycetes Isolated from Petroleum Contaminated Soil

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Abstract

Uncontrolled release of hydrocarbon compounds that are carcinogenic, mutagenic and are potent immunotoxicants into soil and ground water poses a serious threat to human and animal health. Due to their extensive use, they cause serious environmental pollution which has drawn attention towards the research. In the present study total 134 indigenous actinomycetes isolates were obtained from different Petrol contaminated sites (N=40). Fifty one actinomycetes strains were able to grow on 5% crude oil containingineral salt Medium showingaximum growth at temperature 30°C and pH 7.5. They were identified on basis of cultural, morphological and biochemical characteristics as *Streptomyces* sp.1, *Streptomyces* sp.3, *Streptomyces* sp.2, *Rhodococcus* sp., *Nocardia* sp.2 and *Nocardia* sp.1. Isolates were tested for their growth potential on Mineral Salt Broth/Agar supplemented with hydrocarbons *viz.* Crude oil, Anthracene, Coronene, Napthacene, Acenapthene at concentrations 5%, 10% and 15% incubated for 5 days, 10 days and 15 days. All the isolates utilized the hydrocarbons as sole carbon and energy sources in an unequal rate thus suggesting genetic disimilarities in respect of oil degradation capabilities. The study clearly demonstrates that Gram-positive actinomycetes showed good growth potential on hydrocarbon as substrate and support its effective use in hydrocarbon degradation.

Keywords: Actinomycetes; Crude oil; Anthracene; Coronene; Napthacene; Acenapthene

Introduction

The extensive use of petroleum products lead to severe contamination of the environment, becoming a great threat to the natural habitat [1]. One of the most urgent problems of the modern world is environment purification from different toxic residues. Oil hydrocarbons are the principal pollutants of the environment. Today great attention is paid for the working out of ecologically safe biological technology for rehabilitation and biodegradation of soil contaminated with crude oil [2].

As a result of the increase in automobiles, the number of gasoline/diesel station and automobile service station is ever increasing. In gasoline, diesel station and service stations, oil is spilled during transfer and servicing operations. During accidental spills, action has to be taken to remove or remediate or recover the contaminant immediately, whereas in the gasoline and diesel stations the spills due to leakage may be small but continuous and prolonged. Because of its persistence, the chance for groundwater contamination is greater [3].

Hydrocarbon degradation is a highly oxidative process in which molecular oxygen is necessary. Therefore, for maximal degradation both aeration and agitation are required. Agitation of medium maintains homogenous chemical and physical conditions, disperses the dissolved oxygen into smaller bubbles thereby increasing the interfacial area. As the agitation speed increases, oxygen transfer rate increases resulting in higher degradation [4]. The actinomycetes are important oil degraders and the strains of *Streptomyces rochei, Streptomyces plicatus, Streptomyces diastaticus, Nocardia, Frankia* and *Rhodococcus fascians* have commonly been isolated from oil wells and soils. Actinomycetes possess many properties that make them good candidate for application in bioremediation of soil contaminated with organic pollutants. They play an important role in the recycling of organic carbon and are able to degrade complex polymers. Some reports indicated that *Streptomyces* flora could play a very important role in degradation of hydrocarbons. Many strains have the ability to solubilise lignin and degrade lignin-related compounds by producing cellulose and hemicellulose degrading enzymes and extracellular peroxidases. In some contaminated sites, actinomycetes represent the dominant group among the degraders [5]. Actinomycetes are gram positive filamentous bacteria and, are good choice as biosurfactant producer because of their abundance in soil and their major roles in recycling of material in nature. Moreover, they have been found to produce many kind of metabolites including antibiotics, pigments, enzyme, biosurfactants [6].

Hydrocarbon biodegradation in soil can be limited by many factors, for example microorganism type, nutrients, pH, temperature, moisture, oxygen, soil properties and contaminant presence [7].

They produce extracellular enzymes that degrade a wide range of complex organic compounds and spores that are resistant to desiccation. In addition, the frequently occurring filamentous growth favours the colonization of soil particles [8]. In the case of the actinomycetes, the surfactant activity is due to the production of extracellular biosurfactants, specially glycolipids, like trehalose lipids produced by *Rhodococcus* species [9-11] or the lipopeptide produced by *Arthrobacter* sp. strain MIS38 [12], cellular biosurfactants such as mycolic acids that give adherence of the microbial cells to hydrophobic phases in two-phase systems [13]. Many actinomycetes can degrade different pollutants, including several pesticides. For example, members of the genus *Arthrobacter* degrade 4-Chlorophenol [14], Atrazine [15] and Monocrotophos [16] and *Streptomyces* sp. degrades Alachlor

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[17]. The nocardioform actinomycetes (*e.g. Rhodococcus*, *Gordonia*, and *Mycobacterium*) are known hydrocarbon degraders [18-20] and degrade PAH in soil. In some contaminated sites they also represent the dominant group among the degraders [18,21].

The purpose of this study was to examine the hydrocarbon degradation capabilities of actinomycetes in contaminated soil and also to determine the capabilities of the recovered actinomycetes to grow on petroleum and their derivatives.

Materials and Methods

Screening of samples

The soil samples (100 g each) were collected from fuel oil pumps at different location of North India like, Mathura Oil Refinery, Lucknow Railway carriage wagon sites and different petrol pumps of Lucknow for the isolation of oil degrading microorganisms. The samples were collected in pre-sterilised glass bottles and transported to the laboratory for analysis. Enumeration and isolation of heterotrophic bacteria and actinomycetes was carried out through serial dilution agar plating technique using Casein Glycerin (Starch) agar, and Mineral salt medium (HIMEDIA) respectively [5].

Isolation of actinomycetes from hydrocarbon contaminated soil

Isolation of actinomycetes was carried out through tenfold serial dilution agar plate technique using Casein Glycerin (starch) Agar medium (CGA) at pH 8.0. 1g of air dried soil sample was mixed with 9 ml sterilized distilled water. The mixture was shaken vigorously for 15 minutes and allowed to settle for 5 min. 1 ml aliquots of soil suspension (diluted to 10^{-1}) was transferred to 9 ml sterilized distilled water and subsequently diluted upto $10^{-4\cdot}$ Aliquots from 10^{-4} dilution was pour plated using CGA medium in duplicates. Uninoculated plate served as control. Plates were incubated at 28 ± 2°C for 7 days. Morphologically different colonies were selected and streaked over Mineral Salt Agar medium supplemented with 5% for screening and incubated at 28 ± 2°C for 7 days. Growth was examined regularly. Isolates were maintained on CSA slant subcultured regularly at 15 days interval and incubated at 28 ± 2°C for 5 to7 days and then stored at 4°C [5].

S. No.	Sample sites	Sample size N=40	Average cfu/g (10^5)
1	Mathura Oil refinery	10	**4.3**
2	HP Petrol Pump, Lucknow	10	2.7
3	Railway carriage wagon, Lucknow	10	1.7
4	Bharat Petrol Pump, Lucknow	10	1.1

Table 1: Enumeration of actinomycetes isolated from hydrocarbon contaminated soil.

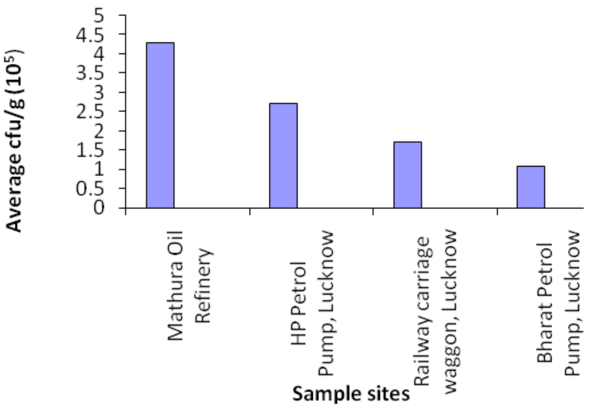


Figure 1: Distribution of population of actinomycetes in hydrocarbon contaminated soil samples.

Identification of actinomycetes isolated from hydrocarbon contaminated soil

The identification was done by on the basis of morphological and biochemical characteristics as per Bergeys Manual of Systemic Bacteriology [2].

Effect of temperature and pH on growth

The effect of temperature and pH on the growth of isolates was studied using Mineral salt medium supplemented with (5%) crude oil inoculated with the isolates and exposed to different temperatures (10°C, 20°C, 30°C, 40°C and 50°C) and pH (5.5, 6.5, 7.5, 8.5 and 9.5). Uninoculated tubes served as control. At 5-days intervals, total viable count (log cfu/ml) was observed [22,23].

Growth potential of actinomycetes isolates on model hydrocarbons

Biodegradation capability of the actinomycetes was determined by the method given by Mills et al. [24]. Selected petroleum derivatives as representative hydrocarbon were assessed to check the growth of isolated actinomycetes on individual hydrocarbons supplemented medium. For these overnight cultures (1%) of actinomycetes were transferred to tubes, containing 5 ml Mineral Salt medium with 5%, 10%, and 15% model hydrocarbons (Crude oil, Anthracene, Coronene, Napthacene, Acenapthene). All the tubes were diluted upto 10^{-5} dilution and plated on mineral salt agar medium. An uninoculated plate was used as control. The plates were incubated at 28˚C for 15 days. Growth was measured in terms of cfu/ml.

Results and Discussion

Enumeration of actinomycetes [Average cfu/g (10^5)] from hydrocarbon contaminated soil

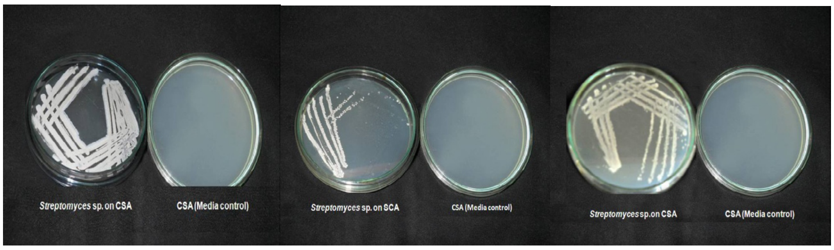
Highest population of actinomycetes was found in the sample collected from Mathura oil refinery sites, Mathura (4.3 × 10^5 cfu/g) followed by HP Petrol pump sites, Lucknow (2.7 × 10^5 cfu/g), Railway carriage wagon sites, Lucknow, (1.7 × 10^5 cfu/g) and least populated site was found in samples collected from Bharat Petrol pump site, Lucknow (1.1 × 10^5 cfu/g) (Table 1 and Figure 1).

Isolation and identification of actinomycetes from hydrocarbon contaminated soil

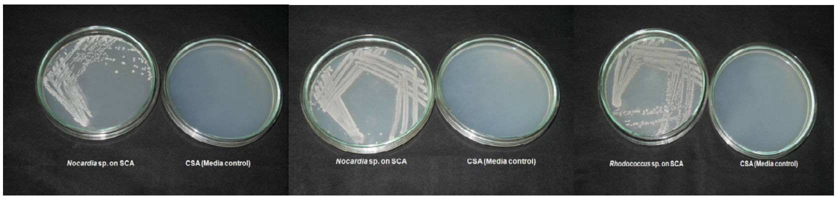
40 samples from hydrocarbon contaminated soil of 4 different sites were used for isolation of different Hydrocarbon degrading actinomycetes. In the present study six species of actinomycetes were identified as *S. sp1, S. sp2, S. sp3, N.sp1, N. sp2 and R. sp* 1 (Figure 2).

Distribution of actinomycetes isolated from different sites potent for growth at 5% concentration of hydrocarbon contaminated soil (Petrol)

Among the 40 samples screened for growth of actinomycetes in Mineral Salt agar supplemented with 5% crude oil, 51 isolates showed positive result. Six different isolates were identified that showed positive result namely *Streptomyces* sp.1, *Streptomyces* sp.3, *Streptomyces* sp.2, *Rhodococcus* sp., *Nocardia* sp.1 and *Nocardia* sp.2. Out of ten samples of Railway fuel oil installation sites, Allahabad, 20 actinomycetes were



Streptomyces sp 1 **Streptomyces sp 2** **Streptomyces sp 3**



Nocardia sp 1 **Nocardia sp 2** **Rhodococcus sp 1**

Figure 2: Bacterial species isolated from oil contaminated sites.

Sample site	Sample size n=40	No.of actinomycetes isolated	No. of isolate positive for growth at 5% supplement	Incidence of isolates capable for hydrocarbon degradation					
				Streptomyces sp.1	*Streptomyces* sp.3	*Streptomyces* sp.2	*Nocardia* sp.2	*Nocardia* sp.1	*Rhodococcus* sp.
Mathura Oil refinery	10	**59**	**20**	**7(35%)**	6(30%)	1(5%)	4(20%)	2(10%)	0(0%)
HP Petrol Pump, Lucknow	10	24	12	3(25%)	2(16.6%)	1(8.3%)	**4(33.3%)**	1(8.3%)	1(8.3%)
Railway carriage wagon, Lucknow	10	40	15	**7(46.6%)**	4(2.66%)	1(6.6%)	2(13.3%)	0(0%)	1(6.6%)
Bharat Petrol Pump, Lucknow	10	11	4	**3(75%)**	1(25%)	0(0%)	0(0%)	0(0%)	0(0%)

Table 2: Distribution of isolates from hydrocarbon contaminated soil.

screened as potent actinomycetes for growth in Mineral Salt agar medium supplemented with 5% crude oil (hydrocarbon). Highest incidence of Streptomyces sp.1 were found from the samples of Bharat Petrol Pump site (46.6%), Mathura oil refinery site (35 %), Mathura and Railway carriage wagon site, Lucknow (75 %) while at HP Petrol Pump, Lucknow *Nocardia* sp.2 (33.3%). had highest incidence. In a similar study conducted by Milic et al. [22]; Jayabarath et al. [25]; Sharma and Pant [4] the results are comparable showing high occurrence of *Streptomyces*, *Nocardia*, and *Rhodococcus* which were measures of incidence and growth of actinomycetes; They represent the dominant group among the degraders (Table 2 and Figure 3).

Optimization of physical growth parameter of isolates

Effect of temperature on growth of actinomycetes at 5% hydrocarbon (Crude oil): Actinomycets isolated from different hydrocarbon contaminated soil showed minimum growth at high temperature and low temperatures. All isolates namely *Streptomyces* sp.1, *Streptomyces* sp.3, *Streptomyces* sp.2, Nocardia sp.2, *Nocardia* sp.1, and *Rhodococcus* sp. showed highest growth at temperature 30°C (6.49 log cfu/ml, 5.52 log cfu/ml, 5.52 log cfu/ml, 6.548 log cfu/ml, 6.56 log cfu/ml and 6.56 log cfu/ml) statistical analysis of data reveled significant differences due to isolates and temperature (Table 3 and Figure 4).

Effect of pH on growth of actinomycetes at 5% hydrocarbon (Crude oil): The optimal pH that supported growth of isolates from hydrocarbon contaminated soil range between 6.5 to 8.5 while maximum growth occurred at pH 7.5 and minimum growth at pH 5.5 and 9.5 in Mineral salt Agar medium supplemented with 5% Petrol after 7 days of incubation. All isolate namely *Streptomyces* sp.1, *Streptomyces* sp.3, *Streptomyces* sp.2, *Nocardia* sp.2, *Nocardia* sp.1 and *Rhodococcus* sp. showed highest growth at pH 7.5 (7.77 log cfu/ml, 7.71 log cfu/ml, 6.63 log cfu/ml, 6.45 log cfu/ml, 6.59 log cfu/ml and 6.68 log cfu/ml) (Table 4 and Figure 5).

Growth potential of actinomycetes

Growth of *Streptomyces sp1:* Growth of *Streptomyces sp1* was analyzed for different hydrocarbons namely Crude oil, Anthracene, Coronene, Napthacene, Acenapthene at 5%, 10% and 15% concentrations incubated for three different time intervals *viz.* 5 days, 10 days and 15 days. *Streptomyces* sp.1 had highest growth for Coronene (4.67 log cfu/ml) followed by Napthacene (4.62 log cfu/ml),

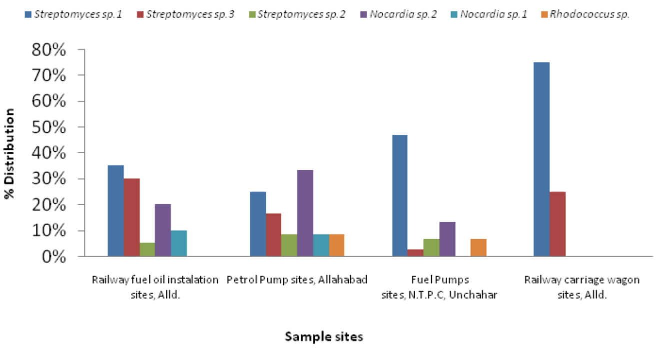


Figure 3: Percent distribution of actinomycetes isolates from different hydrocarbon contaminated soil.

Isolates	Growth of actinomycetes at 5% crude oil concentration cfu/ml				
	Incubation Temperature °C				
	10°C	20°C	**30°C**	40°C	50°C
Streptomyces sp1	2.27	3.36	**6.49**	6.35	4.32
Streptomyces sp3	1.34	3.35	**5.52**	5.36	4.34
Streptomyces sp2	2.37	4.37	**6.48**	5.41	3.37
Nocardia sp2	2.32	4.32	**6.56**	5.29	3.23
Nocardia sp1	1.38	4.44	**6.63**	4.43	4.32
Rhodococcus fascians	2.37	3.46	**6.67**	5.45	3.37

Table 3: Effect of temperature on growth of actinomycetes at 5% hydrocarbon (Crude oil).

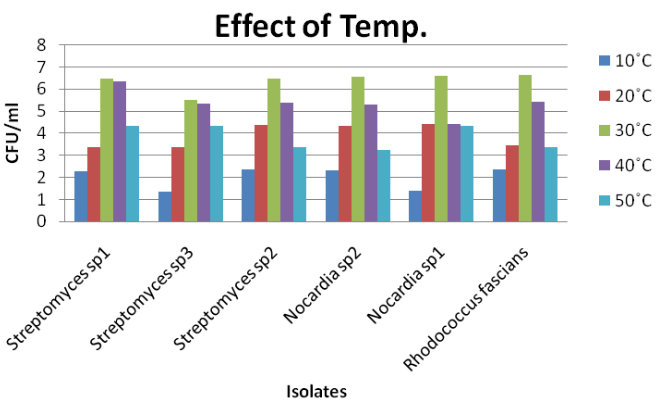


Figure 4: Effect of temperature on growth of actinomycetes at 5% hydrocarbon (Crude oil).

Anthracene (3.69 log cfu/ml), Crude oil (3.60 log cfu/ml) and least was shown by Acenapthene (3.59 log cfu/ml) at 10% and 10 days incubation respectively (Table 5 and Figure 6).

Growth of *Streptomyces sp2:* Growth of *Streptomyces sp2* was analyzed for different hydrocarbons namely Crude oil, Anthracene, Coronene, Napthacene, Acenapthene at 5%, 10% and 15% concentrations incubated for three different time intervals *viz.* 5 days, 10 days and 15 days. *Streptomyces* sp.2 had highest growth for Anthracene (7.69 log cfu/ml) followed by Crude oil (7.60 log cfu/ml), Acenapthene (7.59.59 log cfu/ml), Coronene (7.24 log cfu/ml), and least was shown by Napthacene (6.62 log cfu/ml) at 10% and 10 days incubation (Table 6 and Figure 7).

Growth of *Streptomyces sp3:* Growth of *Streptomyces sp3* was analyzed for different hydrocarbons namely Crude oil, Anthracene, Coronene, Napthacene, Acenapthene at 5%, 10% and 15% concentrations incubated for three different time intervals *viz.* 5 days, 10 days and 15 days. Highest growth of *Streptomyces* sp.3 was recorded at 10% concentration for Crude oil (7.80 log cfu/ml) followed by Anthracene (7.79 log cfu/ml), Napthacene (7.68 log cfu/ml), Acenapthene (7.59 log cfu/ml) and least was shown by Coronene (7.24 log cfu/ml) at 10% and 10 days incubation respectively (Table 7 and Figure 8).

Growth of *Nocardia sp1:* Growth of *Nocardia sp1*was analyzed for different hydrocarbons namely Crude oil, Anthracene, Coronene, Napthacene, Acenapthene at 5%, 10% and 15% concentrations incubated for three different time intervals *viz.* 5 days, 10 days and 15 days. *Nocardia* sp.1 had highest growth for Crude oil (7.68 log cfu/ml) followed by Anthracene (7.39 log cfu/ml), Coronene (7.37 log cfu/ml), Napthacene (7.36 log cfu/ml) and least was shown by Acenapthene (7.15 log cfu/ml) at 10% and 10 days of incubation respectively (Table 8 and Figure 9).

Growth of *Nocardia sp2:* Growth of *Nocardia sp2* was analyzed for different hydrocarbons namely Crude oil, Anthracene, Coronene, Napthacene, Acenapthene 5%, 10% and 15% concentrations incubated for three different time intervals *viz.* 5 days, 10 days and 15 days. Growth of *Nocardia* sp.2 was analyzed for different hydrocarbons had highest growth for Crude oil (7.80 log cfu/ml) followed by Anthracene (7.79 log cfu/ml), Coronene (7.68 log cfu/ml), Napthacene (7.63 log cfu/ml) and least was shown by Acenapthene (7.59 log cfu/ml) at 10% and 10 days incubation (Table 9 and Figure 10).

Growth of *Rhodococcus sp:* Growth of *Rhodococcus sp.* was analyzed for different hydrocarbons namely Crude oil, Anthracene, Coronene,

Isolates	Growth of actinomycetes at 5% crude oil concentration cfu/ml				
	pH				
	5.5	6.5	**7.5**	8.5	9.5
Streptomyces sp1	4.44	6.63	**7.77**	6.60	4.59
Streptomyces sp2	2.45	7.48	**7.71**	6.51	5.15
Streptomyces sp3	3.36	6.39	**7.63**	5.36	6.26
Nocardia sp2	3.40	6.42	**7.45**	7.47	5.30
Nocardia sp1	3.39	7.40	**7.59**	5.43	5.21
Rhodococcus fascians	3.46	6.46	**7.68**	5.47	5.32

Table 4: Effect of pH on growth of actinomycetes at 5% hydrocarbon (Crude oil).

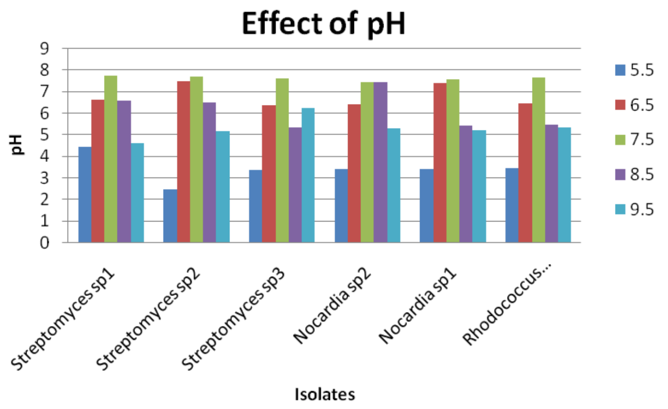


Figure 5: Effect of pH on growth of actinomycetes at 5% hydrocarbon (crude oil).

