BIODEGRADATION OF SOME MODEL POLYCYCLIC AROMATIC HYDROCARBONS BY BACTERIAL STRAINS ISOLATED FROM SOIL AND WATER

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A dissertation submitted to the University of Peshawar in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology and Microbiology.

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This dissertation is submitted by Nadia Bibi in partial Fulfillment of the requirements for the Degree of Doctor of Philosophy in Microbiology

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CERTIFICATE OF APPROVAL

This dissertation titled **"BIODEGRADATION OF SOME MODEL POLYCYCLIC AROMATIC HYDROCARBONS BY BACTERIAL STRAINS ISOLATED FROM SOIL AND WATER"**

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Nadia Bibi is hereby approved and recommended as partial fulfillment for the award of Degree of Doctor of Philosophy in Biotechnology and Microbiology

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AUTHOR'S DECLARATION

I solemnly declare that the research work presented in this dissertation was carried out in accordance with the requirement of the University of Peshawar's regulation for research Degree programs. The author has not submitted this or any part of this work for any other academic award. The work is original and of author's own data, while work done in collaboration with, or with assistance of, other is indicated as such. The views expressed in the dissertation, belongs to the author's.

Date: 23-12-2016

Signature of Scholar 

DEDICATED

TO MY LOVING, CARING, AND DEVOTED

HUSBAND

SYED MOHAMMAD ISLAM SHAH, MY

ABBA JAN, AMMI JAN

AND LOVING FAMILY

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LIST OF ABBREVIATIONS

HCs	Hydrocarbons
PAHs	Polycyclic aromatic hydrocarbons
U.S. EPA	United State environmental protection agency
SCPCB	State and Central Pollution Control Board
LMW	Low molecular weight
HMW	High molecular weight
BTEX	Benzene, toluene, ethylbenzene, xylenes
WHO	World Health Organization
U.V	Ultra violet
PNRG	Mineral media with 5 mM glucose
PNR	Mineral media
NB	Nutrient Broth
Phe	Phenanthrene
Pyr	Pyrene
Ant	Anthracene
Ace	Acenaphthene
Naph	Naphthalene
B	Isolates isolated from *Brassica campestris,*
S	Isolates from *Sisymbrium irio*
M	*Morus alba* isolates
1H2N	1-Hydroxy-2 Naphthoic acid
3H2N	3-Hydroxy-2 Naphthoic acid
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
HPLC	High Performance Liquid Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
LRT	Likelihood Ratio Tests
Kbps	Kilobase pairs

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ACKNOWLEDGMENTS

I am grateful to Almighty ALLAH, the most merciful and beneficent; His blessings enabled me to achieve my goals. Tremulous veneration is for His Holy Prophet Hazrat Muhammad (PBUH) who is forever torch of guidance and knowledge for humanity.

I have great reverence & admiration for my research supervisor Dr. Sumera Afzal Khan. I am highly indebted for her patience, moral support, sincere criticism and valuable guidance throughout the study. Sincere thanks to Dr. Muhammad Hamayun Chairman, Department of Botany Abdul Wali Khan University Mardan (AWKUM) for providing me an opportunity to work under his kind supervision and valuable guidance.

I am also grateful to Prof. Dr. Ghosia Lutfullah, Director, Centre of Biotechnology and Microbiology, University of Peshawar for providing research facilities at the Department.

Thanks are to Dr. Anwar Hussain, Prof. Dr. Bashir Ahmad, Dr. Rasool Khan, Dr. Ziarat Shah, Dr. Rubeela, Dr. Ibrar, Dr. Sadiq, Dr. Farman, Dr. Irshad, Dr. Sehrun, and Dr. Kafeel for suggestions & guidance that helped me towards the completion of this work.

Special thanks to Prof. In-jung Lee, Crop Physiology Laboratory, School of Applied Bioscience, College of Agriculture and Life Science, Kyungpook National University, Daegu, Korea for provision of chemicals for molecular analysis.

I highly appreciated the efforts, guidance, and help of Dr. Mohammad Waqas for his cooperation and guidance in molecular analysis. I am in depth thanks to Dr. Humaira Inayat (my dearest friend) for provision of facilities for GC-MS analysis at PCSIR laboratories Peshawar.

A great depth and loving thanks to my lab fellows, Ghazala Yasmeen, Dr. Aabid, Sajid, Shabeena, Aaqib Sayyed, Husna, Mahwah and Husnara. I sincerely acknowledge the friendly attitude & assistance of technical staff of botany lab

(AWKUM), Rashad, Aadil, Naeem, Ishaq, Adnan, Tanveer and the office staff of the department throughout my research work.

I also want to acknowledge Higher Education Commission (HEC), Pakistan who provided me the financial support through indigenous scholarship for my studies.

A non-payable debt to my family members, specially, Syed Mohammad Islam Shah (Husband) for his care, devotion and courageous support that help me complete the task. I am grateful for the valuable suggestions of Dr. Shabbir Ahmad (brother) that motivated me in striving for higher education. Special thanks to my in-laws for their prayers, constant support and co-operation. My siblings; Humayun Ahmed, Bilal Ahmed, Faisal Zahir, Aaqib Zahir, Andaleeb and Sunbul, who prayed a lot for me, shared the burden and made sure that I sailed through smoothly. Last but not the least; my endless thanks are to my Abba Jan and Ammi Jan (Zahir Ullah & Ulfat Zahir) and Nana (Hafi Ullah) whose motivation and precious prayers are of great value to me.

Nadia Bibi

ABSTRACT

Biodegradation of polycyclic aromatic hydrocarbons (PAHs), using soil microorganism is the most efficient, cost effective and environment friendly remediation strategy. In the present study, bacterial strains were isolated from four different sites including rhizospheric soil of three native species and sewage waste water using serial dilution and spread plate technique in order to isolate wild strains. Initial screening was done on simple nutrient agar via streak and spray method. The isolates were adapted to biodegradation through changing PAHs concentration, different media and physical mutation. It was observed that exposure to UV-light for a short period of 15 minutes results in enhanced growth rate but, some isolates lose the degrading ability of a certain compound.

A total of 360 isolates were initially screened for growth on nutrient agar medium containing up to 1100 and 1200 ppm on selected PAHs (anthracene, naphthalene, acenaphthene, phenanthrene and pyrene). Among which 101 of these isolates with best growth, were then screened on PNRG and PNR medium for the utilization of PAHs and selection of best degrading isolates was done. Eight bacterial isolates were selected on the basis of best growth in liquid static media condition, namely; B_4, B_{30}, B_{36}, S_{13}, S_{21}, S_{51}, M_3 and W_2. These were characterized on the basis of morphology and biochemical characterization. S_{13} was most closely related to *Bacillus cereus* and M_3 to *Mycobacterium* sp. on the basis of key provided by Bergey's manual classification. Identification was finalized on the basis of molecular analysis i.e., 16S rRNA analysis and construction of phylogenetic tree. All the selected isolates except M_3 were identified as *Bacillus* sp., isolates that were closely related to one another, and probably were sub-species of *Bacillus cereus*.

Before, carrying out biodegradation studies of selected PAHs, various physico-chemical conditions were optimized as concentration of PAHs (phenanthrene 500ppm for B_{30} and S_{21}) and 1000ppm for the rest of isolates, p^H (7), temperature (30ºC) and inoculum concentration was 10%, for degradation and growth. Isolate S_{21} was able to carry out phenanthrene degradation at both acidic and alkaline pH while, isolate S_{13} degrade anthracene at a high shaking speed of 180rpm. Isolate B_{36} and M_3 were able to grow at lowest temperature of 4ºC and high temperature 40ºC

respectively but, maximum degradation was observed at 35ºC for and M_3 and 30ºC for B_{36}. S_{13} degraded anthracene upto 86.26% with cell viability of 1.4×10^{-23} $CFUml^{-1}$ after 120 hours. Isolate B_{30} removes 100% naphthalene, 78.82% phenanthrene and 84.68% acenaphthene while, S_{21} degrade phenanthrene upto 96.60% and pyrene 76.52% in 120 hours. Three isolates were capable of pyrene degradation of B_4 (57.41%), S_{51} (71.99%) and M_3 (76.3%) at a concentration of $1000mgL^{-1}$. The mean and standard deviation of the replicates were calculated and the mean values were compared by parametric one-way ANOVA tests. The level of significance for all degradation experiment was $p\leq0.05$.

Co-metabolic biodegration experiment was also carried out for eight isolates on PAHs mixture (naphthalene, acenaphthene, phenanthrene, anthracene and pyrene) and analysed by HPLC. Naphthalene and acenaphthene were completely converted in 24 hours and salicylic acid was recovered which is the main oxidation product of LMW PAH compounds. The mixture of PAHs was transformed mainly to catechol, gentisic acid and salicylic acid. Isolate S_{51} produce yellow coloration which is normally produced from the meta-clevage of catechol to muconic semi-aldehyde. It can be concluded from this study that bacterial isolates from contaminated areas were most efficient and those of un-contaminated area can be adapted to biodegradation under laboratory conditions.

In order to locate the biodegrading gene, plasmid gel electrophoresis and curing experiment was also made. It was found that the degradation genes for naphthalene, acenaphthene, phenanthrene and anthracene were located on mobile genetic elements. Isolates, B_{30} and B_{36} completely lost the degradation ability while, B_4 degradation efficiency was reduced after plasmid curing.

INTRODUCTION

Polycyclic or polynuclear aromatic hydrocarbons (PAHs) also known as polyarenes is the largest class of organic molecules. It is a well-known class of organic compounds having special stability and persistent nature associated with numerous environmental problems. These are chemical compounds that are universally dispersed in the ecosphere with a similar structure comprising of fused benzene rings [Hassan *et al.* 2009; Dabestani *et al.* 1999; Gao & Zhou, 2004; Vane *et al.* 2009; Fetzer *et al.* 2007; Kristine *et al.* 2005; Hamdi *et al.* 2007; Vinas *et al.* 2005]. Their skeleton is composed of fused aromatic rings having linear, angular or cluster arrangement which is responsible for biochemical persistence due to low solubility. These are resistant to neuclophilic attack because of presence of free electron in their ring structure [Zhou *et al.* 2007; Jonsen *et al.* 2005; Santos *et al.* 2008; Nikolaou *et al.* 2009; Rodrigo *et al.* 2008; Javid *et al.* 2005; Kafilzadeh *et al.* 2011].

Types of Polyaromatic Hydrocarbons

PAHs can exist in over 160 different combinations, among these 16 PAHs are listed by U.S. EPA and State and Central Pollution Control Boards as priority pollutants due to their suspected carcinogenicity [Seoud *et al.* 2003; Garbeth *et al.* 2004; Luch, 2005; Guinness et al. 2009; Laumann *et al.* 2011]. These are naphthalene, acenaphthene, acenaphthylene, anthracene, fluorene, phenanthrene, benz[*a*]anthracene, chrysene, fluoranthene, pyrene, benzo[*b*] fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene, benzo[*g,h,i*] perylene, and indeno[*1,2,3-c,d*] pyrene. These compounds have been classified pollutant because of their toxicity, carcinogenicity and teratogenic effects on living body [Kumar *et al.* 2012; Hassan *et al.* 2009; Juliana *et al.* 2003; Ruma *et al.* 2007].

These compounds containing two to three rings are known as low molecular weight (LMW) and high molecular weight (HMW) with more than four or more fused rings. The physical and chemical properties of these compounds as well as the angularity of molecule depend on the molecular weight. HMW PAHs are hydrophobic and persist in nature due to their molecular stability [Corgie *et al.* 2006]. LMW PAHs are volatile in nature and have higher solubility in water, degraded more rapidly than

HMW PAHs [Arulazhagan *et al.* 2009; Wick *et al.* 2011]. The solubility of these compound is reduced with increase in number of aromatic rings with enhance affinity which limits their bioavailability and thus the efficiency of a bioremediation process [Lubecki *et al.* 2010]. The aqueous solubility of naphthalene ($30mgL^{-1}$), phenanthrene ($1\text{-}1.6mgL^{-1}$), pyrene ($0.132mgL^{-1}$) and benzo [a] pyrene ($0.0038mgL^{-1}$) and there is variation in the half-life ranging from 16-126 days in case of HMW [Liang *et al.* 2009].

Environmental Sources of PAHs

Sources of PAHs can be natural, mainly produced from thermal geological reactions, natural fires and petrogenic spills. The erosion of bituminous rocks and decaying organic matter adds to the levels of PAHs in the atmosphere [Luan *et al.* 2006; Yahaya *et al.* 2005; Saha *et al.* 2009]. Anthropogenic sources can be stationary and mobile including, heating, cooking, burning and pyrolysis of coal, oil, gas, garbage, wood fuel, charcoals, and coal briquettes in a stove, wood fuel, or other organic substances are the main domestic sources. PAHs in the indoor air of smoking residences tend to be higher than those of non-smoking residences. Combustion of fossil fuels, power generation, municipal and industrial waste incineration also adds a huge quantity of these pollutants [Ravindra *et al.* 2006; Chen *et al.* 2007; Hassan *et al.* 2009; Haritash & Kaushik, 2009; Wick *et al.* 2011].

Industrial and waste water treatment plants, coal-gasification sites, smoke houses, asphalt roads, roofing tar, aluminum production plants, and atmospheric contamination of leafy plants, charbroiled meat also adds in PAHs's emissions. Sources of PAHs from industrial activities are dyes, drugs, semiconductors, and incandescent reagents, polychromatic and antistatic additives for plastics. Different synthetic chemicals like surfactants and insecticide, varnishes rubber tires and cement manufacturing plants also cause release of these hydrocarbons [Nording *et al.* 2006; Chadhain *et al.* 2006]. Building wreckage, previous gas and catering plants, cable pole coatings and railway ridge are also known to accumulate PAHs from human intervention [Guinness *et al.* 2009].

The major cause of PAH in urban areas are mostly exhaust fumes of transport, including automobiles, railways, ships, aircrafts, and other motor vehicles. These

sources are categorized as mobile sources. PAH emissions from these sources are associated with petroleum products, road dust, medical waste incineration, municipal wastes and biomass burning at high temperature [Ravindra *et al.* 2006; Luke *et al.* 2012; Haritash & Kaushik, 2009; Onwurah *et al.* 2007].

Transport of PAHs

PAHs can be released into the environment and distributed worldwide as a result of transportation irrespective of the source of production. These can be detected in air, soil, sediments and dispersed to vegetation. Soil is the part of the environment that acts as the ultimate reservoir of these persistent organic pollutants. These can enter aquatic ecosystems by numerous routes and due to their hydrophobicity usually bound and transported by fine particles which undergo sedimentation [Yu *et al.* 2005; Claudia *et al.* 2005; Adebusoye *et al.* 2007; Das *et al.* 2011].

Effect of PAHs on Soil

The accumulation of PAHs in the soil environment is of increasing concern because of their impacts on soil health, food safety and potential health risks. Vegetables cultivated on the wastewater polluted soils may take up these pollutants in sufficient quantities and cause health problems for the consumers [Khan *et al.* 2008]. Pollutants enter human organisms by means of food cause severe damage to living organism at DNA level [Das *et al.* 2011].

Toxic effect of PAHs in Food Chain

PAHs are also present at different levels of food chains as in green leaves of plants and in fish. Food chain contamination is one of the important pathways for the entry of these toxic pollutants into the human body [Guinness *et al.* 2009; Leungsakul *et al.* 2006; Hafez *et al.* 2008; Ting *et al.* 2011; Li *et al.* 2011; Garbeth *et al.* 2004; Arulazhagan *et al.* 2009]. Reduced metabolic rate, biomass production and even death of plants are caused by these contaminants [Onwurah *et al.* 2007]. Some are lethal while, others are carcinogenic to aquatic life and can reach to human through food consumption [Johnsen *et al.* 2005].

Effects of PAHs on Humans

The portal of entry of PAHs into human bodies can be dermal, nasal and oral. Once these enter the human body, can reach the kidneys, liver, fat tissues and can also be absorbed by the gastrointestinal tract of other mammals. Once these enter the living tissue convert to carcinogenic substances that can cause cancers in different tissues like lungs, livers, pancreas, skins, breast, colon and bladder in humans [Johnsen *et al.* 2005; Rodrigo *et al.* 2005; Santos *et al.* 2008; Nour *et al.* 2010; Perugini *et al.* 2007; Kishida *et al.* 2010; Hassan *et al.* 2009; Rodrigo *et al.* 2005; Mohammadi *et al.* 2009]. These contaminants can also cause protein synthesis inhibition; irregular nervous synaptic signals and plasma membrane lose its function. Regardless of their physical properties and initial routes of exposure, the tumor inducing effects of carcinogenic PAHs depend on their conversion in the body into compounds called as genotoxic carcinogens. These compounds can react with and damage DNA in the nuclei of cells [Bin *et al.* 2007; Luke *et al.* 2012; Dhananjayan *et al.* 2012; Yahaya *et al.* 2005; Crisafully *et al.* 2008; Muratova *et al.* 2003; Patel *et al.* 2012; Onwurah *et al.* 2007].

Naphthalene having two fused benzene rings is the simplest and has long been used as a model compound. Anthracene and phenanthrene are three ring aromatic hydrocarbons that are found in polluted areas in huge quantity [Das *et al.* 2008; Hassan *et al.* 2009; Zeinali *et al.* 2008]. Anthracene and phenanthrene can cause tumerogenesis and allergic reaction and disruption at cellular level respectively [Othman *et al.* 2010; Xiang *et al.* 2006]. Pyrene and its urinary metabolite, 1-hydroxypyrene is commonly used in laboratory test to reveal exposure to PAHs [CDC, 2005].

Physical Processes for PAHs Removal from Environment

The fate of polyaromatic compounds in the atmosphere depends upon not only on their physical and chemical properties but on various living and non-living environmental factors. The common methods for contaminants removal can be alteration or incineration which are expensive and can harm the environment by making hazardous compound bioavailable [Edward *et al.* 2008; Leungsakul *et al.* 2006; Soojhawon *et al.* 2005].

The physico-chemical methods in use for PAHs removal are volatilization, evaporation, photooxidation, adsorption and hydrolysis. But, these aromatic compounds when comes in contact to light produce partially oxidized intermediates which are more susceptible to biodegradation. On exposure to UV light from sunrays cause formation of ozone and various nitrogen and sulphur substituted compounds [Hassan *et al.* 2009]. These techniques are not always effective, are very expensive and sometimes lead to generation of other contaminants [Palm *et al.* 2004].

Biological Process for Removal of PAHs

Bioremediation strategies rely on the catabolic capacities of microbes to transform harmful pollutants into harmless compounds. It is a technique used for biotransformation of organic pollutants into less complex inorganic substances, using indigenous microbiological community of the polluted areas [Trindade *et al.* 2005; Mohammadi *et al.* 2009; Haritash & Kaushik, 2009]. It is an emerging ecologically harmonious remediation technology that removes or detoxifies the pollutants by utilization of rhizospheric microflora. Plants can either bio-accumulate the contaminants or even degrade [Clayton *et al.* 2005; Ho *et al.* 2006].

Phytoremediation and PAHs Removal

Phytoremediation is the utilization of synergistic effects of soil microflora for the contaminants removal [Erik *et al.* 2006]. The rhizospheric microbial populations play a crucial role in PAHs removal from soil. Plant assisted PAHs withdrawal is linked well with the abundance of root associated bacteria that are able to metabolize a contaminant of concern [Kamath *et al.* 2004; Olson *et al.* 2003; Clayton *et al.* 2005]. Before, the choice of plant to be used in phytoremediation of PAH polluted soil, an economical, simple and fast method for quantification of rhizospheric microflora must be a part of initial screening [Holger *et al.* 2006].

Effect of Bioavailability on PAHs Removal

PAHs also undergo bioaccumulation, leaching and microbial degradation. *Mycobacterium* sp., *Pseudomonas* sp., *Sphingomonas*, and *Rhodococcus* sp. have been well documented for the degradative ability [Odd & Bonaunet, 2006; Li *et al.*

2008; Li *et al.* 2009; HuiJie *et al.* 2011; Lors *et al.* 2012; Mohammed *et al.* 2012; Mohammed *et al.* 2014]. The degradation of PAHs mostly occurs under oxic condition but degradation in the absence of oxygen is also reported. The biodegradation of organic matter in anaerobic conditions can be the result of bacterial respiration without oxygen supply while, denitrification, occurs at a relatively high redox potential having nitrate as electron acceptor [Ting *et al.* 2011]. Organic contaminants must be released to the aqueous phase prior to enter the microbial cell and subsequent intracellular transformation by the necessary catabolic enzymes [Kumar *et al.* 2006].

Biodegradation as an Efficient Source of PAHs Removal

Biodegradation is the most cost-effective and eco-friendly remediation strategy of obnoxious chemicals by microorganisms. These organisms have the ability of mineralization, transformation and immobilization of pollutants [Onwurah *et al.* 2007; Kallimanis *et al.* 2009; Ting *et al.* 2011; Luciana Pereira, 2013].

Naturally, biodegradation of polyaromatics depends largely on rates are highly dependent on their various environmental conditions, geochemical and microbial community structure. Microbial biodegradation has been found as an attractive alternative to engineering built methods for remediation of PAHs contaminated sites [Clayton *et al.* 2005]. The removal of PAHs from soil is the combine efforts of bacterial communities and metabolism of microbial populations as well as individual isolates [Helmi *et al.* 2007]. Biodegradation of PAHs is limited by the low bioavailability of these hydrophobic organic contaminants [Zeinali *et al.* 2008].

Bacterial biodegradation

Fortunately, bacteria are able to feed upon the wide variety of compounds found in petroleum products [Mesdaghinia *et al.* 2005; Liangli & Hungchen, 2009; Sarikhani *et al.* 2011]. An enormous number of bacteria exist in the atmosphere that can exploit aromatic compounds as their energy source to carry out metabolic processes [Arun *et al.* 2012]. The diversity of bacterial collections in enrichment cultures has not been monitored by molecular techniques using primers or probes for their potential of PAHs degradation [Lies *et al.* 2007; Edward *et al.* 2008].

Mycobacterium and *Sphingomonas* are the two genera of soil bacteria are significant PAHs degrading colonizers of contaminated soil. These bacteria exhibit several physiological adaptations to low bioavailability by attachment to the PAHs substrate and astonishing affinity to bind these contaminants [Maarten *et al.* 2006].

Oil degradation of various genera of gram +ive and –ive including *Pseudomonas, Rhodococcus, Acinetobacter, Alcaligenes, Bacillus, Flavobacterium,* and *Nocardia* is also reported [Shafiee *et al.* 2006; Nasrollahzadeh *et al.* 2007; Ebrahimi *et al.* 2012]. Biodegradation of phenanthrene by different bacterial species from polluted marine sites has also been described [Isola *et al.* 2008]. *Pseudomonas* is among familiar class of bacteria with high potential to remediation of different types of hydrocarbons [Hong *et al.* 2005].

Some of the bacterial genera capable of degrading low-molecular weight PAHs are *Bacillus, Pseudomonas, Rhodococcus, Aeromonas, Alcaligenes, Acinetobacter, Berjerinckia, Micrococcus, Corynebacterium, Burkholderia, Mycobacterium, Sphingomonas, Streptomyces, Stenotrophomonas, Paenibacillus, Marinobacter* sp. and *Vibrio* [Ramadan *et al.* 2012]. Solid and liquid HCs degradation by *Staphylococcus, Burkholderia, Corynebacterium, Bacillus, Pseudomonas, Klebsiella* and *E. coli* is also reported. *Acinetobacter* from oil-polluted soil and investigated its ability in degradation of PAHs in liquid media [Bamforth & Singleton, 2005; Maarten *et al.* 2006; Ashmasg *et al.* 2009].

Enzymes Involved in Biodegradation

Biodegradation of PAHs is catalyzed by a wide range of enzyme capable to break the ring structure of polyaromatics under oxic condition. Initial ring oxidation is carried by dioxygenase genes which are possible indicators of PAH degrading bacteria. Naphthalene degrading genes were used in the identification of *Pseudomonas* and *Burkholderia* sp. while, NidA and pdoB genes were used for detection of *Rhodococcus sp., Nocardioides sp.* and *Mycobacterium sp*. [Zhou *et al.* 2006; Guo *et al.* 2010].

Plasmid encoded biodegradation

Genes located on transposons and plasmids are liable for catabolism of aromatic compounds. The first known reported study on plasmid mediated naphthalene degradation was described by Dunn in 1973. It is revealed that *Pseudomonad putida* isolate has NAH plasmid that carry's naphthalene degrading genes. Later on another plasmid pDTG1 capable of naphthalene degradation was isolated from *Pseudomonas putida* 9816 [Kurkela *et al.* 1988]. *Pseudomonas flourscence* possess three ring PAHs naphthalene, anthracene and phenanthrene degrading plasmid pLPa and pKG2 [Kiyohara *et al.* 1983; Obayuri & Salam, 2010]. *Terrabacter* sp. DBF63 contains a plasmid pDBF1 that degrade fluorine, while *Sphingomonas* sp. HS362 possesses p4 plasmid for Phenanthrene degradation [Habe *et al.* 2005; Hwa *et al.* 2005]. Three species of *Mycobacterium* i.e., MC, PYR-GCK and KMS have the ability to degrade pyrene and two species of *Pseudomonas* ARP26, ARP28 and *Alcaligenes faecalis* has PHK2 plasmids with phenanthrene degrading capability [Miller *et al.* 2007; Coral & Karagol, 2005].

PUC18 plasmid located on *Pseudomonas* C18 isolates, *Pseudomonas* 112 and *Pseudomonas R007* and pNL and pKS14 and was isolated from *Sphingomonas aromaticoviran F119 and Sphingomonas* KS14. pPNY from *Staphylococcus* spp. PN/Y also has the ability to degrade naphthalene and phenanthrene. Reported genes responsible for naphthalene include a number of nahAa, nahAb, nahAc, nahAd, nahB, nahC, nahF, nahG, nahE, nahD, nahN, nahL, nahO, nahM, nahK, nahJ, nahT, nahH, nahN, nahL, nahl, nahR [Mallick *et al.* 2007]. *Pseudomonas putida* degraded napthalene genes pahA/ pahAb/ pahB/ pahC/ pahD/ pahE/ pahF located on chromosomes while plasmid was responsible for phenanthrene degradation in *Alcaligenes faecalis* AFK2 were, phnAa/ phnAc/ phnAd/ phnB/ phnC/ phnD/ phnE/ phnF/ phnG, phnH and phnI [Habe & Omori, 2003; Chauhan *et al.* 2008]. Pyrene degrading genes were also shown to be located on chromosome pbhD in *Sphingomonas paucimobilis* Var.EPA505 [Kanaly & Harayama, 2000; Sinha *et al.* 2009].

Co-Metabolism and High Molecular Weight PAHs Degradation

Co-metabolism is an important degradation process is most effective in case of recalcitrant polyaromatics [Pizzul *et al.* 2006; Guo *et al.* 2010]. Both LMW and HMW PAHs may affect the degradation of one another by co-metabolism which can pose either stimulatory or inhibitory effects. The metabolites produced by one microbe can be possible substrate for the other [Serrano *et al.* 2009]. Co-metabolic bioremediation is the breakdown of a contaminant by an enzyme or cofactor that is formed during microbial metabolism of another compound. The bacteria involved in co-metabolic degradation do not receive a carbon or energy benefit from the contaminants. In addition, intermediate products that act as inhibitors of microbial metabolism can be produced during co-metabolic bioremediation [Sipkema *et al.* 2000; Rui *et al.* 2004; Semprini *et al.* 2005; Powell *et al.* 2011].

Microbial community structure can be improved from alpha, beta to gamma-proteobacteria when exposed to PAHs. Molecular-based techniques have also been used to for phylogenetic study of PAHs degrading bacteria from different areas were found independent of geographic location [Sinead *et al.* 2006]. Microbial transformation and degradation are the chief process for successful eradication of PAHs from the environment and it is thought to be one of the main processes of removal of these contaminants [Jie *et al.* 2011]. The use of natural or indigenous microflora for bioremediation is of great interest as it is often more convenient and beneficial than commercial inoculums. Diverse groups of indigenous microorganisms accomplished PAHs degrading potency have been isolated from polluted sites which were able to mineralize several PAHs. Research on the isolation, identification, and characterization of different bacterial species capable of degrading LMW and HMW PAHs based on 16S rRNA gene sequence phylogeny has been carried out [Clavijo *et al.* 2012; Hesham *et al.* 2014].

Soil is an excellent source for unknown microorganisms the most commonly isolated genus has been *Bacillus.* The use of 16S rRNA gene sequences to study bacterial evolution has been the most commonly used genetic marker used for a number of reasons. These are large enough operons that are present universally in all bacteria as a multigene family which does not change their function through the process of evolution [Triman *et al.* 1998; Sarma *et al.* 2004; Gregory *et al.* 2005]. It is

proved that physiological constituents arising from the use of multiple copy plasmids encoded rRNA operons required by the existence of seven chromosomal rRNA operons. Thus, interpretation of the phenotypes of rRNA mutants should take into account the potential impact of hyperexpression of rRNA on regulation of ribosome synthesis and overall growth physiology [Rodon *et al.* 2000; Gregory *et al.* 2001; Carr *et al.* 2005].

Table 1.1 Properties of the 16 US-EPA PAHs [Mackay *et al.* 1992].

PAHs	No. of rings	Mol. wt.	Aqueous solubility (mgl^{-1})	Vapor pressure (Pa)	Log Kow
Naphthalene	2	128	31	$1.0x10^{2}$	3.37
Acenaphthylene	3	152	16	$9.0x10^{-1}$	4.00
Acenaphthene	3	154	3.8	$3.0x10^{-1}$	3.92
Fluorene	3	166	1.9	$9.0x10^{-2}$	4.18
Phenanthrene	3	178	1.1	$2.0x10^{-2}$	4.57
Anthracene	3	178	0.045	$1.0x10^{-3}$	4.54
Pyrene	4	202	0.13	$6.0x10^{-4}$	5.18
Fluoranthene	4	202	0.26	$1.2x10^{-3}$	5.22
Benzo[a]anthracene	4	228	0.011	$2.8x10^{-5}$	5.22
Chrysene	4	228	0.006	$5.7x10^{-7}$	5.91
Benzo[b]fluoranthene	5	252	0.0015	-	5.80
Benzo[k]fluoranthene	5	252	0.0008	$5.2x10^{-8}$	6.0
Benzo[a]pyrene	5	252	0.0038	$7.0x10^{-7}$	5.91
Dibenzo[a,h]anthracene	6	278	0.0006	$3.7x10^{-10}$	6.75
Indeno[1,2,3-cd]pyrene	6	276	0.00019	-	6.50

A study carried out on the workers of petrol pump and mechanical workshop professional of twin city of Pakistan showed that, their blood and urine contains naphthalene, phenanthrene and pyrene 93.1, 5.96 and 143.23μgL^{-1} [Ikhtiar Uddin, 2013]. Further sources of PAH exposure include inhalation of vehicular exhaust gases, smoke from burning solid fuels and cigarettes [Zhong *et al.* 2011; Li *et al.* 2011; Adetona *et al.* 2012].

Quantification of PAHs in soil with waste water irrigation of different areas of Khyber Pakhtunkhwa was found to contain 223-929mgkg^{-1} in soils & 51.6-402mgkg^{-1} in vegetables especially green leaves vegetable of metropolitan areas. While, the heighest toxicity factor of naphthalene and pyrene was recorded for Mardan [Waqas *et al.* 2014]. The presence of PAHs in fresh vegetables, fruits, and cereals has been detected due to the deposition form airborne sources in urban areas particularly near industries and densely traffic area. These have also been found in sea animals and in animal products like meat, milk and fat, 27ngg^{-1}, 37ngg^{-1} and 70µgkg^{-1} respectively [Kishikawa *et al.* 2003; Abou-Arab *et al.* 2010]. In a case study in Peshawar near brick klin using low quality coal and rubber tires, it was found that the soil and medicinal plants accumulates a huge quantity of low molecular weight PAHs (naphthalene, anthracene and phenanthrene). In a related study, on the roadside soil of the Peshawar city with high traffic burden was found to contain 0.1-18mgkg^{-1} mostly three and four ringed PAHs [Khan *et al.* 2008; Fazalurrahman 2011].