Growth of *Streptomyces sp1* Log cfu/ml												
Hydrocarbon	**0 days**			**5 days**			**10 days**			**15 days**		
	5%	10%	15%	5%	10%	15%	5%	**10%**	15%	5%	10%	15%
Crude Oil	0.6	1.77	1.84	1.91	2.48	1.44	2.12	**3.60**	2.01	1.98	2.19	1.97
Anthracene	0.84	0.77	1.77	2.07	2.53	1.98	2.16	**3.69**	2.22	2.08	2.27	2
Coronene	1.6	0.77	1.77	2.06	2.56	1.83	2.06	**4.67**	2.24	2.97	2.25	2.08
Napthacene	0.90	0.90	0.95	2.07	2.46	2.30	2.04	**4.62**	2.15	2.01	2.25	2.04
Acenapthene	0.6	1.6	1.6	1.92	2.48	1.89	2.99	**3.59**	2.25	2.12	2.26	1.98

Table 5: Growth of *Streptomyces sp1.*

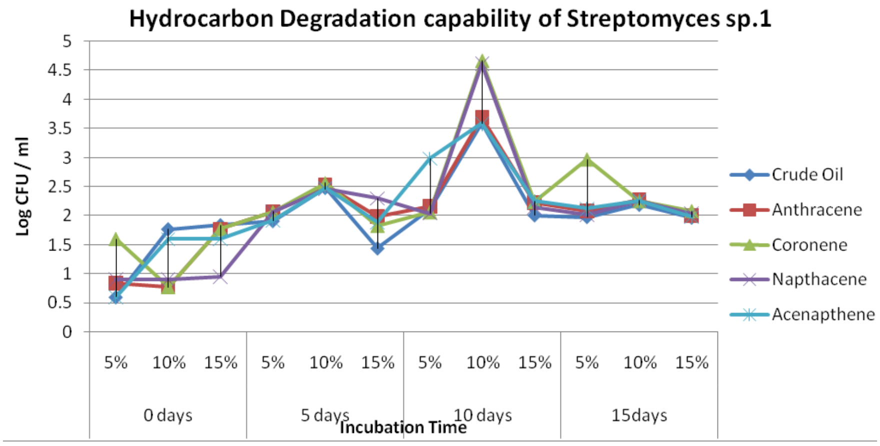


Figure 6: Growth of *Streptomyces sp1.*

Growth of *Streptomyces sp2* Log cfu/ml												
Hydrocarbon	**0 days**			**5 days**			**10 days**			**15 days**		
	5%	10%	15%	5%	10%	15%	5%	**10%**	15%	5%	10%	15%
Crude Oil	1.8	1.77	1.84	2.91	3.48	2.44	4.12	**7.60**	6.01	3.98	5.19	2.97
Anthracene	0.94	1.77	2.77	3.07	3.53	2.98	4.16	**7.69**	6.22	4.08	5.27	3
Coronene	0.9	1.77	2.77	3.06	4.56	2.83	3.06	**7.24**	6.67	4.97	4.25	3.08
Napthacene	0.90	0.90	0.95	3.07	4.46	2.30	4.04	**6.62**	6.15	5.01	4.25	2.04
Acenapthene	1.6	1.6	2.6	2.92	3.48	2.89	3.99	**7.59**	6.25	5.12	3.26	3.98

Table 6: Growth of of *Streptomyces sp2.*

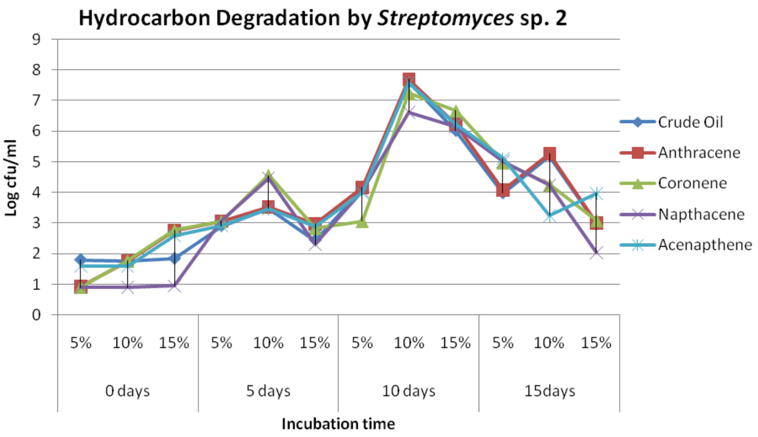


Figure 7: Growth of *Streptomyces sp2.*

Napthacene, Acenapthene at 5%, 10% and 15% concentrations incubated for three different time intervals *viz.* 5 days, 10 days and 15 days. *Rhodococcus* sp. had highest growth for Crude oil (7.50 log cfu/ml) followed by Anthracene (7.39 log cfu/ml), Coronene (7.38 log cfu/

Growth of *Streptomyces sp3* Log cfu/ml												
Hydrocarbon	**0 days**			**5 days**			**10 days**			**15 days**		
	5%	10%	15%	5%	10%	15%	5%	**10%**	15%	5%	10%	15%
Crude Oil	1.5	1.87	1.84	3.91	4.48	2.44	4.12	**7.80**	6.01	3.98	5.19	2.97
Anthracene	0.94	1.97	2.77	3.07	4.53	2.98	4.16	**7.79**	6.22	4.08	5.27	3.54
Coronene	0.96	1.67	2.77	3.06	4.56	2.83	3.06	**7.24**	6.68	4.97	4.25	3.08
Napthacene	0.93	0.95	1.95	3.07	4.46	2.30	4.04	**7.68**	6.15	5.01	4.25	2.04
Acenapthene	1.7	1.7	2.6	3.92	3.98	2.89	3.99	**7.59**	6.25	5.12	3.26	3.98

Table 7: Growth of of *Streptomyces sp3.*

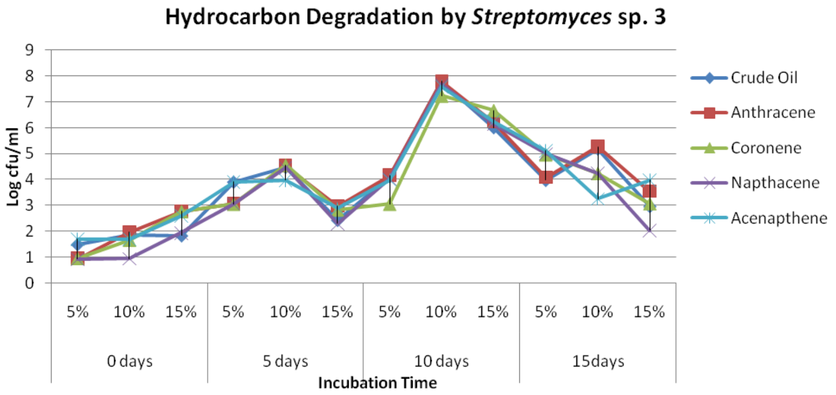


Figure 8: Growth of *Streptomyces sp3.*

Growth of *Nocardia sp1* Log cfu/ml												
Hydrocarbon	**0 days**			**5 days**			**10 days**			**15 days**		
	5%	10%	15%	5%	10%	15%	5%	**10%**	15%	5%	10%	15%
Crude Oil	0.9	1.67	1.84	3.91	4.58	2.44	4.22	**7.68**	6.01	3.98	5.19	2.97
Anthracene	1.04	1.8	2.77	3.37	4.83	2.98	4.36	**7.39**	6.22	4.08	5.27	3.54
Coronene	1.16	1.77	2.67	3.46	4.56	2.83	5.46	**7.37**	7.24	4.97	5.25	3.08
Napthacene	0.99	0.95	1.85	3.37	4.96	2.30	4.54	**7.36**	6.15	5.01	4.25	2.04
Acenapthene	1.07	1.67	2.16	3.92	3.98	2.89	3.99	**7.15**	6.25	5.12	5.26	3.98

Table 8: Growth of *Nocardia sp1.*

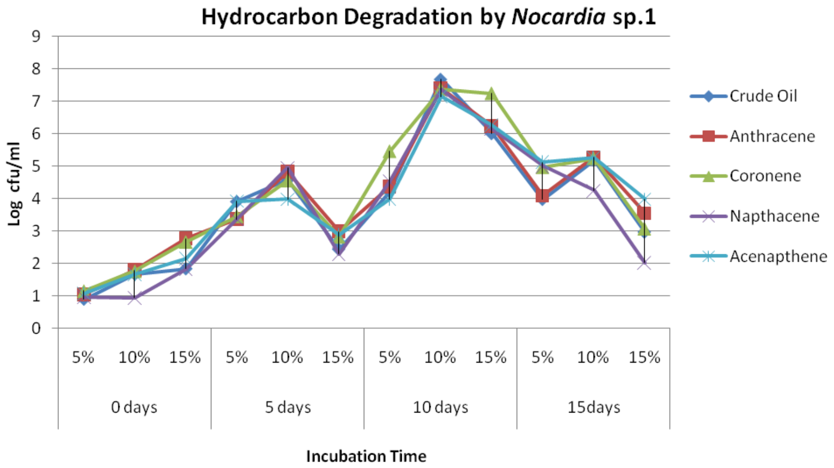


Figure 9: Growth of *Nocardia sp1.*

ml), Napthacene (7.29 log cfu/ml) and least was shown by Acenapthene (7.17 log cfu/ml) at 10% and 10 days incubation respectively (Table 10 and Figure 11).

Summary and Conclusion

For the investigation ten soil samples from four different hydrocarbon contaminated sites. The soil from four sites were used

Hydrocarbon utilizing capacity of *Nocardia sp2* Log cfu/ml

Hydrocarbon	**0 days**			**5 days**			**10 days**			**15 days**		
	5%	10%	15%	5%	10%	15%	5%	**10%**	15%	5%	10%	15%
Crude Oil	0.98	1.98	1.84	3.91	4.58	3.44	6.22	**7.80**	6.01	3.98	5.49	3.97
Anthracene	0.94	1.84	1.97	3.67	5.83	2.98	6.36	**7.79**	6.22	4.08	5.47	3.54
Coronene	0.96	1.77	2.64	3.36	5.56	3.83	6.46	**7.68**	6.24	4.97	5.25	3.08
Napthacene	0.99	1.35	1.95	3.11	5.96	3.30	6.54	**7.63**	6.15	5.01	4.25	3.04
Acenapthene	1.17	1.57	2.16	3.33	4.98	2.89	6.99	**7.59**	6.25	5.12	5.46	3.98

Table 9: Growth of *Nocardia sp2.*

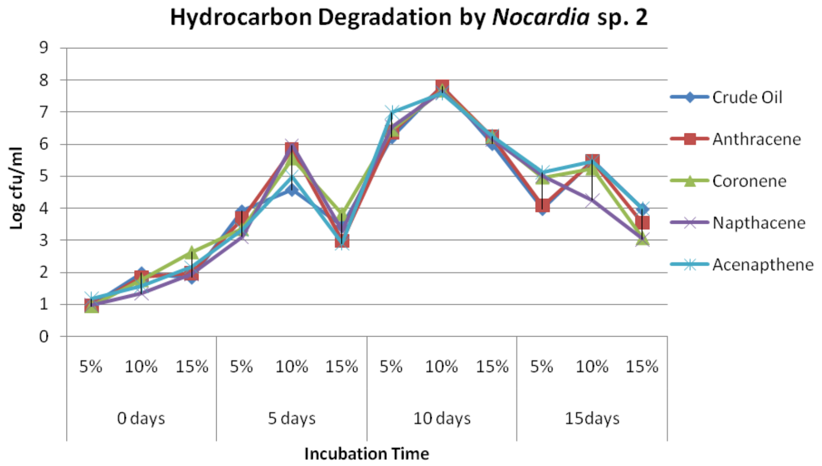


Figure 10: Growth of *Nocardia sp2.*

Growth of *Rhodococcus sp.* Log cfu/ml

Hydrocarbon	**0 days**			**5 days**			**10 days**			**15 days**		
	5%	10%	15%	5%	10%	15%	5%	**10%**	15%	5%	10%	15%
Crude Oil	0.78	1.98	1.94	3.91	5.58	4.44	6.22	**7.50**	6.41	3.98	5.49	3.87
Anthracene	1.04	1.74	1.96	4.67	5.83	3.98	6.36	**7.39**	6.42	4.48	5.47	3.54
Coronene	1.06	1.77	2.04	4.36	5.56	3.83	5.46	**7.38**	6.24	4.97	5.25	3.78
Napthacene	1.09	1.65	1.99	4.11	5.96	4.30	6.45	**7.29**	6.54	5.01	4.25	3.74
Acenapthene	1.07	1.47	2.16	4.33	5.98	3.89	6.33	**7.17**	6.47	5.12	5.46	3.78

Table 10: Growth of *Rhodococcus sp.*

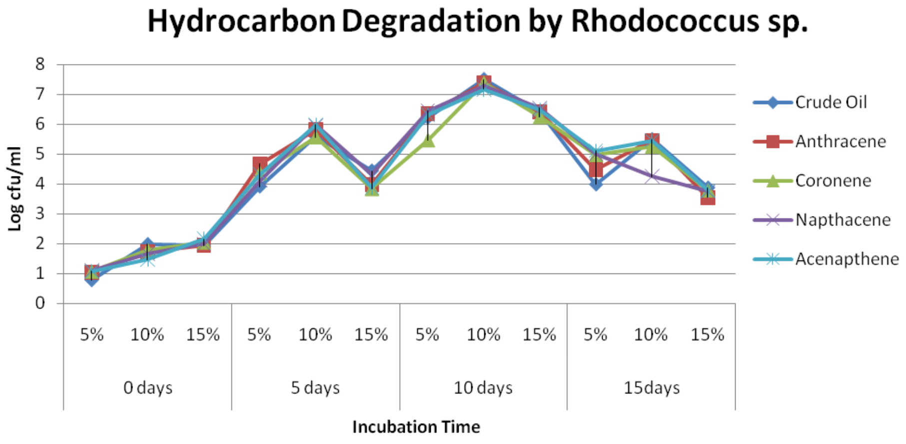


Figure 11: Growth of *Rhodococcus sp.*

for isolation and enumeration of actinomycetes after which the morphologically different colonies were purified and screened for hydrocarbon utilization as sole source of carbon and energy at 5% concentration of Crude oil (hydrocarbon). The isolate found positive for growth on screened plates were identified on the basis of cultural, morphological and biochemical analysis and were further assessed for their growth potential for selected hydrocarbons at varying concentrations (5%, 10%, and 15%) incubated for three different time intervals *i.e.* 5 days, 10 days, and 15 days.

The present investigation revealed that indigenous actinomycetes isolated from hydrocarbon contaminated sites could be used for *in situ* bioremediation purpose. Among the 134 isolates screened, 51 efficient oil (crude oil) degraders, out of which *Streptomyces* sp.1 39% *followed by Streptomyces* sp.3 25%, *Streptomyces* sp.2 5.5%, *Nocardia* sp.2 5.8%, *Nocardia* sp.119.6%, and *Rhodococcus* sp. 3.9% found from four different sites namely Mathura Oil Refinery, Mathura, HP Petrol Pump, Lucknow, Bharat Petrol Pump, Lucknow and Railway carriage wagon, Lucknow were able to grow efficiently on model hydrocarbons *i.e.* Crude oil, Anthracene, Coronene, Napthacene, Acenapthene. Upon analyzing the growth potential of isolates at different concentration (5%, 10%, and 15%) of hydrocarbons and different time of incubation (5, 10 and 15 days). It was found that the actinomycetes isolates had efficient growth at 10% hydrocarbon concentration after prolonged incubation for 15 days. The study highlighted the potential of actinomycetes isolated from hydrocarbon contaminated soil for bioremediation of hydrocarbon polluted area, spills as it offers effective degradation of various fractions of hydrocarbons at wide range of concentration and time duration. Therefore, bioremediation of toxicant hydrocarbons in soil or spill have a better option of environmentally adopted microflora that effect detoxification and stabilization of processes of biological degradation with low economical expenses and of no danger for environment.

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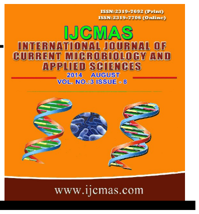

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Original Research Article

Hydrocarbon Bioremediation Efficiency by five Indigenous Bacterial Strains isolated from Contaminated Soils

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ABSTRACT

Keywords

Bioremediation, Petrol, Benzene, Toluene, Xylene and Cyclohexane

Twenty hydrocarbon degrading microorganism were isolated from four hydrocarbon contaminated sites and were identified on the basis of morphological and biochemical characteristics as *Bacillus cereus, Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*. The study revealed high density of bacteria acclimatized for biodegradation of hydrocarbon (Petrol) in soil. The isolates were examined for other hydrocarbon degradation in media supplemented with Benzene, Toluene, Xylene and Cyclohexane at three different concentrations *viz* 5%, 10% and 15% incubated for 3 different time intervals 5, 10 and 15 days. The results indicated that all the isolates possessed potential to degrade the wide variety of hydrocarbons. The most efficient among them was *Pseudomonas aeruginosa* which degraded all tested hydrocarbon showing maximum growth at 5% concentration and 10 days incubation. It could be concluded that native flora of hydrocarbon contaminated site adapt to the environmental condition and could be implicated to remove hydrocarbons.

Introduction

In the last years, a large number of ecosystems have been changed by the growing influence of human activity. As a result, many people have become aware of the need to protect ecosystems as well as to evaluate the damage caused by contamination. During the previous years, the frequency and risk of oil pollution has lead to extensive research. Most of the petroleum goes in the ecosystem via leak of coastal oil refineries. Approximately five million tons of crude oil and refined oil enter the environment each year as a result of anthropogenic sources such as oil spills (Hinchee and Kitte, 1995).

Hydrocarbon pollutants are amongst the most reported pollution worldwide (Shukor *et al*, 2009). Polyaromatic hydrocarbons in the environment originate from anthropogenic source like mineral oil. Numerous bacteria, fungi and algae have been isolated for the breakdown of aromatic hydrocarbons as carbon and energy sources (Cerniglia, 1992; Lal et al., 2004; Pathak et al. 2008). The degradation pathways have been -elucidated

PAHs present as natural constituents in fossils fuels, are formed during the incomplete combustion of organic materials (Lee et al., 1981; Wang et al., 1999; Desche Anes et al., 1996). PAHs can exert toxic effects or possess mutagenic, teratogenic, or carcinogenic properties (Heitkamp and Cerniglia, 1987).

Some microorganisms can utilize the hydrocarbons as sole carbon sources for getting their energy and metabolic activities (Jyothi et al.,2012). Biodegradation is a complex process that depends on the nature of petroleum and also on the amount of the hydrocarbons present (Das et al.,2011). The microbes can utilize the hydrocarbons depending on the chemical nature of the compounds within the petroleum mixture (Adeline et al.,2009). Hydrocarbon degrading microorganisms usually exist in very low abundance in aquatic environments (Sivaraman et al., 2011). Biodegradation of bacteria is considered as the most active process in petroleum degradation and they are the primary degraders of spilled oil (Rahman et al.,2003; Brooijmans et al.,2009) and this is specially carried out largely by diverse bacterial populations, mostly by Pseudomonas species (Boboye et al.,2010; Dubey et al.,2009).

There are so many known consortia of microorganisms which can degrade mineral oil hydrocarbons under laboratory or field conditions (Ratajczak et al.,1998; Wikstrom et al.,1996). This work is concentrated on isolation and identification of hydrocarbon degrading bacteria associated with petrol and diesel oil contaminated sites in India and also to test their ability to degrade different hydrocarbons. The expected interpretation of this study will provide information on the bacterial population, hydrocarbon-degrading microorganisms and their degrading ability of diesel because these bacteria can utilize the hydrocarbons as carbon source. Biodegradation by indigenous populations of microorganisms is one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment (Ulrici et al.,2000) and this process is also cheaper than the other remediation technologies (Leahy et al.,1990).

Materials and Methods

Samples were collected randomly from Fuel oil pumps of Mathura oil refinery, Barauni oil refinery, Haldia oil refinery, Paradip oil refinery at a depth within 1-5cm from the surface of the soil using sterile spatula and were placed in pre sterilized polythene bags and tightly packed. Samples were immediately transferred to the laboratory for analysis and stored at 4°C for further processing.

Isolation of Hydrocarbon degrading Bacteria

One gram of dried soil sample was dissolved in 9ml of distilled water and agitated vigorously. A 10 fold serial dilution was done followed pour plate method. Soil sample was serially diluted upto 10^{-7} dilution and 1 ml from each dilution poured in Petri plate followed by addition of 20ml of molten Bushnell Haas-Agar medium at around 50°C. After gently rotating, the plates were incubated at 37°C for 24 hours and uninoculated plate was serve as media control and then enumeration of different isolates were carried out (Santhini*et al.*, 2009). Culturally different colonies were selected and streaked over Bushnell Hass-Agar medium supplemented with 5% petrol. Uninoculated media plate was serve as control. Incubation was done at 28˚C for upto7 days and growth were examined. Isolates were maintained on Nutrient agar slants which were subcultured at 15 days interval and were incubated at 37°C for 24-42 hours and then stored at 4°C.

Identification of hydrocarbon utilizing bacteria

The identification was done by cultural (margin, colour, texture and elevation), morphological and biochemical analysis as per Bergey's Manual of Systemic Bacteriology (Holt *et al*., 1994).

Effect of Temperature and pH

The effect of temperature and pH on the growth and degradation will be studied by using Bushnell-Haas broth supplemented with petrol (5%) will inoculate with the isolates and incubate at different temperatures (10°C, 20°C, 30°C, 40°C, 50°C) and different pH (5.5, 6.5, 7.5, 8.5 and 9.5) for this uninoculated tubes will be serve as control. Growth and degradation of the organism will be assayed by optical density (O.D) measurement at 600nm. (Rahman and Rahman, 2002).

Evaluation of the specific degradation capacity of selected solid and liquid hydrocarbons

Biodegradation capability of the organism were determined the method given by Santhini*etal*., (2009). In order to monitor the liquid hydrocarbon degradation, overnight cultures were inoculated on Bushnell-Haas medium at pH 7.0 supplemented with hydrocarbon (5-15%v/v) then the tubes were incubated at 37°C. Uninoculated medium with hydrocarbon were served as a control. Growth of the organism was assayed by optical density (O.D) measurement at 620nm. The inoculated and uninoculated tubes were incubated at 37°C for 5-15 days and examined regularly for growth in Benzene, Toluene, Xylene, Cyclohexane.

Results and Discussion

Isolation of Hydrocarbon degrading Bacteria

The samples of hydrocarbon contaminated soil supplemented with various hydrocarbons showed the growth of *Pseudomonas aeruoginosa, Bacillus subtilis, Bacillus cereus, E.coli and Staphylococcus aureus.* These organisms were found to be actively growing in petrol during the study of the 40 hydrocarbon contaminated soils. A total of twenty positive isolates of bacterial species were identified out of which 9 (45%) were isolated from Mathura oil refinery, 6 (30 %) from Barauni oil refinery, 4 (20 %) from Haldia oil refinery and 1 (5 %) from Paradip oil refinery. Maximum isolates were of *Psuedomonas aeruginosa* 9 (45%) followed by *Bacillus subtilis* 6 (30 %), *Bacillus cereus* 3 (15 %), *E. Coli* 1 (5 %) and *Staphylococcus aureus* 1 (5%).

Microbial distribution in Hydrocarbon contaminated soil samples.

The highest population was observed from the soil sample of Mathura oil refinery. The bacterial counts were recorded as 4.26 x 10^6 in this sample. Plate count of viable bacteria from Barauni oil refinery and Haldia oil refinery was determined to be in order of 3.4 x 10^6 and 3.04 x 10^6. The least count of bacteria was determined in the soil sample from Paradip oil refinery as 2.58 x 10^6**.**

Identification of hydrocarbon utilizing bacteria

After evaluation of colony morphology, cell morphology, utilization of carbon source and biochemical characteristics the isolates were identified as *Pseudomonas aeruoginosa, Bacillus subtilis, Bacillus cereus, E.coli and Staphylococcus aureus.*

Effect of Temperature on hydrocarbon degrading bacteria

Hydrocarbon degrading bacteria grow optimally in a wide range of temperature ranging from 27^0C to 37^0C. Growth decreases dramatically at higher temperature. *Pseudomonas aeruoginosa* showed highest growth at 30°C temperature at media supplemented with 5% petrol while *Bacillus cereus* and *Bacillus subtilis* showed maximum growth at 40°C whereas *E.coli* and *Staphylococcus aureus* showed high growth at 30°C. All these bacteria show less growth at low as well as high temperature.

Effect of pH on hydrocarbon degrading bacteria

Maintenance of pH in bacterial medium is important since pH strongly affect bacterial growth. The optimal pH that supported growth of bacteria was range between 6.5 to 7.5 *Pseudomonas aeruoginosa Bacillus subtilis, Bacillus cereus, Staphylococcus aureus and E. coli* showed highest growth at pH 7.5 at media supplemented with 5% petrol. All these bacteria show very low growth at low as well as high pH.

Hydrocarbon degradation capacity of *Pseudomonas aeruoginosa.*

Among the hydrocarbons used for the degradation studies by *Pseudomonas aeruoginosa* degradation was observed maximum in petrol (0.68) followed by cyclohexane (0.65), toluene (0.64), xylene (0.62) and with a minimum hydrocarbons degradation with benzene (0.53).

The degradation process was observed to gradually increase with the peak value at 10 days and then a gradual decrease in the optical density was observed. While testing the concentrations of hydrocarbons, 5% hydrocarbons concentration was found to be most effective for degradation as compared to 10% and 15% hydrocarbons concentration.

Hydrocarbon degradation capacity of *Bacillus subtilis.*

Among the hydrocarbons used for the evaluation of the degradation ability for *Bacillus subtilis*, benzene (0.67) was maximally degraded, followed by the hydrocarbons toluene (0.65), cyclohexane (0.54), xylene (0.51) and petrol (0.47). The concentration of 5% was found to be more pronounced for the degradation of the hydrocarbons by *Bacillus subtilis* as compared to 10% and 15% concentrations.

There was significant increase in the degradation of hydrocarbons by *Bacillus subtilis* with respect to the time, *i.e.* from 0 days towards 15 days.

Hydrocarbon degradation capacity of *Bacillus cereus.*

The degradation capacity of *bacillus cereus* was observed maximum with petrol (0.52) followed by other hydrocarbon *viz* xylene (0.5), cyclohexane (0.48), toluene (0.47) and benzene (0.46). The degradation was found to gradually increase up to 15 days of incubation.

A decreasing trend in optical density was observed. Even with *bacillus cereus* 5% hydrocarbon concentration was found to be optimum for hydrocarbon degradation. Significant increase in the degradation of hydrocarbon by *bacillus cereus* was found with respect to time, *i.e.*, from 0 hour to 15 days, then a decrease towards 15 days at 10% and 15% concentration was observed.

Hydrocarbon degradation capacity of *E.coli*

The degradation capacity of *E.coli* was observed maximum with cyclohexane (0.49) followed by other hydrocarbon *viz* petrol (0.43), toluene (0.43), xylene (0.42) and benzene (0.41). A decreasing trend in optical density was observed. Even with *E.coli 5*% hydrocarbon concentration was found to be optimum for hydrocarbon degradation at 10 days. Significant increase in the degradation of hydrocarbon by *E.coli was* found with respect to time, *i.e.*, from 0 hour to 15 days, then a decrease towards 15 days at 10% and 15% concentration was observed.

Hydrocarbon degradation capacity of *Staphylococcus aureus.*

Among the hydrocarbons used for the degradation studies by S*taphylococcus aureus* degradation was observed maximum in petrol (0.39) followed by xylene (0.35), benzene (0.34), cyclohexane (0.33) and toluene (0.26). While testing the concentrations of hydrocarbons, 10% hydrocarbons concentration was found to be most effective for degradation as compared to 5% and 15% hydrocarbons concentration. A significant increase in the degradation of Hydrocarbon by *Staphylococcus aureus* was observed with increase in time (*i.e.* from 0 days to 10 days) and a decrease at 15 days at 10% and 15% concentrations.

Summary and Conclusion

For the investigation ten soil samples each from four different hydrocarbon contaminated sites were obtained and used for isolation and enumeration of bacteria after which the culturally different colonies were purified and screened for hydrocarbon utilization as sole source of carbon and energy at 5% concentration of petrol. The isolate found positive for growth on screened plates were identified on the basis of cultural, morphological and biochemical analysis and were further assessed for their growth potential for selected hydrocarbons at varying concentrations (5%, 10%, and 15%) incubated for three different time intervals *i.e*. 5 days, 10 days, and 15 days.
The present investigation revealed that indigenous bacterial population isolated from hydrocarbon contaminated sites could be used for *insitu* bioremediation purpose. A total of 20 positive isolates were obtained, out of which *Psuedomonas aeruginosa* showed maximum occurrence while *E. coli* and *Staphylococcus aureus* showed least occurrence. Analysis of temperature and pH optimization showed that all the bacterial species were most active at 40^0C-50^0C and pH 7.5 respectively. Upon analyzing the growth potential of isolates at different concentration (5%, 10%, and 15%) of hydrocarbons and different time of incubation (5, 10 and 15 days) it was found that the bacterial species showed maximum growth at 15 days incubation and 5 % concentration and also at 10 days incubation and 5 % concentration

The study highlighted the potential of bacterial population isolated from hydrocarbon contaminated soil for bioremediation of hydrocarbon polluted area, spills as it offers effective degradation of various fractions of hydrocarbons at wide range of concentration and time duration. Therefore, bioremediation of toxicant hydrocarbons in soil or spill have a better option of environmentally adopted microflora that effect detoxification and stabilization of processes of biological degradation with low economical expenses and of no danger for environment.

Table.1 Incidence of hydrocarbon degrading bacteria

Sample Sites n=40	**No of positive isolates**	**Individual Incidence**				
		P. aeruginosa	***B. subtilis***	***B. cereus***	***E. coli***	***S. aureus***
Mathura oil refinery (n=10)	9	4(45%)	2(22.2%)	1(11.1%)	1(11%)	1(11%)
Barauni oil refinery (n=10)	6	2(33.3%)	3(50%)	1(16.6%)	0(0%)	0(0%)
Haldia oil refinery (n=10)	4	2(50%)	1(25%)	1(25%)	0(0%)	0(0%)
Paradip oil refinery (n=10)	1	1(100%)	0(0%)	0(0%)	0(0%)	0(0%)
Total sample (n=40)	20	9(45%)	6(30%)	3(15%)	1(5%)	1(5%)

Table 2: Microbial distribution

Sample sites	**Soil sample size n=40**	**Average cfu g/ml(10^6)**
Mathura oil refinery	n=10	4.26
Barauni oil refinery	n=10	3.4
Haldia oil refinery	n=10	3.04
Paradip oil refinery	n=10	2.58

Fig 1: Effect of temperature on growth of bacteria at 5% petrol

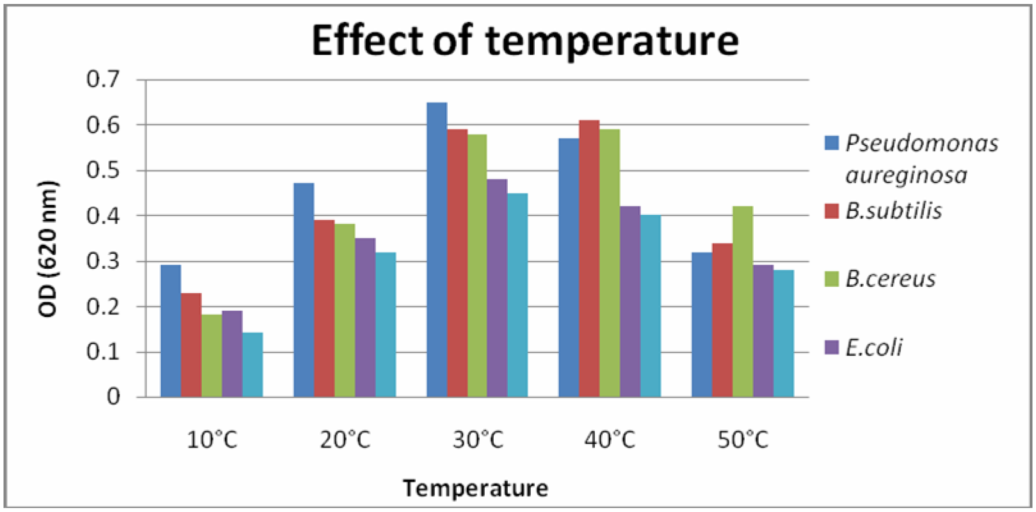


Table 3: Biochemical chart

Characteristics		**Hydrocarbon degrading bacteria from soil**				
		Pseudomonas aeruginosa	***Bacillus subtilis***	***Bacillus cereus***	***E. coli***	***Saphylococcus auries***
Colony morphology	Color	White	Creamy white	White waxy	White	Shiny yellow
	Margin	Entire	Undulate	Undulate	Regular	Entire
	Elevation	Flat	Unbonate	Flat	Convex	Convex
	Shape	Irregular large	Circular flat	Irregular large	Circular large	Circular
	Opacity	Opaque	opaque	Opaque	Smooth Glistening	Opaque
	Pigmentation	Blue-green	No	No	No	Golden yellow
Carbon source	Glucose	_	+ A	+ A	+ AG	+ A
	Sucrose	_	+ A	+ A	+ A	+ A
	Mannose	+	+	_	+	+
	Xylose	_	+	_	+	_
	Arabinose	_	+	_	+	_
	Lactose	_		_	+ AG	+ A
Cell morphology	Gram stain reaction	_	+	+	_	+
	Cell shape	Cylindrical rods	Rods	Rods	Rods	Coccus
Biochemical characteristics	Oxidase test	+	+	+	_	_
	Catalase test	+	_	_	+	+
	Starch hydrolysis test	–	–	+	–	–
	M.R	_	_	_	+	+
	V.P	_	_	_	_	_
	Indole test	_	_	_	+	_
	Citrate utilization test	–	–	–	–	–
	Gelatin liquefication	+	+	+	_	+
	Lipid Hydrolysis	+	–	+	–	+
	H_2S Reduction	_	_	_	_	_
	Nitrate Reduction	+	+	+	+	+
	Urease Activity	_	_	_	_	_

A = Acid, AG = Acid with gas

Table 4: Effect of temperature on growth of bacteria in 5% petrol

Temperature	**10°C**	**20°C**	**30°C**	**40°C**	**50°C**
P. aeruginosa	0.29	0.47	**0.65**	0.57	0.32
B. subtilis	0.23	0.39	0.59	**0.61**	0.34
B. cereus	0.18	0.38	0.58	**0.59**	0.42
E. coli	0.19	0.35	**0.48**	0.42	0.29
S. aureus	0.14	0.32	**0.45**	0.40	0.28

Table 5: Effect of pH on growth of bacteria at 5% petrol

pH	**5.5**	**6.5**	**7.5**	**8.5**	**9.5**
Pseudomonas aeruginosa	0.43	0.58	**0.65**	0.38	0.2
B. subtilis	0.48	0.56	**0.64**	0.32	0.10
B. cereus	0.45	0.50	**0.62**	0.30	0.14
E. coli	0.3	0.45	**0.59**	0.42	0.18
S. aureus	0.28	0.43	**0.58**	0.48	0.19

Fig 2: Effect of pH on growth of bacteria at 5% petrol

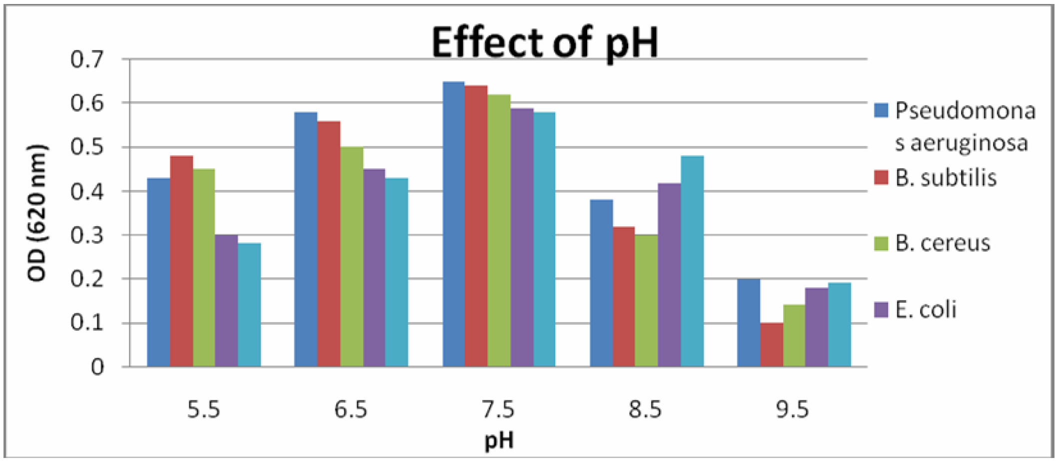


Table 6: Hydrocarbon degrading ability of *Pseudomonas aeruginosa*

	0 days			5 days			10 days			15 days		
	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%
Petrol	0.02	0.05	0.06	0.52	0.29	0.3	0.67	0.57	0.39	**0.68**	0.51	0.44
Benzene	0.01	0.04	0.06	0.39	0.48	0.39	0.49	0.52	0.43	**0.53**	0.50	0.45
Toluene	0.02	0.04	0.06	0.57	0.49	0.36	0.59	0.56	0.46	**0.64**	0.59	0.42
Xylene	0.02	0.04	0.06	0.5	0.41	0.25	0.59	0.49	0.45	**0.62**	0.57	0.42
Cyclohexane	0.02	0.04	0.05	0.59	0.46	0.45	0.64	0.48	0.48	**0.65**	0.53	0.54

Fig 3: Hydrocarbon degradation ability of *Psuedomonas aeruginosa*

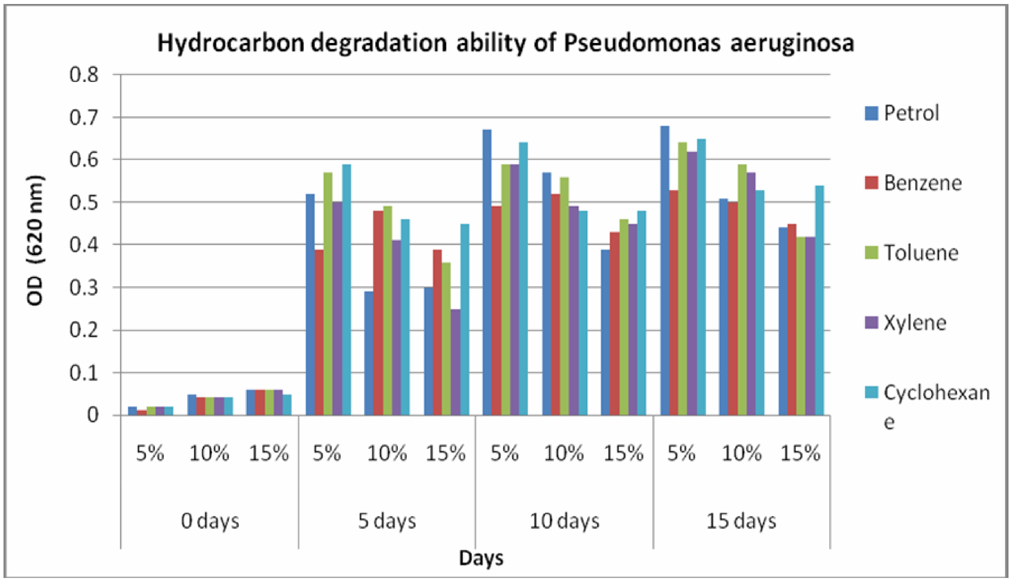


Table 7: Hydrocarbon degrading ability of *Bacillus subtilis*

	0 days			5 days			10 days			15 days		
	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%
Petrol	0.03	0.04	0.06	0.39	0.42	0.19	0.46	0.46	0.27	0.44	**0.47**	0.28
Benzene	0.01	0.04	0.06	0.50	0.36	0.15	**0.67**	0.42	0.23	0.62	0.44	0.24
Toluene	0.01	0.03	0.05	0.58	0.44	0.28	0.63	0.50	0.34	**0.65**	0.43	0.31
Xylene	0.02	0.03	0.05	0.41	0.43	0.35	0.48	0.48	0.38	**0.51**	0.50	0.39
Cyclohexane	0.02	0.04	0.05	0.45	0.37	0.30	**0.54**	0.39	0.33	0.52	0.40	0.35

Fig 4: Hydrocarbon degradation ability of *Bacillus subtilis*

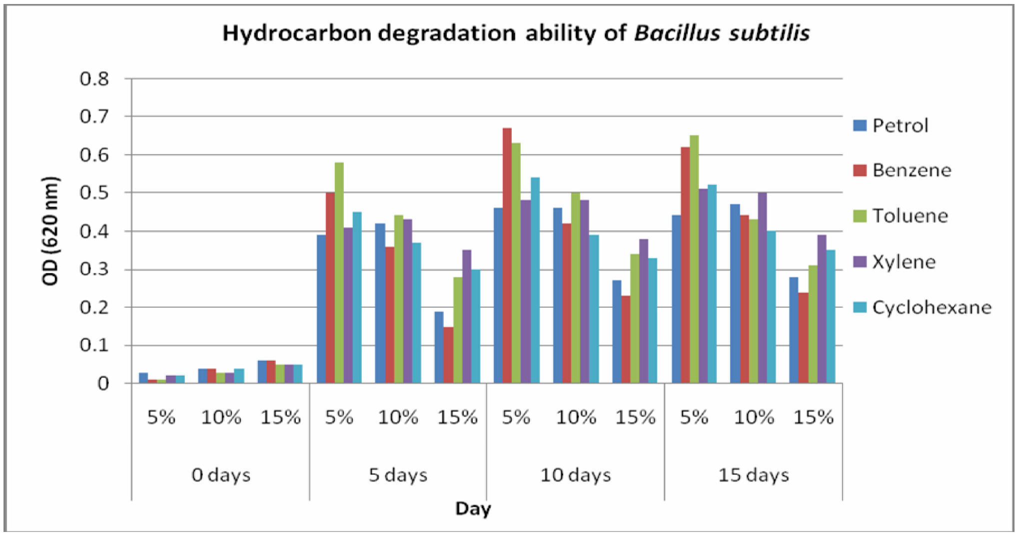


Table 8: Hydrocarbon degrading ability of *Bacillus cereus*

	0 days			5 days			10 days			15 days		
	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%
Petrol	0.03	0.04	0.05	0.4	0.43	0.34	0.49	0.48	0.38	**0.52**	0.5	0.44
Benzene	0.02	0.04	0.06	0.31	0.27	0.05	0.43	0.35	0.21	**0.46**	0.43	0.26
Toluene	0.02	0.05	0.06	0.32	0.28	0.1	**0.47**	0.34	0.18	0.44	0.27	0.22
Xylene	0.02	0.05	0.06	0.3	0.28	0.06	0.49	0.37	0.16	**0.5**	0.29	0.34
Cyclohexane	0.03	0.04	0.05	0.37	0.4	0.24	0.44	0.44	0.31	**0.48**	0.46	0.3

Fig 5: Hydrocarbon degradation ability of *Bacillus cereus*

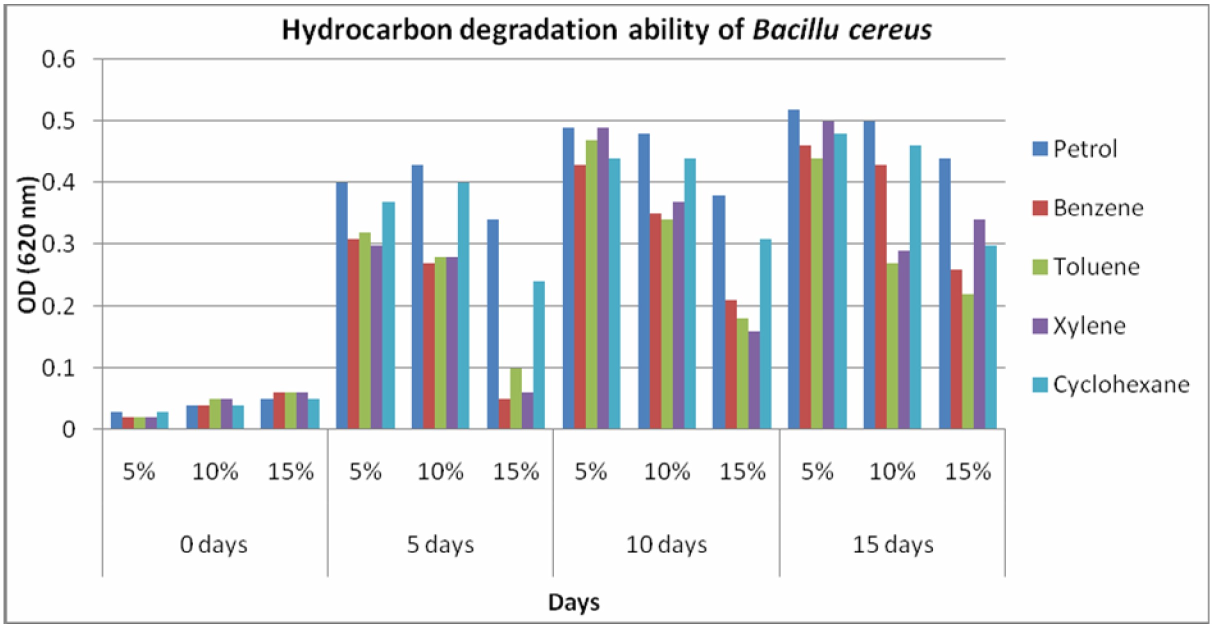


Table 9: Hydrocarbon degrading ability of *E. coli*

	0 days			5 days			10 days			15 days		
	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%
Petrol	0.02	0.04	0.06	0.41	0.28	0.11	**0.43**	0.31	0.17	0.36	0.22	0.20
Benzene	0.02	0.04	.05	0.37	0.29	0.18	0.39	0.29	0.22	**0.41**	0.24	0.25
Toluene	0.02	0.03	0.04	0.38	0.35	0.18	**0.43**	0.37	0.26	0.41	0.29	0.29
Xylene	0.02	0.04	0.05	0.37	0.26	0.22	**0.42**	0.30	0.25	0.35	0.29	0.29
Cyclohexane	.02	.04	.06	0.36	0.22	0.21	**0.49**	0.35	0.20	0.44	0.42	0.31

Fig 6: Hydrocarbon degradation ability of *E. coli*

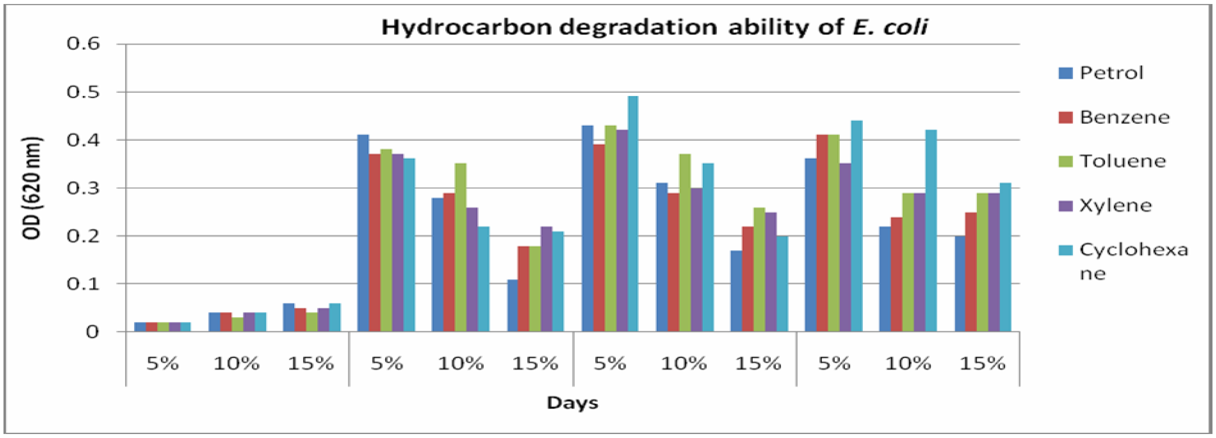
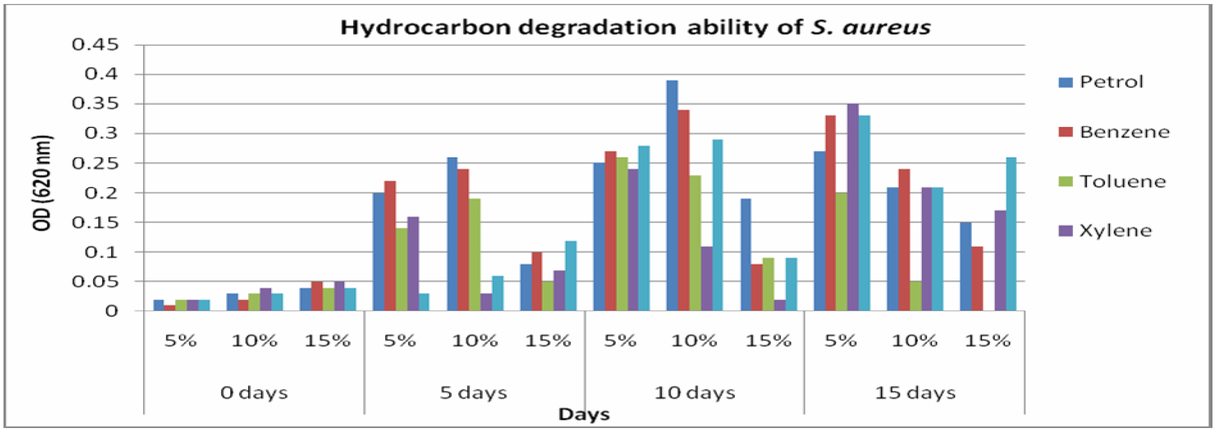


Table 10: Hydrocarbon degrading ability of *Staphylococcus aureus*

	0 days			5 days			10 days			15 days		
	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%
Petrol	0.02	0.03	0.04	0.2	0.26	0.08	0.25	**0.39**	0.19	0.27	0.21	0.15
Benzene	0.01	0.02	0.05	0.22	0.24	0.1	0.27	**0.34**	0.08	0.33	0.24	0.11
Toluene	0.02	0.03	0.04	0.14	0.19	0.05	**0.26**	0.23	0.09	0.2	0.05	0
Xylene	0.02	0.04	0.05	0.16	0.03	0.07	0.24	0.11	0.02	**0.35**	0.21	0.17
Cyclohexane	0.02	0.03	0.04	0.03	0.06	0.12	0.28	0.29	0.09	**0.33**	0.21	0.26

Fig 7: Hydrocarbon degradation ability of *Staphylococcus aureus*



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Research article

THE STANDARDIZATION OF PROTOCOL FOR LARGE SCALE PRODUCTION OF SUGARCANE (CO-86032) THROUGH MICRO PROPAGATION

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ABSTRACT: Sugarcane belongs to the grass family (Poaceae), an economically important seed plant family that includes maize, wheat, rice, and sorghum and many forage crops. The main product of sugarcane is sucrose, which accumulates in the stalk internodes. India is the second largest producer of sugar in the world. Sugarcane is an important agro-industrial crop in India, occupying 4.0 million hectare area. The average cane yield in India is about 70.0 tonnes per hectare while the sugar recovery is around 10.0 percent. The present study was carried out to standardize micro-propagation of sugarcane CO-86032 for large scale production of sugarcane planting material. Standardization and optimization of shoot induction and root induction medium were the major objectives of the study. Initiation of explant was found maximum in BAP (1 mg/lit) in MS medium. For shoot proliferation & multiplication SM - II media i.e. MS salts supplemented with 0.3 mg/lit BAP, was found to be the best compared to other media prepared. Though number of multiple shoots were more in SM – III media, but was not appropriate for sub-culturing. Best rooting result obtained in RM – III media i.e. ½ strength MS media supplemented with 3 mg/lit NAA & 3 mg/lit IBA. Maximum survival percentage (97.5 %) was found in SM – II media. From the present study it can be concluded that MS media supplemented with 0.3 mg/lit is best for shoot multiplication and for root induction ½ strength MS media supplemented with 3 mg/lit NAA & 3 mg/lit IBA is best. For commercial production of sugarcane ½ strength MS is found to be more economically viable.