Keeping in view, the importance of microbial degradation of PAHs compounds, the current project was designed because such, work had never been conducted in our region. Soil and water of our city, Peshawar, is contaminated with different PAHs along with many other pollutants from industries and household waste.

AIMS AND OBJECTIVES

Aim of current study includes biodegradation of some model polycyclic aromatic compounds was achieved by following the objectives:

- Isolation screening and preservation of PAHs degrading rhizospheric bacteria from native plants and water sample was carried out.
- Various physic-chemical conditions such as concentration of PAHs, pH of the media and temperature were optimized for biodegradation.
- Adaptation of bacterial isolates by changing conc. of PAHs, changing media and physical mutation was also carried out.
- Biodegradation of individual and PAHs mixture was also studied.
- End product identification of bacterial degradation was also done.
- Most efficient isolates were identified on the basis of morphology and phylogeny.

LITERATURE REVIEW

PAHs were first detected on the surface of agricultural soils [Blumer, 1961]. Oil spill land in 1970's adversely affected the soil fertility, crop production and aquatic life. An incidence, of terrestrial oil spill occurs in Nigeria in 1978-79 cause soil sterility which adversely affected the growth and germination of various crops of economic importance. Some plants growing in the area of spill specially, *Dioscorea deltoidea* produced steroidal sapogenin from tuber tissues. While, some vegetation cover was removed from the affected area and even a drift of agriculture dependent farmers was also observed. Accumulation of contaminants from oil spills changes the pH range of soil making condition which favors heavy metal accumulation and water logging from anaerobic condition [Onwurah *et al.* 2007]. In Pakistan MV Tasman, the Greek ship spilled thousand barrels ton of oil cause destruction in sea environment. This spill in Clifton endangered aquatic life and PAHs were detected in almost all aquatic animals. About 90% of 8.8 million metric tons released, 30% of which enters the fresh water bodies [Khan *et al.* 2006]. There is seasonal and climatic variation in PAHs level which is higher in the winter months due to reduction of combustion, thermal and photo-decomposition and higher than in summer months [Latimer & Zheng, 2003].

Soils can be contaminated with 1μg-300gkg^{-1} PAHs, depending on the source of contaminant production. Levels of PAHs concentrations in urban industrial soils can be 10-100 times higher than in remote soils [Wick *et al.* 2011]. According to a recent World Health Organization (WHO) report, PAHs emissions from cooking account for 32.8% of total indoor PAHs [Liu *et al.* 2008; Zhu *et al.* 2009]. Overall, PAHs concentrations of 5863mgkg^{-1}, 18,704mgkg^{-1} and 821mgkg^{-1} at creosote, at a wood and petrochemical site respectively [Latimer & Zheng, 2003; Bamforth *et al.* 2005; Wick *et al.* 2011]. There is seasonal, climatic and regional variation of PAHs level in atmosphere which is normally more in winter than in summer [Latimer & Zheng, 2003].

A main contributor to PAHs concentrations in road dust as well as urban areas is vehicle exhaust [Abrantes *et al.* 2009]. Emission from heavy and light duty automobiles were 11.4–82.1μgkm^{-1} and 28.2μgkm^{-1} respectively [Wick *et al.* 2011].

PAHs concentrations released from wood combustion depend on wood type, kiln type and combustion temperature. 80-90% of PAHs emitted from biomass burning are LMW PAHs, including naphthalene, acenaphthylene, phenanthrene, fluoranthene and pyrene [Lu *et al.* 2009]. It is expected that a significant amount of PAHs are produced from the open burning of biomass. Total emissions of 16 PAHs from the burning of rice and bean straw varied from 9.29-23.6μgg^{-1} and from 3.13-49.9μgg^{-1}, respectively. Power generation activities like, refuse burning, and coke production provide 50% of the annual benzo[a]pyrene (BaP) emissions, which are widely used as a standard of PAHs emissions [Wick *et al.* 2011]. A study in Taiwan revealed that human exposure through inhalation to be between 0.4 and 1.55$ngday^{-1}$ and found PAHs levels to be 15-50ngm^{-3} in urban areas [Kuo *et al.* 2003]. Tobacco smoke contains more than 150 compounds in the gas phase and more than 2000 in the particulate phase, making tobacco use, the single greatest factor contributing to respiratory cancers. Phenanthrene and pyrene are commonly found in high concentrations from 0.25-12.9mgg^{-1} dry weight in river sediments in Taiwan [Liang *et al.* 2009; Wick *et al.* 2011].

PAHs concentrations of 5863$mgkg^{-1}$ at a creosote production site, 18,704$mgkg^{-1}$ at a wood preserving site, 821$mgkg^{-1}$ at a petrochemical site and 451$mgkg^{-1}$ at a gas manufacturing plant site [Latimer & Zheng, 2003]. Acenaphthene was found in various types of beans and different food items upto 0.50$ugkg^{-1}$. Drinking water has been found to contain 1-10ngL^{-1} PAHs [Wick *et al.* 2011]. Concentrations of PAHs compounds in particular sediment can range from μgkg^{-1} to gkg^{-1}. High levels, 10's of ng PAH kg^{-1} has been found in leafy vegetables, grains, fats, oils [Wick *et al.* 2011]. Vegetables contains PAHs in the range of 59.6-194.3 while, fruits have 25.5-51.7μgkg^{-1} [Bishoni *et al.* 2006].

The very first reported study on toxicity of PAHs on humans was carried out by the physician John Hill in 1761. He reported that use of powdered tobacco sniffing cause lung cancer [Bamforth *et al.* 2005]. Following, this discovery Percival Pott 1775 published his data concerning soot from chimney's sweeps was probably responsible for the increased incidence of scrotal cancer among the workers [Brown & Thornton, 1957; Searle *et al.* 1976]. Studies of PAHs toxicity in animals and carcinogenecity effects of PAHs from occupational workers exposed to PAHs during

coke production, roofing, oil refining and coal gasification was reported in the mid 1930 from human exposure [Abbey *et al.* 2011].

The earliest human PAHs related epidemiologic study was reported in 1936 by investigators in Japan and England who studied lung cancer mortality among workers in coal carbonization and gasification processes. Following U.S. studies carried on occupational coke oven confirmed an excess of lung cancer mortality, with the recommendation of extreme genitourinary system cancer mortality [Bamforth *et al.* 2005]. Later experimental studies showed that contaminants in particulate matter containing PAHs are a foremost contributor to accumulation of these compounds in urban areas (www.atsdr.cdc.gov/csem/exphistory).

Almost each and every person is exposed to PAHs on a daily basis take about 1-5ng PAHs through nasal, oral or dermal contact [Onwurah *et al.* 2007, Wick *et al.* 2007]. Pyrene is commonly found in PAHs mixtures and its urinary metabolite, 1-hydroxypyrene, has been used as an indicator of exposure to PAH chemicals [CDC 2005]. Measurement of 1-hydroxypyrene in the urine samples as a biological exposure index (BEI) is used for the assessment of exposure to mixtures containing PAHs [Story *et al.* 2004; Arab *et al.* 2010]. Due to deposition of vehicles emission the roadside soils are heavily polluted by traffic activities. A comparative study on the PAHs concentration level of roadside soils in Peshawar with France and Jordan revealed that the PAHs contamination of the roadside soil of Peshawar was much high [Khan *et al.* 2008].

Phytoremediation of polluted soil depend upon the capacity of microbes in the rhizosphere. Microorganisms equipped with metabolic machinery along with plants have complementary role in phytoremediation of oil-polluted soil [Muratova *et al.* 2003; Diab *et al.* 2010]. Rhizoremediation is proposed as the most potential approach for PAHs remediation in soil [Ma *et al.* 2009]. Bioremediation techniques are typically more economical than traditional methods of waste treatment such as incineration, absorbent/adsorbent techniques, and catalytic destruction (US EPA Office of Solid Waste and Emergency Response, 2007), cultivation-based techniques have been used to evaluate the microbial ecology of PAH degradation [Maiysha *et al.* 2011]. Many researchers have undertaken fundamental investigations for the degradation of benzene by microorganisms [Nicholson *et al.* 2004]. Bioremediation of

benzene has been studied using activated sludge, pure and mixed microbial cultures [Otenio *et al.* 2005; Anuradha *et al.* 2008]. Soil microflora plays a vital role in the rhizoremediation of xenobiotics [Joner *et al.* 2004; Johnsen *et al.* 2005; Semple *et al.* 2007; Hanan *et al.* 2009]. Bioremediation is more economical and bring about contaminants removal in more efficient ways [Jeon *et al.* 2003; Willison, 2004; Gaskin & Bentham, 2005; Zhou *et al.* 2008]. Microbial degradation has been found to be most efficient among remediation techniques [Padmanabhan *et al.* 2003; Nicholson *et al.* 2004; Fulekar *et al.* 2005; Singleton *et al.* 2005; 2006; 2007; Jones *et al.* 2008; Huang *et al.* 2009]. Grass species due to presence of fibrous root systems with huge root length are effective plants for phytoremediation oil polluted soils. The growth of some plant produce aliphatic hydrocarbons to the soil stimulates the degradation of PAHs [Paul *et al.* 2006].

The potential of various organisms to catabolize and metabolize organic compounds has been recognized as potentially effective means of disposing of hazardous wastes. Although most microorganisms particularly, bacteria play a crucial role in detoxification through mineralization, transformation and immobilization of pollutants [Ojumu *et al.* 2005; Diaz *et al.* 2004; Oelho *et al.* 2010]. Bioremediation, which is accomplished by adding exogenous microbial populations or stimulating indigenous ones, attempts to raise the rates of degradation found naturally to significantly higher rates. Most of bioremediation studies have been carried out using pure-cultures and the roles of these bacteria in a natural environment remain substantially unknown [Indah *et al.* 2007].

Bioremediation techniques are typically more economical than traditional methods of waste treatment such as incineration, absorbent/adsorbent techniques, and catalytic destruction (US EPA Office of Solid Waste and Emergency Response, 2007). Cultivation-based techniques have been used to evaluate the microbial ecology of PAH degradation [Maiysha *et al.* 2011]. This application can be more effective where environmental conditions permit microbial growth and activity; its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate [Jeon *et al.* 2003; Padmanabhan *et al.* 2003; Willison, 2004; Gaskin & Bentham, 2005; Fulekar *et al.* 2005; Singleton *et al.* 2005; 2006; 2007; Zhou *et al.* 2008; Jones *et al.* 2008; Huang *et al.* 2009]. Many

researchers have undertaken fundamental investigations for the degradation of benzene by microorganisms. Bioremediation of benzene has been studied using activated sludge, pure and mixed microbial cultures [Nicholson *et al.* 2004; Otenio *et al.* 2005; Anuradha *et al.* 2008].

Biodegradation efficiency can be enhanced through natural attenuation, bioaugmentation and biostimulation. Bioaugmentation, inoculation of microorganisms with desired degradation capability is a possible mean to enhance biodegradability of toxic contaminants. Biostimulation, supplying additional nutrients or substrates to stimulate degradation of native microorganisms, is another strategy to promote biodegradation, especially in environments such as mangrove sediments where nutrients are often limiting, reported that the microbial activity to degrade oil contamination was stimulated by addition of soluble inorganic fertilizer to mangrove sediments. However, the effectiveness of these strategies varies from sediments to sediments and from contaminants to contaminants [Mills *et al.* 2003; Yu *et al.* 2005; Maiysha *et al.* 2008].

Biodegradation of PAHs plays a crucial role in cleaning the crude oil contaminated marine environment. PAHs have ubiquitous distribution and deleterious effects on human health. The hydrophobic nature of polyaromatic hydrocarbons makes their clean-up extremely difficult as they persist for a long period of time. In addition to increasing environmental persistence with increasing molecular size, evidence suggests that in some cases PAHs genotoxicity also increases with size, up to at least four or five fused benzene rings. Several bacterial species were isolated from areas of different salt concentrations and their effects on biodegradation were analysed [Arulazhagan *et al.* 2009]. The microbial degradation of PAHs having two or three rings is well documented. Microbial degradation has been proposed as an inexpensive and efficient method to remove PAHs from the environment. Unlike HMW PAHs, phenanthrene and anthracene do not pose a risk to human health, since they exhibit no genotoxic or carcinogenic effects. However, they have been shown to be toxic to fish and algae [Adebusoye *et al.* 2007; Sinha *et al.* 2009; Hassan *et al.* 2009].

Bacteria have developed strategies of obtaining energy from virtually every compound under oxic or anoxic conditions by means of alternative final electron

acceptors such as nitrate, sulfate, and ferric ions. Several bacteria have been isolated and characterized which possess the ability to act on aromatic hydrocarbons. Some *Pseudomonas* species have also been identified as being efficient for degradation of aromatic compounds including benzene and its derivatives [Dipty Singh, 2010]. Some genera of the PAH-degrading bacteria have been found to reside in specific environments, for instance, *Cycloclasticus, Sphingomonas* and *Vibrio* isolates were suggested to be common phenanthrene-degraders in marine systems. A large group of PAH-degrading *Paenibacillus* sp. isolates were isolated from the rhizosphere of salt marsh plants. The LMW PAHs are relatively easy to be degraded, while the HMW PAHs are recalcitrant [Wei *et al.* 2008].

Studies suggested that a range of bacteria have been discovered to be capable of degrading in particular LMW PAHs, such as naphthalene and phenanthrene. These isolates belong to genera like *Agmenellum*, *Aeromonas, Alcaligenes, Acinetobacter, Bacillus, Berjerinckia, Burkholderia, Corynebacterium, Cyclotrophicus, Flavobacterium, Micrococcus, Moraxella, Mycobacterium, Nocardioides, Pseudomonas, Lutibacterium, Rhodococcus, Streptomyces, Sphingomonas, Stenotrophomonas, Vibrio* and *Paenibacillus.* Conversely, fewer bacteria are known to degrade HMW PAH such as fluoranthene, pyrene and benzo (a) pyrene. These include members of the genera *Bacillus, Burkholderia, Cycloclasticus, Flavobacterium, Pseudomons, Mycobacterium,* and *Stenotrophomonas.* The relative abundance and activity of different groups of PAH-degrading microorganisms in the environment could lead to different persistence and accumulation of HMW and LMW PAHs [Wei *et al.* 2008].

PAHs have motivated efforts to develop bioremediation technologies to eliminate its sources of exposure [Warshawsky *et al.* 2005]. Naphthalene is commonly used as a model for studying PAHs metabolism by bacteria because it is the simplest and most soluble PAHs, as well as a frequent constituent of PAH-contaminated environments. Naphthalene degradation has shown that there are two main pathways for the metabolism of naphthalene to catechol or gentisate by the transformation of salicylate. Metabolism of naphthalene by catechol has been studied in two *Pseudomonas* species that carry the archetypal catabolic plasmids NAH7 [Sota *et al.* 2006]. Naphthalene is usually shown to convert naphthalene diol, salicylic acid

and catechol [Chauhan *et al.* 2008]. Six bacterial isolates with pyrene and phenanthrene degrading potentials were also isolated so far [Johnsen & Karlson, 2007].

An organism isolated from soil sediments of municipal wastes *Pseudomonas* sp. had the capability to degrade PAHs with enhanced rate in the presence of surfactant [Kumar *et al.* 2012]. Isolation from mangrove sediments was done in which a total of 53 degraders isolates including of 14 isolates of phenanthrene,13 isolates of pyrene and Bap and mixed PAH degrading bacteria each [HuiJie *et al.* 2011]. These isolates were grouped into the alpha, beta, gamma-proteobacteria, *Sphingomonas Flavobacterium, Actinobacteria* and *Bacilli* [Feitkenhauer *et al.* 2003; Brakstad & Lodeng, 2005; Brito *et al.* 2006; Kumara *et al.* 2006; Zhou *et al.* 2006; Grant *et al.* 2007; Baboshin *et al.* 2008; Chen *et al.* 2008; Huang *et al.* 2008].

Comparative analysis of dioxygenase enzymes, in *Novosphingobium pentaromativorans* US6-1 in response to polycyclic and monocyclic aromatic hydrocarbons was pre-cultured to obtain sufficient biomass to determine dioxygenase activity and proteomic analysis. The activities of four major dioxygenases namely, CD1, 2, CD2, 3, PCD3,4 and PCD4, 5 were assayed using protein extracts from *N. pentaromativorans* US6-1 cultured in the presence of three PAHs to determine which biodegradation pathways were induced. Analysis of the *N. pentaromativorans* US61 genome indicated the presence of only extradiol oxygenase genes. In an attempt to determine if *N. pentaromativorans* US6-1 also expressed intradiol oxygenase activities, four dioxygenase enzymes that were thought to cover most degradation pathways in aerobic cultures. Intradiol dioxygenases CD1, 2 and PCD3, 4 showed no activity while, that of CD2, 3 was high in cells cultured in media containing phenanthrene ($1.59 Umg^{-1}$ at 12 h cultivation). This suggests that the CD2, 3 pathways could be a primary pathway for the degradation of PAHs in *N. pentaromativorans* US6-1 [Yun *et al.* 2014].

A research study revealed the hazard of PAHs to human health from their presence in placenta, maternal blood, umbilical cord blood and milk of Indian women. Umbilical cord blood and breast milk samples showed relatively high concentrations of all the three PAHs and thus demonstrated that the developing fetus or new born were exposed to these carcinogenic environmental contaminants. PAHs are toxic air

pollutants and people exposed to it at sufficient concentrations and durations may have an increased chance of getting cancer or other serious health effects. These health effects include damage to the immune as well as neurological systems; reduce fertility, developmental, respiratory problems. Like humans, animals may experience health problems if exposed to sufficient quantities of toxic air [Madhavan & Naidu, 1995; Yun *et al.* 2014].

There are a number of studies suggests that 16S rRNA gene sequencing provides 90% genus and about 83% species level identification. The 16S rRNA and its gene have revealed to be valuable and dominant indicators for the occurrence of bacteria in nature. The 16S rRNA gene sequence has been defined for a large number of isolates. Gene Bank, the largest databank of nucleotide sequences has 90,000 16S rRNA gene out of more than 20 million deposited sequences. This means that there are many formerly deposited sequences against which the sequence of an unidentified isolate can be compared. The 16S rRNA gene is universal in bacteria and on its basis, bacterial relationships can be measured among all bacteria [Jill & Clarridge, 2004; Zhang *et al.* 2012].

Characterization of bacteria that were capable of LMW PAHs utilization by 16S rRNA analysis were found to belongs to the genus *Pseudomonas*, *Clostridium* and *Bacillus* sp. [Han *et al.* 2003; Gomare & Lahane, 2011; Attallah *et al.* 2014]. *Bacillus* species is abundant and generally more modified to grow in different situations inside the environment. Several *Bacillus* species were categorized based on 16S rRNA and separated into diverse phylogenetically different groups [Said *et al.* 2008]. Most other spore forming bacteria from the rhizosphere of a wide range of plants were identified as *B. cereus* and *B. subtilis.* Initially, the species diversity is so great the available libraries of 16S rRNA genes permit an initial analysis of the universal soil bacterial community structure. The gene encoding the small subunit rRNA serves as a prominent tool for the phylogenetic analysis and classification of bacteria and archaea owing to its high degree of preservation and its essential function in living organisms [Rasool *et al.* 2003; Puangkaew *et al.* 2013]. In this study, the taxonomic position of soil bacteria to find out partial gene amplification and sequencing through 16S rRNA phylogenetic identification was carried out.

ISOLATION, GENERAL SCREENING AND ADAPTATION OF BACTERIA

3.1 INTRODUCTION

Polycyclic aromatic hydrocarbons are one of the most important classes of chemicals found in petroleum products [Farahani *et al.* 2010]. Some of these PAHs are toxic, mutagenic and carcinogenic to human. Accumulation of PAHs in agricultural soils may result in an increased uptake by crops, and threat the quality and safety of food. Hydrocarbons absorbed by roots are accumulated and metabolize in plant tissues of contaminated soil. PAHs have been detected in various crops, cereals and different animal products, milk ($37ngg^{-1}$), fats ($27ngg^{-1}$) and meat ($70\mu gkg^{-1}$) [Wang *et al.* 2011; Binet *et al.* 2000; Gunther *et al.* 2000].

Isolation of different bacterial species including, *Alcaligenes paradoxus, Aeromonas* sp., *B. licheniformis* and *P. fluorescens, Achromobacter, Arthrobacter* and *Pseudomonas* sp. for efficient biodegradation of diesel oil from polluted marine sites had been reported [Kayode-Isola, 2008]. Isolation, identification and biodegradation of different hydrocarbons have been studied [Mesdaghinia *et al.* 2005; Hong *et al.* 2005; Shafiee *et al.* 2006; Tang *et al.* 2006; Nasrollahzadeh *et al.* 2007; Ebrahimi *et al.* 2012]. Enrichment culture has long been used as a method of isolation of contaminant degrading capable microbes. However, isolation of bacteria from soil and adaptation process can also prove to be very helpful in contaminants removal from environment [Gaskin & Bentham, 2005; Mesdaghinia *et al.* 2005; Fazlurrahman, 2011; Neeraja *et al.* 2013].

3.2 MATERIALS AND METHODS

3.2.1 Chemicals and Media

Analytical grade pyrene (99%), anthracene (99%), naphthalene (99%) phenanthrene (98%), acenaphthene (99%) salicylic acid, salicylaldehyde, catechol, gentisic acid, 1-hydroxy-2, naphthoic acid and 2-hydroxy-1, naphthoic acid, 6,7 benzo-caumarin, 1-naphthol, indole, ampicillin were purchased from Sigma-Aldrich and Fluka (St. Louis, MO, USA). Other chemical and media components were of high

purity and analytical grade from Oxide, Merck and BDH. Nutrient agar and Mineral salt medium (PNR) was used for initial screening.

3.2.2 Collection of samples

Four sites were selected for collection of rhizospheric soil and water samples for isolation of bacteria. Based on the possibility for presence of PAH pollutants, three different sites were randomly selected for the isolation of rhizospheric bacteria of native plants. These sites were filling station of university road Peshawar, road side soil at university of Peshawar, agricultural soil. Plants collected from gas station were *Sisymbrium irio,* from agriculture field *Brassica capmpestris* and from road side soil was *Morus alba.* Moreover, sewage water also contains PAH compounds as well as large number of potential degrader microbes. For this reason sewage waste water sample was collected from PCSIR laboratories complex colony Peshawar.

All the plant samples were dig out as a whole, identified and placed in sterile polythene bags and immediately brought to the microbiology laboratory of COBAM, University of Peshawar and stored at 4°C till further processing. The water samples were collected in sterile tubes and brought to laboratory and processed immediately for bacterial isolation.

3.2.3 Growth medium and culture conditions

The composition of PNR and PNRG (PNR + 5 mM glucose) per liter of distilled water was [Khan *et al.* 2006; Pan *et al.* 2008; Ebrahimi *et al.* 2012], PN (20x) 50mL used as 50 mlL^{-1}: KH_2PO_4 13.6% (w/v), $(NH_4)_2SO_4$ 2.4% (w/v), NaOH 2.5% (w/v). The R salts were used as $7mlL^{-1}$: $MgSO_4.7H_2O$ 8% (w/v), $FeSO_4.7H_2O$ 0.2% (w/v), HCl 0.4% (w/v), Agar (2%) was used as solidifying agent.

3.2.4 Isolation of rhizospheric bacteria from the plant samples

Bacteria were isolated from rhizospheric soil using serial dilution and spread plate technique on nutrient agar media and incubated at 28°C for 72 hours. Inoculated plates were purified repeatedly by sub culturing and streak plate method. Colonies appeared were picked and cultured on new nutrient agar plates. Multi steps of purification were performed until pure isolates were obtained. Purification process

involving streak plate method was done to ensure isolation of single bacterial isolate. Pure cultures obtained were stored in slants of enrichment medium at 4°C [Zhao *et al.* 2000]. The isolated isolates of the selected sites were properly labeled as B, S and M for *Brassica capmpestris, Sisymbrium irio* and *Morus alba* respectively.

3.2.5 Isolation of bacteria from sewage waste water samples

Water samples were processed immediately after collection for isolation of bacteria using serial dilution and spread plate technique. The plates were incubated at 28°C for 72 hours. Isolated colonies were purified and labeled as W and stored in slants at 4^{o}C.

3.2.6 Screening of the isolated bacteria on solid media

A qualitative assay by the spray-plate method was used to check the degradation potential of the isolated bacterial isolates for growth on anthracene [Kiyohara *et al.* 1982; Othman *et al.* 2010]. Initially, the isolates were screened on nutrient agar and then on mineral salt medium with (PNRG) or without glucose (PNR). Concentration range was 100-1200ppm (anthracene), plates were incubated until the appearance of growth. PNR plates were prepared and inoculated with bacteria and sprayed with 0.1% of anthracene which was appropriately labeled. The plates were incubated at room temperature (about 28°C) for four days. Appearance of growth on mineral plates was an indication of the utilization of anthracene as source of carbon and energy. Isolates were also screened for the other four selected PAHs (naphthalene, acenaphthene, phenanthrene and pyrene with different range of concentration. Concentration range was 25 ppm-1200ppm for anthracene, 100-1000ppm (phenanthrene), 100-1000ppm (naphthalene), 100-900ppm (acenaphthene) and 10-1000ppm (pyrene) [Alias *et al.* 2011]. The isolates with best growth were selected for further studies.

3.2.7 Screening of the isolated bacteria in liquid media

To confirm the degradation capability, PAHs were added to liquid mineral media as sole carbon and energy source.

3.2.8 Screening in liquid media and selection of best degraders

A loopful of each isolate was inoculated into large test tubes containing 25mL of screening medium. The screening medium was the same as the enrichment medium, except 15mg of PAHs (acenaphthene, naphthalene, phenanthrene, anthracene and pyrene) separately dissolved in acetone was added to each tube after autoclaving, as sole source of carbon. Thereafter, the test tubes were statically incubated by keeping on the laboratory bench at room temperature (23–25°C) for three days [Thani *et al.* 2009; Othman *et al.* 2010; Ahmed *et al.* 2010].

3.2.9 Adaptation of bacteria for degradation

The isolates were adapted to PAHs degradation by increasing concentration of selected compound like anthracene from 50-1200ppm while, in case of pyrene the range was 10-1000ppm**.** The isolates were tested against selected PAHs using different media like simple nutrient agar, then on mineral media supplemented with glucose (PNRG). Finally, the isolates degradative ability was confirmed on PNR with only PAHs as carbon source as a result of adaptation. Physical mutation of the isolates was also done on exposure to UV-light for 15 minutes in order to enhance degradative capability [Top & Springael, 2003].

3.3 RESULTS AND DISCUSSION

3.3.1 Isolation and general screening

In this study, isolation of 360 bacterial isolates from the rhizospheric soil and sewage wastewater on simple nutrient agar is an indication that a large number of bacteria can exists in our environment. Screening of these isolates was done using anthracene as a model compound because it is a part of ring structure of many HMW PAHs. Moreover, bacteria isolated on anthracene were found more adapted to degrade a range of PAHs than those isolated on enrichment media containing naphthalene or phenanthrene. Bacteria that have been isolated on naphthalene as substrate generally metabolize narrower range of PAHs. So isolates that show best growth on anthracene were more adopted to degrade a wide range of PAHs as also reported previously [Kiyohara *et al.* 1982; Singleton *et al.* 2005; Khan *et al.* 2006; Kumar *et al.* 2010; Othman *et al.* 2010; Dastgheib *et al.* 2011].

It is also interesting that, there was no substantial similarity between organisms capable of growing on naphthalene and those growing on phenanthrene. A number of phenanthrene degrading isolates were found to grow on naphthalene while, others cannot. The ability of bacteria in water, soil or sediment to degrade PAHs depends largely on the complexity of the PAHs chemical structure and the extent of enzymatic adaptation by indigenous bacteria in response to chronic exposure to aromatic hydrocarbons. As previously reported, that various bacteria were isolated from soil and wastewater contains PAHs degrading isolates like *Pseudomonas* sp., *Bacillus* sp., *Enterobactor* and *Clostridium* sp. [Heitkamp *et al.* 1988; Zhao *et al.* 2000; Christensen *et al.* 2004].

3.3.2 Preliminary screening of the isolated bacteria on solid media using anthracene

101 isolates were able to grow on anthracene amended media. This capability was further confirmed on mineral salt media with PNRG and without glucose PNR. The number of resistant isolates was 68 that showed best growth up to 1200ppm. The isolated bacteria were screened on solid media individually on the selected compounds are discussed below in consecutive sections:

3.3.3 Screening of anthracene degrading bacteria isolated from *Brassica campestris*

Among the 59 B-isolates, 11 were able to grow on anthracene concentration of 1200ppm among which 4 isolates showed rich growth indicated by (+++) at this very high concentration as shown in Table 3.1.

Table 3.1: Screening of bacterial isolates from *Brassica campestris* (B-isolates) on anthracene amended nutrient agar

S. No.	Isolate	Anthracene concentration in ppm													
		25	50	100	200	300	400	500	600	700	800	900	1000	1100	1200
1.	B_1	+++	+++	+++	+++	++	++	+	+	+	+	-	-	-	-
2.	B_2	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	B_3	+	+	+	+	-	-	-	-	-	-	-	-	-	-
4.	B_4	+++	+++	+++	++	+++	+++	+++	+++	+++	+	+	+	+	+
5.	B_5	+++	+++	+++	++	++	++	++	++	++	++	++	++	++	++
6.	B_6	+++	+++	+++	++	++	+++	+++	+++	++	++	++	++	++	++
7.	B_7	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
8.	B_8	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	-	-	-

9.	B_9	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	-	-
10.	B_{10}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	+	+
11.	B_{11}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	-	-
12.	B_{12}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	+	+
13.	B_{13}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+
14.	B_{14}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	-	-
15.	B_{15}	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	+	-	-
16.	B_{16}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	-	-
17.	B_{17}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
18.	B_{18}	++	+	++	++	+++	+++	+++	+++	+++	+++	+++	++	+	+
19.	B_{19}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	-
20.	B_{20}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
21.	B_{21}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
22.	B_{22}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
23.	B_{23}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+
24.	B_{24}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+
25.	B_{25}	+++	+++	+++	+++	+++	++	++	+++	++	++	+	+	-	-
26.	B_{26}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+
27.	B_{29}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+
28.	B_{30}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+
29.	B_{34}	+++	+++	+++	+++	+++	++	++	++	+	+	+	+	-	-
30.	B_{35}	+++	+++	+++	+++	++	++	++	++	++	+	+	+	-	-
31.	B_{36}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
32.	B_{37}	+++	+++	+++	+++	++	++	++	+	+	+	+	-	-	-
33.	B_{38}	+++	+++	+++	+++	++	+++	+++	+++	++	++	++	++	+	+
34.	B_{39}	+++	+++	+++	+++	++	+++	+++	++	++	++	+	+	+	-
35.	B_{40}	+++	+++	+++	+++	++	+++	+++	++	++	++	+	+	+	+
36.	B_{41}	+++	+++	+++	+++	++	+++	+++	++	++	++	++	+	+	+
37.	B_{42}	+++	+++	+++	+++	+++	++	+++	++	++	++	+	+	-	-
38.	B_{43}	+++	+++	+++	+++	+++	+++	+	+	+	+	+	+	+	-
39.	B_{44}	+++	+++	+++	+++	+++	++	++	++	++	+	+	+	-	-
40.	B_{45}	+++	+++	+++	+++	+++	++	++	++	+	+	+	+	-	-
41.	B_{46}	+++	+++	+++	+++	+++	+++	++	++	++	+	+	+	-	-
42.	B_{47}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+	+
43.	B_{49}	+++	+++	+++	+++	+++	+++	++	++	++	+	+	+	+	-
44.	B_{53}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++
45.	B_{55}	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+	-	-
46.	B_{56}	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+	-	-	-
47.	B_{57}	+++	+++	+++	+++	+++	++	++	++	+	+	+	+	-	-
48.	B_{58}	+++	+++	+++	+++	+++	++	++	++	+	+	+	-	-	-
49.	B_{59}	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+	+
50.	B_{60}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+	-
51.	B_{61}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	-	-
52.	B_{62}	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+	+	+
53.	B_{63}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+	+
54.	B_{64}	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+	+	-	-
55.	B_{65}	+++	+++	+++	+++	+++	++	++	++	+	+	+	+	-	-
56.	B_{66}	+++	+++	+++	+++	+++	++	++	++	++	+	+	-	-	-
57.	B_{67}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+	+
58.	B_{68}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++
59.	B_{69}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.4 Screening of naphthalene degrading bacteria isolated from *Brassica campestris*

59 B-isolates were found to show growth on nutrient agar amended with different concentrations of naphthalene. 7 isolates showed rich growth while 11 were found to have medium growth on nutrient media supplemented with naphthalene concentration of 1100ppm as shown in Table 3.2.