Key words: Sugarcane, micro propagation, MS, BAP, NAA, IBA.

INTRODUCTION

Sugarcane belongs to the grass family (Poaceae), an economically important seed plant family that includes maize, wheat, rice, and sorghum and many forage crops. The main product of sugarcane is sucrose, which accumulates in the stalk internodes. Sucrose, extracted and purified in specialized mill factories, is used as raw material in human food industries or is fermented to produce ethanol. Ethanol is produced on a large scale by the Brazilian sugarcane industry. It is native to the warm temperate to tropical regions of South Asia, and used for sugar production. In 2012, FAO estimates it was cultivated on about 26.0 million hectares, in more than 90 countries, with a worldwide harvest of 1.83 billion tons. Brazil was the largest producer of sugar cane in the world. The next five major producers, in decreasing amounts of production, were India, China, Thailand, Pakistan and Mexico. The world demand for sugar is the primary driver of sugarcane agriculture. Cane accounts for 80% of sugar produced; most of the rest is made from sugar beets. Sugarcane predominantly grows in the tropical and subtropical regions, and sugar beet predominantly grows in colder temperate regions of the world. Other than sugar, products derived from sugarcane include falernum, molasses, rum, *cachaça* (a traditional spirit from Brazil), bagasse and ethanol.

Development of tissue culture method for sugarcane production was initiated almost three decades before in late 1970s using meristem culture (1). Till date several somaclones has been developed through tissue culture with improved productivity and eliminating certain defects like spines, leaf drying, disease susceptibility etc. Somaclones with smut resistance were developed from susceptible clones. Improvement in agronomic characters also have been reported in somaclones and some of the recent 'Co' releases like Co 86032, Co 92007, Co 92029, Co 93005, Co 94003, Co 94012, Co 94003, Co 95016, Co 99011 and Co 99012.

The sugarcane variety Co 94012 is a somaclonal variant that was released for cultivation in Maharashtra and is found to give high sugarcane recovery. However there is still a large gap between demand and supply of tissue culture raised sugarcane plants developed from above mentioned somaclones. Difficulty in standardizing tissue culture protocols for large scale production of sugarcane and lack of commercial interest in the same due to poor survival of the in-vitro regenerated plantlets of sugarcane during primary and secondary hardening procedures are the major reason behind such a large difference. The project entitled "Standardization of Protocol for Large Scale Production of Sugarcane (CO-86032) through Micro-propagation" was thus undertaken to study and understand important growth parameters of in-vitro regenerated sugarcane plants in order to standardize tissue culture protocol for large scale production of sugarcane.

Distribution of sugarcane world wide

Sugarcane area and productivity differ widely from country to country. Brazil has the highest area, while Australia has the highest productivity. Out of 90 sugarcane producing countries, 15 countries (Brazil, India, China, Thailand, Pakistan, Mexico, Cuba, Columbia, Australia, USA, Philippines, South Africa, Argentina, Myanmar, Bangladesh) 86% of area and 87.1% of production.

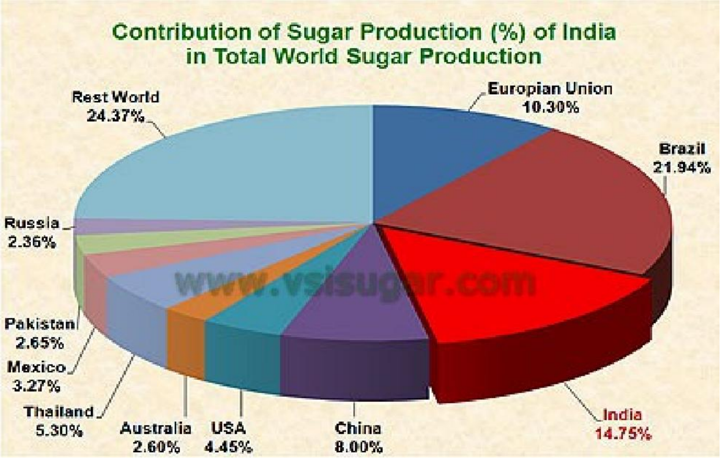


Fig. 1: Average production of Sugarcane during 2002-2012. (www.google.com)

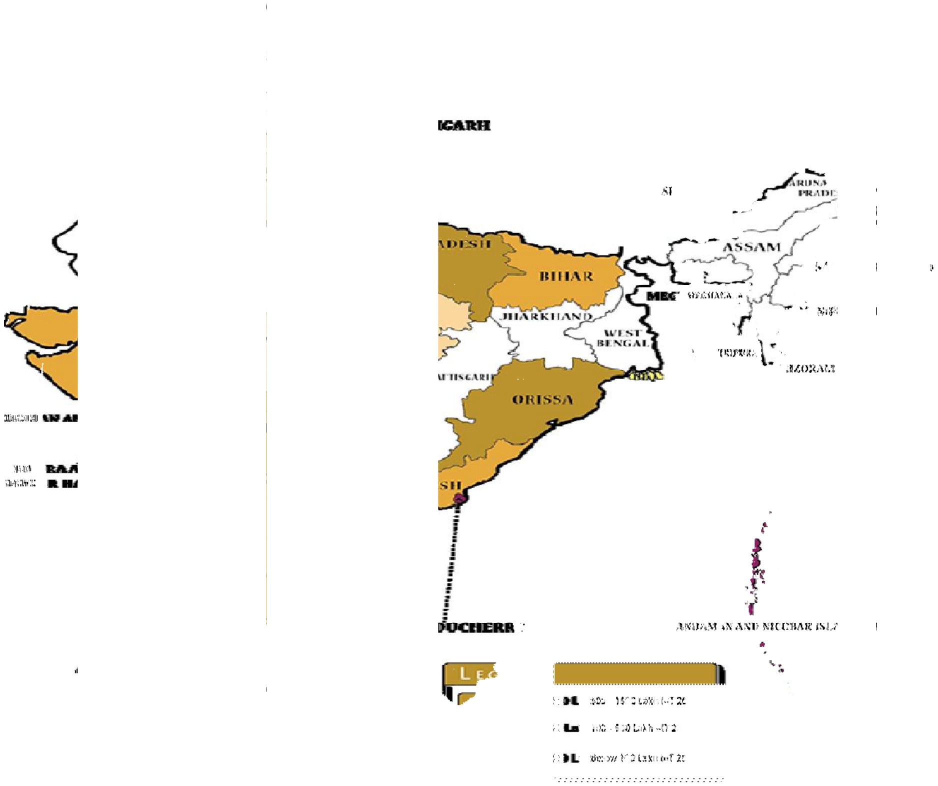
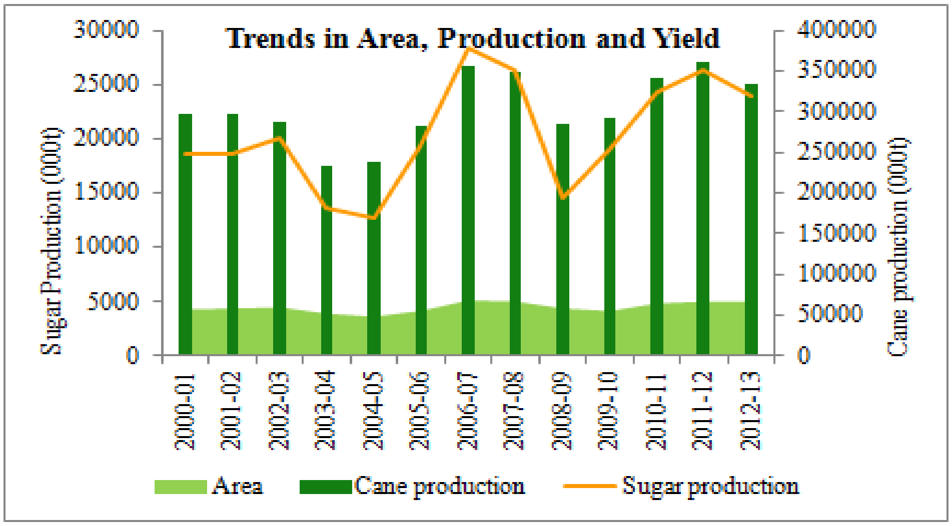


Fig. 2: Production of Sugarcane in different states of India (www.sugarcane.res.in)

Sugar industry in India

India is the second largest producer of sugar in the world. Sugarcane is an important agro-industrial crop in India, occupying 4.0 million hectare area. The average cane yield in India is about 70.0 tonnes per hectare while the sugar recovery is around 10.0 percent. However, there is potential of increasing the average cane yield to 100 tonnes per hectare and sugar recovery to 11.0 percent, if new technologies are transferred to the farmers fields.



Source: Ministry of Agriculture, GoI

Fig. 3: Trends in area, Production and Yield (Govt. Of India)

The various sugar canes are native to tropical locations around the world. Africa, South America, parts of Asia, and India are examples of some of the areas where one or more types of canes are cultivated, grown, and harvested. Just under two hundred different countries around the world are involved in the production of the canes and various Cane sugar products of some type. Sugar is a broad term applied to a large number of carbohydrates present in many plants and characterized by a more or less sweet taste. The primary sugar, glucose, is a product of photosynthesis and occurs in all green plants. In most plants, the sugars occur as a mixture that cannot readily be separated into the components.

In vitro Regeneration of planting material

The family poeceae includes grasses consisting of about 620 genera and 10,000 species, occupying maximum land and water bodies across the globe. Constitute a natural and homogeneous family (2) and are among the most successful of all angiosperms in their spread. They provide the most important source of food for human and animal. Over 70% of the calories consumed by humans come from grasses, either directly in the form of' flour, cereals, sugar, and alcohol, or indirectly, in the Corm of meat form animals that consume grass (3).

Tissue culture is based on the principle of totipotency, first described by German plant physiologist Haberland in1902. He predicted the indefinite culture of tissues, cells, and organs. However, it was in 1939 Nobecourt and Gautheret in France and White in the United States independently reported the indefinite culture of plant callus tissue on a synthetic medium supplemented by auxin. Thus the first requirement for micropropagation was provided, that is the ability to grow plants in artificial culture. A second requirement for successful micropropagation was the regulations of shoot and root regeneration. This was elegantly presented in the classic work by Skoog and coworkers utilizing tobacco pith callus (4). In recent years, tissue and cell culture techniques have been recognized as potentially valuable tool in crop improvement program. It is now possible to regenerate plants from single cells and protoplasts of' numerous species (5), foreign genes can be introduce in to cultured cells where they are expressed (6, 7).

Hendre *et al,* 1983, (8) reported that about 2 lac plants can be produced in six months from a single shoot tip. *In vitro* micropropagation provides for the rapid multiplication of sugarcane. By this technology nearly 10,00,000 healthier, virus-free, type sugarcane plantlets can be produced from single plant in one year. It was also studied micro propagation of sugarcane using two procedures (1) shoot tip culture, (2) indirect somatic embryogenesis from callus and reported that Shoot tip culture produced plants phenotypically similar to the mother plant and gave a much more rapid multiplication rate compared to the other procedure (9).

Chen *et al,* 1988, (10) reported sugarcane spindles of 6-12 months old plant were successful for shoot regeneration. Lal and Singh, 1994, (11) reported use of different gelling agents like (agar and agarose), and support materials (filter paper bridge , cotton cloth bridge and adsorbent cotton) as well as shaken and static liquid (control) cultures to improve in vitro shoot multiplication and vigor in sugarcane. Dhumale *et al,* 1994, (12) demonstrated that 2-3 mm shoot tips of sugarcane plant give significant shoot regeneration. Burner *et al*, 1995 (13) propagated sugarcane in vitro from shoot tip. Mullegadoo and Dookun, 1999, (14) studied the explant source of sugarcane and it's effect on multiplication Explants from three sources, axillary bud, apical bud and shoot apex, were cultured. Severe bacterial contamination occurred in axillary buds resulting in necrosis and death of the explants. Growth responses were better with apical buds than with axillary buds in two of the three varieties cultured. By micropropagation over 1.5 million plants can be produced form a single shoot tip in six months (15). Ali and Afghan, 2001, (16) reported micro propagation of sugarcane using meristem culture method and found that micropropagated plants were phenotypically similar to the mother plants. Noguera *et al*, 2002, (17) described the micropropagation of sugarcane from shoot apical. Hoy *et al,* 2003, (18) studied the effect of tissue culture explant source on sugarcane yield components. Chen *et al*, 1998, (19) has described surface sterilization of leaf explants with 95% ethanol for 5 minutes. Gallo-Meagher, 2000, (20) reported surface sterilization of leaf explant by 0.5% sodium hypochlorite for 20 minutes. Mamun *et al*, 2004, (21) reported that sterilization of leaf explant with 0.1% mercuric chloride for 8 to 10 min gave satisfactory results with, 85-90% of the explants being contamination free. Chen *et al,* 1987, (22) reported that at lower concentration of BAP (0.5 mg/l) and Kinetin (0.5 mg/l), there was loss of proliferation and vigour. Naritoom *et al*, 1993 (23) reported plant regeneration from shoot tip culture of sugarcane using MS medium supplemented with cytokinin. Ali and Afghan, 2001, (16) reported that basal medium (MS) supplemented with benzyl amine purine (BAP) and kinetin (Kn) gave rapid shoot multiplication. It was observed that at lower concentration of BAP, shoot proliferation was significant. Patel *et al*, 2001 (24) also recorded highest multiplication on medium containing 1.5 mg/l Kinetin. The treatment 1.5 mg/l kinetin + 1.0 mg/l BAP + 20% CW gave the highest values for length of main shoot in all cultivar. Pawar *et al,* 2002 (25) reported effect of growth regulators on in vitro multiplication of sugarcane cultivars (Co-86032, Co-740 and Co-8014) and studied the effect of different levels of kinetin, BAP and coconut water (CW) on shoot multiplication , length of main shoot and number of leaves on main shoot using MS medium supplemented with three levels of kinetin (0.5, 1.0 and 1.5 mg/l), four levels of BAP (0.5, 1.0, 1.5 and 2.0 mg/l) and two levels of CW (10 and 20%). The treatment MS + 1.5 mg/l kinetin + 1.0 mg/l BAP + 20% CW gave the highest values for length of main shoot in all cultivars. Baksha *et al*, 2002, (26) reported multiple shoots from shoot tip explant of sugar-cane (*Saccharum officinarum)* by culturing on MS medium supplemented with BAP (0.5-2.0 mg/l), Kn (0.1-0.5 mg/l). Mamun *et al,* 2004 (21) reported that *in vitro* micro propagation for sugarcane variety viz., Isd-28 and Isd-29 showed best shooting when media were supplemented with BA 1.5 mg /l +0.5 mg /l NAA. Shenk and Hildebrandt, 1972, (27) have reported requirement of high concentration of auxin for rooting in sugarcane. Nadar and Heinz, 1977, (28) reported that preferred auxin for root initiation was NAA. Rooting was induced in clumps. Chen *et al*, 1988, (10) reported that rooting in sugarcane takes place in the clumps. Auxins are reported to be essential for root induction in sugarcane, 70 gm/l sugar in MS media supplemented with 500 mg/l casein hydrolysate and 5 mg/l of NAA induced rooting. Naritoom *et al,* 1993, (23) initiated rooting on MS medium with 1 mg IBA/l. Dhumale *et al,* 1994, induced roots on half strength MS medium containing 2 mg/l IBA + 1 mg/l IAA. Cheema *et al,* 1995, (29) demonstrated rooting on MS medium supplemented with 7% sugar and 5 mg/l NAA. Singh *et al,* 2001, (30) transferred clumps with 5-10 well grown shoots on 1/2 strength MS liquid medium supplemented with NAA (5.0 mg/l) and elevated sucrose level (60 g/l). All cultures were incubated at 25 ± 20C under 12 h illuminations at a photon flux density of 50-70 micron. The development of fine roots, which began after 7-15 days, ranged from 75% (CoJ 85) to 95% (CoJ 86). Profuse rooting was achieved within 30-40 days. Plants transplanted in the field 45-60 days after hardening in the greenhouse showed uniform growth and asynchronous tillering within 60 days after transplanting. Pawar *et al,* 2002, (25) induced rooting (85-92%) by transferring shoot clumps on 1/2 MS medium containing 2 mg/l NAA and 1.0 mg/ IBA. Mamun *et al*, 2004, (21) reported that. Best results of rooting ware observed on modified MS with auxins. Eighty to ninety percent regenerated plantlets were viable at normal temperature with 85% humidity while transferred sterilized.

MATERIALS AND METHODS

Explant preparation

Healthy explants of CO-86032 were collected from the mother plant nursery of sugarcane from Aditya Biotech Lab's field. The mother plants of Sugarcane variety CO-86032 were obtained from National Sugarcane Research Institute, Coimbatore. Selection of explants was done from mother plant nursery protected with insect proof nets.

Healthy mother plants with maximum numbers of tillers, good girth, appropriate color were selected and tagged with respective accession numbers. Soil was removed from the explants and washed with running tap water. The explants were then dipped in bavistin solution (1 gm/liter) 5 minutes followed by washing in running tap water to remove all traces of detergent. Then the explants were treated with 10 % sodium hypo chloride solution for 15 minutes. The explants were taken to the laminar airflow bench. Sodium hypo chloride solution was discarded and the explants were washed 3 times with sterilized distill water.

Media preparation and Initiation of Culture

The Murashige and Skoog`s (MS) (1962) basal medium was used for the study. Stock solutions were prepared by using required quantities of macronutrient and micronutrient for convenience. Medium pH was adjusted to 5.8 and solidified with 0.7 % agar prior to autoclaving at 121ºC and 15 psi pressure for 20 minutes. Media was stored at media storage room for at least 3 days prior to inoculation. Initiation of *in-vitro* culture of *Sachharun officinarum* is an important step. For culture initiation Murashige and Skoog MS) medium + Benzyl Amino Purine (BAP) (1 mg/L) was used. For multiplication of shoots MS media was prepared and was supplemented with 3 different concentrations BAP and designated SM – I, SM – II and SM – III. For root induction ½ strength MS media was prepared. After 3 - 4 cycle of sub-culturing, well developed shoots were induced for root multiplication in ½ strength MS medium with different concentrations and combinations of Indole Butyric Acid (IBA) and Napthalene acetic acid (NAA). These cultures were designated as RM1, RM2 and RM3.

RESULT AND DISCUSSION

Initiation of *in-vitro* regeneration

Shoot tip containing auxiliary meristem of cultivar Co-86032 were used as explants for *in-vitro* regeneration. The shoot tips containing axillary meristem were inoculated in MS medium with a fixed concentration of BAP (1mg/lit) for shoot tip initiation and establishment. Small shoots started appearing with in 7-10 days in all cultures bottles. Similar results have been reported by Dhumale *et al* (1994) for initiation where BAP @ 3 mg/lit. and NAA 1mg/lit. were used. Biradar *et al* (2008) also reported that axillary bud is the most suitable explant for initiation with MS medium containing BAP at 2 mg/lit concentrations.

Fig. 4: Sugarcane Node used as Explant

Fig. 5: Culture Initiation in MS

Shoot Elongation

Ten random bottles were selected for recording average shoot length of the shooting cultures during three cycles of multiplication. It was observed that maximum shoot lengths were obtained in the medium coded as SM-II i.e. MS supplemented with 0.3mg/lit BAP (Table 4.1). After 3o days of incubation the average shoot length recorded in SM-II medium was found to be 2.88cm, whereas minimum shoot length of 1.44cm was observed in SM-I i.e. MS supplemented with 0.1mg/lit BAP. It was also recorded that shoot growth in SM-3 were as elongated as SM-II but the shoot quality was relatively poor being succulent and weak. The shoots obtained in SM-III were not appropriate for further sub culturing due to curled morphology and fragile nature.

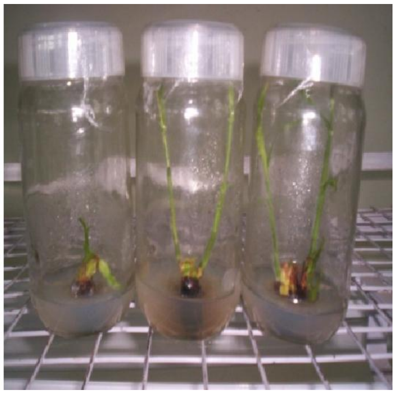


Fig. 6: Shoot Elongation.

Fig.7: Initiation of multiple shooting

Fig. 8: First sub culturing for shoot multiplication

Fig. 9: Second sub culturing for shoot multiplication in sugarcane

Multiplication of Shoots

For recording average no of multiple shoot similar procedure was followed as in shoot elongation. After 4-5 week of shoot growth, actively growing shoots were transferred to fresh medium in jars for further growth and proliferation. Proliferation of shoot started & during secondary proliferation stage, lateral shoots developed from base of newly initiated shoot. As a result a dense mass of shoot (25-30) was developed in each culture. After 30 days of incubation at about 25-28ºC temperature and 60% relative humidity the dense shoot growth were separated and sub-divided in bunches containing 4-5 shoots each. Each of bunches was then cleaned and transferred in to fresh shooting medium in jars. In these way shoot multiplication was maintained for several passages by regular transfer to fresh medium. The best shoot multiplication response for sugarcane variety Co-86032 was obtained in SM-2 medium i.e MS Medium supplemented with 0.3mg/lit. BAP (Table-1). The medium showed about 13.18 shoots in an average after 1 month of incubation in 3rd cycle. Though SM-3 medium i.e MS medium supplemented with .5mg/lit. BAP also showed high numbers of multiple shoot the shoots were succulent and curly in nature that made them highly unsuitable for rooting.

Fig.10: Profused multiple shooting in Sugarcane

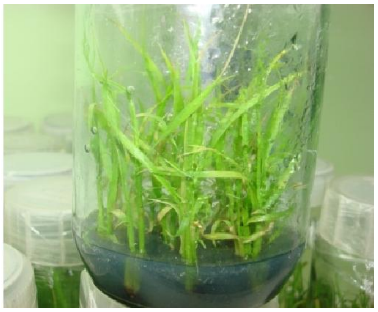


Fig. 11: Initiation of rooting

Survival percentage

The percent of survival varied between different shooting media carrying different levels of BAP. Maximum survival rated was found in medium containing BAP @ 0.3mg/lit (97.5%), on which shoots were active and healthy compared to other treatments. It was observed that survival percentage increased with increase in no of sub culturing.

Rooting of regenerated shoots

The regenerated shoots were used for root induction in root forming media. Half strength MS medium supplemented with 3 different combinations of NAA & IBA was used. The frequency of root formation was different in all media. Best root formation response was obtained in RM-III (table-2). MS medium having 3 mg/lit NAA & 3 mg/lit IBA. Average number of plants showing roots were 9.2 in RM-III, where as In RM-I & RM-II was 5.6 & 5.3 respective. Frequency of root formation was always recorded best in RM-III. Heavy bunch, healthy roots more than 20 roots per plant & whitish color.