Table 3.2: Screening of B-isolates on naphthalene amended nutrient agar media

S. No.	Isolate	Naphthalene concentration in ppm											
		50	100	200	300	400	500	600	700	800	900	1000	1100
1.	B_1	+++	+++	+++	++	++	+	+	+	+	-	-	-
2.	B_2	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	B_3	+++	+++	+++	++	++	++	+	+	+	-	-	-
4.	B_4	+++	+++	++	+++	+++	+++	+++	+++	+	+	+	+
5.	B_5	+++	+++	++	++	++	++	++	++	+	+	-	-
6.	B_6	+++	+++	++	++	+++	+++	+++	+	+	-	-	-
7.	B_7	+++	+++	++	++	+++	+++	+++	+++	++	-	-	-
8.	B_8	+++	+++	++	++	+++	+++	+++	+++	++	-	-	-
9.	B_9	+++	+++	++	++	+++	+++	+++	+++	+++	+++	+++	++
10.	B_{10}	+++	+++	++	++	+++	+++	+++	+++	+++	+++	+++	++
11.	B_{11}	+++	+++	++	++	+++	+++	+++	+++	+++	++	++	-
12.	B_{12}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+
13.	B_{13}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
14.	B_{14}	+++	+++	+++	+++	+++	+++	+++	++	+	+	-	-
15.	B_{15}	+++	+++	+++	+++	+++	+++	++	++	+	+	+	-
16.	B_{16}	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+	-
17.	B_{17}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+
18.	B_{18}	+	++	++	++	+++	+++	+++	+++	+++	+++	++	++
19.	B_{19}	+++	+++	+++	++	+++	+++	+++	+++	++	++	+	+
20.	B_{20}	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++
21.	B_{21}	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++
22.	B_{22}	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++
23.	B_{23}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
24.	B_{24}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+
25.	B_{25}	+++	+++	+++	+++	++	++	+++	++	++	+	-	-
26.	B_{26}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	-
27.	B_{29}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
28.	B_{30}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
29.	B_{34}	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	-
30.	B_{35}	+++	+++	+++	++	++	++	++	+	+	+	-	-
31.	B_{36}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+
32.	B_{37}	+++	+++	+++	++	++	++	+	+	+	+	-	-
33.	B_{38}	+++	+++	+++	++	+++	+++	+++	++	+	+	-	-
34.	B_{39}	+++	+++	+++	++	+++	+++	+++	+++	+++	++	++	++

35.	B_{40}	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	++	++
36.	B_{41}	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	++
37.	B_{42}	+++	+++	+++	+++	++	+++	+++	+++	++	++	+	+
38.	B_{43}	+++	+++	+++	+++	+++	++	++	++	+	+	+	+
39.	B_{44}	+++	+++	+++	+++	++	++	+++	+++	++	++	-	-
40.	B_{45}	+++	+++	+++	+++	+++	+++	+++	+	+	+	-	-
41.	B_{46}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
42.	B_{47}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++
43.	B_{49}	+++	+++	+++	+++	+++	++	++	++	+	+	+	+
44.	B_{53}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	-
45.	B_{55}	+++	+++	+++	+++	+++	+++	++	++	++	+	+	-
46.	B_{56}	+++	+++	+++	+++	+++	+++	++	+	+	+	-	-
47.	B_{57}	+++	+++	+++	+++	+++	+++	++	++	+	+	+	-
48.	B_{58}	+++	+++	+++	+++	++	++	++	++	++	++	++	+
49.	B_{59}	+++	+++	+++	+++	+++	+++	++	++	++	++	++	+
50.	B_{60}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+
51.	B_{61}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++
52.	B_{62}	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	++	++
53.	B_{63}	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	-
54.	B_{64}	+++	+++	+++	+++	++	++	++	+	+	+	+	-
55.	B_{65}	+++	+++	+++	+++	++	++	++	++	+	+	-	-
56.	B_{66}	+++	+++	+++	+++	+++	+++	+++	++	+	+	-	-
57.	B_{67}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
58.	B_{68}	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	++
59.	B_{69}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.5 Screening of phenanthrene degrading bacteria isolated from *Brassica campestris*

Results of 59 B-isolates for growth are shown in Table 3.3. As clear from table, 7 isolates showed rich growth and 14 showed moderate growth on 1100ppm phenanthrene concentration.

Table 3.3: Screening of B-isolates on phenanthrene amended nutrient agar media

S. No	Isolate	Phenanthrene concentration in ppm											
		50	100	200	300	400	500	600	700	800	900	1000	1100
1.	B_1	+++	+++	+++	++	++	+	+	+	+	-	-	-
2.	B_2	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	B_3	+++	+++	+++	++	++	++	+	+	+	-	-	-
4.	B_4	+++	+++	++	+++	+++	+++	+++	+++	+	+	+	+

5.	B_5	+++	+++	++	++	++	++	++	++	+	+	-	-
6.	B_6	+++	+++	++	++	+++	+++	+++	+	+	-	-	-
7.	B_7	+++	+++	++	++	+++	+++	+++	+++	++	-	-	-
8.	B_8	+++	+++	++	++	+++	+++	+++	+++	++	-	-	-
9.	B_9	+++	+++	++	++	+++	+++	+++	+++	+++	+++	+++	++
10.	B_{10}	+++	+++	++	++	+++	+++	+++	+++	+++	+++	+++	++
11.	B_{11}	+++	+++	++	++	+++	+++	+++	+++	+++	++	++	-
12.	B_{12}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+
13.	B_{13}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
14.	B_{14}	+++	+++	+++	+++	+++	+++	+++	++	+	+	-	-
15.	B_{15}	+++	+++	+++	+++	+++	+++	++	++	+	+	+	-
16.	B_{16}	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+	-
17.	B_{17}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+
18.	B_{18}	+	++	++	++	+++	+++	+++	+++	+++	+++	++	++
19.	B_{19}	+++	+++	+++	++	+++	+++	+++	+++	++	++	+	+
20.	B_{20}	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++
21.	B_{21}	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++
22.	B_{22}	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++
23.	B_{23}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
24.	B_{24}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+
25.	B_{25}	+++	+++	+++	+++	++	++	+++	++	++	+	-	-
26.	B_{26}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	-
27.	B_{29}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
28.	B_{30}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
29.	B_{34}	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	-
30.	B_{35}	+++	+++	+++	+++	++	++	++	+	+	+	-	-
31.	B_{36}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+
32.	B_{37}	+++	+++	+++	+++	++	++	+	+	+	+	-	-
33.	B_{38}	+++	+++	+++	+++	+++	+++	+++	++	+	+	-	-
34.	B_{39}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++
35.	B_{40}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++
36.	B_{41}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
37.	B_{42}	+++	+++	+++	+++	++	+++	+++	+++	++	++	+	+
38.	B_{43}	+++	+++	+++	+++	+++	++	++	++	+	+	+	+
39.	B_{44}	+++	+++	+++	+++	++	++	+++	+++	++	++	-	-
40.	B_{45}	+++	+++	+++	+++	+++	+++	+++	+	+	+	-	-
41.	B_{46}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
42.	B_{47}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++
43.	B_{49}	+++	+++	+++	+++	+++	++	++	++	+	+	+	+

44.	B_{53}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	-
45.	B_{55}	+++	+++	+++	+++	+++	+++	++	++	++	+	+	-
46.	B_{56}	+++	+++	+++	+++	+++	+++	++	+	+	+	-	-
47.	B_{57}	+++	+++	+++	+++	+++	+++	++	++	+	+	+	-
48.	B_{58}	+++	+++	+++	+++	++	++	++	++	++	++	++	+
49.	B_{59}	+++	+++	+++	+++	+++	+++	++	++	++	++	++	+
50.	B_{60}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+
51.	B_{61}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++
52.	B_{62}	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	++	++
53.	B_{63}	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	-
54.	B_{64}	+++	+++	+++	+++	++	++	++	+	+	+	+	-
55.	B_{65}	+++	+++	+++	+++	++	++	++	++	+	+	-	-
56.	B_{66}	+++	+++	+++	+++	+++	+++	+++	++	+	+	-	-
57.	B_{67}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
58.	B_{68}	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	++
59.	B_{69}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.6 Screening of acenaphthene degrading bacteria isolated from *Brassica campestris*

Results of 59 B-isolates on acenaphthene amended media are shown in Table 3.4. 19 isolates showed rich growth upto 800ppm while 14 isolates were resistant to 900ppm.

Table 3.4: Screening of B-isolates on Acenaphthene amended nutrient agar media

S. No.	Isolate	Acenaphthalene concentration in ppm									
		50	100	200	300	400	500	600	700	800	900
1.	B_1	+++	+++	+++	++	++	++	++	+	+	-
2.	B_2	+++	+++	++	+++	+++	+++	+++	+++	+++	+++
3.	B_3	+++	+++	+++	++	++	++	+	+	+	-
4.	B_4	+++	+++	++	+++	+++	+++	+++	+++	+	+
5.	B_5	+++	+++	+++	++	++	++	++	++	+	+
6.	B_6	+++	+++	+++	+++	+++	+++	++	++	+	-
7.	B_7	+++	+++	++	++	+++	+++	+++	+	+	-
8.	B_8	+++	+++	++	++	+++	+++	+++	++	+	-
9.	B_9	+++	+++	++	++	+++	+++	+++	+++	+++	+++

10.	B_{10}	+++	+++	++	++	+++	+++	+++	+++	+++	+++
11.	B_{11}	+++	+++	++	++	+++	+++	+++	+++	++	++
12.	B_{12}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
13.	B_{13}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
14.	B_{14}	+++	+++	+++	+++	+++	+++	+++	++	+	-
15.	B_{15}	+++	+++	+++	+++	+++	+++	++	++	+	+
16.	B_{16}	+++	+++	+++	+++	+++	+++	++	+	-	-
17.	B_{17}	+++	+++	+++	+++	+++	+++	+++	+++	++	+
18.	B_{18}	+++	+++	+++	+++	+++	+++	+++	+++	++	++
19.	B_{19}	+++	+++	+++	+++	+++	+++	+++	++	++	+
20.	B_{20}	+++	+++	+++	+++	+++	+++	+++	++	++	++
21.	B_{21}	+++	+++	+++	+++	+++	+++	+++	++	++	++
22.	B_{22}	+++	+++	+++	+++	+++	+++	++	++	++	+
23.	B_{23}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
24.	B_{24}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
25.	B_{25}	+++	+++	+++	+++	++	++	+++	++	++	+
26.	B_{26}	+++	+++	+++	+++	+++	+++	+++	+++	++	++
27.	B_{29}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
28.	B_{30}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
29.	B_{34}	+++	+++	+++	+++	+++	+++	+++	++	++	++
30.	B_{35}	+++	+++	+++	++	++	++	++	+	+	+
31.	B_{36}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
32.	B_{37}	+++	+++	+++	++	++	++	+	+	+	+
33.	B_{38}	+++	+++	+++	++	+++	+++	+++	++	+	+
34.	B_{39}	+++	+++	+++	++	+++	+++	+++	+++	+++	++
35.	B_{40}	+++	+++	+++	++	+++	+++	++	+	-	-
36.	B_{41}	+++	+++	+++	++	+++	+++	++	++	+	-
37.	B_{42}	+++	+++	+++	+++	++	+++	+++	+++	+	+
38.	B_{43}	+++	+++	+++	+++	+++	++	++	++	+	+
39.	B_{44}	+++	+++	+++	+++	++	++	+++	+++	+	+
40.	B_{45}	+++	+++	+++	+++	+++	+++	+++	+	+	+
41.	B_{46}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
42.	B_{47}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
43.	B_{49}	+++	+++	+++	+++	+++	++	++	++	++	++
44.	B_{53}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
45.	B_{55}	+++	+++	+++	+++	+++	+++	++	++	++	+
46.	B_{56}	+++	+++	+++	+++	+++	+++	++	+	+	+
47.	B_{57}	+++	+++	+++	+++	+++	+++	++	++	+	+
48.	B_{58}	+++	+++	+++	+++	++	++	++	++	++	++

49.	B_{59}	+++	+++	+++	+++	+++	+++	++	++	++	++
50.	B_{60}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+
51.	B_{61}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
52.	B_{62}	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
53.	B_{63}	+++	+++	+++	+++	+++	+++	+++	++	++	+
54.	B_{64}	+++	+++	+++	+++	++	++	++	+	+	+
55.	B_{65}	+++	+++	+++	+++	++	++	++	++	+	+
56.	B_{66}	+++	+++	+++	+++	+++	+++	+++	++	+	+
57.	B_{67}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
58.	B_{68}	+++	+++	+++	+++	+++	+++	+++	++	++	++
59.	B_{69}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.7 Screening of pyrene degrading bacteria isolated from *Brassica campestris*

The isolated 101 bacteria when grow on pyrene amended media 61 B-isolates were able to show rich growth (+++) as shown in Table 3.5. 16 isolates grow upto 1000ppm, half with rich and half with moderate growth rate.

Table 3.5: Screening of B-isolates on pyrene amended nutrient agar media

S. No.	Isolate	Pyrene concentration in ppm												
		10	20	50	100	200	300	400	500	600	700	800	900	1000
1.	B_1	+++	+++	+++	+++	++	++	+	+	+	+	-	-	-
2.	B_2	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	B_3	+	+	+	+	-	-	-	-	-	-	-	-	-
4.	B_4	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
5.	B_5	+++	+++	+++	++	++	++	++	++	++	++	++	++	++
6.	B_6	+++	+++	+++	++	++	+++	+++	+++	++	++	++	++	++
7.	B_7	+++	+++	+++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
8.	B_8	+++	+++	+++	++	++	+++	+++	+++	+++	++	++	-	-
9.	B_9	+++	+++	+++	++	++	+++	+++	+++	+++	+++	++	+	-
10.	B_{10}	+++	+++	+++	++	++	+++	+++	+++	+++	+++	++	+	+
11.	B_{11}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	-
12.	B_{12}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	+

13.	B_{13}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+
14.	B_{14}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	-
15.	B_{15}	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	+	-
16.	B_{16}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	-
17.	B_{17}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
18.	B_{18}	++	+	++	++	++	+++	+++	+++	+++	+++	+++	++	+
19.	B_{19}	+++	+++	+++	+++	++	+++	+++	+++	+++	++	++	+	+
20.	B_{20}	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++
21.	B_{21}	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++
22.	B_{22}	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++
23.	B_{23}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
24.	B_{24}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
25.	B_{25}	+++	+++	+++	+++	+++	++	++	+++	++	++	+	+	-
26.	B_{26}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++
27.	B_{29}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+
28.	B_{30}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
29.	B_{34}	+++	+++	+++	+++	+++	++	++	++	+	+	+	+	-
30.	B_{35}	+++	+++	+++	+++	++	++	++	++	++	+	+	+	-
31.	B_{36}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
32.	B_{37}	+++	+++	+++	+++	++	++	++	+	+	+	+	-	-
33.	B_{38}	+++	+++	+++	+++	++	+++	+++	+++	++	++	++	++	+
34.	B_{39}	+++	+++	+++	+++	++	+++	+++	++	++	++	+	+	+
35.	B_{40}	+++	+++	+++	+++	++	+++	+++	++	++	++	+	+	+
36.	B_{41}	+++	+++	+++	+++	++	+++	+++	++	++	++	++	+	+
37.	B_{42}	+++	+++	+++	+++	+++	++	+++	++	++	++	+	+	-
38.	B_{43}	+++	+++	+++	+++	+++	+++	+	+	+	+	+	+	+
39.	B_{44}	+++	+++	+++	+++	+++	++	++	++	++	+	+	+	-
40.	B_{46}	+++	+++	+++	+++	+++	++	++	++	+	+	+	+	-
41.	B_{30}	+++	+++	+++	+++	+++	+++	++	++	++	+	+	+	-
42.	B_{45}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+
43.	B_{46}	+++	+++	+++	+++	+++	+++	++	++	++	+	+	+	+
44.	B_{47}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++

45.	B_{49}	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+	-
46.	B_{53}	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+	-	-
47.	B_{55}	+++	+++	+++	+++	+++	++	++	++	+	+	+	+	-
48.	B_{56}	+++	+++	+++	+++	+++	++	++	++	+	+	+	-	-
49.	B_{57}	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+
50.	B_{58}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+
51.	B_{59}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	-
52.	B_{60}	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+	+
53.	B_{61}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+
54.	B_{62}	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+	+	-
55.	B_{63}	+++	+++	+++	+++	+++	++	++	++	+	+	+	+	-
56.	B_{64}	+++	+++	+++	+++	+++	++	++	++	++	+	+	-	-
57.	B_{65}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+
58.	B_{66}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
59.	B_{67}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+
60.	B_{68}	+++	+++	+++	+++	+++	++	+	+	+	+	-	-	-
61.	B_{69}	+++	+++	+++	+++	+++	++	+	+	+	+	-	-	-

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.8 Screening of anthracene degrading bacteria isolated from *Sisymbrium irio*

Among the isolated bacteria, 25 S-isolates when grown on anthracene amended media, 3 isolates showed rich growth and 3 medium growths at 1200ppm concentration as shown in Table 3.6.

Table 3.6: Screening of S-isolates on anthracene amended nutrient agar media

S. No.	Isolate	Anthracene concentration in ppm													
		25	50	100	200	300	400	500	600	700	800	900	1000	1100	1200
1.	S_2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	S_5	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	S_6	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
4.	S_7	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
5.	S_{11}	+++	++	+++	+++	+++	+++	+++	++	+	+	+	-	-	-
6.	S_{12}	+++	+++	+++	+++	+++	+++	+++	++	+	+	+	-	-	-

7.	S_{13}	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
8.	S_{15}	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+	+	-	-
9.	S_{16}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+
10.	S_{18}	+++	+++	+++	+++	+++	+++	+++	++	+++	++	++	++	+	+
11.	S_{19}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	++	+
12.	S_{20}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	++	++
13.	S_{21}	+++	++	+++	+++	+++	+++	+++	++	++	++	+	+	+	-
14.	S_{22}	+++	+++	++	+++	+++	+++	+++	+++	++	+	+	+	-	-
15.	S_{23}	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+	-	-
16.	S_{24}	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	-
17.	S_{30}	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	-
18.	S_{31}	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+	+
19.	S_{33}	+++	++	+++	+++	+++	+++	+++	+++	+++	++	++	-	-	-
20.	S_{34}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	+	+
21.	S_{36}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	-	-
22.	S_{37}	+++	+++	+++	+++	++	++	++	+	+	+	+	-	-	-
23.	S_{39}	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	+
24.	S_{40}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	-
25.	S_{51}	+++	+++	+++	++	++	++	+	+	+	+	+	-	-	-

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.9 Screening of naphthalene degrading bacteria isolated from *Sisymbrium irio*

25 S-isolates on naphthalene (1100 ppm) amended media, 12 isolates showed rich growth and 5 were able to grow moderately as shown in Table 3.7.

Table 3.7: Screening of S-isolates on naphthalene amended nutrient agar media

S. No.	Isolate	Naphthalene concentration in ppm											
		50	100	200	300	400	500	600	700	800	900	1000	1100
1.	S_2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	S_5	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	S_6	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
4.	S_7	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
5.	S_{11}	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+
6.	S_{12}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+
7.	S_{13}	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
8.	S_{15}	+++	+++	+++	+++	+++	+++	++	+++	+++	++	+	+
9.	S_{16}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

10.	S_{18}	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
11.	S_{19}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
12.	S_{20}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++
13.	S_{21}	++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++
14.	S_{22}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
15.	S_{23}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
16.	S_{24}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
17.	S_{30}	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
18.	S_{31}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
19.	S_{33}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	-
20.	S_{34}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++
21.	S_{36}	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	-
22.	S_{37}	+++	+++	+++	++	++	++	++	++	++	+	+	-
23.	S_{39}	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	++	++
24.	S_{40}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
25.	S_{51}	+++	+++	+++	+++	+++	++	++	++	++	+	+	-

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.10 Screening of phenanthrene degrading bacteria isolated from *Sisymbrium irio*

9 out of 25 S-isolates when grow on phenanthrene amended media were able to show rich growth upto 1100ppm as shown in Table 3.8. 9 isolates were observed to show medium growth rate at this concentration.

Table 3.8: Screening of S-isolates on phenanthrene amended nutrient agar media

S. No.	Isolate	Phenanthrene concentration in ppm											
		50	100	200	300	400	500	600	700	800	900	1000	1100
1.	S_2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	S_5	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	S_6	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
4.	S_7	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
5.	S_{11}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+
6.	S_{12}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+
7.	S_{13}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
8.	S_{15}	+++	+++	+++	+++	+++	+++	++	+++	+++	++	+	+
9.	S_{16}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
10.	S_{18}	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
11.	S_{19}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
12.	S_{20}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++
13.	S_{21}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

14.	S_{22}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
15.	S_{23}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
16.	S_{24}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
17.	S_{30}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++
18.	S_{31}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++
19.	S_{33}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	-
20.	S_{34}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++
21.	S_{36}	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	-
22.	S_{37}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	+
23.	S_{39}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++
24.	S_{40}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
25.	S_{51}	+++	+++	+++	+++	+++	++	++	++	++	+	+	-

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.11 Screening of acenaphthene degrading bacteria isolated from *Sisymbrium irio*

23 S-isolates were grown on acenaphthene, 13 were found to show rich growth and 5 with medium growth rate as shown in Table 3.9.

Table 3.9: Screening of S-isolates on acenaphthene amended nutrient agar media

S. No.	Isolate	Acenaphthene concentration in ppm									
		50	100	200	300	400	500	600	700	800	900
1.	S_2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	S_5	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	S_6	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
4.	S_7	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
4.	S_{11}	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
5.	S_{12}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
6.	S_{13}	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
7.	S_{15}	+++	+++	+++	+++	+++	+++	++	+++	+++	++
8.	S_{16}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
9.	S_{18}	+++	+++	+++	+++	+++	+++	++	+++	+++	+++
10.	S_{19}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
10.	S_{20}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
11.	S_{21}	++	+++	+++	+++	+++	+++	+++	+++	+++	++
12.	S_{22}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
13.	S_{23}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
14.	S_{24}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
15.	S_{30}	++	+++	+++	+++	+++	+++	+++	+++	+++	+++

16.	S_{31}	+++	+++	+++	+++	+++	+++	+++	+++	++	+
17.	S_{33}	+++	+++	+++	+++	+++	+++	+++	+++	++	++
18.	S_{34}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
19.	S_{36}	+++	+++	+++	+++	+++	+++	+++	++	++	++
20.	S_{37}	+++	+++	+++	++	++	++	++	++	++	+
21.	S_{39}	+++	++	+++	+++	+++	+++	+++	+++	+++	+++
22.	S_{40}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
23.	S_{51}	+++	+++	+++	+++	+++	++	++	++	++	+

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.12 Screening of pyrene degrading bacteria isolated from *Sisymbrium irio*

Among the 25 S-isolates isolates grown on pyrene, 4 isolates showed rich growth and 2 were able to grow moderately upto 1000ppm pyrene concentration as shown in Table 3.10.

Table 3.10: Screening of S-isolates on pyrene amended nutrient agar media

S. No.	Isolate	Pyrene concentration in ppm												
		10	20	50	100	200	300	400	500	600	700	800	900	1000
1.	S_2	+++	+++	+++	+++	+++	+++	++	++	+	+	+	-	-
2.	S_5	+++	++	+++	+++	+++	+++	+++	++	++	++	+	+	-
3.	S_6	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
4.	S_7	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
5.	S_{11}	+++	++	+++	+++	+++	+++	+++	++	+	+	+	-	-
6.	S_{12}	+++	+++	+++	+++	+++	+++	+++	++	+	+	+	-	-
7.	S_{13}	+++	++	+++	+++	+++	+++	+++	+++	+++	+	+	+	+
8.	S_{15}	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+	+	-
9.	S_{16}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	+
10.	S_{18}	+++	+++	+++	+++	+++	+++	+++	++	+++	++	++	++	+
11.	S_{19}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	++
12.	S_{20}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	++
13.	S_{21}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
14.	S_{22}	+++	+++	++	+++	+++	+++	+++	+++	++	+	+	+	-
15.	S_{23}	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+	-
16.	S_{24}	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+
17.	S_{30}	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+
18.	S_{31}	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+
19.	S_{33}	+++	++	+++	+++	+++	+++	+++	+++	+++	++	++	-	-
20.	S_{34}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	+

21.	S_{36}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	-
22.	S_{37}	+++	+++	+++	+++	++	++	++	+	+	+	+	-	-
23.	S_{39}	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	++	+
24.	S_{40}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+
25.	S_{51}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.13 Screening of anthracene degrading bacteria isolated from *Morus alba*

Among the isolated 8 bacterial isolates (M-isolate) when grown on anthracene amended media 3 isolates showed best growth upto 1200ppm concentration as shown in Table 3.11.

Table 3.11: Screening of M-isolates on anthracene amended nutrient agar media

S. No	Isolate	Anthracene concentration in ppm													
		25	50	100	200	300	400	500	600	700	800	900	1000	1100	1200
1.	M	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	M_1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	M_2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
4.	M_3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+	+	+
5.	M_4	+++	+++	+++	+++	+++	++	++	+	+	+	+	+	-	-
6.	M_5	+++	+++	+++	+++	+++	+	+	+	+	+	+	+	+	-
7.	M_6	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+	+	-
8.	M_7	+++	+++	+++	+++	+++	+++	+++	+++	++	+	+	-	-	-

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.14 Screening of naphthalene degrading bacteria isolated from *Morus alba*

Half of isolates showed rich growth upto only 600 ppm from the 8 bacterial isolates (M-isolates) as shown in Table 3.12.

Table 3.12: Screening of M-isolates on naphthalene amended nutrient agar media

S. No.	Isolate	Naphthalene concentration in ppm											
1.	M	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+
2.	M_1	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+
3.	M_2	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+
4.	M_3	+++	+++	+++	+++	+++	+++	++	++	++	+	+	+
5.	M_4	+++	+++	+++	+++	++	++	++	++	+	+	+	-
6.	M_5	+++	+++	+++	+++	+	+	+	+	+	+	-	-
7.	M_6	+++	+++	+++	+++	+++	++	++	++	++	-	-	-
8.	M_7	+++	+++	+++	+++	+++	+++	+++	+	+	+	-	-

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.15 Screening of phenanthrene degrading bacteria isolated from *Morus alba*

8 M-isolates on phenanthrene only a one isolate was able to show rich growth at 700ppm phenanthrene concentration as shown in Table 3.13.

Table 3.13: Screening of M-isolates on phenanthrene amended nutrient agar media

S. No.	Isolate	Phenanthrene concentration in ppm											
		50	100	200	300	400	500	600	700	800	900	1000	1100
1.	M	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+
2.	M_1	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+
3.	M_2	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+
4.	M_3	+++	+++	+++	+++	+++	+++	++	++	++	+	+	+
5.	M_4	+++	+++	+++	+++	++	++	++	++	+	+	+	-
6.	M_5	+++	+++	+++	+++	++	++	++	+	+	+	-	-
7.	M_6	+++	+++	+++	+++	+++	++	++	++	++	-	-	-
8.	M_7	+++	+++	+++	+++	+++	+++	+++	+	+	+	-	-

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.16 Screening of acenaphthene degrading bacteria isolated from *Morus alba*

Only one M-isolate was able to grow on acenaphthene amended media upto700 ppm as shown in Table 3.14.

Table 3.14: Screening of M-isolates on acenaphthene amended nutrient agar media

S. No.	Isolate	Acenaphthene concentration in ppm											
		50	100	200	300	400	500	600	700	800	900	1000	1100
1.	M	+++	+++	+++	+++	+++	+++	+++	+++	++	+	+	+
2.	M_1	+++	+++	+++	+++	+++	+++	++	++	++	+	+	+
3.	M_2	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+
4.	M_3	+++	+++	+++	+++	+++	+++	++	++	+	+	+	+
5.	M_4	+++	+++	+++	+++	++	++	++	++	+	+	+	-
6.	M_5	+++	+++	+++	+++	+	+	+	+	+	+	-	-
7.	M_6	+++	+++	+++	+++	+++	++	++	++	++	-	-	-
8.	M_7	+++	+++	+++	+++	+++	++	++	+	+	+	-	-

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.17 Screening of pyrene degrading bacteria isolated from *Morus alba*

5 isolates from the 8 M-isolates when grown on pyrene amended media showed rich growth upto 1100ppm as shown in Table 3.15.

Table 3.15: Screening of M-isolates on pyrene amended nutrient agar media

S. No.	Isolate	Pyrene concentration in ppm												
1.	M	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	M_1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	M_2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
4.	M_3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
5.	M_4	+++	+++	+++	+++	+++	++	++	+	+	+	+	+	-
6.	M_5	+++	+++	+++	+++	+++	+	+	+	+	+	+	+	+
7.	M_6	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+	+
8.	M_7	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.18 Screening of anthracene degrading bacteria isolated from sewage water

Among the isolated 11 bacteria (W-isolates) from sewage water when grown on anthracene amended media as shown in Table 3.16. 2 isolates showed rich growth upto1200ppm.

Table 3.16: Screening of W-isolates on anthracene amended nutrient agar media

S. No	Isolate	Anthracene concentration in ppm													
		25	50	100	200	300	400	500	600	700	800	900	1000	1100	1200
1.	W_1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	W_2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	W_3	+	-	-	-	-	-	-	-	-	-	-	-	-	-
4.	W_4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+	+	+
5.	W_5	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+	-	-
6.	W_6	+++	+++	+++	+++	+++	+++	+++	+++	++	+	+	+	-	-
7.	W_7	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	-
8.	W_8	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	-	-	-
9.	W_9	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	-	-
10.	W_{10}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	+	+
11.	W_{11}	+++	+++	+++	++	++	++	++	++	++	++	+	+	+	-

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.19 Screening of naphthalene degrading bacteria isolated from sewage water

2 out of 11 W-isolates from sewage water on naphthalene amended media were able to show rich growth upto 1100ppm as shown in Table 3.17.

Table 3.17: Screening of W-isolates on naphthalene amended nutrient agar media

S. No.	Isolate	Naphthalene concentration in ppm											
		50	100	200	300	400	500	600	700	800	900	1000	1100
1.	W_1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	W_2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	W_3	+++	+++	+++	+++	++	++	++	++	+	+	-	-
4.	W_4	+++	+++	+++	+++	+++	+++	+++	+++	+	-	-	-
5.	W_5	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	-
6.	W_6	+++	+++	+++	+++	+++	+++	+++	++	+	-	-	-
7.	W_7	+++	+++	+++	+++	+++	+++	++	++	+	+	-	-
8.	W_8	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++
9.	W_9	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++
10.	W_{10}	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+
11.	W_{11}	+++	+++	++	++	++	++	++	++	++	++	++	++

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.20 Screening of phenanthrene degrading bacteria isolated from sewage water

11 W-isolates) on phenanthrene 2 isolates showed rich growth while, 3 isolates showed medium growth as shown in Table 3.18.

Table 3.18: Screening of W-isolates on phenanthrene amended nutrient agar media

S. No.	Isolate	Phenanthrene concentration in ppm											
		50	100	200	300	400	500	600	700	800	900	1000	1100
1.	W_1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	W_2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	W_3	+++	+++	+++	+++	++	++	++	++	+	+	-	-
4.	W_4	+++	+++	+++	+++	+++	+++	+++	+++	+	-		-
5.	W_5	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	-
6.	W_6	+++	+++	+++	+++	+++	+++	+++	++	+	-	-	-
7.	W_7	+++	+++	+++	+++	+++	+++	++	++	+	+	-	-
8.	W_8	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++
9.	W_9	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++
10.	W_{10}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+
11.	W_{11}	+++	+++	++	++	++	++	++	++	++	++	++	++

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.21 Screening of acenaphthene degrading bacteria isolated from sewage water

11 W-isolates on acenaphthene amended media, 2 isolates showed rich growth and 3 showed moderate growths at 1100ppm as shown in Table 3.19.

Table 3.19: Screening of W-isolates on acenaphthene amended nutrient agar media

S. No.	Isolate	Acenaphthene concentration in ppm											
		50	100	200	300	400	500	600	700	800	900	1000	1100
1.	W_1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	W_2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	W_3	+++	+++	+++	+++	++	++	++	++	+	+	-	-
4.	W_4	+++	+++	+++	+++	+++	+++	+++	+++	+	-		-
5.	W_5	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	-
6.	W_6	+++	+++	+++	+++	+++	+++	+++	++	+	+	-	-
7.	W_7	+++	+++	+++	+++	+++	+++	++	++	+	+	-	-
8.	W_8	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++
9.	W_9	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++
10.	W_{10}	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+
11.	W_{11}	+++	+++	++	++	++	++	++	++	++	++	++	++

+++ = Rich growth ++ = Medium growth+ =Less growth (-) = No growth

3.3.22 Screening of pyrene degrading bacteria isolated from sewage water

11 W-isolates) on pyrene 3 isolates rich growth showed medium growth at 1000 ppm as shown in Table 3.20.

Table 3.20: Screening of W-isolates on pyrene amended nutrient agar media

S. No.	Isolate	Pyrene concentration in ppm												
		10	20	50	100	200	300	400	500	600	700	800	900	1000
1.	W_1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	W_2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	W_3	+	-	-	-	-	-	-	-	-	-	-	-	-
4.	W_4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+	+
5.	W_5	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+	-
6.	W_6	+++	+++	+++	+++	+++	+++	+++	+++	++	+	+	+	-
7.	W_7	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+
8.	W_8	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
9.	W_9	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	-
10.	W_{10}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++
11.	W_{11}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

3.3.23 Adaptation of bacterial isolates

Adaptation processes, which occur as a result of an increase in the hydrocarbon-oxidising potential of the microbial community, allow the development of microbial populations with the ability to degrade PAHs [Macleod & Semple, 2006; Ye *et al.* 2011].

3.3.23.1 Adaptation through changing PAHs concentration

Adaptation was carried out through changing PAHs concentration in the growth media. The bacterial isolates subjected to lower concentration showed medium or less growth first and then rich growth with increasing concentration as shown in Table 3.21. Microbes can be adapted for biodegradation of PAHs through following methods:

Table 3.21: Adaptation of bacterial isolates on selected PAHs amended PNRG media

S. No.	Isolate	PAHs Conc. (ppm) /PNRG				
		Acenaphthene	Naphthalene	Anthracene	Phenanthrene	Pyrene
		100-900	100-1000	50-1200	100-1100	10-1000
1.	B_2	+++	+++	+++	+++	+++
2.	B_3	+++	+++	+++	+++	+++
3.	B_4	+++	+++	+++	+++	+++
4.	B_5	+++	+++	+++	+++	+++
5.	B_9	+++	+++	+++	+++	+++
6.	B_{10}	+++	+++	+++	+++	+++
7.	B_{11}	+++	+++	+++	+++	+++
8.	B_{12}	+++	+++	+++	+++	+++
9.	B_{13}	+++	+++	+++	+++	+++
10.	B_{15}	+++	+++	+++	+++	+++
11.	B_{16}	+++	+++	+++	+++	+++
12.	B_{20}	+++	+++	+++	+++	+++
13.	B_{21}	+++	+++	+++	+++	+++
14.	B_{23}	+++	+++	+++	+++	+++
15.	B_{24}	+++	+++	+++	+++	+++
16.	B_{26}	+++	+++	+++	+++	+++
17.	B_{27}	+++	+++	++	+++	+++
18.	B_{29}	+++	+++	+++	+++	+++
19.	B_{30}	+++	+++	+++	+++	+++
20.	B_{33}	+++	+++	+++	+++	+++
21.	B_{36}	+++	+++	+++	+++	+++
22.	B_{46}	+++	+++	+++	+++	+++
23.	B_{47}	+++	+++	+++	+++	+++
24.	B_{49}	+++	+++	+++	+++	+++
25.	B_{57}	+++	+++	+++	+++	+++
26.	B_{58}	+++	+++	+++	+++	+++
27.	B_{59}	+++	+++	+++	+++	+++

28.	B_{60}	+++	+++	+++	+++	+++
29.	B_{61}	+++	+++	+++	+++	+++
30.	B_{62}	+++	+++	+++	+++	+++
31.	B_{67}	+++	+++	+++	+++	+++
32.	B_{68}	+++	+++	+++	+++	+++
33.	S_2	+++	+++	+++	+++	+++
34.	S_5	+++	+++	+++	+++	+++
35.	S_6	+++	+++	+++	+++	+++
36.	S_7	+++	+++	+++	+++	+++
37.	S_{11}	+++	+++	+++	+++	+++
38.	S_{12}	+++	+++	+++	+++	+++
39.	S_{13}	+++	+++	+++	+++	+++
40.	S_{15}	+++	+++	+++	+++	+++
41.	S_{16}	+++	+++	+++	+++	+++
42.	S_{18}	+++	+++	+++	+++	+++
43.	S_{19}	+++	+++	+++	+++	+++
44.	S_{20}	+++	+++	+++	+++	+++
45.	S_{21}	+++	+++	+++	+++	+++
46.	S_{22}	+++	+++	+++	+++	+++
47.	S_{23}	+++	+++	+++	+++	+++
48.	S_{24}	+++	+++	+++	+++	+++
49.	S_{30}	+++	+++	+++	+++	+++
50.	S_{31}	+++	+++	+++	+++	+++
51.	S_{34}	+++	+++	+++	+++	+++
52.	S_{36}	+++	+++	+++	+++	+++
53.	S_{37}	+++	+++	+++	+++	+++
54.	S_{39}	+++	+++	+++	+++	+++
55.	S_{40}	+++	+++	+++	+++	+++
56.	S_{51}	+++	+++	+++	+++	+++
57.	W_1	+++	+++	+++	+++	+++
58.	W_2	+++	+++	+++	+++	+++
59.	W_5	+++	+++	+++	+++	+++
60.	W_8	+++	+++	+++	+++	+++
61.	W_9	+++	+++	+++	+++	+++
62.	W_{10}	+++	+++	+++	+++	+++
63.	W_{11}	+++	+++	+++	+++	+++
65.	M	+++	+++	+++	+++	+++
66.	M_1	+++	+++	+++	+++	+++
67.	M_2	+++	+++	+++	+++	+++
68.	M_3	+++	+++	+++	+++	+++

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

The isolates must be adapted to concentration as exposure to heighest quantity can be proved lethal to bacterial system. With increasing concentration enhanced growth rate was observed on the media plates is an indication of adaptation of the isolates. As previously reported, these adaptation processes, even though not fully understood, are considered to be controlled by a number of contaminant and soil factors including the concentration of contaminant, interactions with the indigenous microbial populations and length of contaminated soil contact time. In addition, microbial populations may undergo bioavailability-induced adaptations, which serve

to aid contaminated microbe interactions and thus increase contaminant bio-accessibility [Kastner *et al.* 1998; Johnsen *et al.* 2005].

3.3.23.2 Adaptation using multiple media

The screening media was changed from simplest nutrient agar to PNRG and then PNR with selected PAHs as the only carbon source showed that the growth of microbes was not affected by media as shown in Table 3.22. It is clear from this study, the enhanced growth rate was observed even after changing media from that containing glucose to simplest PNR media with only PAHs as carbon source.

Table 3.22: Adaptation of selected bacterial isolates on selected PAHs compounds amended PNR media

S. No.	Isolate	Acenaphthene	Naphthalene	Anthracene	Phenanthrene	Pyrene
		100-900	100-1000	50-1200	100-1100	10-1000
1.	B_2	+++	+++	+++	+++	+++
2.	B_3	+++	+++	+++	+++	+++
3.	B_4	+++	+++	+++	+++	+++
4.	B_5	+++	+++	+++	+++	+++
5.	B_9	+++	+++	+++	+++	+++
6.	B_{10}	+++	+++	+++	+++	+++
7.	B_{11}	+++	+++	+++	+++	+++
8.	B_{12}	+++	+++	+++	+++	+++
9.	B_{13}	+++	+++	+++	+++	+++
10.	B_{15}	+++	+++	+++	+++	+++
11.	B_{16}	+++	+++	+++	+++	+++
12.	B_{20}	+++	+++	+++	+++	+++
13.	B_{21}	+++	+++	+++	+++	+++
14.	B_{23}	+++	+++	+++	+++	+++
15.	B_{24}	+++	+++	+++	+++	+++
16.	B_{26}	+++	+++	+++	+++	+++
17.	B_{27}	+++	+++	+++	+++	+++
18.	B_{29}	+++	+++	+++	+++	+++
19.	B_{30}	+++	+++	+++	+++	+++
20.	B_{33}	+++	+++	+++	+++	+++
21.	B_{36}	+++	+++	+++	+++	+++
22.	B_{46}	+++	+++	+++	+++	+++

23.	B_{47}	+++	+++	+++	+++	+++
24.	B_{49}	+++	+++	+++	+++	+++
25.	B_{57}	+++	+++	+++	+++	+++
26.	B_{58}	+++	+++	+++	+++	+++
27.	B_{59}	+++	+++	+++	+++	+++
28.	B_{60}	+++	+++	+++	+++	+++
29.	B_{61}	+++	+++	+++	+++	+++
30.	B_{62}	+++	+++	+++	+++	+++
31.	B_{67}	+++	+++	+++	+++	+++
32.	B_{68}	+++	+++	+++	+++	+++
33.	S_2	+++	+++	+++	+++	+++
34.	S_5	+++	+++	+++	+++	+++
35.	S_6	+++	+++	+++	+++	+++
36.	S_7	+++	+++	+++	+++	+++
37.	S_{11}	+++	+++	+++	+++	+++
38.	S_{12}	+++	+++	+++	+++	+++
39.	S_{13}	+++	+++	+++	+++	+++
40.	S_{15}	+++	+++	+++	+++	+++
41.	S_{16}	+++	+++	+++	+++	+++
42.	S_{18}	+++	+++	+++	+++	+++
43.	S_{19}	+++	+++	+++	+++	+++
44.	S_{20}	+++	+++	+++	+++	+++
45.	S_{21}	+++	+++	+++	+++	+++
46.	S_{22}	+++	+++	+++	+++	+++
47.	S_{23}	+++	+++	+++	+++	+++
48.	S_{24}	+++	+++	+++	+++	+++
49.	S_{30}	+++	+++	+++	+++	+++
50.	S_{31}	+++	+++	+++	+++	+++
51.	S_{34}	+++	+++	+++	+++	+++
52.	S_{36}	+++	+++	+++	+++	+++
53.	S_{37}	+++	+++	+++	+++	+++
54.	S_{39}	+++	+++	+++	+++	+++
55.	S_{40}	+++	+++	+++	+++	+++
56.	S_{51}	+++	+++	+++	+++	+++
65.	M	+++	+++	+++	+++	+++
66.	M_1	+++	+++	+++	+++	+++
67.	M_2	+++	+++	+++	+++	+++
68.	M_3	+++	+++	+++	+++	+++
69.	W_1	+++	+++	+++	+++	+++

70.	W_2	+++	+++	+++	+++	+++
71.	W_5	+++	+++	+++	+++	+++
72.	W_8	+++	+++	+++	+++	+++
73.	W_9	+++	+++	+++	+++	+++
74.	W_{10}	+++	+++	+++	+++	+++
75.	W_{11}	+++	+++	+++	+++	+++

+++ = Rich growth ++ = Medium growth + =Less growth (-) = No growth

As previously reported, adaptation processes enhance the degradative capability of microbes. The mechanism of adaptation depends not only, on the interaction of microbes with soil but with the amount of bioavailable contaminants as well [Kastner *et al.* 1998; Ye *et al.* 2011]. Previous exposure of indigenous microbes to PAHs can influence degradation capabilities, as suggested by a number of studies [Sartoros *et al.* 2005; Puglisi *et al.* 2007; Ye *et al.* 2011]. Naphthalene and phenanthrene biodegradation in soil always remain the main focus of research in terms of contaminants removal [Macleod & Semple, 2006]. The presence of one contaminant effect the accessibility of the other in a specified area as contaminants always exists in groups [Khan *et al.* 2006; Kumar *et al.* 2010].

3.3.23.3 Adaptation by physical mutation

the bacterial isolate when exposed to UV light to 15 minutes and on addition of 100ppm of anthracene showed improved degradation ability as shown in Table 3.23.

Table 3.23: Adaptation of isolates through physical mutation

Isolate	PAHs	Before mutation	After mutation
S_{13}	Anthracene	0.198	0.42
B_{36}	Anthracene	0.272	0.601
B_{30}	Naphthalene	0.239	0.511
B_{30}	Acenaphthene	0.232	0.601
B_{30}	Phenanthrene	0.222	0.489
B_{30}	Anthracene	0.239	0.101
S_{21}	Phenanthrene	0.134	0.671
S_{21}	Pyrene	0.187	0.497
S_{51}	Pyrene	0.182	0.395
B_4	Pyrene	0.19	0.425
M_3	Pyrene	0.231	0.587

It was observed that the bacterial growth rate was enhanced on exposure to UV-light for 15 minutes. In some cases, microbes lost the degradation ability of certain compound as in this study isolate B_{30} lost anthracene degradation ability after physical mutation. But, showed enhanced degradation ability after mutation for naphthalene, acenaphthene and phenanthrene. Therefore, isolation and maintenance of isolates that utilize anthracene as a sole carbon source can often be difficult, as many isolates grown on minimal medium cannot degrade the anthracene or have lost the ability to degrade it. The selective nature of the isolation media originally used to obtain the wild-type degradative isolate from soil was investigated and mutation using UV light was performed to enhance the degradation ability. Hence, it could be concluded that exposure to UV light for 15 minutes could improve the degradation ability significantly as reported earlier [Leahy & Colwell, 1990]. Microbe can adapt to a changing environment by rapid multiplication or induction of resistant genes that can cope with the degradation of pollutant present in their vicinity. They can also make their cell resistant to contaminants by changing their genome sequencing. They possess specialized genes that can move free within genome and can transfer them within its population [Top & Springael, 2003].

3.3.24 Detection of bacterial growth at the expense of anthracene

Screening of the isolates in liquid media under static condition was also done; as PAHs cannot be mineralize completely on solid media. There was inverse relation between growth of bacteria and disappearance of anthracene as shown in Fig. 3.1.

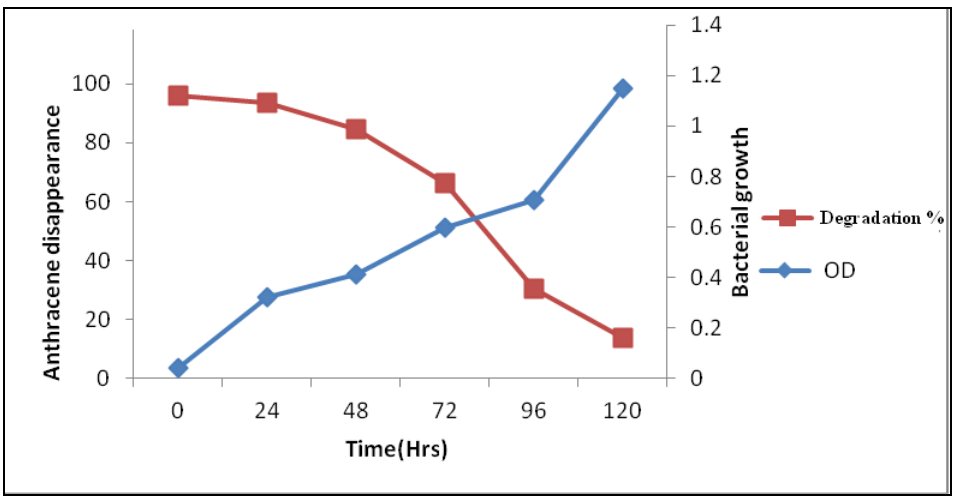

Fig. 3.1: Rate of bacterial growth OD (600nm) (S_{13}) and anthracene disappearance from the medium

The turbidity of the media at OD 600_{nm} was an indicator for utilization of PAHs in liquid assays. The growth of bacteria at the expense of anthracene was confirmed from spectrophotometric analysis. In order to confirm the bacterial growth at the expense of anthracene in liquid media, spectrophotometric analysis of bacterial growth and disappearance of anthracene was observed in PNR media by taking OD at 600_{nm} and 540_{nm} respectively after every 24 hours for 120 hours. As previously reported the degradation of xenobiotics can be the catabolism by individual isolates of microorganisms or from combined metabolism by microbial communities [Sutiknowati, 2007; Nasrollahzadeh *et al.* 2010; Ibrahimi *et al.* 2012].

3.3.25 Screening in liquid media under static lab condition for selection of best degraders

Among 59 bacterial isolates (32) showed growth on anthracene upto 1200ppm in liquid media as shown in Table 3.24.

Table 3.24: Screening of B-isolates in liquid media under static lab conditions

S. No.	Isolate	PAHs/ OD 600nm				
		Anthracene	Naphthalene	Phenanthrene	Acenaphthene	Pyrene
1.	B_2	0.041	0.034	0.039	0.024	0.281
2.	B_3	0.301	0.04	0.134	0.04	0.472
3.	B_4	0.470	0.159	0.238	0.04	0.772
4.	B_5	0.422	0.144	0.304	0.430	0.351
5.	B_9	0.220	0.194	0.449	0.042	0.395
6.	B_{10}	0.154	0.201	0.043	0.027	0.350
7.	B_{11}	0.487	0.026	0.0276	0.028	0.292
8.	B_{12}	0.377	0.044	0.028	0.023	0.039
9.	B_{13}	0.021	0.043	0.045	0.427	0.047
10.	B_{15}	0.117	0.207	0.157	0.201	0.220
11.	B_{16}	0.260	0.112	0.035	0.123	0.271
12.	B_{20}	0.343	0.121	0.264	0.119	0.612
13.	B_{21}	0.388	0.201	0.271	0.193	0.306
14.	B_{23}	0.683	0.223	0.368	0.212	0.177
15.	B_{24}	0.331	0.221	0.298	0.213	0.677
16.	B_{26}	0.448	0.138	0.474	0.281	0.348
17.	B_{27}	0.147	0.114	0.243	0.232	0.357
18.	B_{29}	0.121	0.217	0.156	0.226	0.416
19.	B_{30}	0.470	0.611	0.984	0.427	0.332
20.	B_{33}	0.387	0.132	0.355	0111	0.09
21.	B_{36}	0.497	0.187	0.342	0.213	0.258
22.	B_{46}	0.143	0.178	0.315	0.115	0.225
23.	B_{47}	0.629	0.044	0.419	0.310	0.324
24.	B_{49}	0.304	0.245	0.505	0.263	0.172
25.	B_{57}	0.218	0.135	0.222	0.113	0.739
26.	B_{58}	0.401	0.153	0.297	0.137	0.341
27.	B_{59}	0.608	0.222	0.333	0.128	0.549
28.	B_{60}	0.221	0.111	0.129	0.253	0.104
29.	B_{61}	0.364	0.184	0.760	0.347	0.368
30.	B_{62}	0.243	0.080	0.859	0.372	0.338
31.	B_{67}	0.322	0.113	0.883	0.132	0.388
32.	B_{68}	0.374	0.155	0.514	0.309	0.296

Isolate B_{30} was the only isolate having the ability of three LMW PAHs such as; naphthalene, acenaphthene and phenanthrene degrading capability with growth OD value of 0.611, 0.984 and 0.427 respectively Isolate B36 growth OD of 0.425 was calculated on anthracene. All the remaining isolates showed very small growth with OD value between 0.110 to 0.224 while other were unable to utilize naphthalene and thus showed no growth in the media. In case of phenanthrene, isolates B_9, B_{26}, B_{47}, B_{49}, B_{62}, B_{67} and B_{68} OD values, 0.449, 0.474, 0.419, 0.505, 0.859, 0.883 respectively, after three days of incubation. Pyrene was utilized by B_3, B_9, B_{20}, B_{24}, B_{29}, and B_{57} showing 0.472, 0.395, 0.612, 0.667, 0.416, and 0.793 of OD values, respectively. As reported earlier, bacterial isolates from the rhizospheric soils as well as from water samples were tested for LMW PAHs degradation under static conditions [Othman *et al.* 2010].

Among the S-isolates anthracene degraders, S_{34} and S_{13} exhibited high degradability of 0.652 and 0.425 OD respectively as shown in Table 3.25.

Table 3.25: Screening of S-isolates in liquid media under static lab conditions

S. No.	Isolate	PAHs/ OD 600nm				
		Anthracene	Naphthalene	Phenanthrene	Acenaphthene	Pyrene
1.	S_5	0.321	0.09	0.570	0.278	0.180
2.	S_6	0.125	0.110	0.121	0.113	0.421
3.	S_7	0.115	0.100	0.221	0.09	0.050
4.	S_{11}	0.113	0.090	0.08	0.101	0.395
5.	S_{12}	0.270	0.102	0.119	0.100	0.463
6.	S_{13}	0.425	0.154	0.222	0.344	0.249
7.	S_{15}	0.174	0.143	0.168	0.131	0.365
8.	S_{16}	0.116	0.211	0.111	0.427	0.121
9.	S_{18}	0.239	0.221	0.211	0.233	0.650
10.	S_{19}	0.340	0.09	0.580	0.407	0.311
11.	S_{20}	0.123	0.111	0.131	0.10	0.120
12.	S_{21}	0.342	0.138	0.784	0.222	0.683
13.	S_{22}	0.258	0.217	0.113	0.212	0.218
14.	S_{23}	0.221	0.211	0.125	0.216	0.585
15.	S_{24}	0.270	0.213	0.113	0.197	0.220
16.	S_{30}	0.265	0.185	0.256	0.111	0.288
17.	S_{31}	0.115	0.154	0.210	0.100	0.372
18.	S_{34}	0.652	0.118	0.476	0.363	0.333
19.	S_{36}	0.101	0.08	0.121	0.09	0.07
20.	S_{37}	0.109	0.103	0.110	0.08	0.06
21.	S_{39}	0.160	0.07	0.173	0.123	0.09
22.	S_{40}	0.129	0.123	0.131	0.113	0.153
23.	S_{51}	0.211	0.119	0.223	0.221	0.710

Isolates S_5, S_{21}, S_{22} and S_{30} OD values were 0.321, 0.342, 0.358 and 0.265. No growth was observed on naphthalene at all. OD values of isolates S_5, S_{21}, S_{19} and S_{34} 0.570, 0.784, 0.580, 0.476, on phenanthrene and $S_{13,}$ $S_{16,}$ $S_{19,}$ $S_{34,}$ 0.344, 0.427, 0.407, and 0.363 on acenaphthene, respectively. $S_{6,}$ $S_{18,}$ $S_{12,}$ S_{21} and S_{51} isolates on pyrene showed growth OD values of 0.421, 0.650, 0.463, 0.683 and 0.710 respectively. Likewise, previously reported studies on *Corynebacterium urealyticum* degrade phenanthrene better in liquid culture than in solid medium. Control sample showed clear solution while samples with bacterial culture turned turbid [Tortora *et al.* 2007, Kamil *et al.* 2012].

The isolates isolated from *Morus alba* shows no growth on all the selected compounds except $M_{1,}$ M_2 and M_3 in liquid media growth OD of 0.369, 0.328 and 0.410 respectively as shown in Table 3.26.

Table 3.26: Screening of M-isolates in liquid media under static lab conditions

S. No.	Isolate	PAHs/ OD 600nm				
		Anthracene	Naphthalene	Phenanthrene	Acenaphthene	Pyrene
1.	M	0.135	0.104	0.115	0.101	0.215
2.	M_1	0.121	0.117	0.09	0.132	0.369
3.	M_2	0.141	0.251	0.111	0.09	0.328
4.	M_3	0.199	0.213	0.213	0.08	0.410

The growth with OD values of isolates W_{11} (0.688) on phenanthrene, W_{10} (0.258), W_{11} (0.282) on anthracene, W_1 (0.826), W_2 (0.494), W_5 (0.336), W_8 (0.600) and 0.327 on pyrene as shown in Table 3.27.

Table 3.27: Screening of W-isolates in liquid media under static lab conditions

S. No	Isolate	PAHs/ OD 600nm				
		Anthracene	Naphthalene	Phenanthrene	Acenaphthene	Pyrene
1.	W_1	0.09	0.101	0.210	0.110	0.826
2.	W_2	0.290	0.185	0.180	0.123	0.494
3.	W_5	0.139	0.158	0.126	0.110	0.336
4.	W_8	0.110	0.167	0.121	0.131	0.600
5.	W_9	0.170	0.125	0.101	0.114	0.253
6.	W_{10}	0.258	0.120	0.112	0.121	0.284
7.	W_{11}	0.282	0.07	0.668	0.257	0.327

Isolates with best growth as revealed from the OD values were selected for further studies namely; B_4, B_{30}, B_{36}, S_{13}, S_{21}, S_{51}, M_3 and W_2.

BIODEGRADATION OF ANTHRACENE

4.1 INTRODUCTION

Anthracene is a hydrophobic chemical having three fused aromatic ring structure [Hyotylainen & Oikari, 1999]. Environment receive these compounds after incomplete combustion of fuels, production of synthetic fibers, dyes, plastics, pesticides, coal tar [Johnsen *et al.* 2005; Ni-Chadhain *et al.* 2006; Hafez *et al.* 2008]. Anthracene has been reported toxic to aquatic life. Target tissues are skin hematopoietic system, lymphoid and gastrointestinal tract, adverse dermatologic effect such as burning itching and edema. Pigmentation, cornification of skin surface layer and telangiectasis are caused after prolonged dermal exposure [Neeraja *et al.* 2013]. Due to its hazardous nature a number of methods have been used for its removal which can abiotic or biotic [Kastner *et al.* 1996; Trindade *et al.* 2005].

Degradation activity of microbes depends largely on their mobile genetic machinery and accessibility to pollutants. Microbial community play very crucial role in degradation of anthracene but is limited by its persistence in environment due to hydrophobicity [Doick *et al.* 2005; Macleod & Semple, 2006; Li *et al.* 2008; Vinas *et al.* 2005]. In a previously reported study, on hydrocarbon degrading efficiency of some isolated bacteria from oil polluted sites, such as *Pseudomonas stutzeri, Pseudomonas alcaligenes, Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Sphingobacterium* sp., *Acinetobacter johnsonii, Achromobacter, Serattia odorifera, Xylosoxidans, Entrobacter cloacae, Ralstonia* sp., *Vibrio* sp., *Zymomonas* sp., *Paracoccus* sp., *Pantoea* sp. and *Chryseobacterium* sp. were determined in a solid and liquid medium. Bacterial isolate, *Bacillus vallismortis* capable of degrading phenanthrene, anthracene and pyrene was isolated from the polluted soil [Ling *et al.* 2011; Ibrahimi *et al.* 2012].

4.2 MATERIALS AND METHODS

4.2.1 Chemicals and Media

Analytical grade chemicals and high purity media were purchased from Flukaand Sigma-Aldrich, Merck and BDH. Chemicals were anthracene (99%),

Salicylic acid, salicylaldehyde, catechols, gentisic acid, 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid, 6, 7-benzo-caumarin, 1-naphthol. Nutrient agar and Mineral salt medium was used for initial screening. The composition of PNR and PNRG is given in detail (Chapter 3).

4.2.2 Inoculum preparation

Two selected isolates B_{36} and S_{13} were grown in broth media containing anthracene were incubated at 28±2°C and 150rpm. Cells were harvested at exponential phase according to protocol [Guo *et al.* 2010].

4.2.3 Optimization of various physico-chemical conditions for PAHs degradation

Different parameters such as anthracene conc., temperature, p^H, substrate (alternate carbon and energy source) and agitation speed and effect of inoculum were optimized for degradation studies. A wider range of concentration i.e. 100, 150, 500 and 1000mgL^{-1} was studied for effect on bacterial growth and degradation. Selected pH range was 4, 5, 6, 7, 8 and 9 and temperature 28, 30, 35, 40 and 45°C. To study the effect of shaking different speeds 120, 150 and 180 rpm as well as without shaking was also observed for maximum activity [Naveenkumar *et al.* 2010; Abdelhay *et al.* 2008].

4.2.3.1 Optimization of anthracene concentration

The enrichment media was prepared and dispensed in 100 mL volumes into 250mL Erlenmeyer flasks before autoclaving. Prior addition of media, different concentration of anthracene dissolved in acetone 100, 150, 500 and 1000mgL^{-1} were added to flasks. Acetone was allowed to evaporate. Thereafter, flasks were inoculated with isolate S_{13} and B_{36} containing (anthracene) were then incubated for five days. One mL sample was collected from each flask and OD (600nm) was noted for growth of bacteria. All experiments were performed in triplicate.

4.2.3.2 Optimization of growth temperature

The growth of the bacterial isolates on selected PAHs was observed at different temperature 28, 30, 35, 40 and 45ºC for 120 hour. One mL sample was taken after 24 hours and observed for bacterial growth at 600_{nm}.

4.2.3.3 Optimization of p^H

Degradation of anthracene was optimized at different pH range of 4, 5, 6, 7, 8 and 9 and was observed for maximum activity.

4.2.3.4 Optimization of agitation speed

To study the effect of shaking different speeds 120, 150 and 180 rpm as well as without shaking was also observed to determine the maximum degradation rate.

4.2.4 Effect of carbon sources

Different carbon sources like fructose, glucose and sucrose was also added to anthracene containing media to study their effect on utilization of PAHs by bacteria.

4.2.5 Effect of Nitrogen sources

Nitrogen salts potassium; ammonium, sodium and calcium nitrate were also studied for their effect on PAHs degradability.

4.2.6 Inoculum concentration

Inoculum concentration is one of the important factors to be considered for the maximum degradation of PAHs. The highest inoculum concentration did not stimulate the biodegradation when compared to the minor inoculum concentrations used. 10% vv^{-1} inoculum proved to be optimum for anthracene degradation [Gou *et al.* 2010].

4.2.7 Biodegradation experiment

This experiment was performed in reactor flasks containing 100ml PNR media, 10% bacterial inoculum and 1000mgL^{-1} anthracene dissolved in acetone. Acetone was allowed to evaporate prior adding media. All the experiments were done at p^H of 7.0, 30°C, temperature and shaking speed of 180rpm for 5days [Othman *et al.* 2010].

4.2.8 Determination of bacterial growth by CFUmL^{-1}

The viability of bacteria was determined by CFU study of samples drawn after every 24 hours intervals for 120 hours.

4.2.9 Extraction of PAHs

Samples were collected from reactor flasks for 5 days with an interval of 24 hours each in order to find the remaining concentration of PAHs. PAHs were extracted following established protocol with some modifications [Shokrollahzadeh *et al.* 2010]. The growth medium was extracted twice by dichloromethane (DCM) and acetone10mL each solvent were added to media and left on shaking incubator at 30°C for 24 hours. The extraction phase was stripped of water droplets by pipetting the upper layer after centrifugation at 12,000*g. The remaining moisture was removed by addition of 4gm anhydrous sodium sulphate (Na_2SO_4) and evaporated to dryness in a rotary evaporator and concentrated to around 2ml. The samples were then filtered, using 0.45µm syringe filters.