Table 1 Effect of different concentration of BAP on multiple shoot induction of CO-86032

Media Type	Media Composition (mg/lit.)	Days to Shoot Formation	General Shoot Health	Cycle		
				First	Second	Third
SM - I	MS + 0.1 BAP	10.8	Less multiple shoots, shorter in length.	1.45	1.47	1.54
SM - II	MS + 0.3 BAP	10.3	High multiplication, longer in length.	2.04	2.24	2.88
SM - III	MS + 0.5 BAP	9.8	Very high multiplication, longer in length, succulent & curled leaves	2.11	2.4	3.01

Table 2 Effect of different concentration of BAP on number of multiple shoots in CO-86032

Media Type	Media Composition (mg/lit.)	Days to Shoot Formation	General Shoot Health	Cycle		
				First	Second	Third
SM - I	MS + 0.1 BAP	10.8	Less multiple shoots, shorter in length.	9.41	10.52	11.6
SM - II	MS + 0.3 BAP	10.3	High multiplication, longer in length.	11.46	11.99	13.18
SM - III	MS + 0.5 BAP	9.8	Very high multiplication, longer in length, succulent &curled leaves.	11.66	12.66	13.04

Table 3 Effect of different concentration of NAA & IBA combination on Root Formation

Media Type	Media Composition (mg / Liter)	Days to Root induction (Mean Value)	No.of Plants Showing Roots (Mean Value)	Average Frequency of Roots
RM – I	½ MS + 1 NAA + 1 IBA	16.7	5.6	Low (2-5 Roots, poor health, Blackish color)
RM – II	½ MS + 3NAA + 1 IBA	15.2	8.3	Medium(5-10 Roots, Normal Health, Whitish Color)
RM – III	½ MS + 3NAA + 3 IBA	13.56	9.5	High (<20 Roots, Heavy Bunch, Healthy Roots, Whitish Color)

Hence from the above result & discussion we can summarize the best result obtained in terms of media used for different stages of sugarcane cultures. So, for initiation it was found that BAP 1 mg/lit in MS medium was optimal for regeneration & multiplication. Similarly for shoot proliferation & multiplication SM - II media i.e. MS salts supplemented with 0.3 mg/lit BAP, was found to be the best compared to other media prepared. Best rooting result obtained in RM – III media i.e. ½ strength MS media supplemented with 3 mg/lit NAA & 3 mg/lit IBA.

CONCLUSION

The present study was carried out to standardize micro-propagation of sugarcane CO-86032 for large scale production of sugarcane planting material. Standardization and optimization of shoot induction and root induction medium were the major objectives of the study. It was observed in the present study that an efficient protocol is needed for any new variety or clone to get rapid shoot initiation, shoot multiplication, root induction & elongation. Initiation of explant was found maximum in BAP (1 mg/lit) in MS medium. For shoot proliferation & multiplication SM - II media i.e. MS salts supplemented with 0.3 mg/lit BAP, was found to be the best compared to other media prepared. Though number of multiple shoots were more in SM – III media, but was not appropriate for sub-culturing. Best rooting result obtained in RM – III media i.e. ½ strength MS media supplemented with 3 mg/lit NAA & 3 mg/lit IBA. Maximum survival percentage (97.5 %) was found in SM – II media. From the present study it can be concluded that MS media supplemented with 0.3 mg/lit is best for shoot multiplication and for root induction ½ strength MS media supplemented with 3 mg/lit NAA & 3 mg/lit IBA is best. For commercial production of sugarcane ½ strength MS is found to be more economically viable.

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Mini Review Open Access

Xenobiotic Compounds Present in Soil and Water: A Review on Remediation Strategies

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Abstract

Synthetic chemicals foreign to a particular ecological system and has a biological activity can be called xenobiotic compounds. Xenobiotics include drugs, industrial chemicals, naturally occurring poisons and environmental pollutants. Some microorganisms have the ability of breaking down the xenobiotic compounds partially or entirely. But some xenobiotics are recalcitrant in nature because of various reasons. Some of them cannot be used as substrate by microbes, some cannot transport them due to absence of transporting enzymes and some are in accessible to microbes due to larger structure and insolubility. They can be divided into different groups depending on their chemical composition. Biological and non-biological remediation techniques are the most reliable techniques to degrade these compounds. Bacterial biodegradation used in land filling and composting are most economical methods which uses both the wild type and genetically modified bacterial strains. There are many non-biological techniques which have been grouped under thermal and non-thermal techniques which are suitable for xenobiotic degradation.

Keywords: Xenobiotic; Remediation; Recalcitrant; Bacterial strains

Introduction

Xenobiotic compounds are chemicals which are foreign to the biosphere. Depending on their fate in water and soil xenobiotic compounds may become available to microorganisms [1-5]. Most importantly the dominant means of transformation of these compounds are microorganisms. Polyaromatic hydrocarbons, cyclic biphenyls, nitroaromatic compounds, aliphatic and aromatic halogenated compounds, triazines, azo dyes, organic sulphonic acid and many more have xenobiotic structural features [6-20].

Xenobiotics can exert adverse effects on human health by disrupting or interacting with multiple cellular communication pathways that direct growth, development and normal physiological function [21-40]. These compounds are highly toxic in nature and can affect survival of lower as well as higher eukaryotes. These compounds are persistent and remain in the environment for many years leading to bioaccumulation or biomagnification [41-60]. They also find a way into the food chains and the concentrations of such compounds was found to be high even in organisms that do not come in contact with xenobiotics directly. Certain microbes on continuous exposure to xenobiotics develop the ability to degrade the same as a result of mutations. Mutations resulted in modification of gene of microbes so that the active site of enzymes is modified to show increased affinity to xenobiotics [61-79].

This review gives a brief introduction about the technique, its types with advantages and disadvantages from a detailed list of biological and non-biological techniques.

Biological remediation strategies

There are various biological techniques to use to detoxify or degrade the xenobiotics which are listed in Table 1 and Figures 1-8. Followed by their working diagram.

Non Biological remediation strategies

Thermal strategies (Table 2a and Figures 9-16)

Non thermal strategies (Table 2b and Figures 17-27)

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Remediation Techniques	Types	Advantages	Disadvantages	References
Natural attenuation • Uses natural processes to limit the flow of contaminants from chemical spills and also reduces their concentration at contaminated sites.		• Remediation waste is least which has less impact act on the environment; • Can be easily combined with other technologies.	• Ethical issues remain which needs to be correctly perceived by the people. • Costly and complex site characterization.	[34,35]
Phytoremediation Uses plants in combination with microorganisms to remediate the contaminated area.	**Phytoextraction** • Plants remove dangerous elements or compounds from soil or water, most usually heavy metals, metals that have a high density and may be toxic to organisms even at relatively low concentrations.	• Least environmental disturbance. • Solar energy driven technology. • Used on a large range of contaminants. • Cost-effective for large contaminated sites	• Two growing seasons required • Limited to soils less than one meter from the surface and groundwater less than 3 m from the surface • Contaminants may enter the food chain through animals which eat the plants used in these projects	[44,45,73]
	Phytotransformation • It is the breakdown of organic contaminants sequestered by plants via: (1) metabolic processes within the plant; or (2) the effect of compounds, such as enzymes, produced by the plant.			[14,36]
	Phytovolatalisation • Contaminant is taken in by the plant tissue and then volatlised in the environment			[19,79]
	Rhizofiltration • Involves filtering water through a mass of roots to remove toxic substances or excess nutrients.			[18,48]
	Phytostimulation • Involves the stimulation of microbial degradation through the activities of plants in the root zone			[59]
	Phytostabilisation • Root released compounds enhance microbial activity in the rhizosphere			[46]
	Phytoscreening • plants are used as biosensors of subsurface contamination and is a simple, fast, noninvasive and inexpensive method.			[13,63,70]
Biosparging • Air and nutrients are injected into the saturated zone to increase the biological activity of the indigenous microorganisms		• Readily available equipment; • Cost competitive; • In situ technology	• Biochemical and physiological interactions are very complex and needs to be understood • Migration of constituents can lead to toxicity elsewhere.	[57]
Bioventing process injects air into the contaminated media at a rate designed to maximize in situ biodegradation and minimize or eliminate the off-gassing of volatilized contaminants to the atmosphere		• Very economic and easy to install • can be combined with other technologies	• High concentrations can be toxic for microorganisms • Low soil permeability doesn't allows proper implication. • Good for unsaturated zones of soils.	[23,47]
Bioreactors/ Bioslurry • Uses bioreactors and selected bacteria to biodegrade the contaminants.		• Fast degradation • Effective use of inoculants and surfactant	• Soil transport required • Expensive	[77]
Composting • Uses cow manure and mixed vegetable waste to remove the toxicants upto 90% from the contaminated soil.		• Cheap with rapid reaction rate.	• Treatment time more than other techniques • Requires nitrogen supplementation.	[3]
Biopiling • Involves the piling of petroleum-contaminated soils into piles or heaps and then simulating aerobic microbial activity by aeration and the addition of minerals, nutrients, and moisture		• Insitu technology therefore no transportation cost.	• Need to control abiotic loss • Mass transfer problem • Bioavailability limitation	[25,37]

Land Farming • Bioremediation treatment process that is performed in the upper soil zone or in biotreatment cells.		• Relative simple design and implementation • Short treatment times (six months to two years under optimal conditions).	• Required area is high. • Dust and vapor generation may cause some air pollution.	[29]
Bioslurping • Combines elements of bioventing and vacuum-enhanced pumping to remediate the contaminated site.		• Applied at shallolw as well as deep sites. • Recovers free product, thus speeding remediation	• Low soil permeability hampers remediation. • Soil moisture and oxygen content limits the microbial activities. • Low temperatures slow remediation.	[75]

Table 1: List of biological techniques used in remediation of contaminants with their advantages and disadvantages.

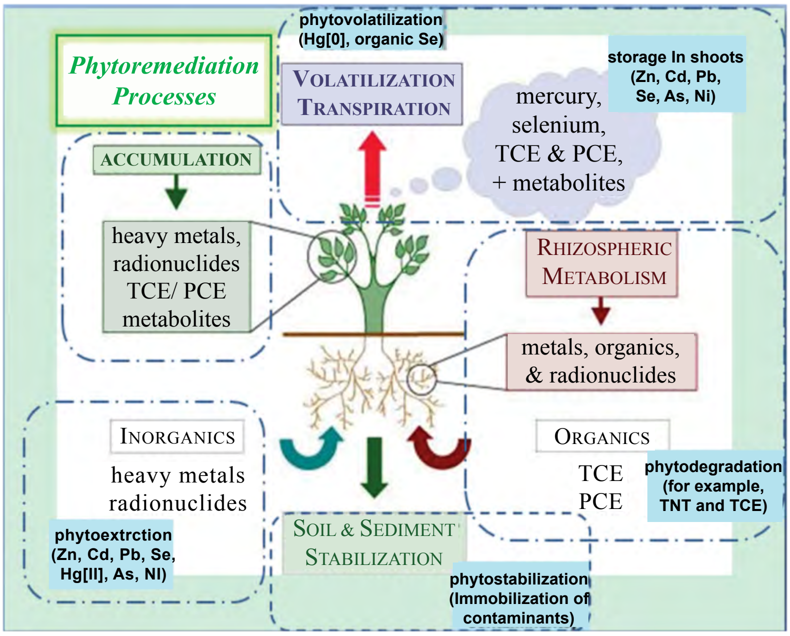

Figure 1: Types of phytoremediation.

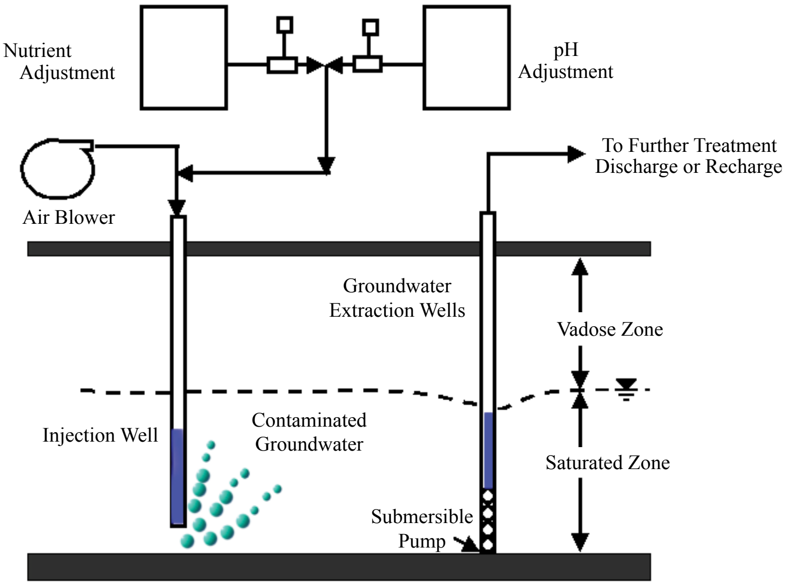

Figure 2: Biosparging.

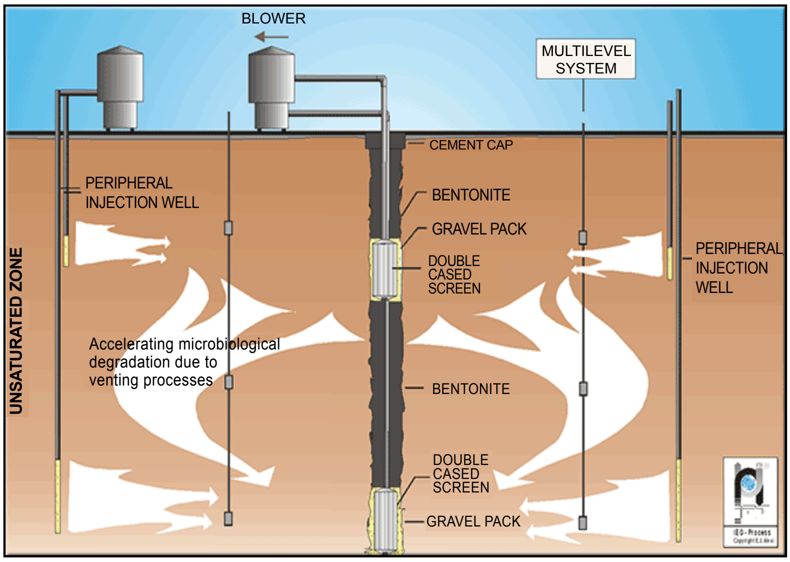

Figure 3: Bioventing.

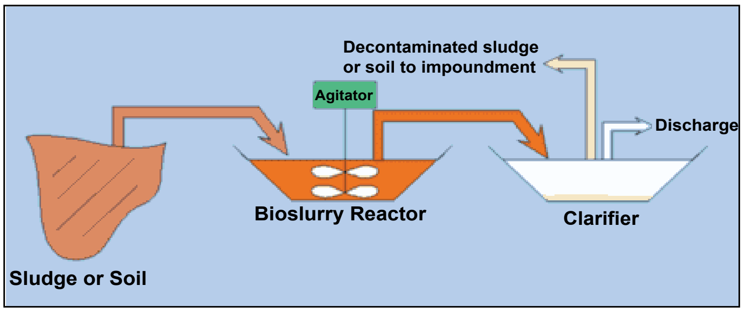

Figure 4: Bioslurry reactors.

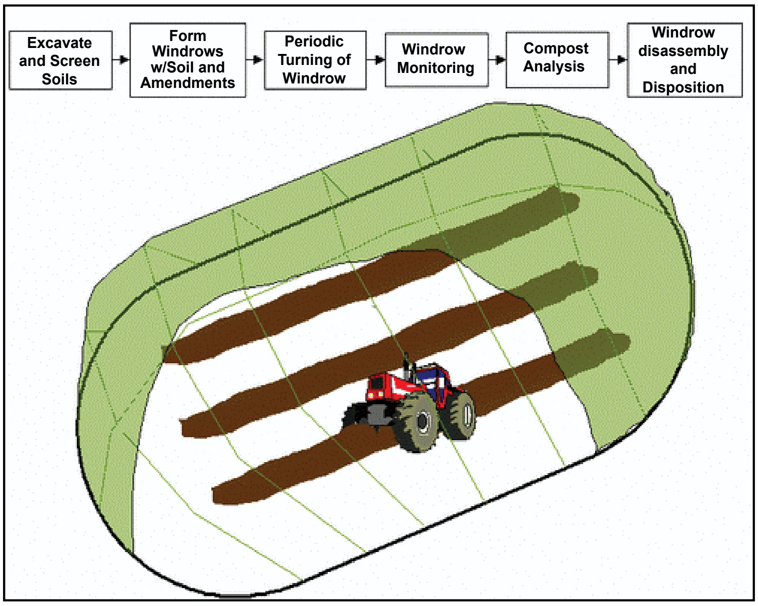

Figure 5: Composting.

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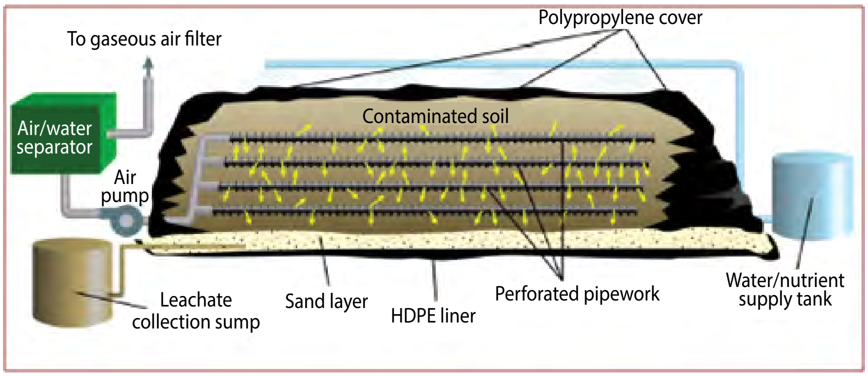

Figure 6: Biopiling.

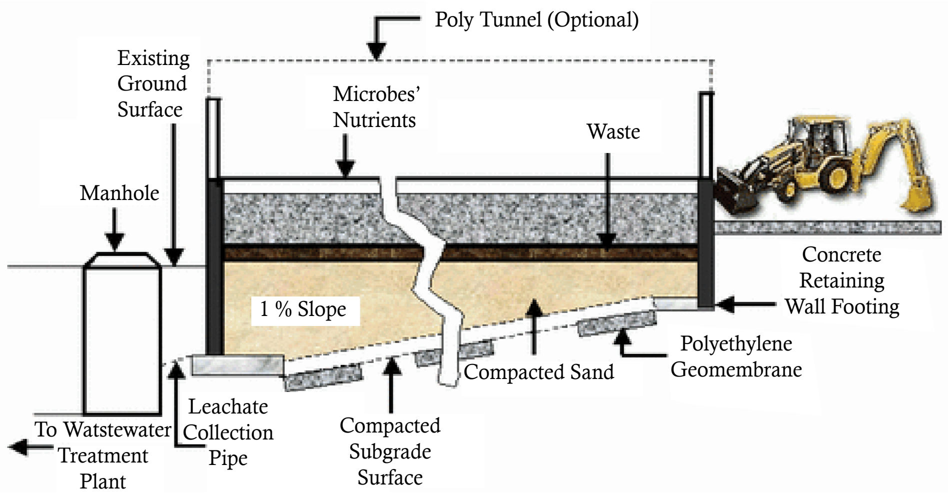

Figure 7: Landfarming.

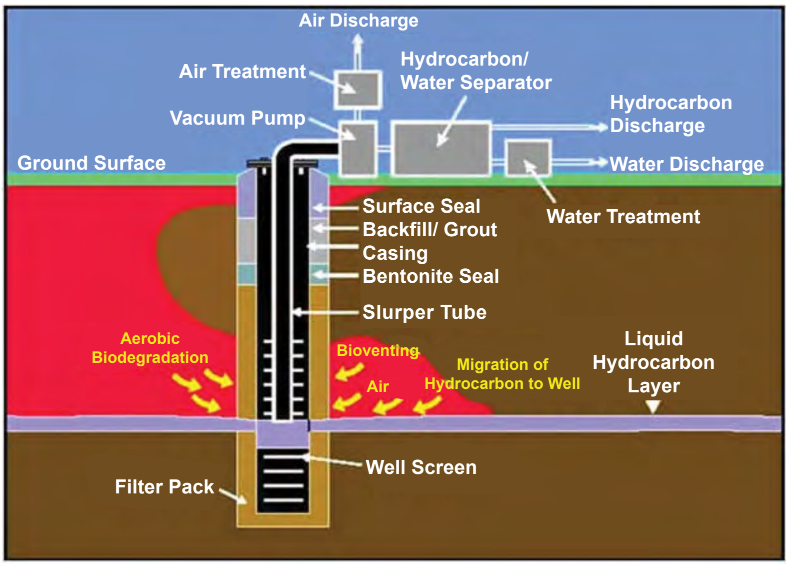

Figure 8: Bioslurping.

Remediation Techniques	Types	Advantages	Disadvantages	References
Thermal treatment (In Situ) • Consists of the following five technologies: electrical resistance heating, steam injection and extraction, conductive heating, radio-frequency heating, and vitrification. 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)** An array of electrodes is used to pass the electrical current through moisture in the soil. As the current flows through the moisture in soil pores, the resistance of the soil produces heat	• Contaminant toxicity as well as its concentration is checked by this technology • Commercially available and widely used.	• Metals are not destroyed and end up in the flue gases or in the ashes. • Rocky soils need to be screened before use.	[7]
	Steam injection and extraction / steam enhanced extraction [SEE]) • Involves injection of steam into injection wells and the removal of contaminants by three methods: Enhanced volatilization, Enhanced mobility and Hydrous pyrolysis oxidation.			[33,61]
	Conductive heating • Uses 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.			[4]
	Radio-frequency heating (RFH) • A high frequency alternating electric field for in situ heating of soils is used.			[27]
	Thermal Desorption • Contaminated soil is excavated, screened, and heated to release petroleum from the soil			[72]
	In situ vitrification (ISV) • Converts contaminated soil to stable glass and crystalline solids.			[22,69]
Thermal treatment (*Ex Situ*) • Involves the destruction or removal of contaminants through exposure to high temperature in treatment cells, combustion chambers, or other means used to contain the contaminated media during the remediation process	**Hot gas decontamination** The temperature of the contaminated area is raised to 260°C for a specified period of time. The gas effluent from the material is treated in an afterburner system to destroy all volatilized contaminants	• Waste is stockpiled Which is easily disposed off later. • Permit reuse or disposal of scrap as nonhazardous material	• Costs of this method are higher than open burning. • Can lead to explosions from improperly demilitarized mines or shells. • Slow rate of decontamination	[33]
	Incineration Uses high temperatures from 870°C to 1200°C to volatilize and combust (in the presence of oxygen) halogenated and other refractory organics in hazardous wastes.	• Used to remediate soils contaminated with explosives and hazardous wastes	• Only one off-site incinerator is permitted to burn • Specific materials and feed size required • Bottom ash produced by heavy metals requires stabilization. • Volatile heavy metals, including lead, cadmium, mercury and arsenic can cause air pollution.	[63]
	Open Burn (OB) and Open Detonation (OD) • Uses self-sustained combustion ignited by an external source, such as flame, heat, or a detonation wave (that does not result in a detonation) to destroy explosives or munitions.	• Very effective for many types of explosives, pyrotechnics and propellants	• Minimum distance requirements for safety purposes. • Emissions are difficult to capture for treatment	[68]
	Pyrolysis • Decomposition induced in organic materials by heat in the absence of oxygen.	• Target contaminant groups for pyrolysis are SVOCs and pesticides. • Can treat organic contaminants in soils and oily sludges.	• Specific feed size and materials handling requirements. • Drying of the soil required • Highly abrasive feed sometimes damage the processor unit. • High moisture content increases treatment costs.	[63]

Table 2a: List of non-biological thermal techniques used in remediation of contaminants with their advantages and disadvantages.