All the flasks were incubated at 30°C for 10 days on rotary shaking incubator at 150rpm and samples were extracted for GC-MS analysis. During incubation the residual concentration of anthracene was monitored spectrophotometrically after 10 days by liquid-liquid extraction method [Survery *et al.* 2009]

4.2.10 High performance liquid chromatography (HPLC) analysis

Followed by the extraction with ethyl acetate, the samples were filtered through 0.2mm syringe filter and analyzed by HPLC (PerkinElmer) with the C18 reverse phase column under isocratic condition using acetonitrile: water (80:20) (vv^{-1}) as mobile phase and detection wavelength –254nm for anthracene. The flow rate of acetonitrile was maintained at 1.2mLmin^{-1}. The samples volume was 2mL in each vial of HPLC tray. Metabolites were studied on the basis of retention time comparison with available authentic standards and literature values.

4.2.11 Gas chromatography–mass spectrometry (GC–MS) analysis

For GC-MS, Shimadzu fused silica capillary column was used for the analysis. The column temperature program was set at 100°C for 1min, 15°Cmin^{-1} to 160°C and 5°Cmin^{-1} to 300°C for 7min. The GC injector was held isothermally at 280°C with a splitless period of 3 min. Helium was used as the carrier gas, at a flow rate of 1mLmin^{-1} by using electronic pressure control. The GC–MS interface temperature was maintained at 280°C. Standards from Sigma Aldrich were used for anthracene and their metabolites. GC-MS library search was used to confirm the metabolites without standards.

4.2.12 Morphological and Biochemical characterization of bacterial Isolates

The bacterial isolates were identified morphologically by Gram's staining and microscopy while biochemically by Bergey's manual of systematic bacteriology.

4.2.13 Isolation of Plasmid and Electrophoresis on Agarose Gel

The plasmid was isolated according to manufacturer instructions of Invitrogen Mini Prep Kit and electrophoreses was done on 0.8 % agarose gel containing ethidium bromide (1μgml^{-1}). DNA bands were visualized under UV light and photographed [Survery *et al.* 2009].

4.2.14 DNA extraction and PCR amplification

The lypholyzed cultures were sent to Crop Physiology Laboratory, School of Applied Bioscience, College of Agriculture and Life Science, Kyungpok National University, Daegu, Korea for extraction of DNA and PCR amplification. The culture were grown in nutrient broth and isolated on agar media. The purified colonies were incubated with reaction mixture for DNA extraction at 37°C for 60min. The composition of reaction mixture is given below:

4.2.14.1 Reaction mixture for DNA extraction

A 1.5ml of lysis buffer**,** 10 mM Tris·Cl, 100 mM EDTA, 15mgml^{-1} lysozyme, pH 8.0, Proteinase K (final concentration 50μgml^{-1}, Sodium dodecyl sulfate (SDS)

1% wv^{-1} final concentration, Phenol-chloroform-isoamyl alcohol (25:24:1), Chloroform-isoamyl alcohol (24:1).

4.2.14.2 DNA extraction

The mixture was incubated with Proteinase K at 55°C for 30min, after which SDS was added, and 3 cycles of freezing (–70°C) and thawing (65°C) were performed. After extractions, with phenol-chloroform-isoamyl alcohol & chloroform-isoamyl alcohol, the DNA was precipitated in isopropanol at –20°C. The extracted DNA was stored in TE buffer at -4°C till further processing.

4.2.14.3 Reaction mixture for PCR

50µl reaction mixture containing 500ng template DNA, 10 mM Tris HCl p^H 8.3, 40 mM KCl, 50µgml^{-1} BSA, 1.5mM $MgCl_2$, Deoxynucleoside triphosphates (2.5mM each) 100pmol of universal primers (16S rRNA) following Sambrook & Russel, 2001 protocol.

4.2.14.4 PCR amplification

Bacterial 16S rRNA genes were PCR amplified in a 50µl reaction mixture with 500ng template DNA, BSA, $MgCl_2$, deoxynucleoside triphosphates, 100 pmol of each primer 27F and 1492R and Taq polymerase [Lane, 1991]. After an initial incubation at 94°C for 4min, 30 cycles were run of 94°C for 1min, 55°C for 1min and 72°C for 2min, followed by a final incubation at 72°C for 10min. Bacterial primers cloning of nearly full length 16S rRNA and sequencing were performed according to the methods described previously [Sambrook & Russel, 2001].

4.2.15 Molecular identification based on 16S rRNA gene and phylogenetic analysis

The 16S rRNA gene sequence of the isolates were processed manually, analysed at NCBI (National Centre for Biotechnology Information) using BLAST tool and compared to the corresponding neighbour sequences from GenBank-NCBI database. Consensus sequence was imported into the Multalin program and multiple alignments were performed with related species (GenBank-NCBI database).

Sequences were compared to those present in the data bank using blast and aligned with the ClustalW program. The results obtained were further imported into MEGA software for the construction of a phylogenetic tree using Bootstrap analysis and maximum likelihood with 500 replicates. The substitution method used was Kimura 2-Parameter model and the statistical method used was maximum likelihood [Tamura & Nei, 1993]. Evolutionary analyses were conducted in MEGA6 [Felsenstein, 1985].

4.3 RESULTS AND DISCUSSION

4.3.1 Optimization of various physico-chemical conditions for anthracene degradation

Concentration of anthracene, p^H of the media, incubation temperature, shaking speed and inoculum concentration affects the degradation rate. So these must be optimized prior to carry out a degradation experiment. The effects of various alternate carbon sources and effects of nitrogen on degradation of anthracene are also important. Optimization of these physico-chemical parameters is discussed below consecutively.

4.3.1.1 Optimization of anthracene concentration

The growth rate of both the isolates was observed at a concentration range of 100-1000mgL^{-1} anthracene, as shown in Fig.4.1.

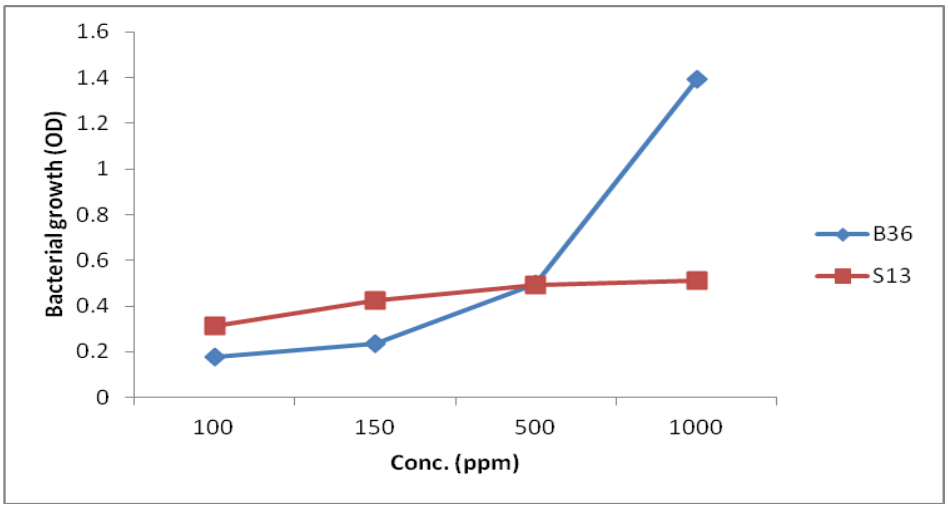

Fig. 4.1: Optimization of anthracene concentration

Heighest growth OD value was recorded for isolate B_{36} followed by S_{13}. As exposure of microbes to high dose may prove lethal so the microbes must be supplemented with low dose first as reported earlier [Neelofur *et al.* 2014].

4.3.1.2 Optimization of growth temperature

For both the isolates the optimum temperature for degradation of anthracene was 30°C as shown in Fig. 4.2. The increased growth rate was observed from 28-30°C which decreased with increasing temperature 35-40°C with no growth between 40°C and 50°C at all for both the strains.

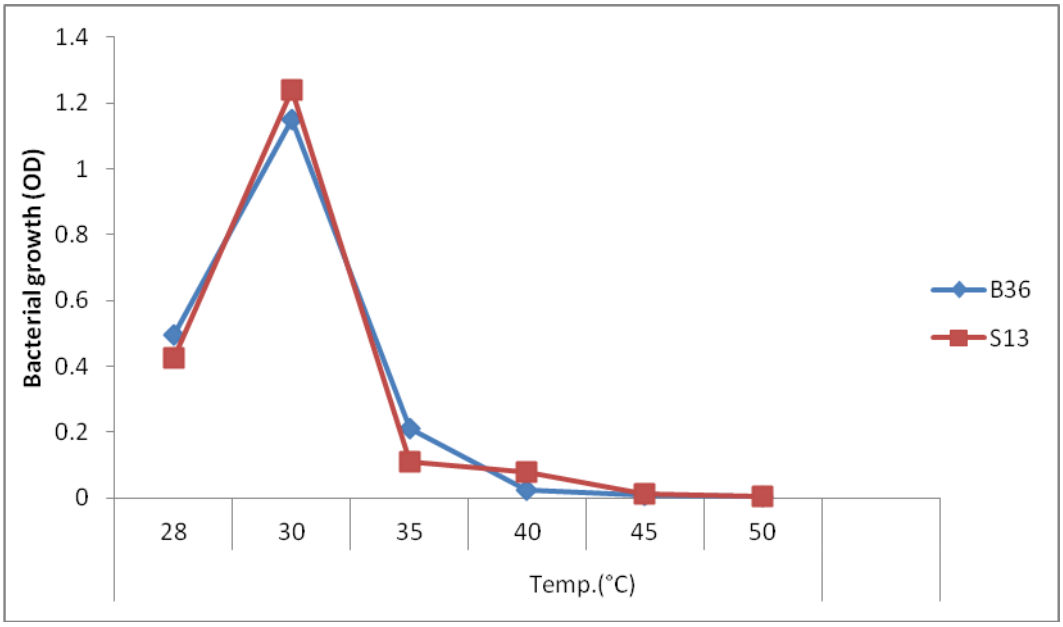

Fig. 4.2: Optimization of temperature for anthracene degradation

Biodegradation of PAHs can occur over a wide temperature range. As temperature influence the solubility of a compound on one hand and enzymatic activity of the microbes on the other hand. Each compound has a certain limit of temperature for its solubility above which it adversely affects oxygen supply and thus inhibits the activity of aerobic microorganisms. The ability of the microorganisms to degrade PAHs is highly dependent on temperature, as it is an important factor in bioavailability of compounds. At low temperature, lower growth is an indication that this temperature is insufficient for the enzymes to speed up the degradation reaction. Enzymes being catalyst have certain optimum temperature for their maximum activity

and every organism has a specific set of enzymes. While, most of the enzymes and specially oxygenase in this case become denatured at temperature above 40°C and unable to cleave the benzene ring which is the main functional group of anthracene

However, most studies tend to focus on mesophilic temperatures rather than the efficiency of transformations at very low or high temperatures. Results of our study regarding temperature for anthracene degradation in the mesophilic range are in line with previously reported work [Ahmed *et al.* 2010; Nour *et al.* 2010; Mukesh *et al.* 2012; Arulazhagan *et al.* 2011].

4.3.1.3 Optimization of media p^H of on anthracene degradation

Present study shows that neutral p^H favored both extensive bacterial growth on anthracene supplemented as sole carbon source. Faster growth rate was observed for isolate S_{13} followed by B_{36}. (Fig.4.3). Growth rate was slowest at both the acidic and basic (p^H of 4-6 and 8-9), and maximum at neutral p^H.

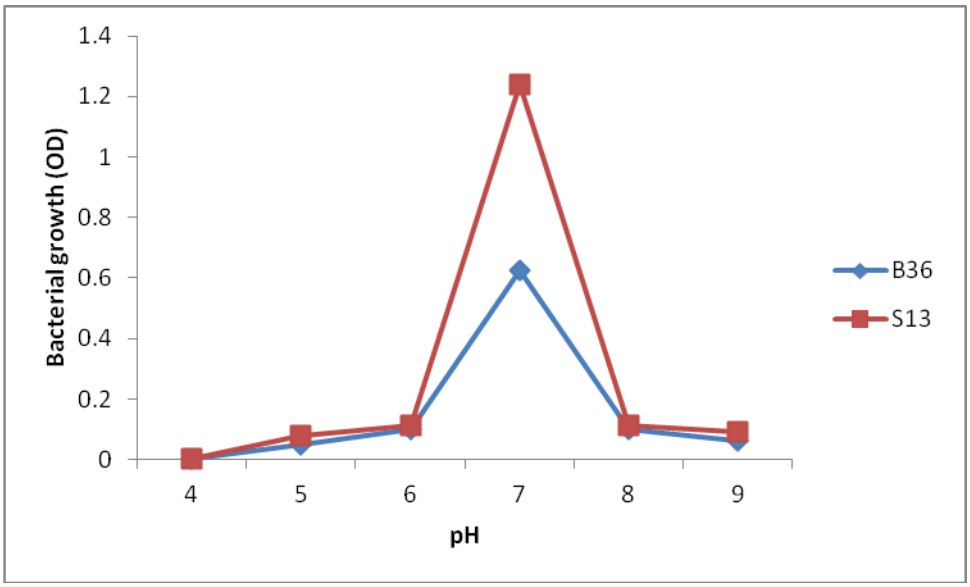

Fig. 4.3: Optimization of p^H for anthracene degradation

p^H of the medium is an important factor in degradation and growth of microorganisms. Each microbe has its own p^H range of metabolic function and is very sensitive to external environment for the maintenance of its internal p^H. The charge imbalance inside and on the surface of the cells was prevented at p^H 7. At extreme p^H,

the proper functioning of the enzymatic machinery involved in PAHs degradation might have been affected because of which the rate of degradation and bacterial growth decreased. Effect of p^H on the disappearance of anthracene was significantly inhibited when p^H fluctuates from acidic to neutral and neutral to alkaline level, as seen in case of phenanthrene and pyrene as previously reported [Nnamchi *et al.* 2006; Sudarat *et al.* 2000].

4.3.1.4 Optimization of agitation speed for anthracene degradation

The optimum shaking speed for isolate B_{36} was 120rpm while, for S_{13} 180rpm for growth on anthracene as shown in Fig 4.4. With increasing shaking speed, isolate S_{13} showed increased growth rate on anthracene upto 180rpm that slows down at 200-220rpm. While, the degradation rate for isolate B_{36} there was steady growth rate on increasing shaking speed. Faster agitation speed resulting in higher degradation rate was probably due to sufficient oxygen supply, anthracene solubility in aqueous phase and nutrients availability for their uptake by microorganism. Better aeration not only helps in temperature maintenance of the medium, nutrient uptake by microorganisms as well as in solubility of compound.

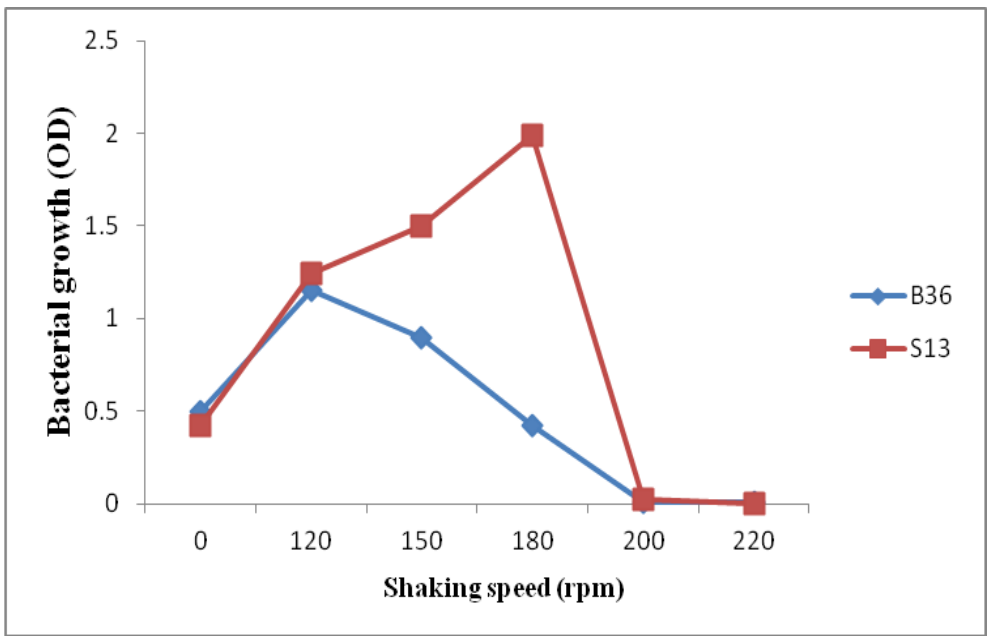

Fig. 4.4: Optimization of agitation speed for anthracene degradation

As aerobic bacteria require oxygen along with better distribution of nutrients and temperature, and low oxygen pressure can be achieved through better aeration. Agitation speed is necessary for ample supply of oxygen, solubility of compounds and nutrient distribution for desired growth of microorganisms in a fermentor. As previously reported, adequate availability of molecular oxygen enhances the level of enzyme production and thus helps in incorporation of molecular oxygen efficiently in aromatic ring of anthracene. This process brings about better initial ring oxidation by oxygenase activity which is typically a rate regulating step in biodegradation of PAHs [Park *et al.* 1990; Margesin *et al.* 2001; Chulalaksananukul *et al.* 2006; Alias *et al.* 2011; Bhattacharya *et al.* 2011].

4.3.2 Effect of different carbon and nitrogen sources

Different carbon sources like glucose, fructose and sucrose were also added to anthracene containing media to study their effect on utilization of anthracene by bacteria. Nitrogen effect, in the form of salts of nitrate, was also studied on anthracene degradability.

4.3.2.1 Effect of different carbon substrates on degradation activity

Growth of bacteria at the expense of anthracene in the presence of different types of carbon sources is shown in Fig. 4.5.

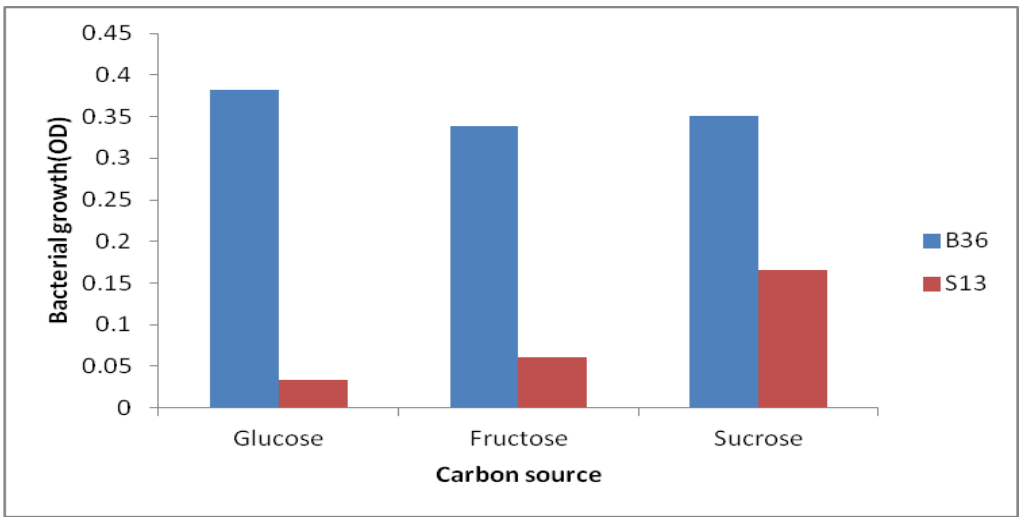

Fig. 4.5: Effect of different carbon sources on growth of isolates B_{36} and S_{13}

In this study, supplement of small amounts of alternate carbon sources had an inhibitory effect on growth rate of bacteria at the expense of anthracene. These results show that S_{13} could use anthracene as its sole carbon source and that additional carbon source in the form of glucose, fructose and sucrose decreased the metabolism of anthracene. While, in case of the isolate B_{36} comparatively increased growth rate was observed. Which may be due to the anthracene degrading enzyme machinery of this isolate becomes activated by the readily available carbon sources. The effects of many kinds of supplements have been studied with the purpose to increase the available contents of pollutants and to mimic population of degradative microorganisms. It is likely due to the assimilation of different carbon sources prior to anthracene by isolate S_{13}, which might cause inhibition of relevant enzymes for anthracene degradation as reported earlier [Kastner *et al.* 1998].

4.3.2.2 Effect of different nitrogen sources on bacterial isolates

Addition of different nitrogen sources (Fig. 4.6), including KNO_3, $NaNO_3$, $CaNO_3$ and NH_4NO_3 has no effect on growth of B_{36} or degradation activity of anthracene.

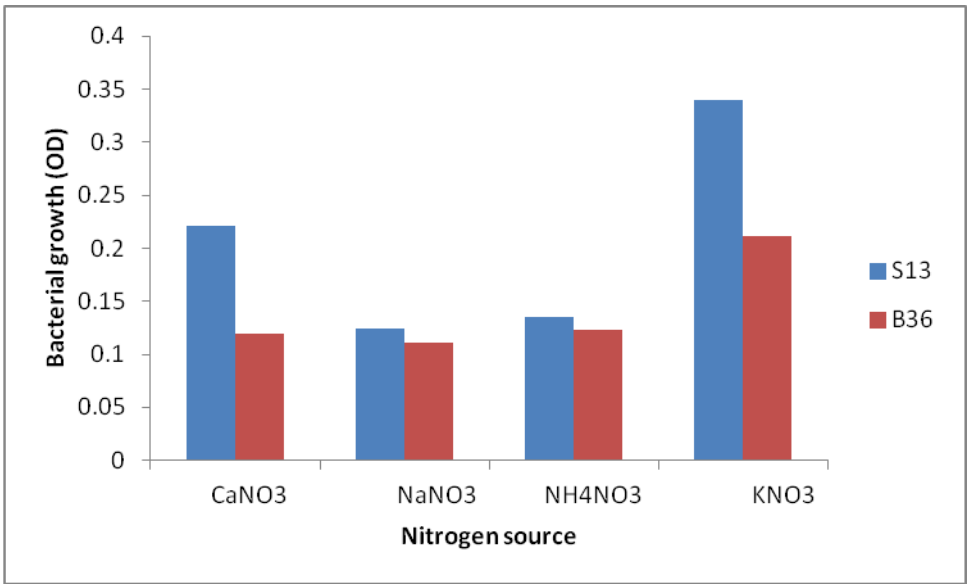

Fig. 4.6: Effect of different nitrogen source on growth of isolate B_{36} and S_{13}

In case of isolate S_{13} KNO_3 growth rate was enhanced in comparison to other the nitrogen salts. On the basis of above observations it can be inferred that both the

carbon and nitrogen sources used in current study may act as competitive substrates in anthracene degradation. Low availability of anthracene as compared to glucose fructose and sucrose in culture media may account for this result [Shabir *et al.* 2008; Ross *et al.* 2001].

4.3.2.3 Effect of Inoculum concentration

Inoculum concentration is one of the important factors to be considered for the maximum degradation of anthracene (Fig.4.7). The higher inoculum concentrations did not stimulate biodegradation as compared to the minor inoculum concentrations used. 10% vv^{-1} inoculum proved to be optimum for anthracene degradation as previously reported [Guo *et al.* 2010].

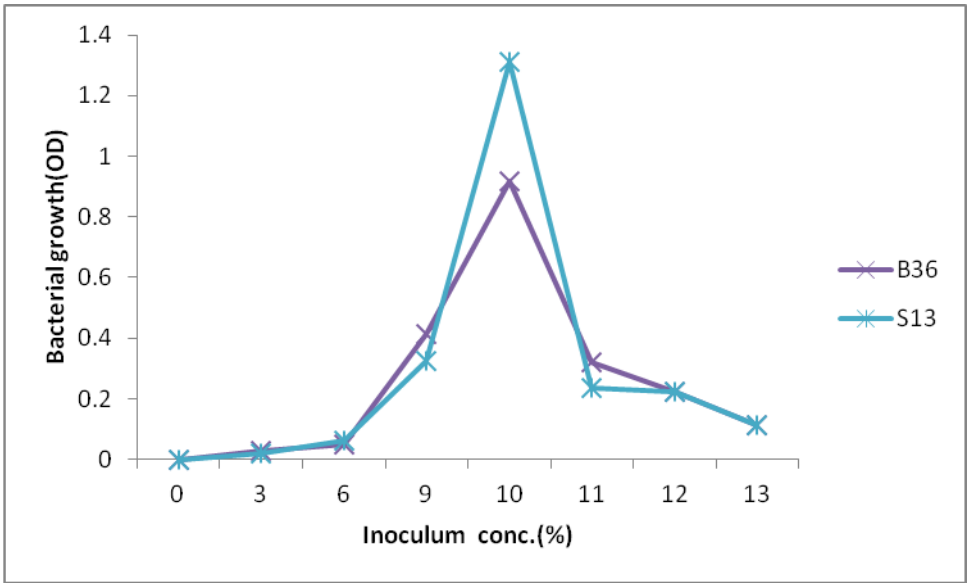

Fig. 4.7: Optimization of inoculum concentration (%) for S_{13} and B_{36}

4.4 Biodegradation analysis

Anthracene is hydrophobic and have slow rate of biodegradation. The bioremediation of a pollutant and its rate depends on the number and type of degrading organisms, environmental conditions and chemical structure of the compound to be degraded. Some microorganisms can consume PAHs as a source of carbon and energy [Johnsen *et al.* 2005].

Isolate S_{13} degraded anthracene efficiently during the incubation period of 24 to 96h with 1000mgL^{-1} in PNR media. While, the growth reaches to its maximum with an OD value of 1.15 after 120 hours of incubation. The growth rate doubles as shown from 0.598 to 1.15 on fourth to fifth day at the utilization of 86.26% anthracene. The significant increase in anthracene degradation was observed with increasing shaking speed up to 180rpm. This can be perhaps due to recrystallization of anthracene from the medium. S_{13} degraded anthracene 82.26% anthracene in 120 hours as shown in Fig.4.8. Microbial degradation is thought to be one of the most efficient processes of contaminants removal [Ye *et al.* 2011; Clayton 2005, Doick 2005].

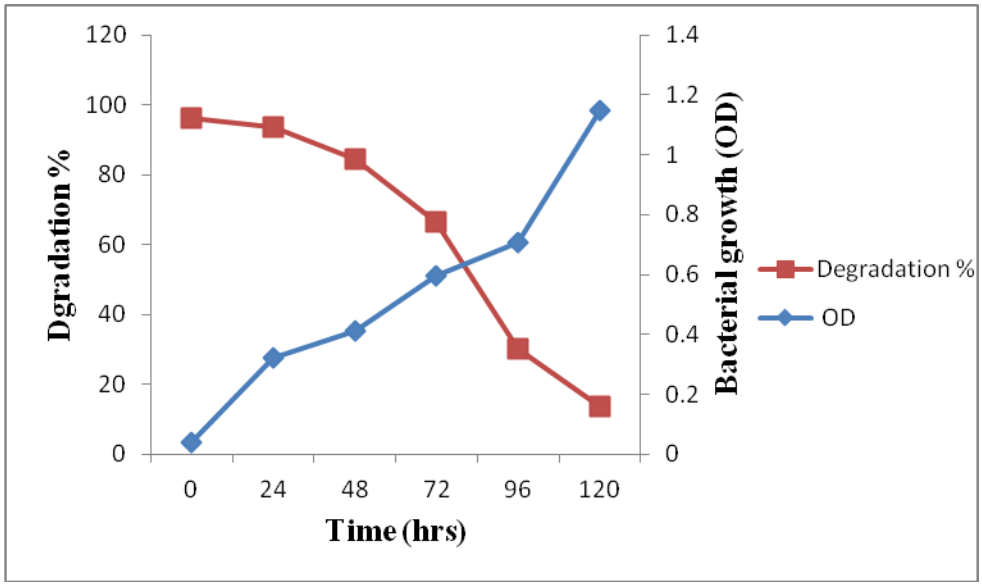

Fig. 4.8: Growth of isolate S_{13} and anthracene degradation

In a previously reported study the complete anthracene degradation of 500ppm after 6 days of incubation showed that after 48 hours *Pseudomonas citronellolis* isolate 222A degraded 72% of anthracene. While, our isolate B_{36} degraded about 60% at 1000mgL^{-1} in only in three days in contrast to other isolates isolated in same studies *Pseudomonas sp.* degraded 74.8% in more than a week time [Rodrigo *et al.* 2005; Somtrakoon *et al.* 2008; Eder *et al.* 2008; Kumar *et al.* 2010].

Another study has stated the complete degradation of added anthracene to autoclaved soil by *Burkholderia* sp. in 20 days. *B. circulans* SBA 12 and *Kurthia* SB A4 degraded 87.5% and 86.6% of anthracene [Bisht *et al.* 2010]. Our results are in

line with previously reported work on *Pseudomonas, Bacillus subtilis* and *Micrococcus luteus* which can utilize anthracene 91.7%. In a previously reported study, *Pseudomonas* sp. was cultured and the maximum anthracene degradation was found to be 74.8% on 10th day and maximum bacterial growth was measured on 9th day. Analogous to these studies have been reported for anthracene degradation and mineralization by *Pseudomonas, Sphingomonas, Nocardia, Beijerinckia, Rhodococcus* and *Mycobacterium* [Bisht *et al.* 2010]. Another stated study on anthracene degradation by BMIT637C in BSM media was observed for 10 days.

Similarly, our results for B_{36} degraded (84.46%) with 1000mgL^{-1} in only five days (Fig. 4.9.) far better than the anthracene degradation by *Pseudomonas* sp. (71%). About 60% degradation occurs in 72 hours and decrease rate was calculated for further 48 hours.

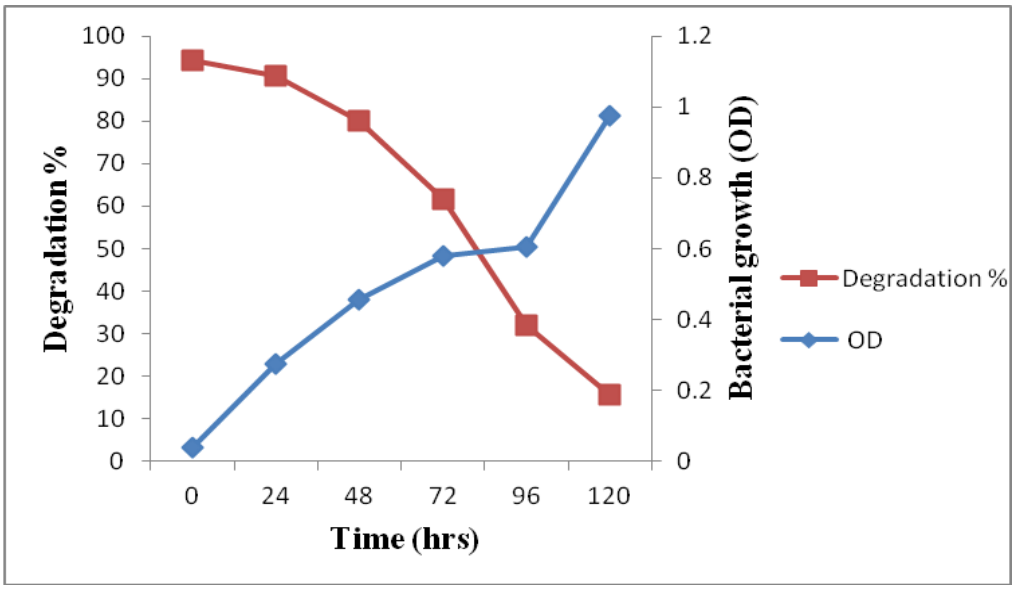

Fig. 4.9: Growth of isolate B_{36} and biodegradation of anthracene

The degradation rate of anthracene was determined after 24 hours and after 72, 120 and 168 hours, was found to be 28.95%, 42.77%, 53.34% and 67.11% respectively. So, it can be concluded that the degradation rate for our isolates is much better in terms of time and degradation rate. It can be attributed to the exposure of these isolates to highly contaminated soil and aerial deposition of PAHs as well as the adaptation process.