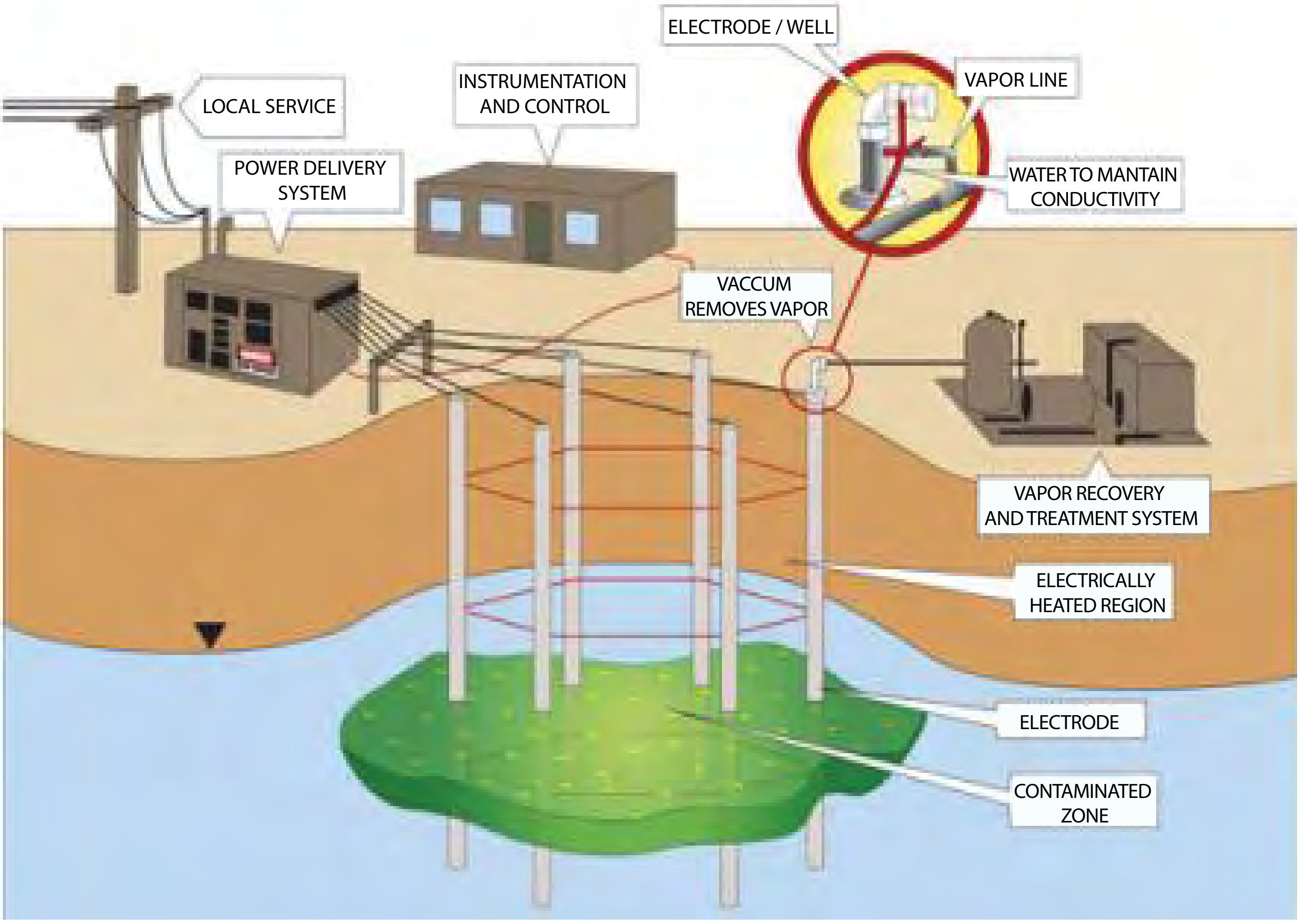

Figure 9: Electrical resistant heating (ERH).

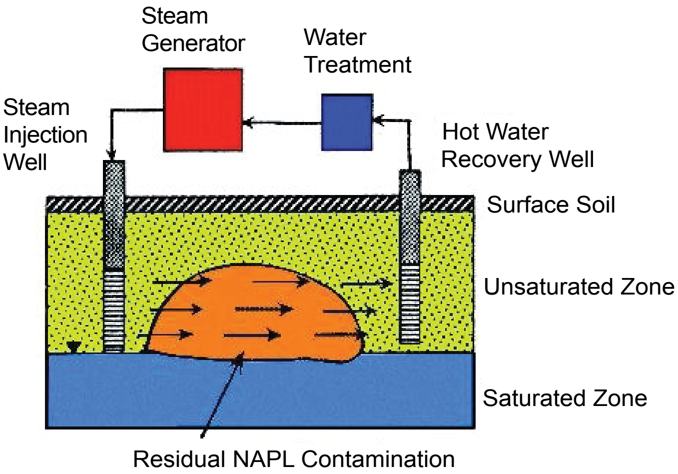

Figure 10: Steam enhanced extraction.

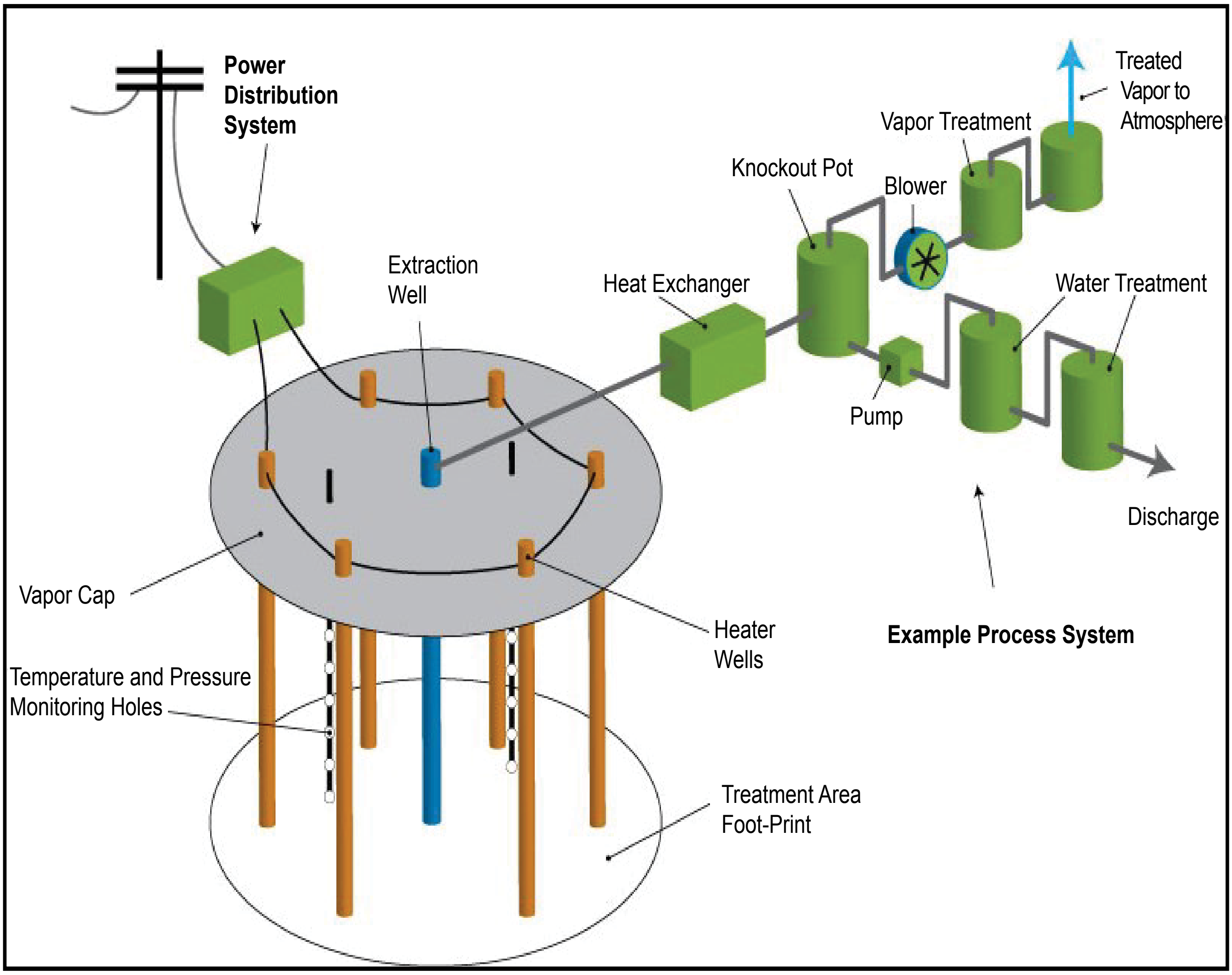

Figure 11: Thermal desorption with conductive heating.

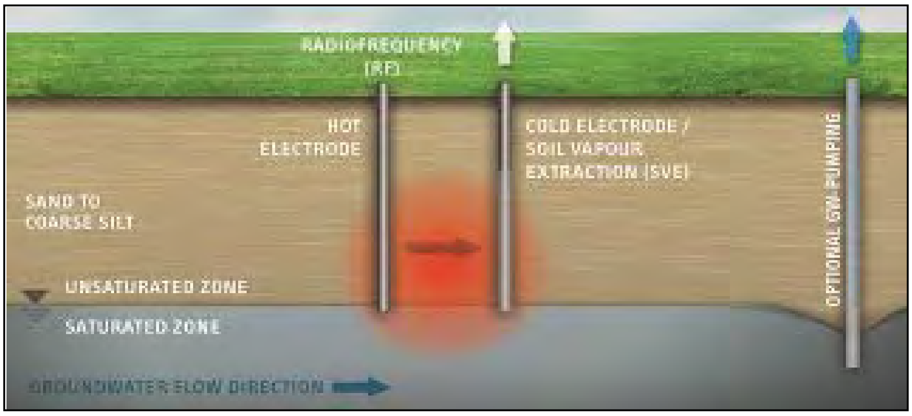

Figure 12: Radio frequency heating.

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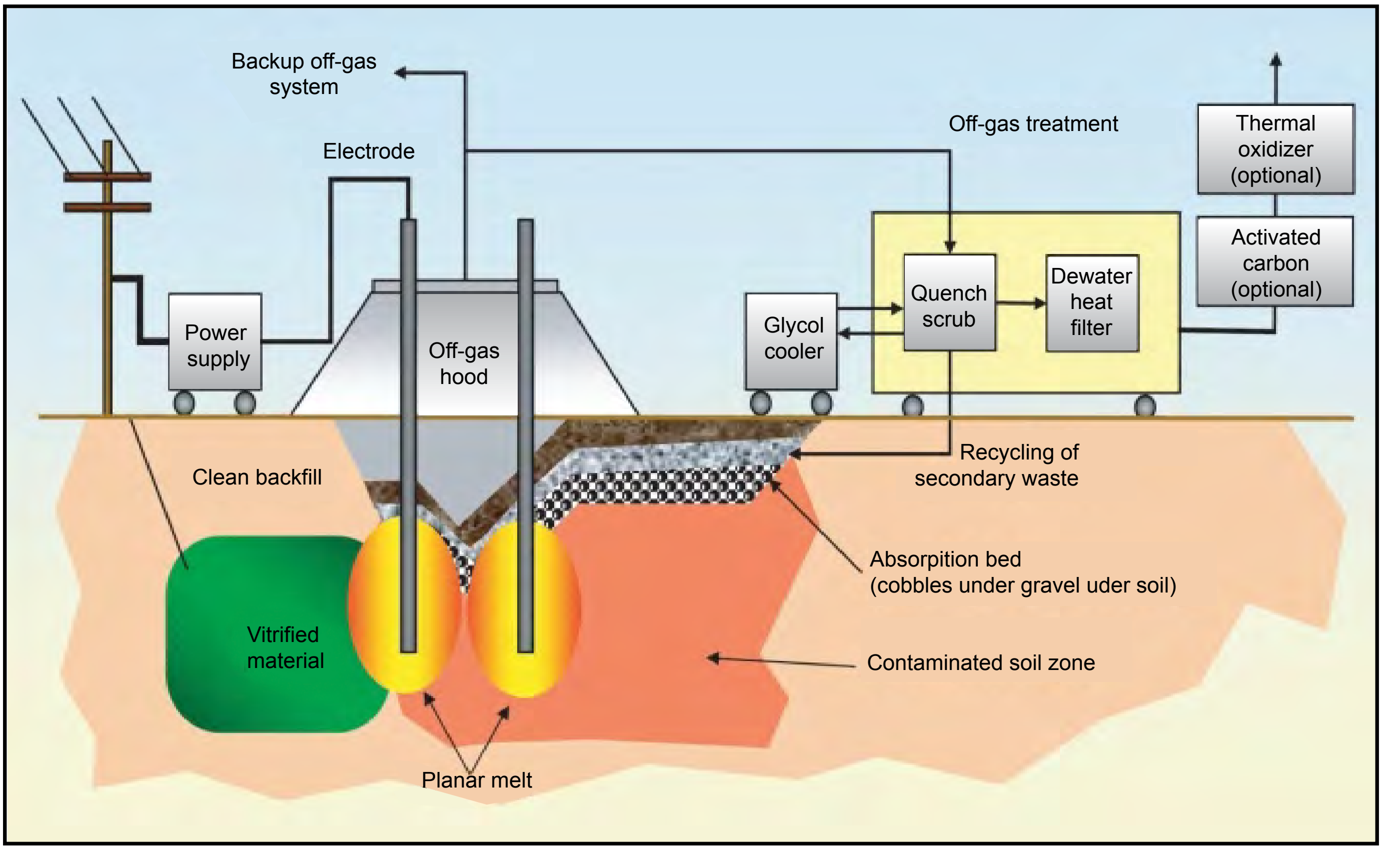

Figure 13: Vitrification.

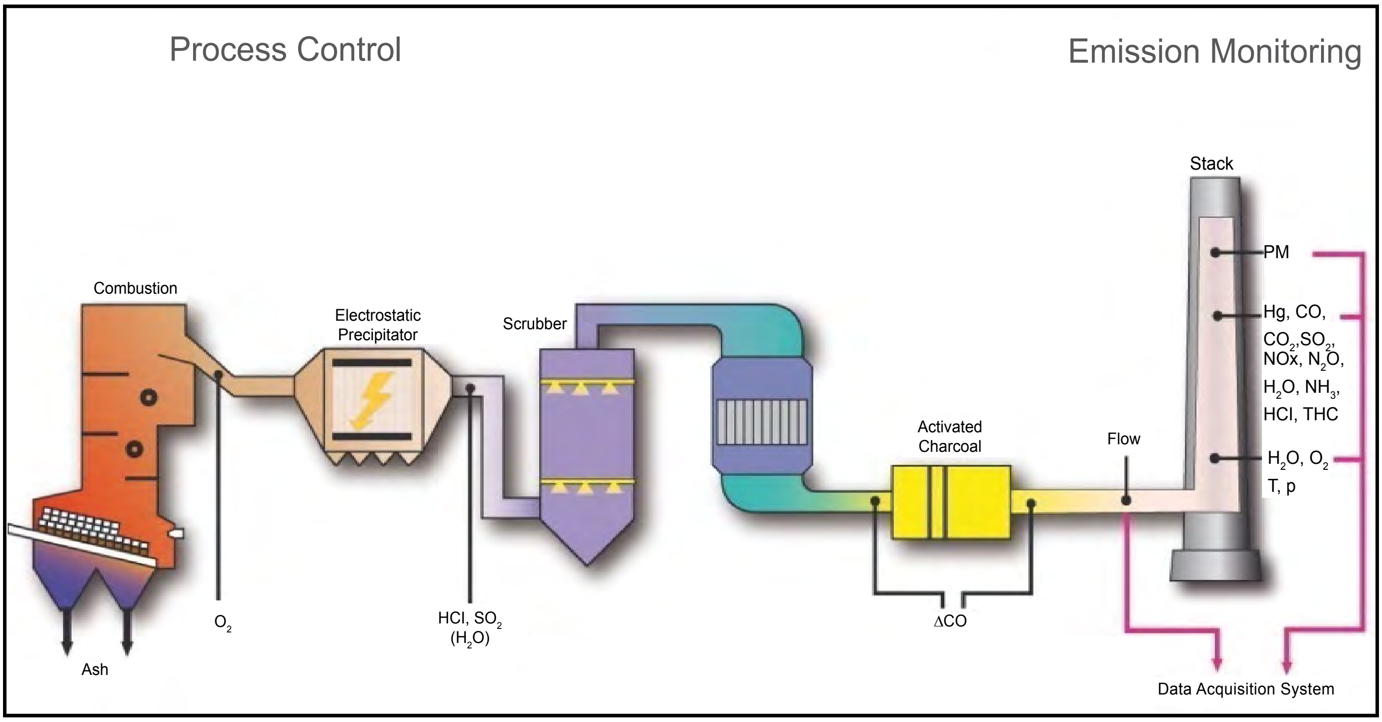

Figure 14: Incineration.

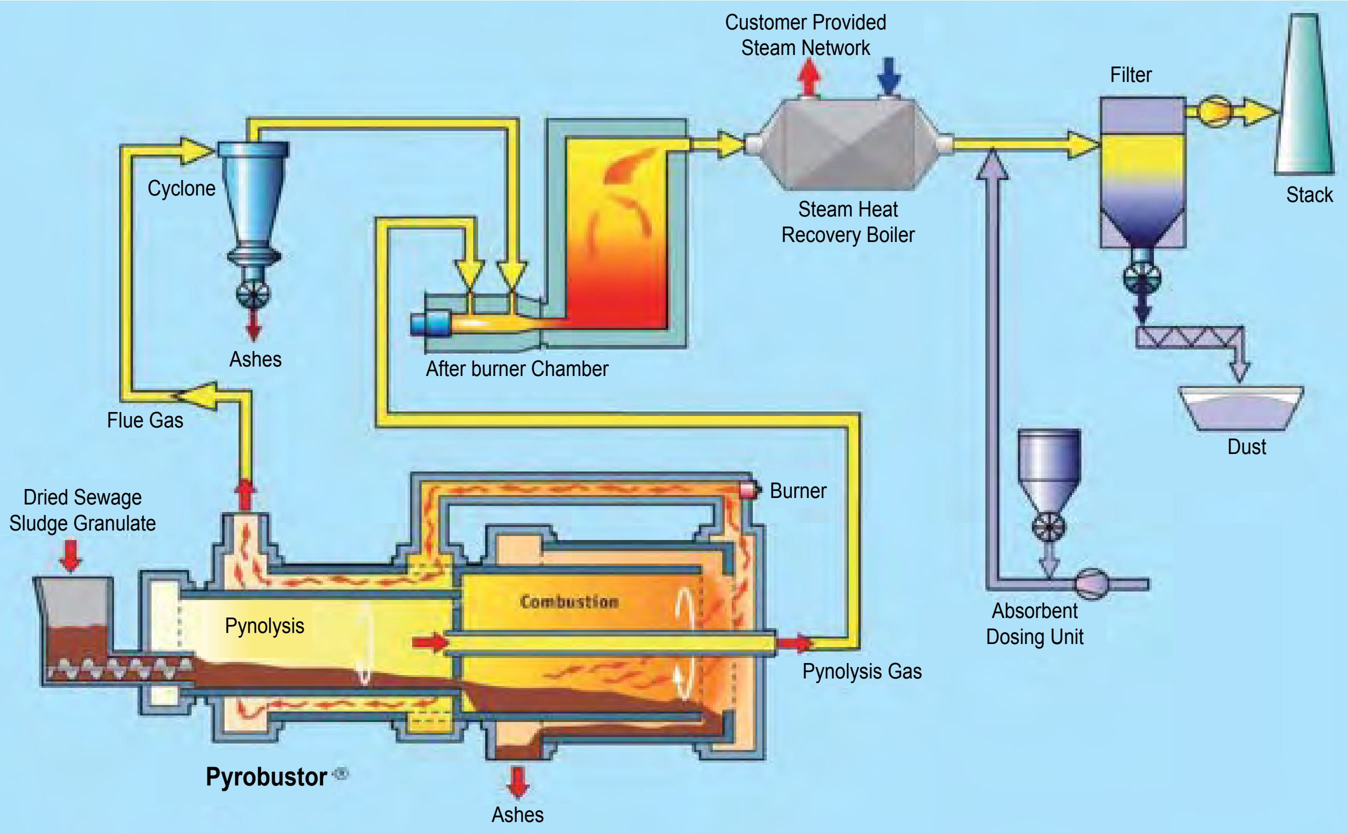

Figure 15: Pyrolysis.

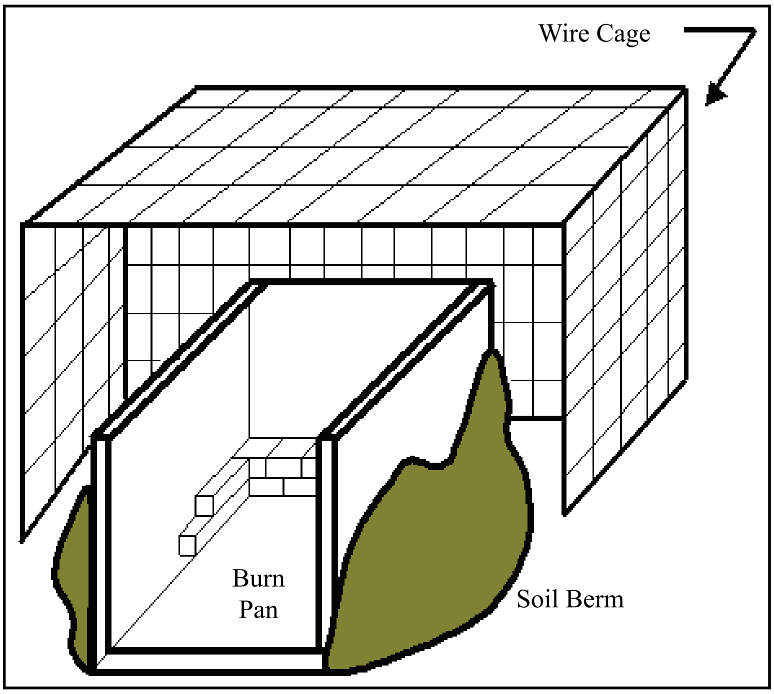

Figure 16: Open dump and open detonation.

Remediation Techniques	Types	Advantages	Disadvantages	References
Soil Vapor Extraction (Soil Venting) • Involves the installation of vertical and/or horizontal wells in the area of soil contamination. Vacuums are then applied through the wells to evaporate the volatile constituents of the contaminated mass which are subsequently withdrawn through an extraction well.		• Very efficient, readily available equipments and easy to install • Requires short treatment times (6-48 months).	• Effectiveness decreases in low soil permeability. • Useful only for the unsaturated zone.	[28,77]
Soil Washing • Uses solvents including water in combination with mechanical processes to scrub soils.		• Effectively reduces the volume of contaminant, therefore, further treatment or disposal is less problematic • Used commercially in large scale.	• Contaminant toxicity is unchanged, although volume is reduced. • Less effective when soil contains a high percentage of silt and clay • After treatment disposal costs are generated.	[17,25]
Soil Flushing • Water is passed through the contaminated soils with a solution that moves the contaminants to an area where they can be removed.		• Useful 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.	• Soils with low permeability or heterogeneity are difficult to treat • Long remediation times. • Requires hydraulic control to avoid the movement of contaminants off-site.	[23,41,56]
Encapsulation • Application of low permeability layers of synthetic textiles or clay caps on contaminated areas. Designed to limit the infiltration of precipitation and thus prevent leaching and migration of contaminants away from the site and into the groundwater		• Comprised of the physical isolation and containment of the contaminated material.	• Lithology of soil site controls the efficacy. • The efficiency of encapsulation decreases with time. • Implemented only with shallow contaminated soils.	[2,59]
Stabilization/solidification (S/S) • Relies on the reaction between a binder and soil to stop/prevent or reduce the mobility of contaminants.		• Useful and established remediation technology for contaminated soils in many countries in the world.	• Lack of expertise on technical guidance. • Uncertainty over the durability and rate of contaminant release. • Residual liability associated with immobilized contaminants remaining on-site	[66]
Stable Isotope Probing • A method to identify active microorganisms without the prerequisite of cultivation which has been widely applied in the study of microorganisms involved in the degradation of environmental pollutants.	Polar lipid derived fatty acid-based stable isotope probing (PLFA-SIP)	• Establishes the identity of microorganisms involved in biodegradation.	• Weaknesses of molecular methods (nucleic acid recovery, PCR bias, etc.) and incubation time may result in cross-feeding.	[11]
	DNA-based stable isotope probing (DNA-SIP)			[50]
	RNA-based stable isotope probing (RNA-SIP)			[23,24]
	Fluorescence in situ hybridization and secondary ion mass spectrometry (FISH-SIMS)			[54,55]
	Stable isotope characterization of small-subunit rRNA			[42]
Nanotech Remediation • Uses nanomaterials and nano-products without toxic ingredients to remove toxic chemicals from environment		• Used to stabilize and guard enzymes against mechanical and biotic degradation. Thus increases their half-life and permits recirculation in their use.	• Yet to be exploited commercially	[5,17,18]
Air Striping • Transferring of volatile components of a liquid into an air stream.		• Can achieve better than 95% removal efficacy for a range of organic compounds which are insoluble or slightly soluble in water.	• The presence of solids in wastewaters can foul steam strippers and therefore it is generally advantageous to remove these solids before stripping	[1,6]

Dehalogenation • This technology involves direct chemical stripping of halogen atoms from organics in soils, sediments, and sludges..		• Target compounds are halogenated organics, halogenated SVOCs and pesticides. • Used for soils and sediments contaminated with chlorinated organic compounds, especially PCBs, dioxins and furans.	• High clay and high moisture content increases treatment costs. • Not cost-effective for large waste volumes. • Sometimes diifficult to capture and treat the residuals.	[66]
Electrokinetic Remediation (EKR) • An in situ soil processing technology using electro-chemical and electro-kinetic processes to desorb (separate) and then remove metals and polar organic contaminants from low permeability soils.		• Has small impact on environment (soil removal is not required). • Metals are actually removed from soil unlike stabilization, which leaves the metals in the soil.	• Efficiency reduced by alkaline soils. • Requires soil moisture.	[60,69]
Electrodialytic soil remediation (EDR) • An electrokinetic method used for removal of heavy metals from soil (and particulate waste products) which uses exchange membranes for separating soil and processing solutions.		• Can treat the soil as a stationary wet matrix (*in-situ* or on-site) • Can treat the soil in a suspension (with the possibility for combining EDR with soil washing and only treat the fine fraction with EDR) (On-site).	• Yet to be exploited commercially	[56]
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	[9,15,72]
Ultraviolet Oxidation • Uses an oxygen-based oxidant (e.g., ozone or hydrogen peroxide) in combination with UV light.		• Chemicals used do not pollute the environment. • Successful with substances such as ferricyanides which cannot be removed by other methods.	• Low turbidity and suspended solids are necessary for good light transmission. • Free radical scavengers may interfere with the reactions.	[11,38]
Precipitation/ Flocculation • Uses non-directed physico-chemical complex cation reaction between dissolved contaminants and charged cellular components (dead biomass).		• Cost-effective	• Yet to be exploited commercially	[50]
Microfiltration • The goal is to separate dispersed oil phase from water using porous membranes.		• Removes dissolved solids effectively.	• Yet to be exploited commercially	[20]
Analytical Biosensors • The biosensors rely 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	[39,51,52]
Chemical Oxidation • Involves reduction/ oxidation (redox) reactions that chemically convert hazardous contaminants to more stable nonhazardous or less toxic compounds.		• Target treatment group is inorganics. • Also used but less effective for non-halogenated VOCs and SVOCs, fuel hydrocarbons and pesticides.	• Incomplete oxidation may occur depending upon the contaminants and oxidizing agent used • Not cost-effective for high contaminant concentrations. • Presence of oil and grease in the media reduces efficiency.	[8,12,28,30,31]

Table 2b: List of non-biological non thermal techniques used in remediation of contaminants with their advantages and disadvantages.