4.4.1 Determination of bacterial growth by CFUmL^{-1}

The viablility of bacteria was determined by CFU study of samples drawn after every 24 hours intervals for 120 hours for bacterial isolate S_{13} from *Sisymbrium irio*. The anthracene degradation rate was 2.54% and cell viability was 2.3×10^{-11} CFUmL^{-1} after 24 hours of incubation. On incubation for another 24 hours viable cell count was 3.8×10^{-14} CFU mL^{-1}and degradation rate was 14.05%. 32.13% anthracene was degraded in 72 hours and viable cell count of 1.8×10^{-17} CFUmL^{-1} was observed. 65.74% degradation was observed with cell viability of 3.2×10^{-20} after 96 hours while 82.29% of anthracene was degraded after 120 hours with 1.4×10^{-23} CFU mL^{-1}. In an earlier reported study, anthracene degradation by the *Pseudomonas sp.* was around 56% after 48 days, while *P. aeruginosa* degraded 71% of the anthracene [Kastner *et al.* 1998]. Decrease in cell viability of these isolates in the anthracene the degradation medium which may due to accumulation of toxic metabolites [Madhuri *et al.* 2013].

The viable cell count of our isolated isolates S_{13} and B_{36} are in line with the previously reported studies for the assessment of viability in presence of anthracene CFUmL^{-1} 2.7×10^{-18} was calculated for B_{36} which revealed that there is more than fourfold increase in cell viability (Table I, Annexure A). Our isolates B_{36} and S_{13} showed (84.46 and 86.26%) degradation in PNR media with a maximum bacterial growth on 5th day of incubation (Fig. 4.10.).

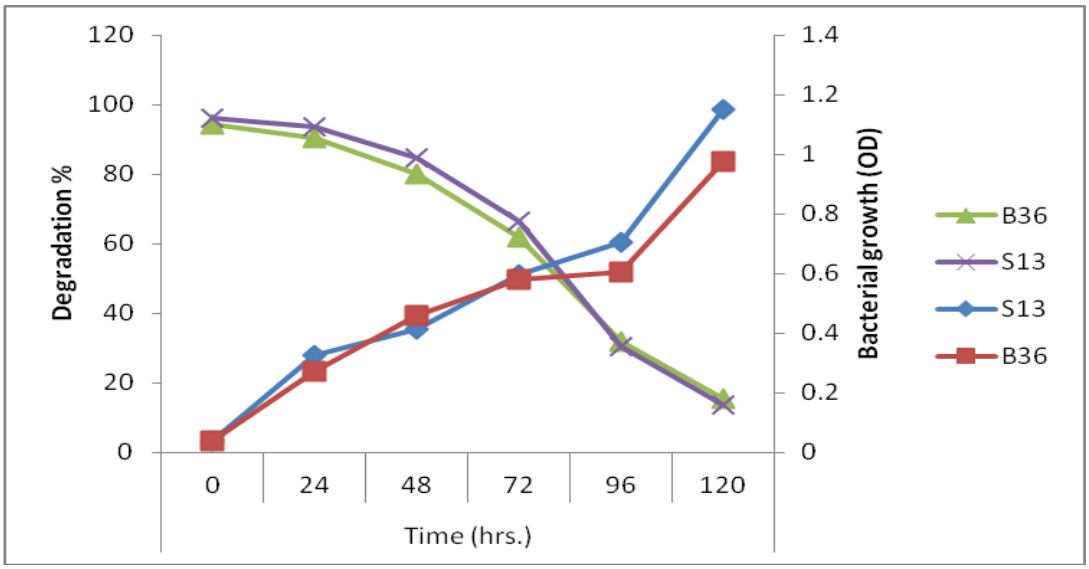

Fig. 4.10: Comparison of growth & degradation of isolates B_{36} and S_{13} on anthracene

Isolate B_{36} degradation of 3.67% and about two-fold increase in growth and degradation was observed after 72 hours and with 77.78% and 88.69% degradation was calculated for 96 and 120 hours respectively.

4.4.2 HPLC and GC-MS analysis

HPLC analysis of metabolites formed by the isolates S_{13} from a contaminated area shows some unidentified peaks with a single sharp peak and some smaller peaks after 72 hours (Fig. 4.11).

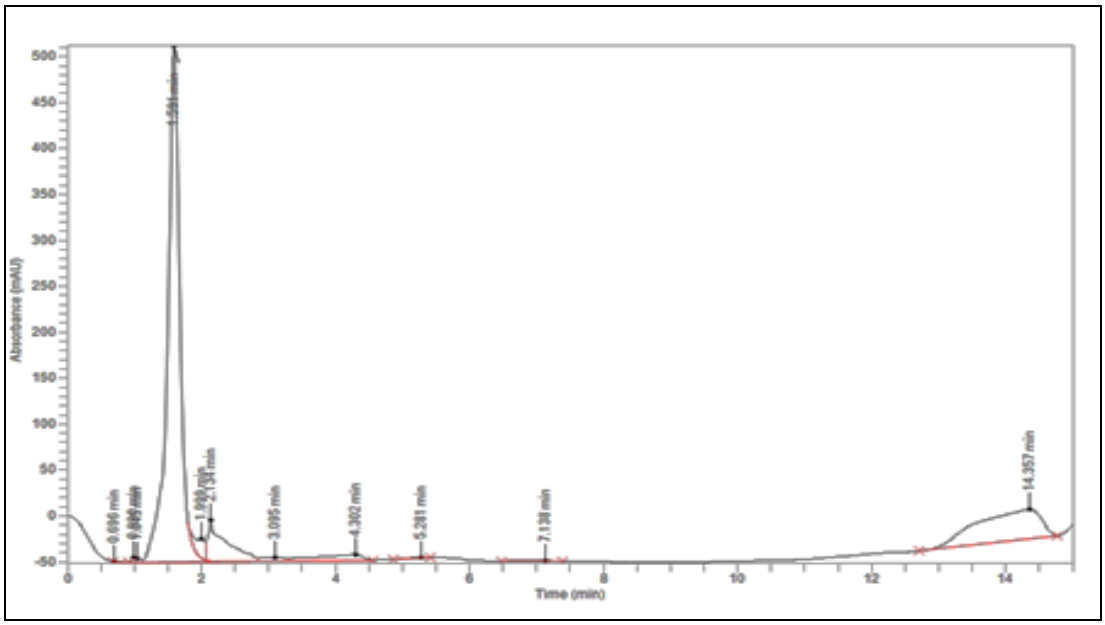

Fig. 4.11: HPLC chromatogram for anthracene degradation by isolate S_{13} after 72 hrs

On further incubation for 48 hours converted to metabolites with retention time and relative abundance (%) in parenthesis are as 1.23min (1.42), 1.55 (30.29), 2.12 (25.40), 4.29 (13.74) 2.003 (5.52), 5.2 (0.65) and 14.37(6.05). The compound with retention time of 14.37 was compared with authentic standard and previously reported work was found to be 9, 10- dihydroxy anthracene [Yu *et al.* 2005].

HPLC analysis of metabolites formed by the isolates B_{36} from a non-contaminated area a single sharp peak with 2.004 retention time previously reported anthracene metabolite [Neelofur *et al*. 2014].

Some unidentified smaller peaks with retention time of 1.508, 1.865 and 5.672 after 72 hours (Fig. 4.12).

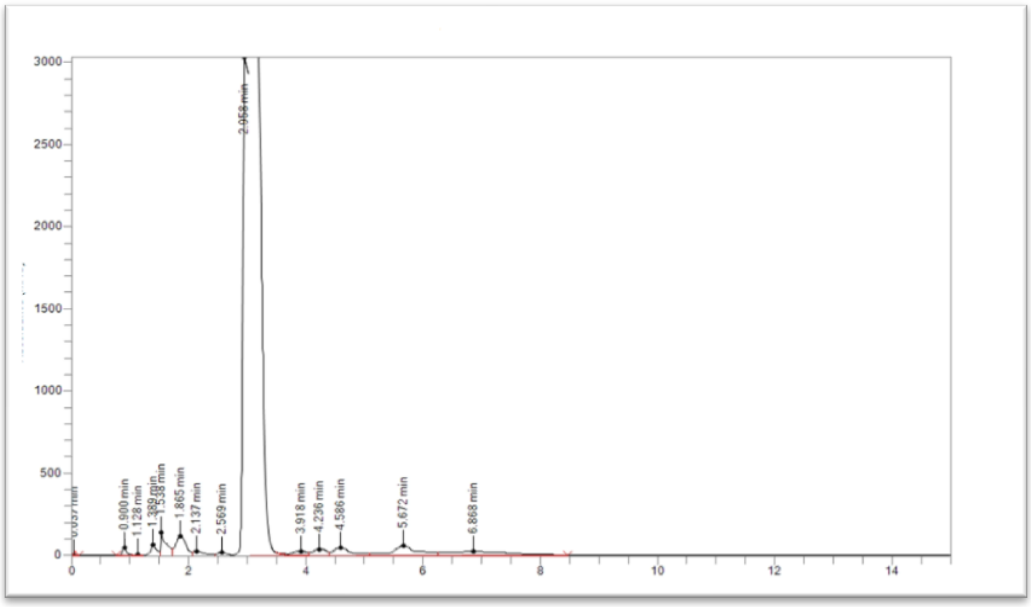

Fig. 4.12: B_{36} on anthracene after 72 hours of incubation

Which on further incubation for 120 hours converted to metabolites with retention time 9.343 and 10.80 (Fig. 4.13), were compared with authentic standard and previously reported work were found to be salicylaldehyde and salicylic acid [Swaathy *et al.* 2014].

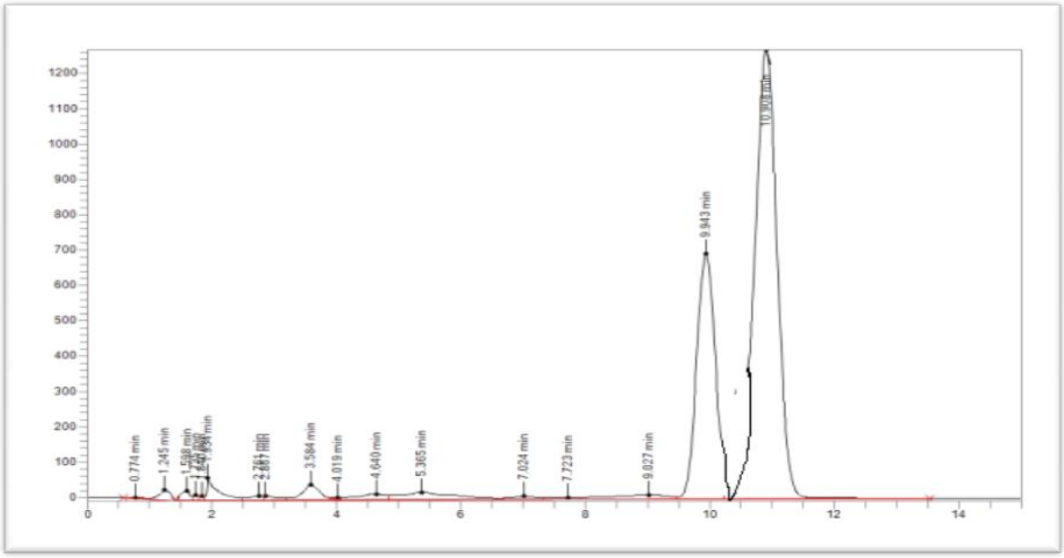

Fig. 4.13: HPLC chromatogram of isolate B_{36} on anthracene after 120 hours of incubation

When the culture was incubated for 10 days in order to study the fate of these metabolites through GC-MS analysis show some unknown peaks (Annexure E, Fig. VI). The oxidation products of our selected PAHs mostly match with that produced during degradation of three LMW and two HMW PAHs via salicylic acid, 1H2N, 3H2N and *o*-hydroxypyrenoic acid, respectively [Wei *et al.* 2009]. The presence of oxygen is vital for benzene ring oxidation as it catalyze dioxygenase for the cleavage of aromatic ring [Cerniglia, 1992; Gibson & Parales, 2000].

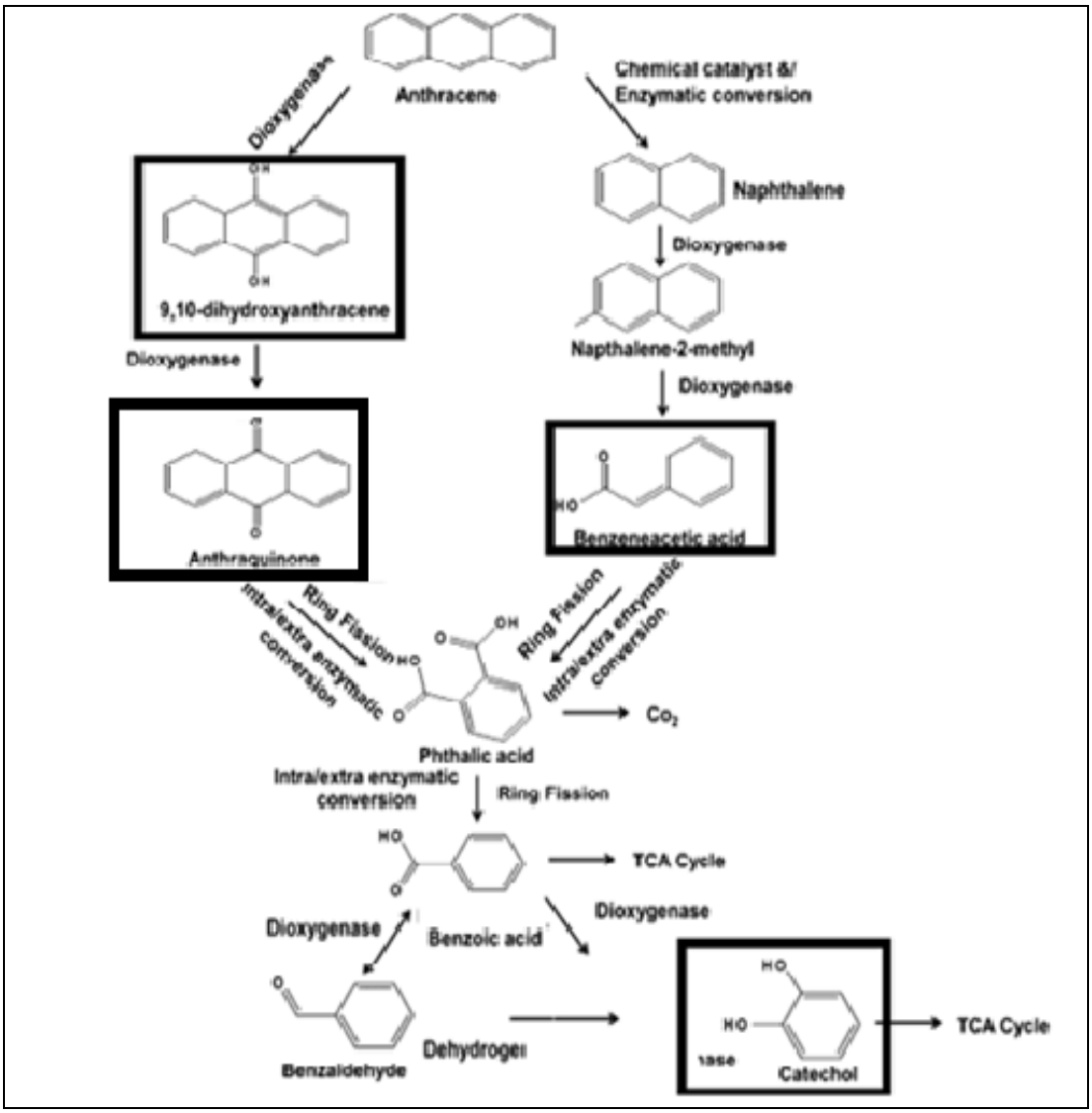

Fig. 4.14: Proposed pathway for Anthracene degaradation, the compounds in rectangles were identified during this study (Simmaro *et al.* 2013)

4.5 Plasmid isolation

Plasmids are mobile genetic elements that carry gene for degradation of polyarenes. These have genes that encode catechol dioxygenase involved in catabolism of hydrocarbons plasmids are extra chromosomal genes and are going to mutate in the environment and becomes adapted to contaminant degradation. As it is clear from gel photograph, that the isolate B_{36} contains more than one plasmid which can be attributed to its ability to degrade anthracene, a comparatively hydrophobic compound. But, when plasmid was cured the isolate still has the capability of growth on anthracene which revealed that the genes responsible for this compound were located on chromosome. The production of naphthoic acid is also an indication of plasmid mediated degradation of PAHs by *Bacillus* as reported earlier [Fu *et al.* 1993]. Moreover, gene rearrangement and expression of specific proteins are among bacterial adaptation strategies. This diverse metabolic response gives an insight into the highly microbial activity occurring in contaminated areas [Coral & Karagoz, 2005; Kumar *et al.* 2010]. In this study, B_{36} and S_{13} have plasmid as shown in Fig.4.15.

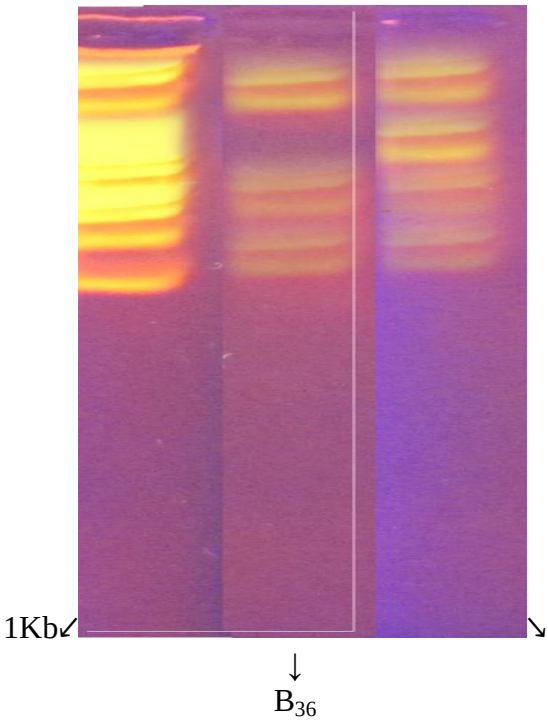

Fig. 4.15: Lane 1. Showing 1 Kb ladder, lane 2 showing plasmid from B_{36} and lane 3 from isolate S_{13}

4.6 Identification of selected bacterial isolates

Identification of isolated bacterial isolates was carried out using both conventional and modern techniques.

4.6.1 Morphological and biochemical characterization of bacterial isolates

The isolates were identified morphologically by Gram's staining and microscopy while biochemically by Bergey's manual of systematic bacteriology. All the isolates were gram positive, S_{13} colony morphology was rod shape and motile, off white/creamy in color with irregular margins while, B36 appeared oblong shape and milky in colour (Table 4.1).

Table 4.1: Morphological identification of isolates B_{36} and S_{13}

S. No	Isolate	Gram's Test	Microscopy	Spore	Motility
1.	S_{13}	+ive	Rods	+	+
2.	B_{36}	+ive	Rods	+	+

The biochemical tests and their results are given (Table 4.2). On the basis of morphological and biochemical analysis, we can identify our isolates B_{36} and S_{13} as species of *Bacillus*.

Table 4.2: Biochemical characterization

S. No.	Biochemical test	Bacterial isolate	
		S_{13}	**B_{36}**
1.	**Catalase**	+ive	+ive
2.	**Starch hydrolysis**	+ive	+ive
3.	**Citrate**	-ive	-ive
4.	**Urease**	+ive	+ive
5.	**Casein hydrolysis**	-ive	+ive
6.	**Gelatin liquification**	+ive	+ive
7.	**Indole production**	-ive	-ive
8.	**Nitrate**	+ive	-ive
9.	**Urease**	+ive	-ive
10.	**Oxidase**	+ive	+ive

4.6.2 Molecular identification and phylogenetic analysis of isolates B_{36} and S_{13}

Partial sequence of 16S rRNA was used for identification of our bacterial isolate B_{36} capable of growth on anthracene. Maximum likelihood phylogenetic tree was constructed using MEGA 6.0 software. 20 sequences were downloaded from BLAST data that were showing maximum relatedness with our isolate. STA16SRR14 *Staphylococcus gallinarum* was used for rooting the tree (Fig.4.16). Our isolate B_{36} formed tree with different species of *Bacillus* bacterial isolates, including *Bacillus subtilis* isolate B7, *B. cereus* isolate B158 and *Bacillus* sp. hb54 as well as *Bacillus* sp. A-BT-nw. Previously reported *Bacillus subtilis* BMT4i has the capability to degrade anthracene as well as naphthalene.

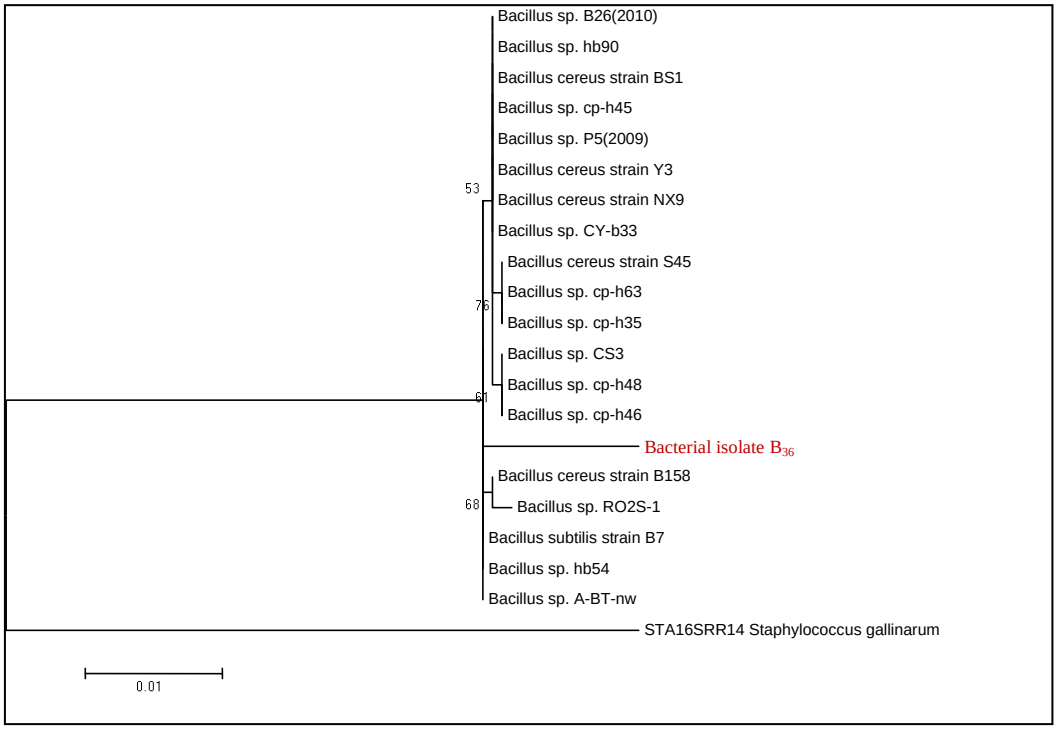

Fig. 4.16: The evolutionary history of bacterial isolate B_{36} was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [Tamura and Nei, 1993]. Evolutionary analyses were conducted in MEGA6 [Felsenstein, 1985].

However, our isolate was unable to degrade naphthalene or phenanthrene, which is structurally similar compound to anthracene. Bacterial isolates from soil that are capable of PAHs degradation typically belong to *Bacillus* sp. [Lily *et al.* 2009;

Fazlurrahman 2011]. On the basis of this analysis, we can identify our isolate B_{36} as *Bacillus* sp.

Bacterial isolate S_{13}, capable of anthracene utilization as carbon and energy source showed 98% similarity with *Bacillus cereus isolate* LP20_03 and *Bacillus cereus RNS-1* when subjected to phylogenetic analysis using MEGA 6.0 software, after BLAST search (Fig.4.17). A number of *Bacillus sp*. isolated so far from rhizospheric sources have astonishing results in terms of PAHs degradative capability [Leung *et al.* 1999; McMahon *et al.* 2007; Dawkar *et al.* 2009; Brown *et al.* 2011]. On the basis of above analysis we can identify our isolate S_{13} as a isolate of *B. cereus.*

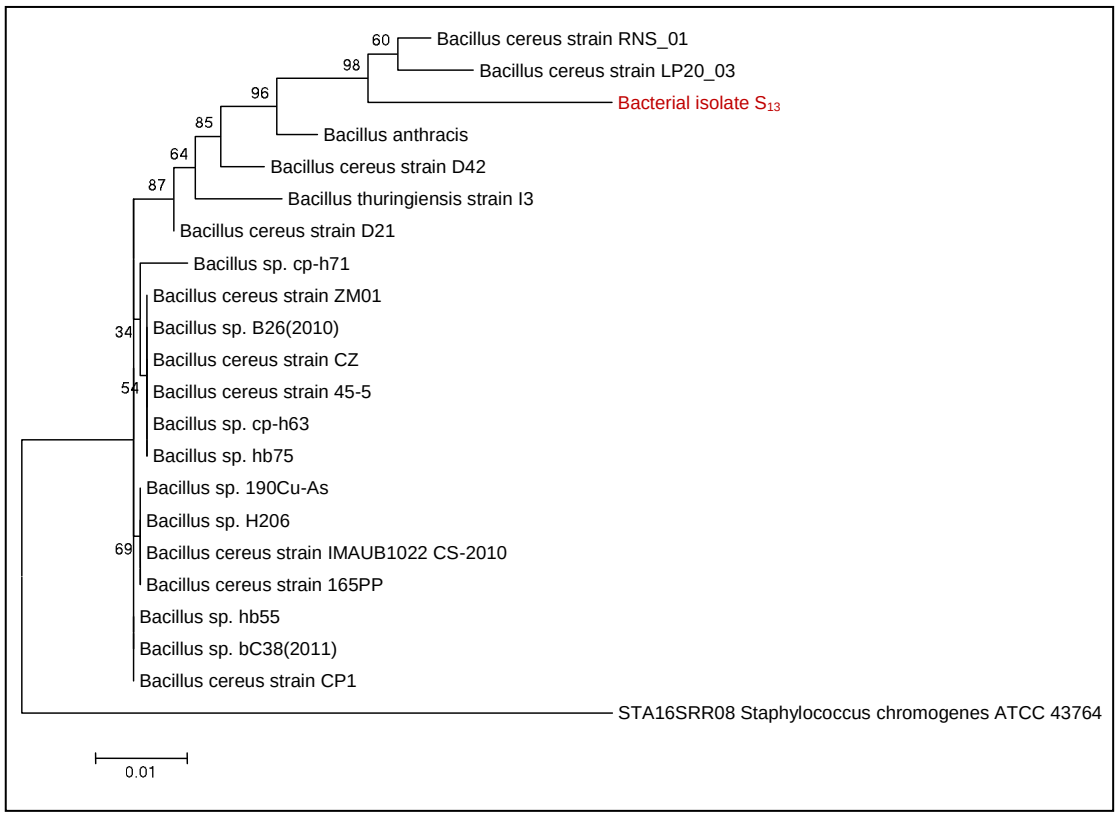

Fig. 4.17: The evolutionary history of bacterial isolate S_{13} was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [Tamura & Nei, 1993]. Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6 [Felsenstein, 1985].

BIODEGRADATION OF NAPHTHALENE

5.1 INTRODUCTION

Naphthalene is a bicyclic aromatic hydrocarbon mainly produced by petroleum products, creosote, and pharmaceutical wastes [Ncibi *et al.* 2007]. It is used as a model PAH compound to explore PAHs degradative capability of bacteria for the reason that it is the simplest PAH [Ahn *et al.* 1998]. Naphthalene is highly volatile and poorly soluble in water. Naphthalene is sprangely soluble in water and has high solid liquid affinity which reduced its bioavailability and enhanced bioaccumulation in water bodies. Its solubility in water is $30mgL^{-1}$ at 25°C. Systematic exposure to naphthalene and its derivatives has been shown to cause several diseases and disturbances to human metabolism [Seoud & Maachi, 2003].

Biodegradation of PAHs in soil is of major and regulatory concern with regard to the bioremediation of contaminated land, with much of the published research focussing on the removal of individual compounds, in particular the LMW PAHs [Puglisi *et al.* 2007; Stroud *et al.* 2008]. Naphthalene degrading bacteria has been used to understand and predict pathways in the degradation of HMW PAHs. Many bacteria that have been isolated and utilize naphthalene as a sole source of carbon and energy belong to the genera *Alcaligenes*, *Burkholderia*, *Mycobacterium*, *Polaromonas*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Sphingomonas* and *Streptomyces* [Baboshin *et al.* 2008; Jouanneau *et al.* 2007; Seo *et al.* 2007]. Microbial degradation of naphthalene has been studied extensively for the elucidation pathway under oxic and anoxic condition [Aranha & Brown, 1981; Rockne & Strand, 2001; Kwok & Loh, 2003; Sheng & Gong, 2006; Gottfried *et al.* 2010; Karamalidis *et al.* 2010].

5.2 MATERIALS AND METHODS

5.2.1 Chemicals and Media

Analytical grade naphthalene (99%), Salicylic acid, salicylaldehyde, catechol, gentisic acid, 1-hydroxy-2, naphthoic acid and 2-hydroxy-1, naphthoic acid, 6, 7-benzo-caumarin, 1-naphthol and were purchased from and other chemicals were purchased from Sigma-Aldrich and Fluka. Other chemicals and media components

were of high purity and analytical grade from Oxide, Merck and BDH. The composition of PNR and PNRG (PNR + 5 mM glucose) per liter of distilled water [Pan *et al.* 2008; Ebrahimi *et al.* 2012; Khan *et al.* 2006], described in detail in chapter 4.

5.2.2 Optimization of various physic-chemical conditions for PAHs degradation

Different parameters such as naphthalene conc., temperature, pH, and substrate (alternate carbon and energy source), agitation speed and effect of inoculum were optimized for degradation studies [Naveenkumar *et al.* 2010; Abdelhay *et al.* 2008].

5.2.3 Biodegradation experiment

The biodegradation experiment was performed with 1000mgL^{-1} naphthalene according to protocol given in chapter 4 in detail [Othman *et al.* 2010].

5.2.4 Determination of bacterial growth by CFUmL^{-1}

The viablility of bacteria was determined by CFU study of samples drawn after every 24 hours intervals for 120 hours.

5.2.5 Extraction of naphthalene and its metabolites

Extraction of naphthalene and its metabolites for HPLC and GC-MS was made according to protocol of [Shokrollahzadeh *et al.* 2010; Survery *et al.* 2009; Neelofur *et al.* 2014].

5.2.6 Morphological and biochemical characterization of bacterial Isolates

The isolates were identified morphologically by Gram's staining and microscopy while biochemically by Bergey's manual of systematic bacteriology.

5.2.7 Plasmid isolation and Agarose Gel Electrophoresis

The plasmid isolation protocol is mentioned in chapter 4 in detail **[**Survery *et al.* 2009**]**.

5.2.8 Molecular identification based on 16S rRNA gene and phylogenetic analysis

DNA extraction, PCR amplification was made according to protocol of (Lane, 1991) and bacterial primers cloning and sequencing were performed according to the protocol of Sambrook and Russel, (2001). The 16S rRNA gene sequence of the targeted isolates were processed manually, analysed at NCBI (National Centre for Biotechnology Information) using BLAST tool and compared to the corresponding neighbour sequences from GenBank-NCBI database, already described in chapter 4.

5.3 RESULTS AND DISCUSSION

5.3.1 Optimization of various physico-chemical conditions

A wider range was selected for optimization of concentration was selected and isolates B_{30} concentration dependent growth naphthalene (Fig.5.1). The growth comparatively slow growth rate was observed with the lower naphthalene concentration of 100 $mgL^{-1}$and maximum growth OD of 0.453 was calculated at 1000 mgL^{-1}. The maximum degradation for this isolate B_{30} was optimized at 1000mgL^{-1} [Othman *et al.* 2010].

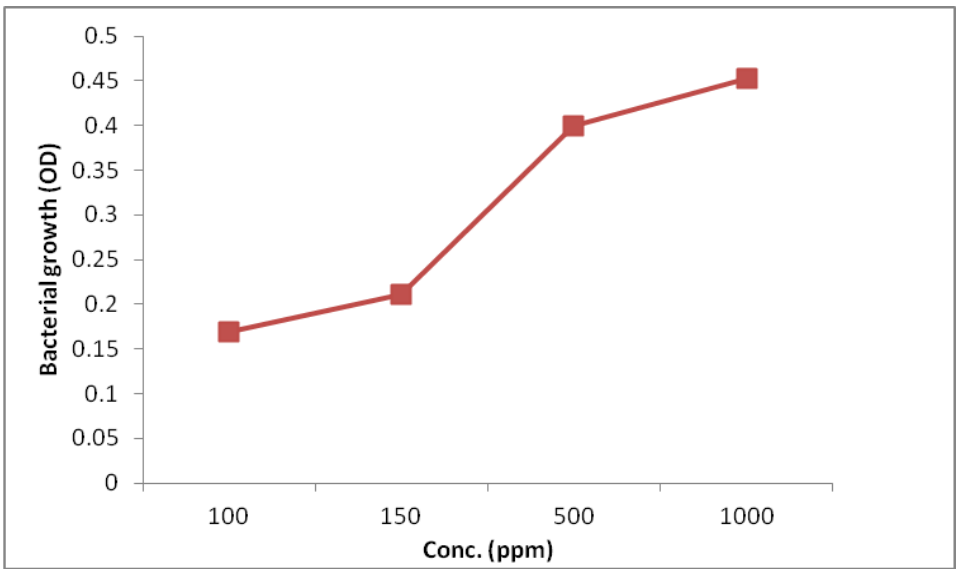

Fig. 5.1: Optimization of naphthalene concentration

5.3.2 Optimization of p^H for naphthalene degradation

p^H was optimized for naphthalene degradation at different p^H range 4-9 and maximum activity was observed at neutral p^H 7 as shown in Fig.5.2. Every microorganism has a specific range of p^H for its growth and metabolism. From our study, it was found that neutral p^H favoured both naphthalene removal and bacterial growth [*Nnamchi et al.* 2006].

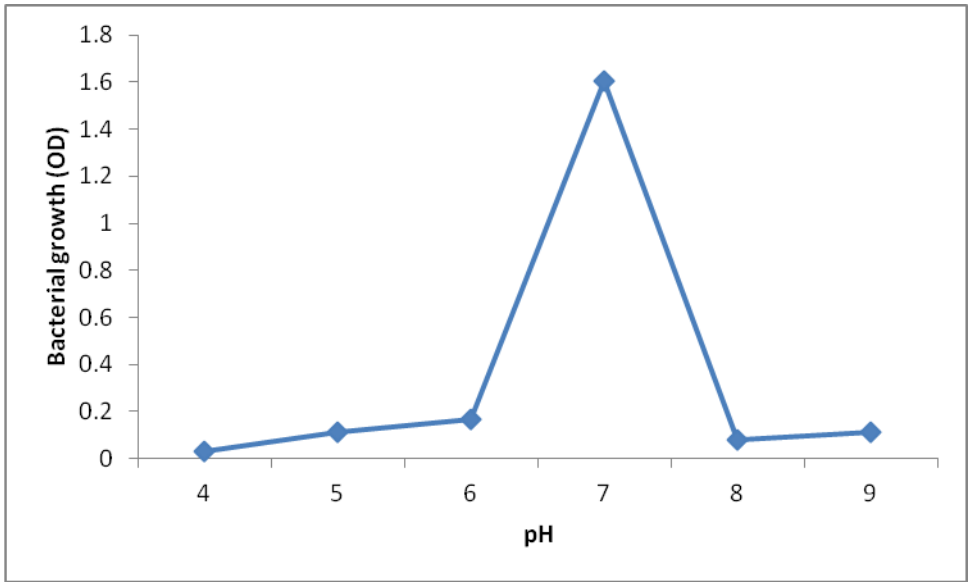

Fig. 5.2: Optimization of the media p^H for isolate B_{30}

5.3.3 Optimization of temperature for naphthalene degradation

Temperature play a pivotal role in the enhance bioavailability of naphthalene but reduce oxygen availability which adversely affects metabolic machinery of mesophilic organism and thus render the low transformation of contaminants [Nour *et al.* 2010].

The enzymes of this microorganism have the potency to bring about maximum activity at this temperature to utilize PAHs as its sole energy source. At low temperature volatility is reduced which cause low aqueous solubility and thus slow rate of onset of biodegradation. This can results in reduced enzymatic activity at low temperature while increasing hydrocarbon metabolism reaches its maximum 40ºC above which membrane toxicity to hydrocarbons is increased [Leahy & Colwell,

1990]. For the isolate B_{30} maximum degradation of naphthalene was found to be in the mesophilic range and was optimized at 30ºC shown in Fig.5.3.

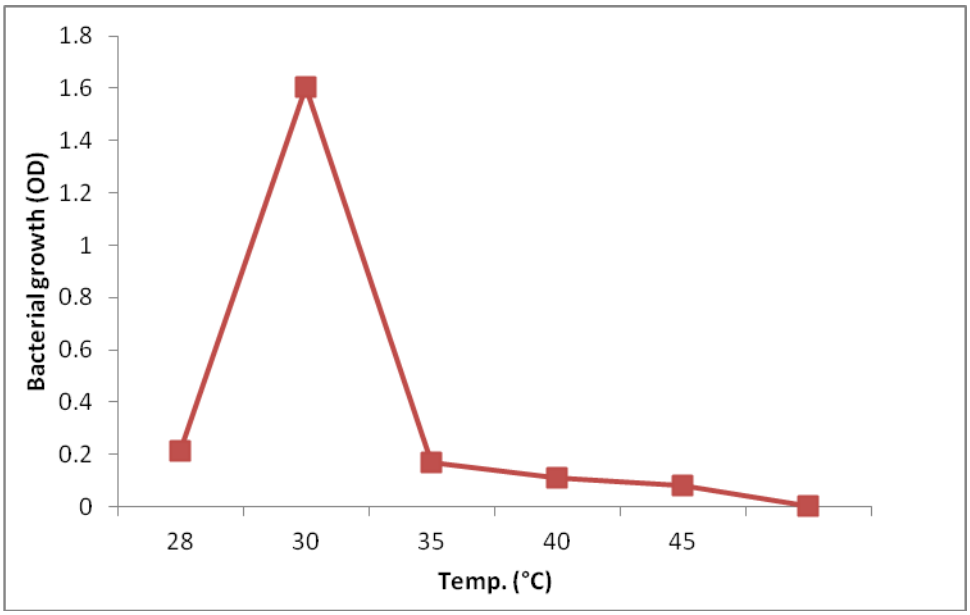

Fig. 5.3: Optimization of temperature for isolate B_{30}

5.3.4 Effect of agitation speed on naphthalene degradation

Shaking speed was optimized at 120rpm for naphthalene degradation as shown in Fig.5.4.

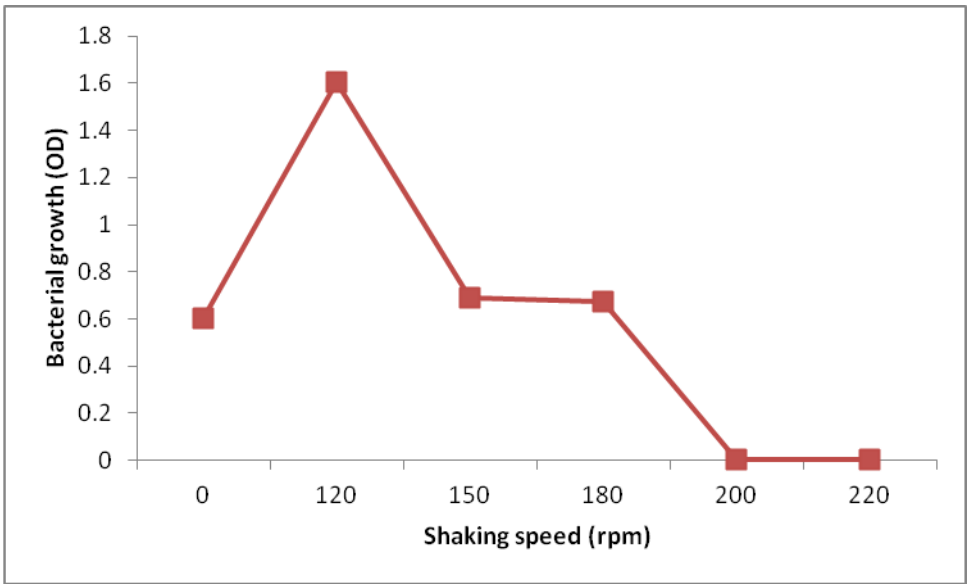

Fig. 5.4: Optimization of shaking speed

Agitation speed is necessary to maintain low oxygen pressure for better aeration and nutrient circulation in the medium as well as for production of degradative enzymes. Adequate availability of molecular oxygen with enhances level of enzymes production to incorporate oxygen in benzene ring [Chulalaksananukul *et al.* 2006; Park *et al.* 1990].

5.3.5 Effect of substrate on degradative activity

Different alternate carbon and nitrogen sources were studied for their effect on degradation rate as shown in Fig.5.5. Bacterial growth on the expense of naphthalene was not affected by glucose.

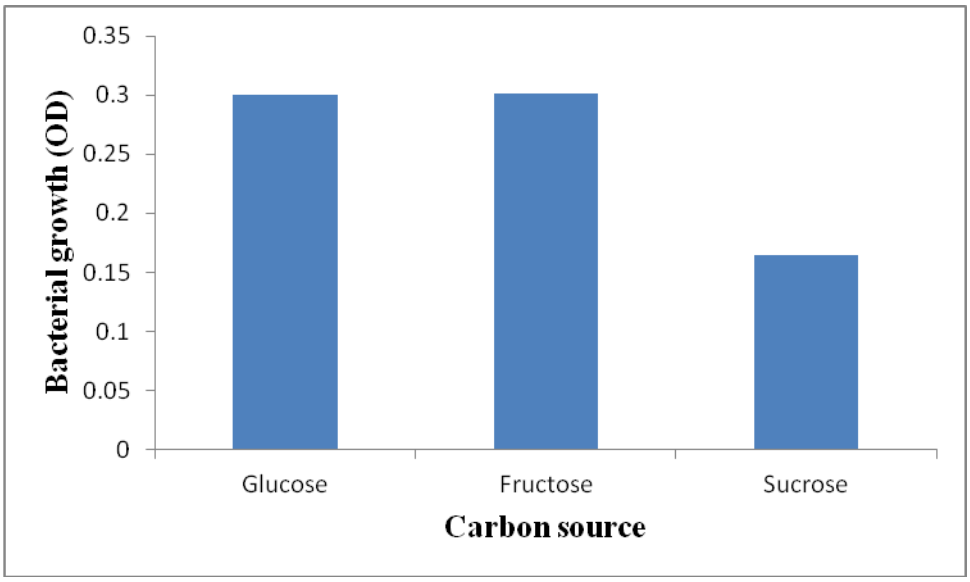

Fig. 5.5: Effect of carbon sources on growth of isolate B_{30}

In present study the maximum degradation of naphthalene is favoured by neutral p^H and nitrogen source can push p^H to alkaline range. Additions of different substrate were found to be competitive inhibitors in naphthalene degradation as they affect the required enzymatic action. These results show that B_{30} could has the ability to assimilate naphthalene as energy source even in the presence of frequent sources like different sugars [Othman *et al.* 2010]. Nitrogen and potassium were proved to have inhibitory effects on naphthalene degradation in contrast to previously reported studies where about 22-32% enhanced oil degradation was observed.

All the nitrate salts pose inhibitory effects on the growth (small OD values) of isolate B_{30} as shown in Fig.5.6.

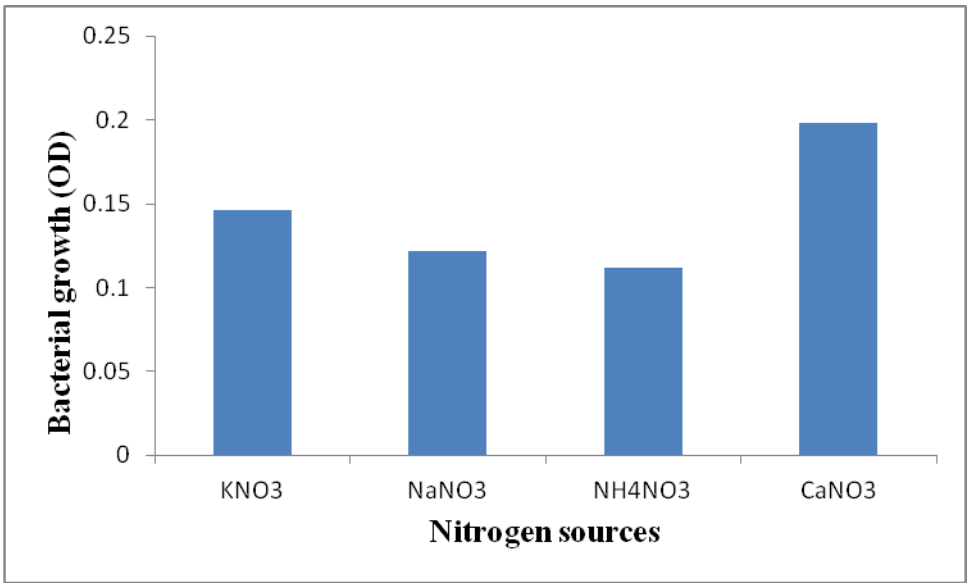

Fig. 5.6: Effect of nitrogen sources on the growth of isolate B_{30}

So the isolate B_{30} might be unable to prevent its charge imbalance of inside with the membrane surface. This can be the possible explanation of inhibitory effect of nitrogen source on naphthalene degradation [Vyas & Dave, 2010].

5.3.6 Optimization of inoculum concentration

The optimized concentration of isolate B_{30} for naphthalene degradation was 10% as shown in Fig.5.7.

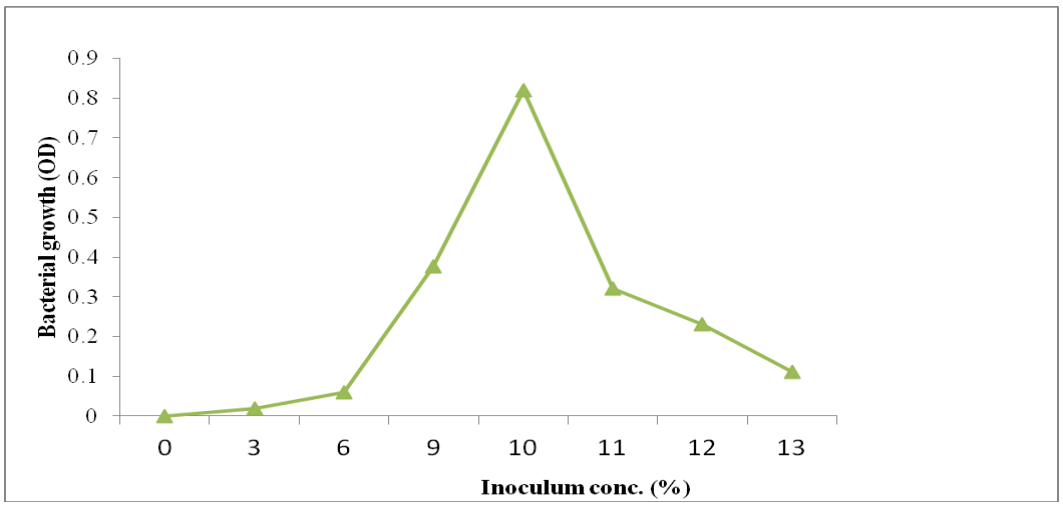

Fig. 5.7: Optimization of inoculum concentration (%) for isolate B_{30}

5.3.7 Biodegradation

Some microorganisms can consume PAHs as a source of carbon and energy [Herwijnen *et al.* 2003; Johnsen *et al.* 2005]. Biodegradation of PAHs in soil is of major and regulatory concern with regard to the bioremediation of contaminated land, with much of the published research focussing on the removal of individual compounds, mostly containing two and three benzene rings **[**Macleod & Semple, 2006**].** However, the impact of the presence of other contaminants on microbial degradation is important in terms of biodegradation and bioremediation, since single contaminants are rarely found at contaminated sites [Sartoros *et al.* 2005; Puglisi *et al.* 2007; Ye *et al.* 2011]. Naphthalene is used as a representative for the degradation of PAHs because of its simplest nature and high aqueous solubility. It can also be utilize for the illustration of degradative pathways of HMW. The genus, that utilize naphthalene belong to the genera *Alcaligenes*, *Mycobacterium*, *Polaromonas*, *Pseudomonas*, *Ralstonia*, *Rhodococcus* and *Streptomyces* [Goyle & Zylstra, 1997].

In this study the growth and degradation of naphthalene for isolate B_{30} was observed and 100 % naphthalene was degraded in 120 hours as shown in Fig 5.8.

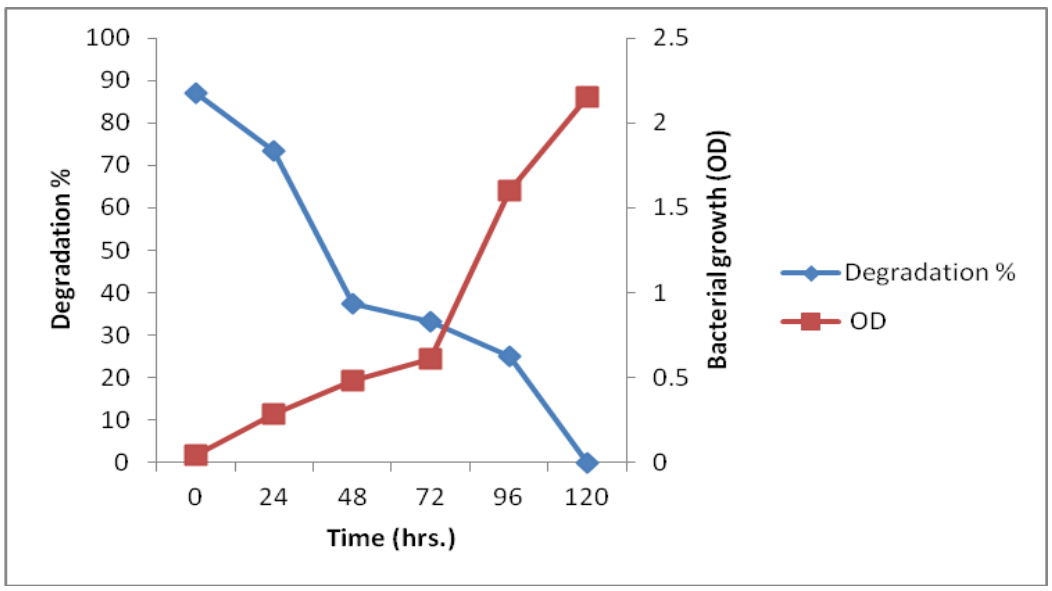

Fig. 5.8: B_{30} growth on naphthalene as sole carbon source

The growth rate was found to be increased fourfold in just 96 hours with OD 1.60 and assimilation of 74.88% naphthalene. The rate of bacterial growth was faster

between 96 to 120 hours of incubation and naphthalene removal drop down due to its transformation. The complete disappearance of naphthalene can be the combined effect of bacterial utilization as well as its volatility [Kumar *et al.* 2010]. In a similar study, *Pseudomonas* isolate PS4040 isolated from contaminated area degrades only 53.1%, naphthalene in about three weeks [Sarma *et al.* 2004].

The 100% naphthalene degradation rate of our studied isolate in just five days are far better than *Kurthia* sp. SBA4, *B. circulans* SBA12 and *Micrococcus varians* SBA8 with 85.3, 95.8 and 86.8% respectively in six days [Bisht *et al.* 2010]. Another reported study on two different isolates of *Pseudomonas aeruginosa* were 77.77% and 61.11% degradation of naphthalene in one week [Bubul *et al.* 2012]. B_{30} isolate degraded 86.89 % naphthalene in four days the highest degradation rate was observed between 48 to 96 hours and slightly decreases on 120 hours of incubation. It can be due to removal of naphthalene, the sole carbon source in the media.

HPLC analysis showed that naphthalene was transformed to other compounds as shown in Fig. 5.9.

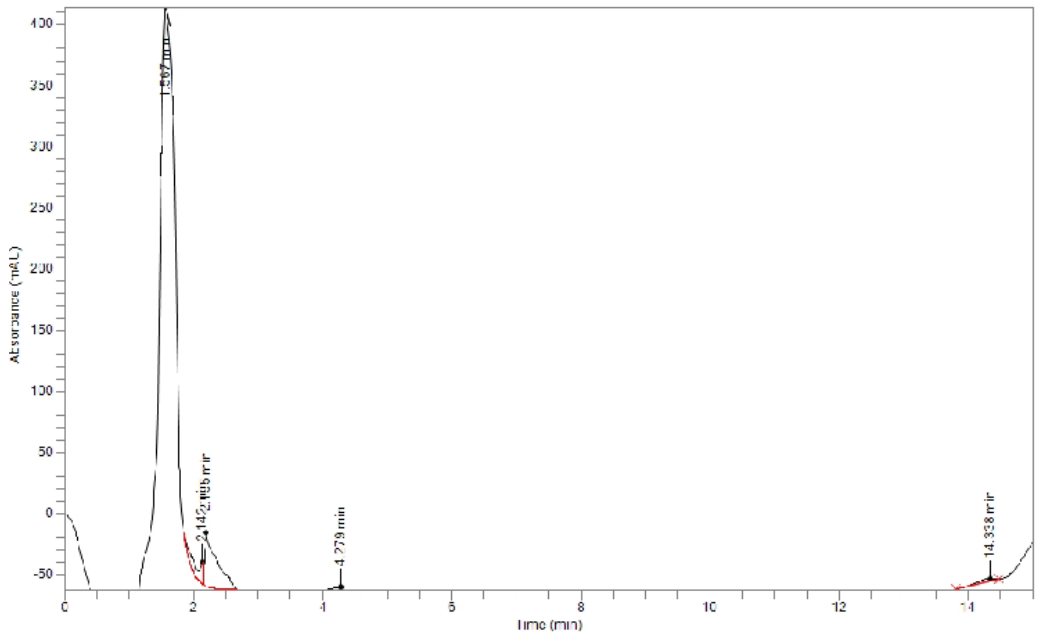

Fig. 5.9: HPLC chromatogram for naphthalene degradation by bacterial isolates B_{30} after 120 hours

It is clear that naphthalene show almost analogous and maximum degradation in single substrate cultures with average 90% after one month in a previously reported work. This might be due to the high capability and higher specificity of microbes to the high evaporation and assimilation rate of naphthalene compounds towards biodegradation [Wei *et al.* 2009].

GC-MS analysis of the end products formed after 10 days of incubation that naphthalene (Annexure E, Fig. III). It is conclded that naphthalene may probably be degraded through catechol pathway and transformed to simpler compounds of metabolic pathway. Studies on the ability of isolate B_{30} was degraded by the formation of gentisic acid or catechol as intermediates are mostly match with the pathway proposed previously as shown in Fig.5.10.

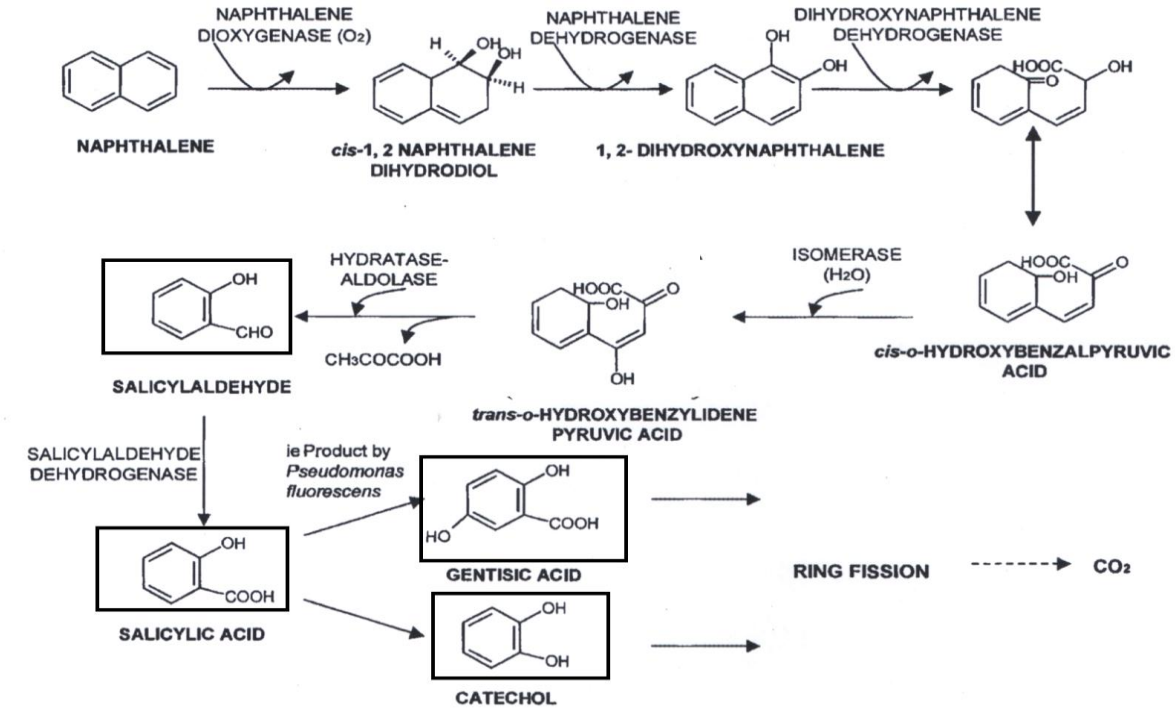

Fig. 5.10: Proposed pathway (Seo *et al.* 2009) for naphthalene biodegradation, the compounds in rectangles were identified during this study

Results of single isolate B_{30} having capabilty of 100 % naphthalene removal in only 5 days are better than reports from same studies available for consortia capable to degrade 97% naphthalene at an initial concentration of 1000 ppm [Alquati *et al.* 2005*;* Sarma *et al.* 2004]. Naphthalene may be oxidize to cis-1, 2 naphthalene dihydrodiol by dioxygenase and dehydrogenated to various intermediates converted to

salicylaldehyde. Salicylaldehyde by enzymatic conversion then converted to salicylic acid and then cleave to catechol which are identified in this study are shown in rectangles in the proposed pathway for naphthalene degradation [Annweiler *et al.* 2000].

5.3.8 Plasmid isolation

Plasmid isolated from isolate B_{30} revealed (Fig. 5.11) that this isolate has gene with degradation ability of LMW PAHs.

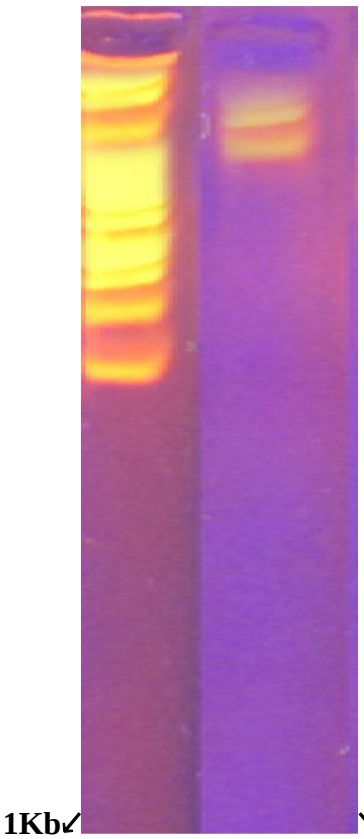

Fig. 5.11: Plasmid isolated from isolate B_{30} and ladder

The naphthalene degradation genes located on plasmid NAH7 of *Pseudomonas putida* PpG7 is most studied. This gene has two operons one encode the formation of salicylate which proceeds catechol formation coded by the other operons. So from our study the degradation of naphthalene through salicylate pathway, it can be concluded that naphthalene degradation plasmids have been studied, in the metabolic pathway, in isolate B_{30} which codes for an ortho cleavage of catechol is partial chromosomally encoded naphthalene, phenanthrene and

acenaphthene degradation. So, it is can be clearly elucidated that in B_{30} the lower pathway was plasmid encoded like *Pseudomonas putida.* While, catechol is the most widely produced metabolite in the degradation of various PAHs with ortho cleavage of catechol is a characteristic of plasmid mediated degradation pathway. The formation of gentisate during co-metabolic degradation of isolate B_{30} further confirm that the degradation genes are located on mobile genetic elements with horizontal gene transfer capability [Mora *et al.* 1994; Fredrickson *et al.* 1991].

PAHs degradation ability of microbes grown in contaminated soil is usually due to presence of genes on mobile genetic elements. Plasmids have been well reported in naphthalene grown isolates with salicylate metabolites. Some plasmid may encode enzymes that have specificity for several substrates. The genes encoding the upper and lower pathways of naphthalene in the NAH plasmids of several *Pseudomonads* have broad specificities, allowing the host to grow LMW [Heitkamp *et al.* 1988].

The plasmids were transmissible, belonged to the p7 incompatibility group and coded for naphthalene degradation via salicyclate and catechol, then, by the catechol metacleavage pathway. Enzymes for the meta-cleavage of catechol were found to be constitutive, in mutant carrying the plasmid NAH, pND140 and pND160. The enzymes involved in the conversion of naphthalene to catechol were found to be inducible during growth on salicylate [Obayori & Salam, 2010]. Furthermore, the bacterial isolates on curing through heat shock of 45°C lose the ability to grow on naphthalene revealed that the degrading gene was located on plasmid [Kumar *et al.* 2010].

5.3.9 Morphological Identification

Isolate B_{30} was Gram's positive and the morphologically appeared oblong and off white with endospores (Table.5.1.).

Table 5.1: Morphological Identification

S. No	Isolate	Gram's Test	Microscopy	Spore	Motility
1.	B_{30}	+ive	Rods	+	+

5.3.10 Biochemical characterization

Biochemical tests revealed gelatin liquification and catalase activity, and the starch hydrolysis, casein hydrolysis and nitrate reduction and oxidase activity were all positive for this isolate. Indole production, urease and Simon citrate test were negative and the sugar fermentation tests results also showed that this isolate B_{30} was unable to ferment lactose and fructose as shown in Table 5.2. On the basis of these results isolate B_{30} can be characterized as *Bacillus* sp. Further identification will be finished on the basis of molecular analysis and phylogeny.

Table 5.2: Biochemical characterization

S. No.	Biochemical test	Bacterial isolate B_{30}
1.	**Catalase**	+ive
2.	**Starch hydrolysis**	+ive
3.	**Citrate**	-ive
4.	**Urease**	+ive
5.	**Casein hydrolysis**	-ive
6.	**Gelatin liquification**	+ive
7.	**Indole production**	-ive
8.	**Nitrate**	-ive
9.	**Urease**	-ive
10.	**Oxidase**	+ive

5.3.11 Molecular identification and phylogenetic analysis

Our isolate showed 99% homology with *Bacillus cereus* 9julio on BLAST search. Phylogenetic analysis however showed that isolate was forming distant clad with all of the isolates, downloaded using MEGA 6. In a related study, a isolate having capability of naphthalene utilization was isolated and was found to have 97.9% homology with *Bacillus* was named as *B. naphthovorans* on the basis of 16S rRNA sequence homology [Zhuang *et al.* 2002]. Isolate B_{30} isolated in this study was

capable of degradation of two (naphthalene) and three ringed (anthracene and phenanthrene) PAHs. We thus identify our isolate B_{30} as isolate of *Bacillus* sp. (Fig.5.12).