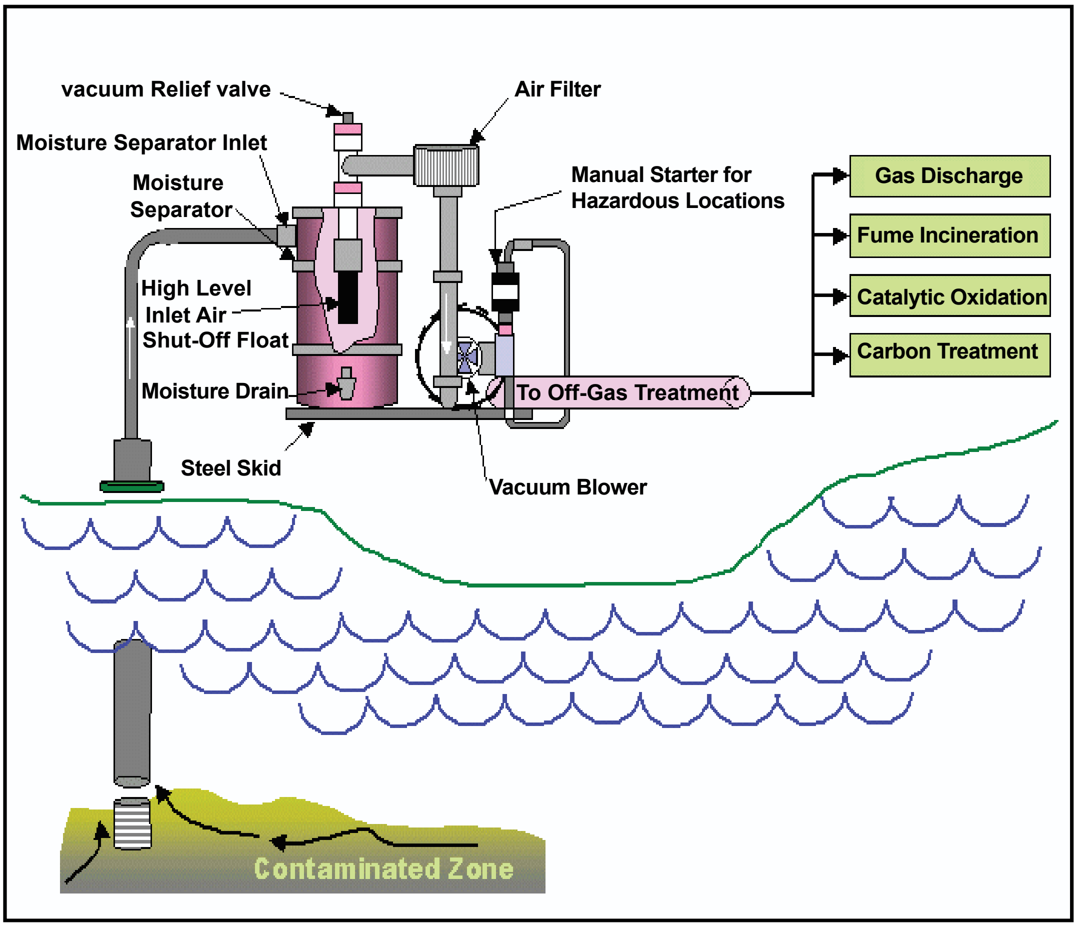

Figure 17: Soil vapor extraction.

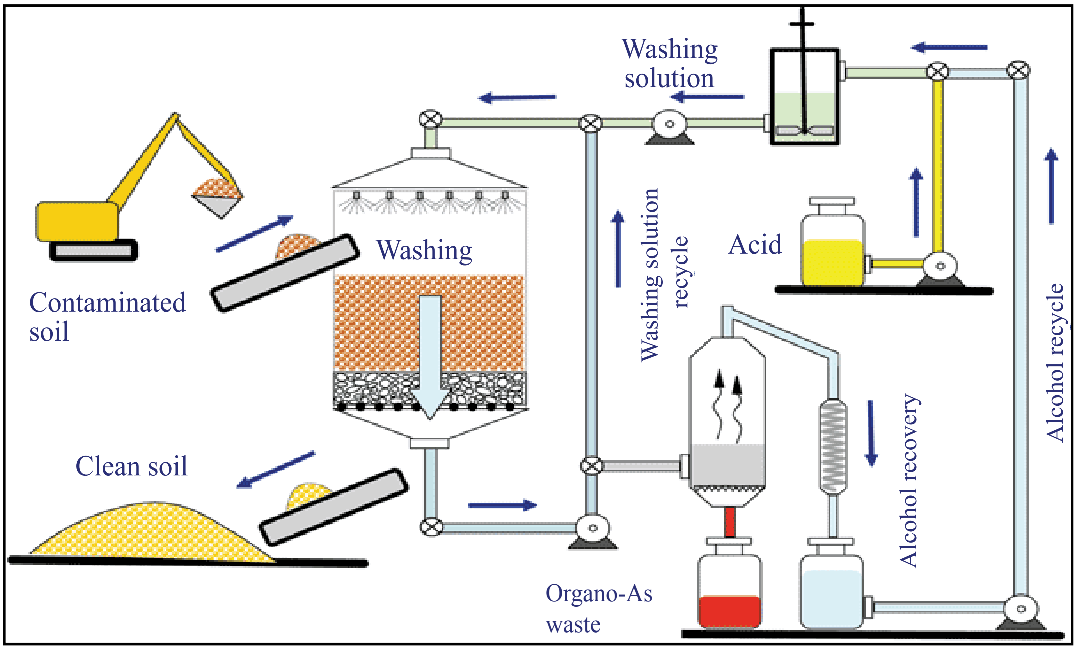

Figure 18: Soil washing.

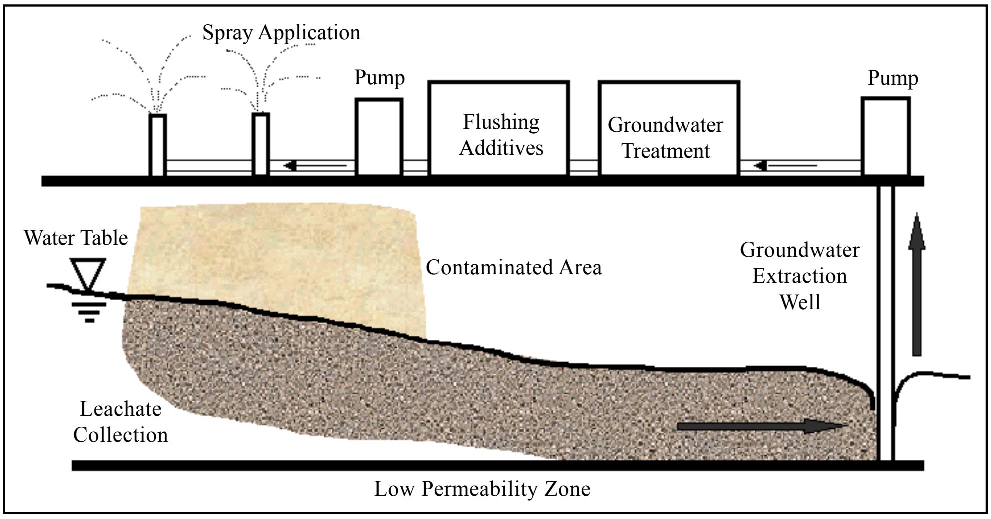

Figure 19: Soil flushing.

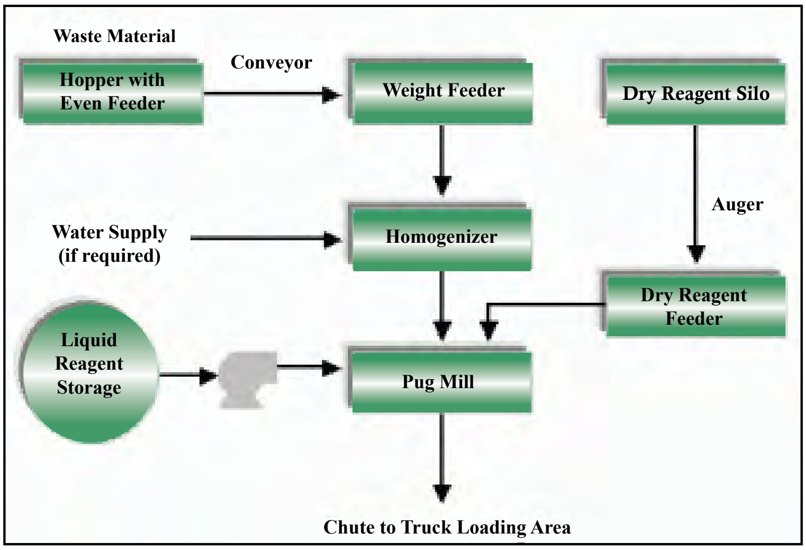

Figure 20: Solidification.

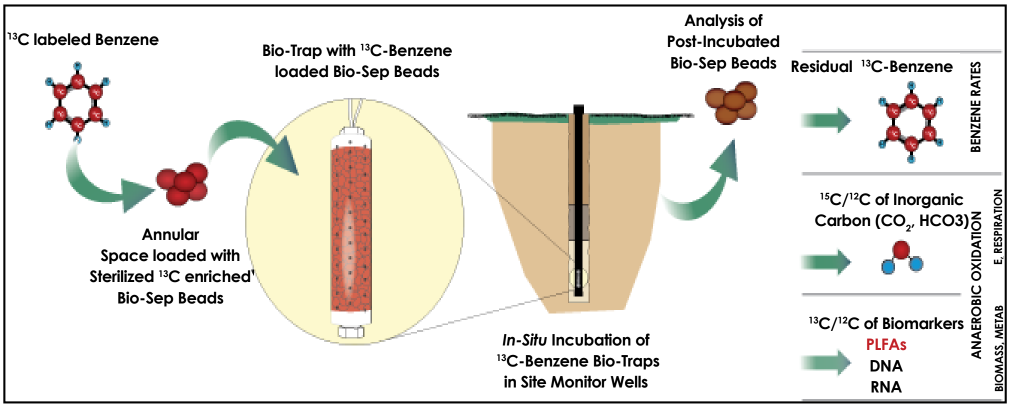

Figure 21: Stable isotope probing.

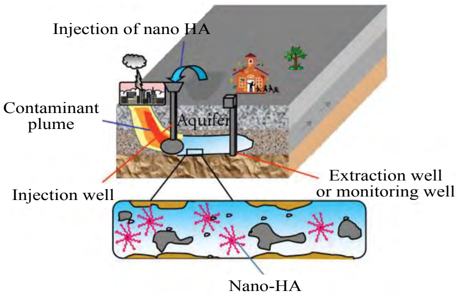

Figure 22: Nanoremediation.

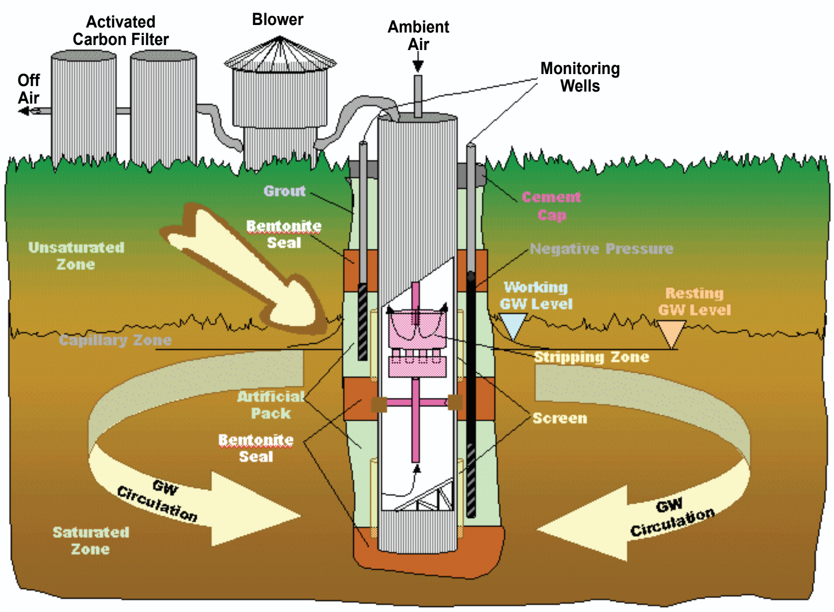

Figure 23: Air stripping.

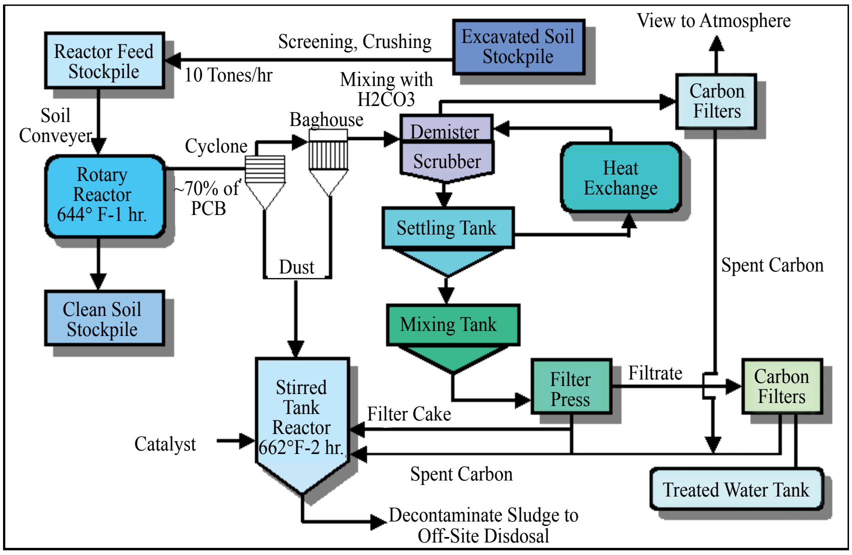

Figure 24: Dehalogenation.

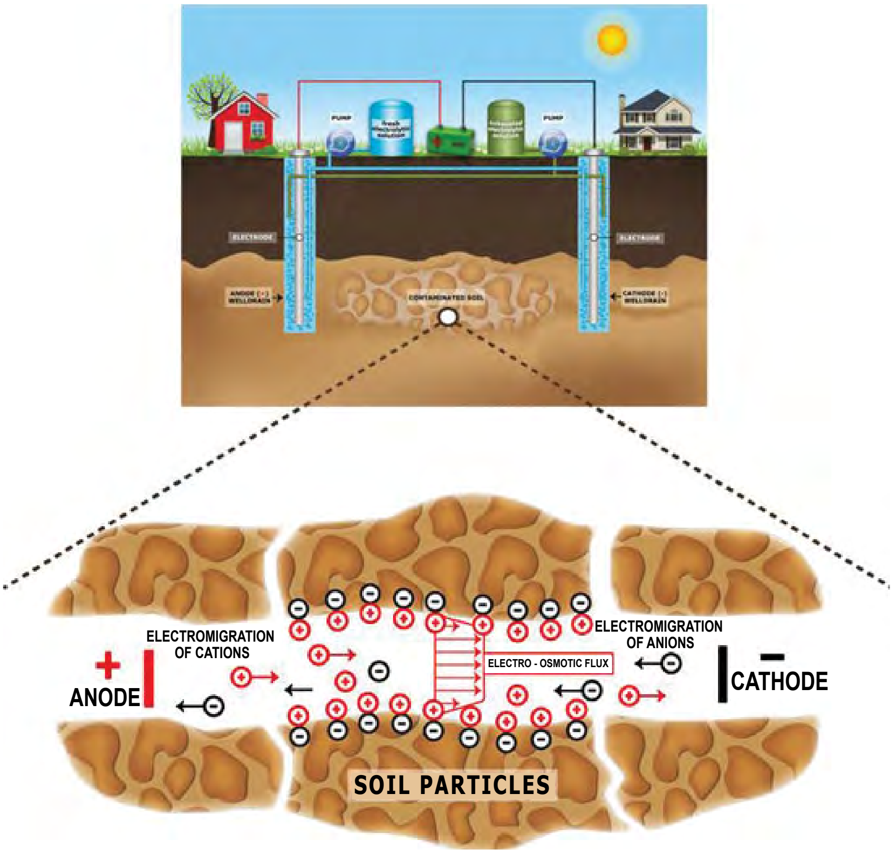

Figure 25: Electrokinetic remediation.

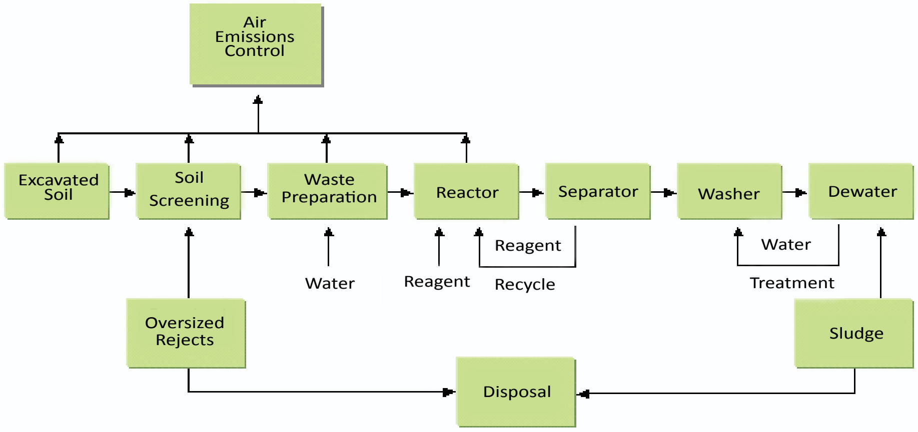

Figure 26: Chemical oxidation.

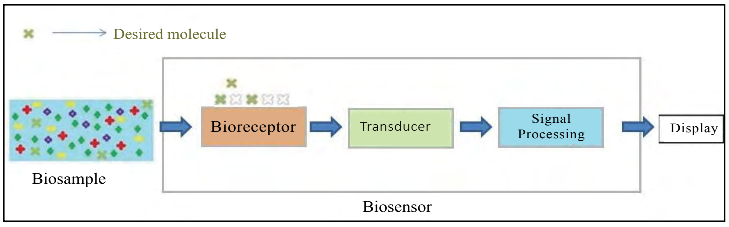

Figure 27: Analytical biosensor.

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Original Research Article

Use of Polymerase Chain Reaction for the Detection of Banana Bunchy Top Virus Infection in Three Successive Generation of Banana

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Abstract: Banana is one of the most important fruit in India. Viral infection causes a great loss in Banana production with a ratio of about 20-30% loss and occasionally reaching 50-80%. The most indigenous banana are infected by BBTV; Banana bunchy top virus, a complex circular single-stranded DNA virus with multiple genomic components. On mature plants infected with BBTV, they appear to be "bunched" at the top of the plant, new leaves emerge with difficulty, are narrower than normal, the symptom for which this disease is named. For the early detection of BBTV, PCR technique was used for the plant samples. Suckers were used for isolating genomic DNA using sodium sulphite method. DNA was quantified (400 ng/µl) and then PCR was used for detecting BBTV by using BBTV specific primers. The agarose gel was loaded with ladder DNA, followed by sample DNA. Among 3 DNA samples all showed amplification by specific primers of BBTV. During the course of my practical it has been clear that the third generation plant of banana was also infected by BBTV. Therefore it is very important to detect the presence of these virus early in infected plant and proper eradication of infectious plant, before it passes to another healthy plants by its vector *Pentagonia nigronervosa*.

Key Words: Banana, BBTV, sucker

Introduction

The word banana is derived from the Arabic word for finger. There are more than 300 kinds of banana but only a few are commercially important. Banana is the man's oldest and most valued fruit crop. It is prized for its nutritive value with high carbohydrates (22.2%), fiber (0.84%) and protein (1.1%) with less fat (0.2%) and water (75.7%). The World production of banana is about 95 million tons and most of the production is consumed locally. In India, it's per hectare yield is also the highest (30 - 35 MT/ha) of all the fruits. Total area under banana is 3.70 lakh ha, next to Mango. Among the States, Maharashtra contributes the maximum area of about 90,000 ha. In Maharashtra, area under banana is concentrated in Jalgaon district with nearly 60% area in that district.

Banana is an important fruit crop in India. Four major viral pathogens viz., bunchy top virus, streak virus, bract mosaic virus and cucumber mosaic virus are known to cause significant yield loss in India and spread vertically through tissue culture plants. Viral diseases threaten banana production with a ratio of about 20-30% and occasionally reach 50-80%. Banana bunchy top viral disease is most severe on banana. It is very destructive viral disease in many countries including India. It causes severe losses because infected plants produce no fruit. It also affects some ornamentals such as *Canna sp.* BBTV, is transmitted by aphids in the persistent manner.

BBTV is the causal agent of banana bunchy top disease (BBTD) (Dale, 1987), and is classified as a member of nanoviruses based on its molecular characterization (Dugdale *et al.,* 1998 and Harding *et al.,* 2000). BBTV has only recently been isolated and characterized (Wu and Su, 1990; Wu and Su, 1991: Harding *et al.*, 1991). Virus diseases are serious, as insect vector are abundant and there are many alternative hosts, (M.K.Dhanya *et al.,* 2007). Bananas infected by BBTV show dash-like streaks as the first observable symptoms. As the disease progresses the leaf blades become narrow, and plants show symptoms such as stunting and bunched leaves. The fruits, if any, are malformed. Finally, the disease (Banana bunchy Top Disease; BBTD) results in plant death. Because of this direct influence on productivity, BBTV is considered to be the

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most economically destructive disease of banana. The disease is widespread in Asia.

BBTV is a phloem-limited virus found in a very low concentration in virus-infected plants, which is a major obstacle in the production of specific polyclonal antibodies (PAbs) for its detection. Dale (1987) recommended a strategy for controlling BBTV based on identifying virus-infected plants as early as possible followed by removing the diseased plants and replanting virus-free banana plants. Banana bunchy top is transmitted in a persistant manner only by banana aphid (*Pentalonia nigronevosa*) (Fig. 1A), its sole biological vector (Lava Kumar, 2009) and through vegetative propagation, but not by artificial manual inoculation.

Figure 1A: Wing less Banana Aphid (*Pentalonia nigronervos*)

The banana aphid, which acquires the virus after at least four (but usually about 18) hours of feeding on an infected plant. The aphid can retain the virus through its adult life, for a period of 15–20 days. During this time, the aphid can transmit the virus to a healthy banana plant by feeding on it, possibly for as little as 15 minutes but more typically for about two hours. Disease symptoms usually appear about a month after infection. Controlling aphids is essential to reducing the spread of BBTV. Aphid populations may increase during the spring or during warm, dry weather, and they can often be found on suckers. To minimize the spread of BBTV, all infected plants and their mats must be destroyed with an approved herbicide. Destroying infected plants with Roundup Ultra Max can help reduce the spread of BBTV.

Bananas and plantains are vegetatively propagated crops, and as such, virus contamination of planting material is a significant issue. Once infected, a plant and its progeny will remain infected thereafter. Although tissue culture is an effective means of freeing planting materials of most pests and diseases, viruses are usually very difficult to eliminate. However, even though some viruses have only been recently been discovered or characterized, functional detection methods are available for all of them. Good summaries of the characteristics, symptoms, and accepted indexing methods for most of the above viruses can be found in (Diekmann and Putter, 1996). While symptomatology is the easiest of the detection methods, it is not always reliable. In some cases, classic symptoms may be displayed allowing fairly certain identification of the virus involved. However, mixed infections are a frequent occurrence and in these cases symptoms are not always clear. Symptoms caused by different viruses can be similar and infected plants may even be symptom less.

Virus indexing is an important step to assure the mother plants free of viruses before mass multiplication and distribution. PCR is widely used for virus testing for many of the plant viruses. Various forms of serological assays are currently available for all seven of the banana viruses discussed. When suitable antisera are available, the most sensitive and efficient practical assay for banana virus indexing is enzyme-linked immunosorbent assay (ELISA). Specific polyclonal and monoclonal antibodies are available for BBTV, BSV, CMV, BBrMV (Diekmann and Putter, 1996) and the banana potexvirus.

Material and Methods

Source of material

The leaf samples of *Musa paradisiaca* were collected from different origin (Fig 1B, IC and ID). First two generation sample were collected from the commercial field of Rajnandgaon and the third generation samples were collected from commercial field of banana from Nanadanvan, Raipur. The leaves samples of all the three generation were collected and were respectively diagnosed for BBTV infection. The leaves were selected on the basis of the characteristic symptoms of Banana Bunchy Top Virus (BBTV), on mature plants infected with BBTV, new leaves emerge with difficulty, are narrower than normal, are wavy rather than flat, and have yellow (chlorotic) leaf margins. They appear to be "bunched" at the top of the plant, the symptom for which this disease is named. Severely infected banana plants usually will not fruit, but if fruit is produced, the banana hands and fingers are likely to be distorted and twisted.

Figure 1B: First Generation Plants

Figure 1C: Second Generation Plants

Figure 1D: Third generation Plants

The samples were selected on the basis of the characteristic symptoms of Banana Bunchy Top Virus (BBTV), on mature plants infected with BBTV, new leaves emerge with difficulty, are narrower than normal, are wavy rather than flat, and have yellow (chlorotic) leaf margins. They appear to be "bunched" at the top of the plant, the symptom for which this disease is so named. Severely infected banana plants usually do not fruit, but if fruiting occurs, the banana hands and fingers are likely to be distorted and twisted. Then 200mg weighed plant materials (Leaves) were grinded in mortar and pestle using liquid nitrogen to a fine powder. The sample was transferred to a presterile eppendorf's tubes.

DNA isolation

There are two successive methods for isolation of Plant Genomic DNA but we had worked on Sodium Sulphite method (V.K. Baranwal, S. Muzumder, 2003). The PCR method was carried out with ready to use chemicals from "Genie, Bangalore" and the BBTV specific reverse and forward primer were made available by IARI, New Delhi. Separation of Amplified DNA was done through gel electrophoresis and the gel was visualized on UV transilluminator and photographed by using digital camera.

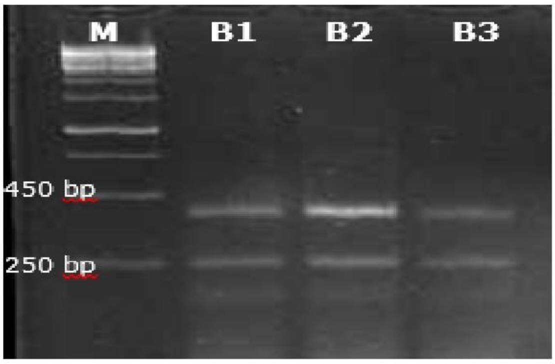
DNA Quantification

The isolated DNAs were loaded in the well of 0.8 % Agarose gel along with known standards and electrophoresis was done under for $1^{1/2}$ hour. The quantity of DNA was estimated using quantitative method. Almost uniform amount of DNA was extracted from each sample. The quantity was estimated to be ~400 ng/µl. The quantified DNA was used for PCR assay by BBTV specific primers.

PCR assay by BBTV specific Primers

An investigation was made to access the occurrence of Banana Bunchy Top Virus in the samples collected from Mother Orchid, Commercial Field. Concentrated samples of DNA isolated from infected plants were used for PCR assay and a set of BBTV specific primers were used to detect integrated viral DNA in Banana genomic DNA in the collected samples. PCR was conducted in the volume of 0.8µl agarose gel electrophoresis is then used for separation of amplified DNA in influence of electric field. The agarose gel was loaded with 1 kb + ladder in 1st Lane, followed by and lane 2-4 carry 3 samples of DNA isolated from respective samples of banana (Fig. 1E).

Figure 1E: The Electrophoretic pattern of the samples showing all the samples infected with BBTV



M: Ladder 1 Kb
B1: Banana Sample from Rajnandgaon field
B2: Banana Sample from Rajnandgaon field
B3; banana Samples from Nanadanvan Field

Detection of BBTV Infection using BBTV specific primers.

The presence of BBTV infection was detected by using BBTV specific primers (450 to 250 bp).

Table 1A: BBTV specific primers

Primer	Forward primer	Reverse Primer	Location
BBTV (~250 amplicon)	TAGATCCATGG TCAGACAAGAA	ATAAAGCTTTCAAA CATGATATGT	206±229
BBTV (~450 Internal control)	GATCTATTGAA GCTGTG	CTAACTTCCATGTCT CT	426±452

Result

The presence of the bands indicates that the selected sample was infected by viral genome. The result was that that all three samples collected from Rajnandgoan and Nandanvan showed the presence of BBTV virus. As shown in fig 5 size of amplified DNA is approximately 450- 200bp when compared with ladder. Among 3 DNA samples of Banana, lane 2, 3 & 4 showed amplification by specific primers of BBTV s. The PCR assay was conducted with one positive and one negative control.

On the basis of PCR assay performed by BBTV specific primer, sample 2, 3 & 4 of banana showed integrated BBTV DNA. In the present investigation it was observed that virus was detected in sample form plant belongs to farmer's field & mother orchid.

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RESEARCH ARTICLE

Biodegradation of keratin from chicken feathers by Bacterial species as a means of sustainable Development

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Abstract

Keratin is an insoluble protein macromolecule with very high stability and low degradation rate. Keratin is mainly present in hair, feather, nails, wool and horns. Feather constitutes over 90% protein, the main component being beta-keratin, a fibrous and insoluble structural protein extensively cross linked by disulfide bonds. This renders them resistant to digestion by animal, insects and proteases leading to serious disposal problems. In addition to this, feather waste is produced at the rate of 22 million kg per year (US alone). Use of keratinolytic microorganisms for feather degradation is an economical, environmental friendly alternative. Keratinases which are produced by these keratinolytic organisms could be used to degrade feather waste and further the digested products could be an excellent material for producing animal feed, fertilizers or natural gas. A feather-degrading bacterium was isolated from poultry waste. This bacterium was grown in basal media with feathers as its primary source of carbon, nitrogen, sulfur and energy. The organism is rod shaped, highly motile, endospore forming, catalase positive and gram negative. Phenotypic characterization carried out in our laboratory showed that this novel gram negative bacterium belongs to *Bacillus* genus. The organism is designated as *Bacillus* and named as *Bacillus* sp PW-1. The isolated strain has activity of 50 U/ml. This novel keratinolytic isolate could be a potential candidate for degradation and utilization of feather keratin.

Key words: Biodegradation, keratin, feather, poultry waste, keratinolytic

Introduction

Insoluble and hard-to-degrade animal proteins are ubiquitously present throughout animal bodies. Enormous numbers of these proteins are generated in the meat industry in a mixture of bones, organs and hard tissues, finally being converted to industrial wastes, the disposal of which is tremendously difficult. Most animal proteins (feathers) are currently disposed of by incineration. This method, however, has ecological disadvantages in terms of an apparent energy loss and the production of a large amount of carbon dioxide.

Thus, an innovative solution to these problems is urgently needed[1]. Feather wastes are generated in large quantities as a byproduct of commercial poultry processing. Feathers represent 5-7% of the total weight of mature chickens. Feathers are made up primarily of keratin, which is also found in the claws and armour of reptiles and the hooves, horns, hide, hair and nails of mammals. These feathers constitute a sizable waste disposal problem.

Several different approaches have been used for disposing of feather waste, including land filling, burning, natural gas production and treatment for animal feed. Most feather waste is land filled or burned, which involves expense and can cause contamination of air, soil and water. Keratin, by virtue of its insolubility and resistance to proteolytic enzymes, is not attacked by most living organisms. Nevertheless, keratin does not accumulate in nature and, therefore, biological agencies may be presumed to accomplish its removal. Several insects, including clothes moth larvae, carpet beetles and

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chewing lice are known to digest keratin. The common occurrence in nature of microorganisms that readily and, in some cases, preferably grow on keratinaceous substrates has supported the general belief that certain microorganisms can digest keratin[2].

Bacterial strains are known which are capable of degrading feathers. These bacterial strains produce enzymes which selectively degrade the beta-keratin found in feathers. These enzymes make it possible for the bacteria to obtain carbon, sulfur and energy for their growth and maintenance from the degradation of betakeratin. An enzyme capable of degrading protein is known as a protease and is described as having proteolytic activity. An enzyme which degrades keratin is a keratinase, while a beta-keratinase is an enzyme capable of degrading beta-keratin. An enzyme which degrades keratin can also be described as having keratinolytic activity.

Keratinases from bacteria are isolated and characterized. For instance, keratinase from *Bacillus sp.*[3 11 12 13.] *Bacillus licheniformis*[4 5 6 7], *Burkholderia*, *Chryseobacterium*, *Pseudomonas*, *Microbacterium sp*[8] *Chryseobacterium sp.*[9 10] (Brandelli, 2005; Riffel *et al*., 2003), *Streptomyces sp.*[11 12] were isolated and was studied with respect to various parameters.

Characterization based on 16S rRNA was carried out for genus *Terrabacter*[12.] On the basis of physiological, morphological and 16S rDNA the new isolate was found to be a member of Thermotogales order and was identified as *Fervidobacterium pennavorans*. The strain was highly related to *Fervidobacterium islandicum* and *Fervidobacterium pullulanolyticum*[13.] Membrane ultrafiltration and carboxymethyl cellulose ion-exchange and Sephadex G- 75 gel chromatographies were used to purify the enzyme from *Bacilus licheniformis* PWD-1[14].

Materials and Methods

Isolation of feather degrading bacteria

Enrichment

1gm of poultry waste was serially diluted in order to reduce the initial number of micro organisms. This dilution was then inoculated into basal feather broth. Feathers were washed, dried and hammer milled prior to being added to the medium. The medium was sterilized by autoclaving. All incubations were done at 37°C with shaking at 120 rpm in a controlled environment shaker[15]

Screening on Skim milk agar plates

Skim milk agar (Himedia) was prepared and the above dilutions were streaked on milk agar plates for testing the caseinolytic activity of the organism. Bacteria were inoculated onto plates and incubated at 37°C for 24 h. Strains that produced clearing zones in this medium were selected[3].

Screening on Keratin agar plates

The colonies obtained from skim milk agar plates were transferred to keratin (Himedia) agar plates.

Subculturing

The organism screened with Keratin agar plates was subcultured by continuously growing the bacterium in basal broth medium (4 days at 37°C, 120rpm) and subsequently streaking on basal agar medium (2% agar, 2 days 37°C).

Identification of Isolated feather degrading bacteria

Table -1: Culture Characteristics

Sl. No.	Agar Plate	Characters	Results
1	Nutrient Agar Plates	Size	Moderate
		Pigmentation	Cream Colored
		Form	Circular
		Margin	Lobate
		Elevation	Raised
2	Nutrient Broth Culture		Uniform with fine turbidity
3	Nutrient Agar Slants	Abundance of growth	Moderate
		Pigmentation	Cream Colored
		Optical Characteristics	Opaque
		Form	Filiform

Gram Stain, Spore staining, Motility test, Catalase Test[16] .

Characterization of the isolate using Biochemical assays

IMViC Test, Hydrogen Sulfide Test, Urease Test, Litmus Milk Reactions, Nitrate Reduction Test, Carbohydrate Fermentation, Starch hydrolysis and Gelatin liquefaction 16.

Assay for keratinase activity The test described below was developed in order to simplify analytical work on keratinase. Azo-keratin hydrolysis provides a colorimetric assay for enzymatic activity on keratin[15] . The inoculum was incubated in 0.9%NaCl at 37°C for 24 hours before inoculating into the basal feather broth for the crude enzyme assay. A 5% inoculum was inoculated in 100 mL of basal feather medium at 37°C and 120rpm.

Synthesis and enzymatic hydrolysis of azo-keratin Synthesis

Ball-milled feather powder was prepared as described in[17]. A 1 g portion of the feather powder (the keratin source) was placed in a 100-ml round-bottomed reaction flask with 20 mL of deionized water. The suspension was mixed with a magnetic stirrer. Two ml of 10% $NaHCO_3$ (weight per volume) were mixed into the 3 feather suspension (Lin *et al.*, 1992). In a separate 10-ml tube, 174 mg of sulfanilic acid were dissolved in 5 mL of 0.2 N NaOH. Sixty-nine mg of $NaNO_2$ were then added to the tube and dissolved. The 2 solution was acidified with 0.4 mL of 5 N HCl, mixed for 2 min and neutralized by adding in 0.4 mL of 5 N NaOH. This solution was added to the feather keratin suspension and mixed for 10 min. The reaction mixture was filtered and the insoluble azo-keratin was rinsed thoroughly with deionized water. The azo-keratin was suspended in water and shaken at 50°C. for 2 hr and filtered again. This wash cycle was repeated until the pH of the filtrate reached 6.0-7.0 and the spectrophotometric absorbance of the washing at 450 nm was less than 0.01 (Burtt and Ichida, 1999). Finally, the wash cycles were repeated at least twice using 50 mM potassium phosphate buffer, pH 7.5. The azo-keratin was washed once again with water and dried in vacuum overnight at 50°C. The resulting product is a chromogenic substrate that can be incubated with

Table 2: Results of additional morphological, physiological and biochemical tests conducted on the isolate

Experimental Details	Observations
Gram Stain	Negative
Shape and arrangement	Rods in single and in chains
Endospore stain	Positive
Litmus milk Reactions	Peptonization
Carbohydrate fermentation with lactose	Acid with gas
Sucrose	Acid with gas
dextrose	Acid with gas
Nitrate Reduction	Positive
Motility Test	Positive
Indole Production	Positive
Methyl Red Test	Negative
Voges – Proskauer Test	Positive
Citrate Utilization	Positive
Catalase activity	Positive
Starch Hydrolysis	Negative
Hydrogen Sulphide Test	Negative
Urease activity	Positive
Gelatin liquefaction	Positive

enzyme solution to produce and release soluble peptide derivatives that cause an increase in light absorbance of the solution[18].

Enzymatic hydrolysis of azo-keratin

This procedure tested the keratinolytic activity of keratinase on azokeratin. To begin the process, 5 mg of azo-keratin was added to a 1.5-ml centrifuge tube along with 0.8 mL of 50 mM potassium phosphate buffer, pH 7.5. This mixture was agitated until the azo-keratin was completely suspended. A 0.2-ml aliquot of supernatant of crude enzyme was added to the azo-keratin, mixed and incubated for 15 min at 50°C with shaking. Assay conditions were the same for each enzyme sample except that pH and temperature were adjusted to their optima for the specific enzyme. The reaction was terminated by adding 0.2 mL of 10% trichloroacetic acid (TCA). The reaction mixture was filtered and analyzed for activity[18].

The absorbance of the filtrate was measured at 450 nm with a UV-160 spectrophotometer (LaboMed.Inc). A control sample was prepared by adding the TCA to a reaction mixture before the addition of enzyme solution. A unit of keratinase activity was defined as a 0.01 unit increase in the absorbance at 450 nm as compared to the control after 15 min of reaction[18].

Results

Isolation and adaptation of feather-degrading microorganism

It was found that a previously enriched, feather - degrading culture contained microorganism exhibiting keratinolytic activity. The isolate is a rodshaped bacterium which appeared singly and in chains. It displayed clearing zone when streaked onto the skim milk agar plates. Cells of the isolate were grown on basal feather agar and transferred at frequent intervals to the basal medium, containing finely chopped feathers. Cells were also grown on keratin containing media and subcultured at regular intervals. Eventually, after several weeks of repeated selection and subculturing a pure culture of the bacterium was obtained.

Identification and characterization of feather degrading isolate

Microscopic observation of the isolate showed a straight rod with endospores. The bacterium grew aerobically, strongly catalase positive, Gram negative and was highly motile. Additional morphological, physiological and biochemical tests were conducted (Table 1 and 2).

The isolate was able to grow on Thrones[19] medium which is specific for *Bacillus licheniformis*. We propose "PW" as the strain designation to indicate isolation of the strain from poultry waste.

Degradation of feathers by isolate

It was observed that aerobic growth by the isolate on feathers, with the feathers as its primary source of carbon, nitrogen, energy and sulfur, resulted in nearly complete degradation of the keratin after 7 to 10 days of incubation at 37°C. Biodegradation was measured as the increase in the absorbance at 450 nm by the Azokeratin hydrolysis enzyme assay.

Isolated strain (PW) has activity of 50.0U/ml as compared to PWD-1 (30.50 U/ml). Change in absorbance at 450 nm with respect to days after inoculation was recorded and it was found that the maximum absorbance is on 12th day (Table 3).

Table 3: Change in absorbance of PW strain with respect to days of incubation

Days	A_{450nm}
0	0
1	0.03
2	0.05
3	0.06
4	0.08
5	0.09
6	0.11
7	0.12
8	0.13
9	0.16
10	0.18
11	0.20
12	0.21
13	0.20
14	0.19
15	0.11

Discussion

A bacterium isolated from poultry waste has been shown to degrade feather keratin by using feathers as a primary source of energy, carbon, nitrogen and sulfur. The bacterium was isolated from an anaerobic habitat; however, it showed maximum growth under aerobic conditions, as would be expected of a member of the family Bacillaceae[7] . One explanation for the presence of this species in a poultry waste may be that the bacterium is indigenous to the chicken gut. However, it is more likely that, it was indigenous to the environment in which poultry excreta are collected. This environment also contains feathers and the isolate may have adapted to utilize this substrate.

When cells of *Bacillus* sp PW were grown aerobically on basal feather medium, they yielded appreciable degradation as measured by the azokeratin hydrolysis assay. Such an increase in absorbance did not occur when the cells were subjected to growth conditions in the nutrient broth in comparison with the basal feather medium. It is noteworthy that the increase in absorbance initially lags behind the growth of the bacterium. This may be due to enzymes liberated by the bacterium following a period of logarithmic growth. Such enzymes have been documented in strains of *B. licheniformis.* These enzymes appear in the extracellular medium only during the post logarithmic phase of growth. The increase in the absorbance of the filtrate of the azokeratin medium during the degradation of azokeratin by this strain of *Bacillus* PW is evidence that this bacterium possesses a protease (s) capable of reducing the disulfide bonds of keratin. The isolated strain was found to be Gram negative[19].

The degradation of feathers by a *Bacillus* sp. has not been reported previously; however, Molyneaux (1959) reported the isolation, from the desmoids cyst of a sheep, of a *Bacillus* sp. which digested wool keratin. He noted that although the growth of various bacilli on wool had been previously reported, his isolate was the first that could degrade nonsteam-sterilized or native wool keratin. Molyneaux (1959) conducted an extensive classification of his isolate but did not assign a species name. Many of the results of biochemical tests he conducted match those we observed for *B. licheniformis* PWD-1. However, the *Bacillus* isolated by[20]grew better under mesophilic temperatures and was also unable to reduce nitrate to nitrite.

Comparison of PW with PWD-1

B. licheniformis strain PWD-1 with ATCC Accession No. 53757[15] is found to be a gram positive (but gram variable) bacteria where as PW is gram negative. Bacterial cells are found both singly and in chains. PW is also rod shaped and found both singly and in chains. One subterminal endospore is formed per cell, the endospore being centrally located and round or oval in shape. As with PWD-1, PW is also endospore forming and motile. PWD-1 produces acid, but not gas, from L-arabinose, D-xylose (weakly), D-glucose, lactose (weakly), sucrose and D-mannitol, where as PW is both acid and gas producer. PWD-1 can utilize both citrate and propionate as a carbon source, similar to PW. PWD-1 liquefies gelatin as that of PW. PWD-1 reduces, but does not reoxidize methylene blue. It reduces nitrate to nitrite, but it does not reduce nitrite. Both PW and PWD-1 is Voges Proskauer positive. It decomposes hydrogen peroxide but not tyrosine, is negative for indole and is positive for dihydroxyacetone. PW is also positive for indole. PWD-1 is negative in the Litmus milk acid test, negative in the Litmus milk coagulation test and negative in the Litmus milk alkaline test, but is positive in both the litmus milk peptonization and litmus milk reduction tests. PW is positive in litmus milk peptonization.

Future Prospective

Purification and characterization of keratinase, studying the kinetics of enzyme, Analysis of reduction in the disulfide bonds, analysis of free amino acids by free amino group assay, testing for the range of substrates, comparison of activity of keratinase with other known and commercially used proteases, testing for the dehairing activity, effect of inhibitors, enhancing the activity of keratinase, submerged state fermentation and large scale production of keratinase, immobilization of keratinase.

Conclusion

A feather-degrading bacterium was isolated from poultry waste. This bacterium was grown in basal media with feathers as its primary source of carbon, nitrogen, sulfur and energy. The organism is rod shaped, highly motile, endospore forming catalase positive and gram negative. Phenotypic characterization carried out in our laboratory showed that this novel gram negative bacteria belongs to *Bacillus* genus. The organism is designated as Bacilus and named as *Bacillus* sp PW. We obtained an activity of 50U/ml as that compared to 30.5U/ml of PWD-1. This novel keratinolytic isolate could be a potential candidate for degradation and utilization of feather keratin.

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NEWS

New Therapeutic Target That Prevents Cell Division

Oct. 8, 2013— Cell division is an essential process for the development of an organism. This process, however, can cause tumour growth when it stops working properly. Tumour cells accumulate alterations in their genetic material, and this makes them divide in an uncontrolled fashion, thus encouraging growth of the tumour. Over the past few years, knowledge of the regulation of this process has led to the discovery of new therapeutic strategies based on blocking cell division or mitosis.

The Cell Division & Cancer Group, led by Spanish National Cancer Research Centre (CNIO) researcher Marcos Malumbres, has managed to decode a new mechanism that regulates cell division, in which the key molecule involved, Greatwall - also known as Mastl- could be a new therapeutic target for oncology treatments. The study is published today in the scientific journal*Proceedings of the National Academy of Sciences*(*PNAS*).

GREATWALL: A KEY PLAYER OF THE CELL DIVISION PUZZLE

The control of cell division or mitosis depends on many proteins, amongst them, Aurora and Polo. Currently, many pharmaceutical companies have shown interest in these molecules, for which inhibitors have already been developed, some of which are currently undergoing clinical trials in oncology.

Greatwall, the protein Malumbres's group has focused their work on, is also a protein that regulates cell division. Until now, almost all of the studies on this protein were carried out on the Drosophila melanogaster fly or on other invertebrate bodies. CNIO's Cell Division & Cancer Group, in collaboration with researchers from the National Centre for Scientific Research (CNRS) in Montpellier, France, has now generated the first genetic model of this protein in mammals, using the mouse as a model.

Thanks to this mouse model, the authors of the work have been able to see that cells lacking Greatwall are not capable of adequately dividing themselves: by eliminating Greatwall, cellular DNA does not form the right structure at the moment of cell division, the cell collapses and this prevents them from continuing to divide.

A NEW TARGET FOR CANCER THERAPY

As Mónica Álvarez Fernández, one of the group's researchers and the first author of the article, says: "the next step now is to explore the potential therapeutic applications of this discovery."

One of the therapeutic advantages Greatwall offers, and one that differentiates it from other mitotic proteins, is that it acts by blocking the function of the PP2A phosphatase, a tumour suppressor frequently altered in human cancer. This implies that the inhibition of Greatwall could, at the same time, slow down cell division and reactivate tumour suppressor PP2A, a protein capable of inhibiting many of the oncogenic molecular pathways involved in cancer development.

The key now is to find out which tumours would benefit from using this strategy, as well as to develop compounds capable of inhibiting this protein. With regard to both of these aspects, CNIO's research group is already actively working with other groups and clinical units.

"Therapeutics development is currently in need of novel targets that attack tumours in a different way," says Malumbres, "and Greatwall offers new strategies amongst which can be found reactivating a very important tumour suppressor, something for which there are no direct therapies at the moment."