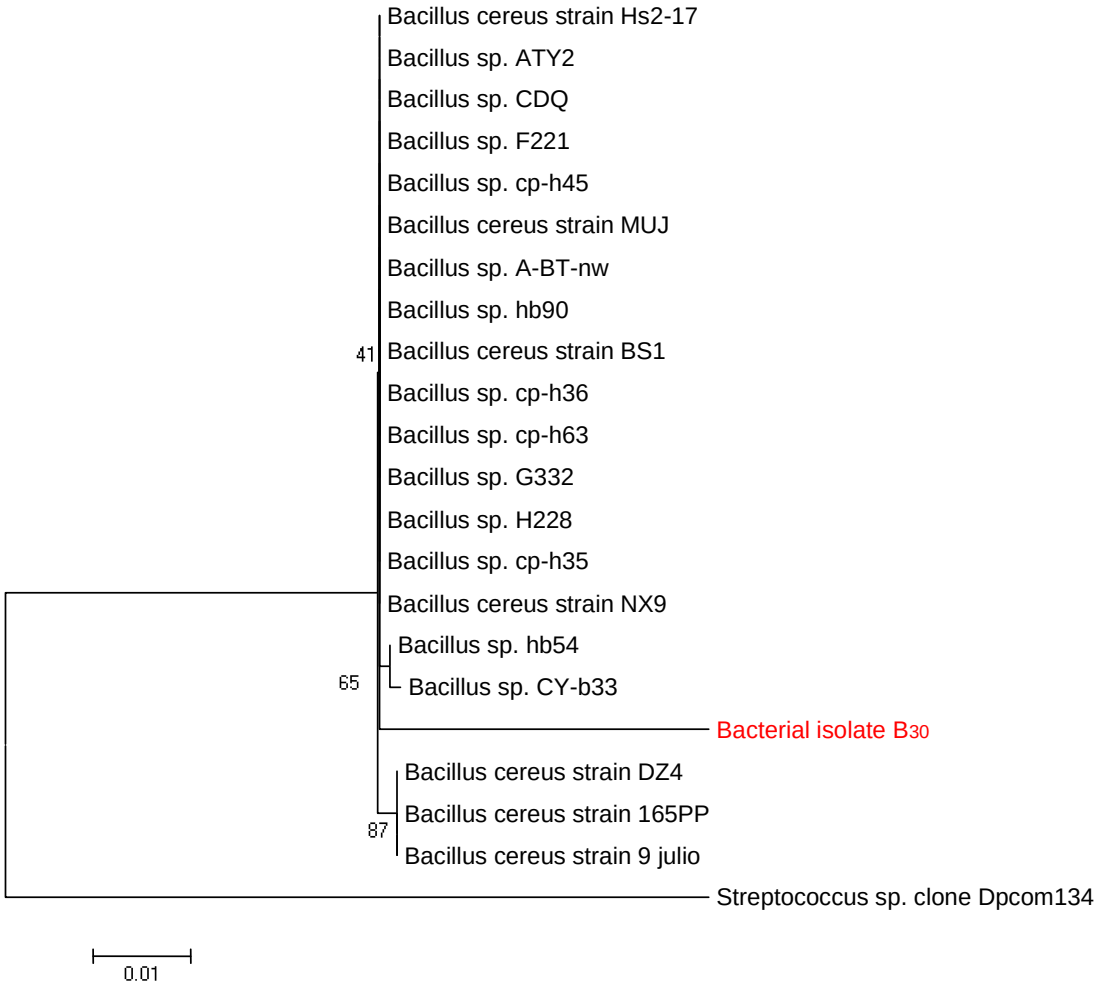

Fig. 5.12: The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [Tamura and Nei, 1993]. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed [Felsenstein, 1985]. The analysis involved 22 nucleotide sequences. Evolutionary analyses were conducted in MEGA6 [Tamura *et al.* 2013].

BIODEGRADATION OF PHENANTHRENE

6.1 INTRODUCTION

Phenantherene promote the research for its remediation from the soil and water resources. It has been reported that the solubility of phenanthrene in water is about 1.1-1.2mgL^{-1}. Phenanthrene has relatively strong carcinogenicity, mutagenicity and toxicity [Zhou *et al.* 2007; Nozaki *et al.* 1982; Nasrollahzadeh *et al.* 2010]. It is human skin photosensitizer and mild allergen and mutagenic to human microbial system under specific condition. It has also been found to be an inducer of sister chromatid exchange and potential inhibitor of gap-junctional intercellular communication [Wodzinksi & Coyle, 1974; Xiang *et al.* 2006].

Removal of phenanthrene influence of the existence of other contaminants on microbial degradation is important in terms of biodegradation and bioremediation, since single contaminants are rarely found at contaminated sites. Enhanced degradation capability has been observed in microbes already exposed to contaminants [Sartoros *et al.* 2005; Macleod & Semple, 2006]. Bacterial degradation of phenanthrene has been studied. Numerous of bacterial isolates such as *Acidovorax, Arthrobacter, Brevibacterium, Mycobacterium* and *Pseudomonas* have been characterised and have the ability to utilize phenanthrene. It is used as a model substrate for studies on the catabolism of bay- and K-region containing carcinogenic PAHs such as benzo[a]pyrene, benzo[a]anthracene, and chrysene [Puglisi *et al.* 2007; Stroud *et al*. 2008; Bamforth & Singleton, 2005].

6.2 Materials and methods

6.2.1 Chemicals and Media

Analytical grade phenanthrene (99%), Salicylic acid, salicylaldehyde, catechol, gentisic acid, 1-H2N, naphthoic acid and 2-H-1N, naphthoic acid, 6, 7-benzo-caumarin, 1-naphthol and were purchased from Sigma-Aldrich and Fluka (St. Louis, MO, USA). Other chemical and media components were of high purity and analytical grade from Oxide, Merck and BDH. The composition of media PNR and

PNRG (PNR+5mM glucose) per liter of distilled water [Pan *et al.* 2008; Ebrahimi *et al.* 2012; Khan *et al.* 2006].

6.2.2 Inoculum preparation

Bacterial isolate was grown in Nutrient Broth following an established protocol [Guo *et al.* 2010].

6.2.3 Optimization of various physic-chemical conditions for PAHs degradation

Different parameters such as naphthalene conc., temperature, pH, and substrate (alternate carbon and energy source) and agitation speed and effect of inoculum were optimized for degradation studies [Naveenkumar *et al.* 2010; Abdelhay *et al.* 2008].

6.2.4 Biodegradation Experiment

The biodegradation experiment was performed in 100ml PNR media, 10% of bacterial inoculum and 1000mgL^{-1} phenanthrene dissolved in acetone at p^H of 7.0 at 30°C and 180rpm [Othman *et al.* 2010].

6.2.5 Extraction of PAHs

Extraction of phenanthrene and its metabolites for HPLC and GC-MS was made according to established protocols [Shokrollahzadeh *et al.* 2010; Survery *et al.* 2009; Neelofur *et al.* 2014].

6.3 RESULTS AND DISCUSSION

6.3.1 Optimization of different parameters

Optimization of different parameters for the degradation phenanthrene by isolate B_{30} is given below:

6.3.1.2 Optimization of Concentration

The optimization of phenanthrene concentration for both the isolates was observed (Fig.6.1). The optimized concentration for maximum growth and degradation was calculated at 1000mgL^{-1}, 500mgL^{-1} for B_{30} and S_{21}. There is a huge gap of phenanthrene assimilation between two isolates as B_{30} has its origin from agricultural field can tolerate more concentration than S_{21} from a contaminated site. This may be due to adaptation process which can enhance the efficiency of bacteria for remediation processes.

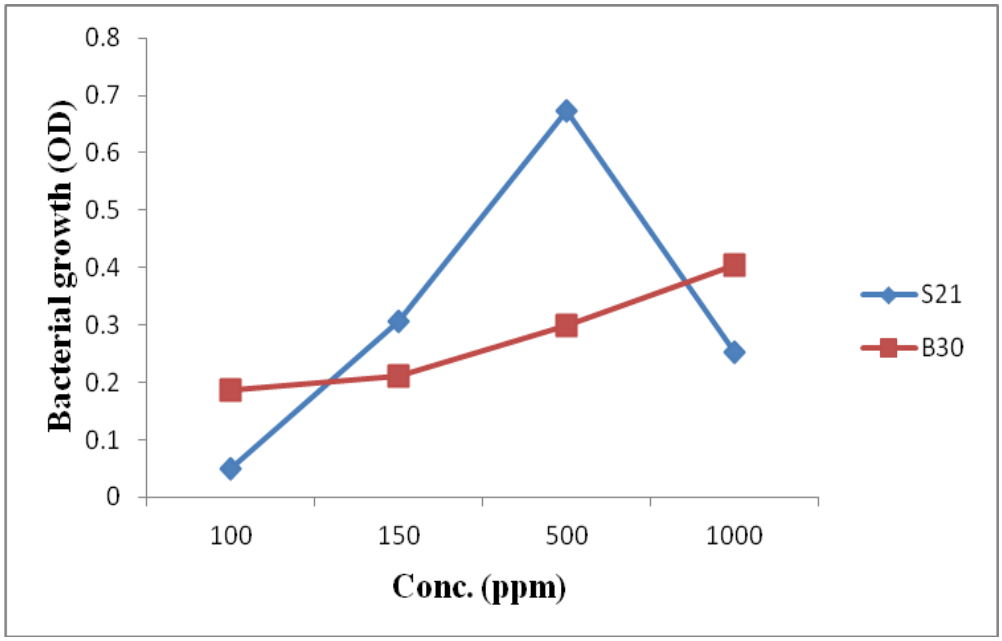

Fig. 6.1: Optimization of phenanthrene concentration

Unlike, previously reported work on phenanthrene removal rate there was no significant difference between 100 and 500mgL^{-1}. Although, phenanthrene at 500mgL^{-1} phenanthrene showed a higher efficiency to remove phenanthrene than that of 100mgL^{-1} phenanthrene culture, which may be due to the fact that too lower concentration may probably recrystalize and beyond the reach of microbes. In fact, bacteria can only degrade those PAHs that exist in soluble form. Therefore, degradation of phenanthrene was determined by the amount of phenantherene remained in the culture broth [Wodzinski & Coyle, 1974; Maiti *et al.* 2012]

6.3.1.3 Optimization of temperature

The optimized temperature for phenanthrene degradation was 30ºC for both the isolates as shown in Fig. 6.2.

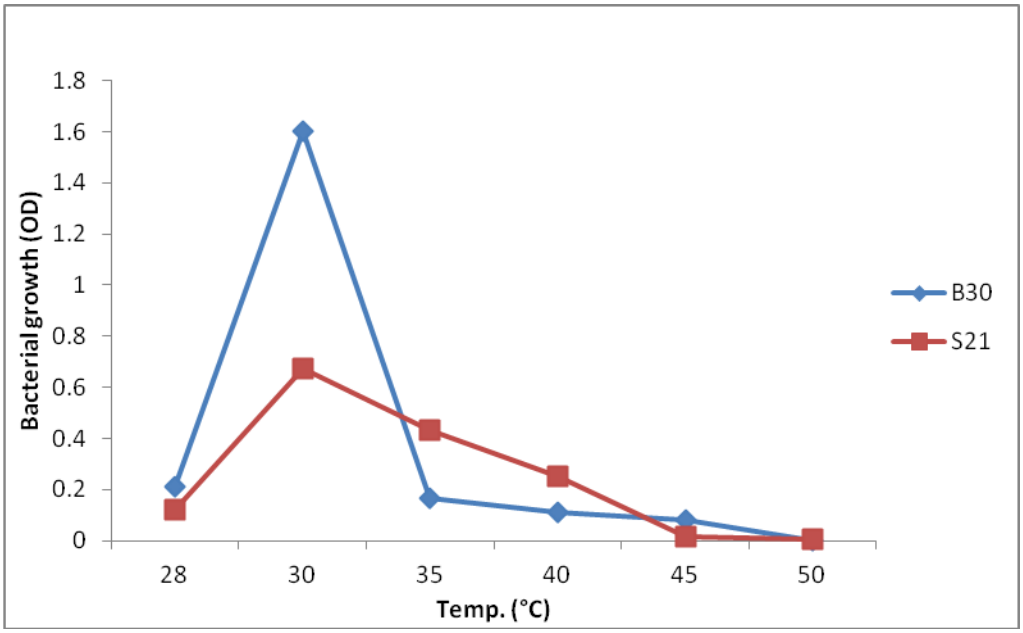

Fig. 6.2: Optimization of temperature

These isolates were isolated from two different sites of almost the same temperature range area. B_{30} have not previously exposed to contaminant possess degradative enzyme that work best at that temperature. Isolate S_{21} was isolated from contaminated area has well tolerated metabolic machinery [Ahmed *et al.* 2010].

6.3.1.4 Optimization of p^H of media on phenanthrene degradation

The growth of bacteria at different range of p^H did not show any significant difference except that at p^H 6 and 9 for S_{21} is an indication of this isolate adoptability to both acidic and alkaline conditions for phenanthrene removal. However, the phenanthrene degradation rate was comparatively higher at p^H 9 than p^H 6. It is reported previously, that the growth of bacteria by changing p^H the efficiency of phenanthrene removal was enhanced. The degradation of phenanthrene resulted in a decrease in p^H, especially between 3 to 6 days, with a relatively lower p^H in the treatments with high phenanthrene concentrations. The decrease in p^H can be explained by the release of 1-hydroxy-2-naphthoic acid, an early intermediate of

phenanthrene metabolism. So it can be elucidated from the results of growth of S_{21} at p^H 6 that the phenanthrene was biotranformed through the above mentioned intermediate [Boldrin *et al.* 1993; Tiehm, 1994]. In an earlier study it was observed that the degradation of phenanthrene was inhibited when pH fluctuates from neutral to acid. But, in present work the isolate S_{21} has the ability to prevent charge imbalance inside and on the surface of the cell even when the external environment p^H is basic or acidic [Sudarat *et al.* 2000]. It can be elucidated from these results that the isolates growing in the vicinity of petroleum product like S_{21} from a filling station develop a strange ability to cope with the changing p^H of the environment. The effects of p^H on bacterial growth at the expense of phenanthrene were optimized at 8 and 9 for B_{30} and S_{21} respectively as shown in Fig. 6.3.

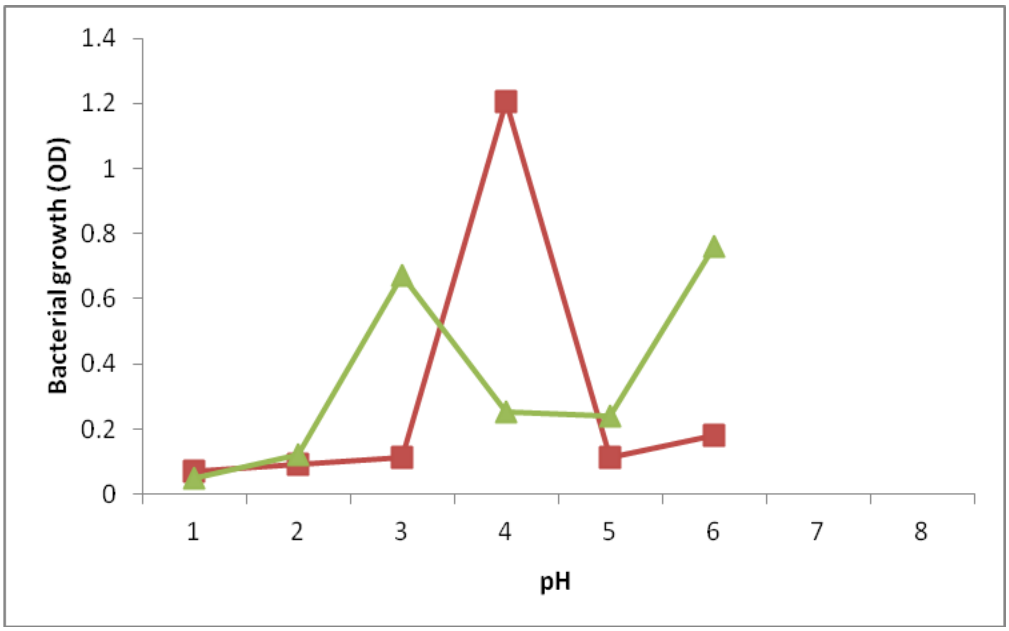

Fig.6.3: Optimization of media p^H

6.3.1.5 Effect of agitation speed on phenanthrene degradation

Better aeration and nutrient distribution is necessary for maximum degradative activity. As the incorporation of molecular oxygen in benzene ring cleavage requires oxygenase. Production of these enzymes depends on oxygen supply which can be provided only at an optimized shaking speed [Park *et al.* 1990].

Shaking speed of both isolates for phenanthrene degradation was optimized at 120 rpm as shown in Fig. 6.4.

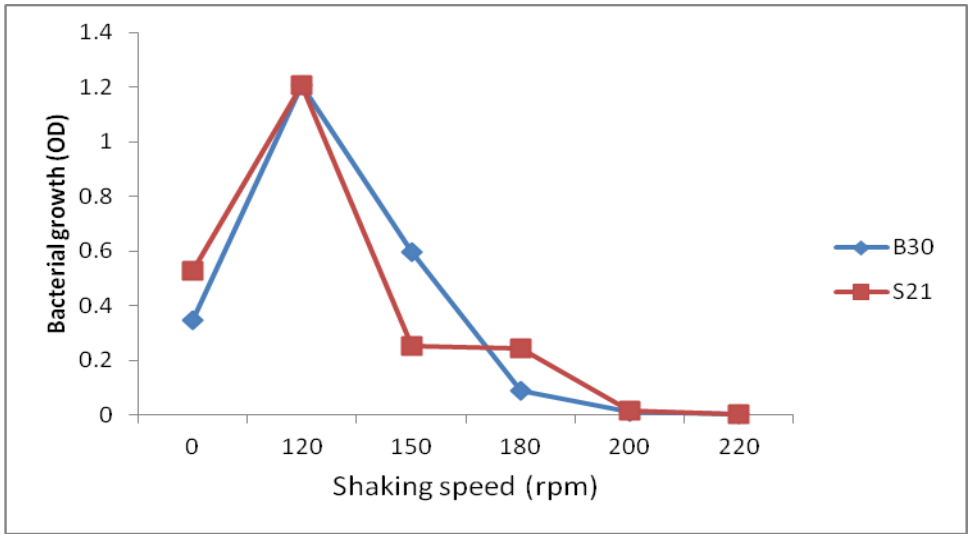

Fig. 6.4: Optimization of shaking for isolates B_{30} and S_{21}

6.3.2 Effect of substrate on degradative activity

Addition of glucose as carbon substitute on the growth of bacteria on phenanthrene degradation is shown in Fig.6.5. It does not affect the assimilation of phenanthrene by bacteria.

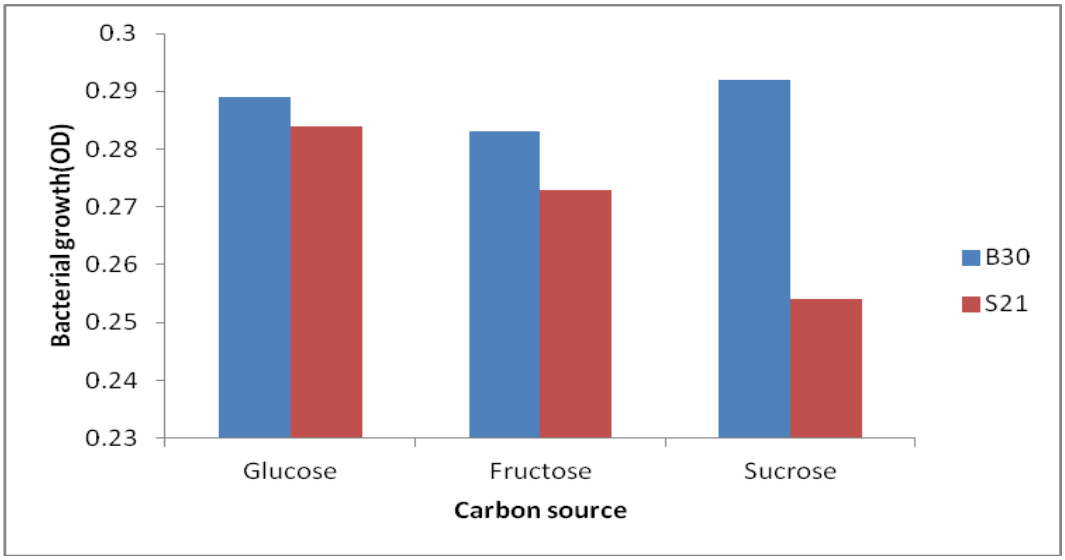

Fig. 6.5: Effect of carbon source for isolates B_{30} and S_{21}

No significant difference was observed in phenanthrene degradation in cultures with and without glucose. These results show that both the bacterial isolates could use phenanthrene as its sole carbon source and that additional carbon source decreased the metabolism of phenanthrene. In this study, glucose has inhibitory effect on degradation activity of phenanthrene. Hence, glucose at high supplement rates became a competitive substrate in phenanthrene degradation. Low availability of phenanthrene as compared to glucose in culture media may account for this result [Wong *et al.* 2002].

To estimate, the influence of nitrogen sources on the growth of bacteria and phenanthrene degradative activity as shown in Fig.6.6. The effect of many kinds of supplements like potassium and nitrogen proved to have an inhibitory effect on phenanthrene degradation by both isolates.

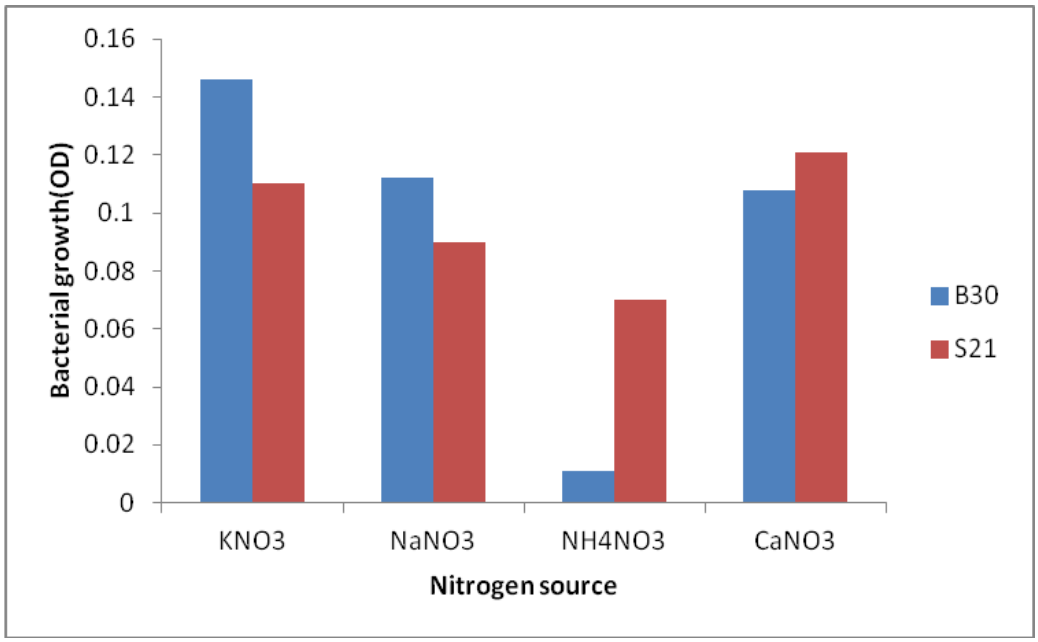

Fig. 6.6: Effect of nitrogen substrate on phenanthrene degradation by isolates B_{30} and S_{21}

Unlike, previously reported studies, addition of nitrogen may supply more available nitrogen and stimulate the growth of bacteria as well as phenanthrene degradation. In this study supplement of nitrogen remarkably reduced the decrease in p^H of the culture media which is unfavorable for both isolates that can utilized

phenanthrene at slightly higher p^H than neutral. It may reduce the availability of phenanthrene for bacterial uptake under p^H shift, resulting in decrease in the growth of bacteria. Nitrogen may have role in maintaining alkaline environment necessary for phenanthrene degradation by both isolates [Cheng *et al.* 2008; Maiti *et al.* 2012].

6.3.3 Optimization of inoculum concentration

Optimized inoculum concentration for both the isolates was 10% as shown in Fig.6.7. The growth of isolate S_{21} was faster than B_{30} on the same substrate (phenanthrene) which may be probably explained by previous exposure of the former to these contaminants present in petroleum products.

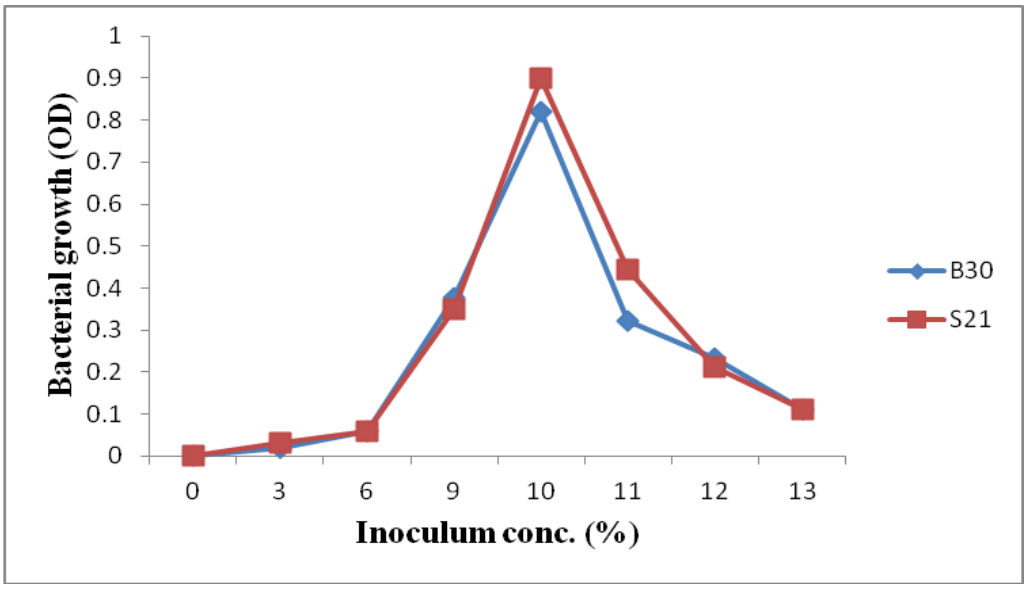

Fig. 6.7: Optimization of inoculum concentration for isolates B_{30} and S_{21}

6.3.4 Biodegradation

Microbial activity of bacterial isolates in particular, efficiently degraded PAHs to a greater extent and has been thought to be the most prominent and significant cause of PAHs removal [Pan *et al.* 2008; Ebrahimi *et al.* 2012; Alias *et al.* 2011; Othman *et al.* 2010; Kumar *et al.* 2010]. The bioavailability of PAHs is thought to reduce with increasing molecular mass. PAHs with LMW are more susceptible to bacterial degradation and more extensive than HMW PAHs [Yu *et al.* 2005; Nour *et al.* 2010; Neelofur *et al.* 2014].

6.3.4.1 Biodegradation of phenanthrene by isolate B_{30}

The degradation rate was also slower 77.88 and 78.82% and the OD of bacterial growth was 0.565 and 0.680 on 4^{th} and 5^{th} day respectively as shown in Fig.6.8.

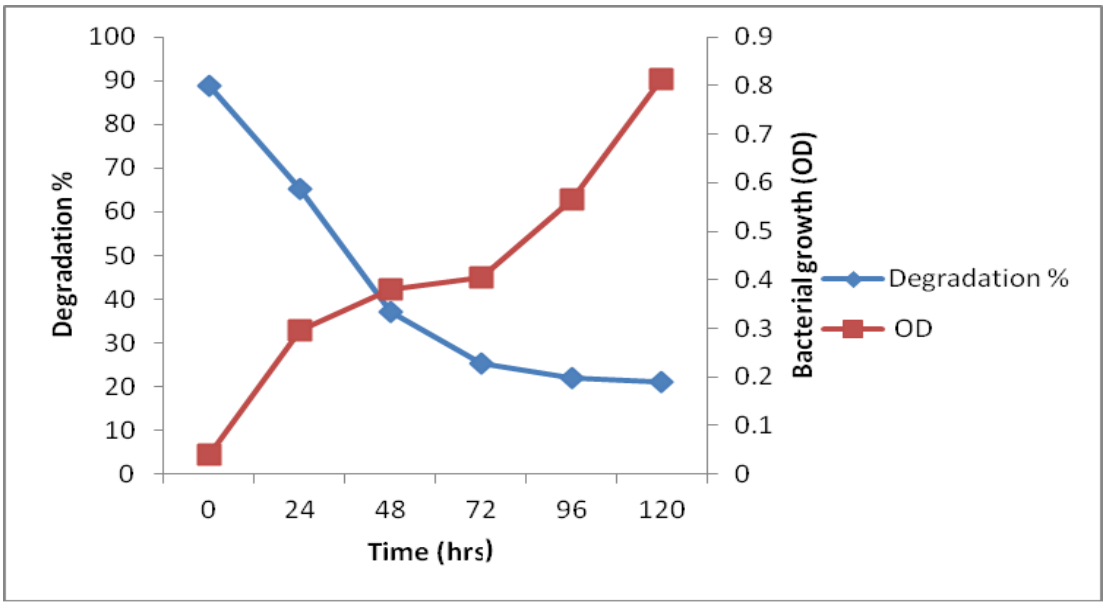

Fig. 6.8: B_{30} growth and degradation on phenanthrene

Results of cell viability we also carried out the bacterial count by measuring $CFUmL^{-1}$ (Table II Annexure, A). There was a four-fold increase in viable cell $CFUmL^{-1}$ count (1.5×10^{-5}-2.4×10^{-20}) during incubation of 0-96 hours. In further 24 hours the increase viability slows down (1.5×10^{-20}-1.8×10^{-23}) which is the characteristic of bacterial growth on phenanthrene. During degradation experiments on phenanthrene it is a normal phenomenon as this compound has an outstanding ability of re-crystallization from the media. It has also been reported that decrease in growth rate and degradation efficiency may be due to formation of some toxic compounds or nutrient deficiency. But, after the substrate reach a specific range of concentrations results in saturation of bacterial cell with organic carbon. This high concentration of substrate may turn out to decreases the efficacy of the degradation process [Boopathy, 2000].

In this study the concentration of $500mgL^{-1}$ was optimum in line with a separate but related study, using $100mgL^{-1}$ and less initial phenanthrene

concentration. Maximum rate of phenanthrene degradation by the isolate B_{30} was observed at 500mgL^{-1} [Boopathy, 2000; Bouchez *et al.* 1995; Romero *et al.* 1998; Othman *et al.* 2010].

Our isolate B_{30} which was not exposed to contamination degrded 78.82% in contrast to previously reported study with only 58% and 50% of phenanthrene by *Pseudomonas* isolates PS1 and PS2 in polluted area. This condition contributes to low bioavailability of the isolates to the phenanthrene due to its hydrophobic and low aqueous compounds and this contributes to limited microbial degradation on PAHs [Pizzul *et al.* 2007; Johnsen *et al.* 2005; Abdul-Ghani 2008]. This isolate from an uncontaminated area was capable of phenanthrene degradation of 78.82% in five days was efficient in terms of time. As *Pseudomonas* sp. isolated from petroleum polluted soil possess high phenanthrene-degrading ability but in a longer period of time [Zhou *et al.* 2007].

A related study is also reported on *Sphingomonas sp*. with capability of 100% phenanthrene degradation in terms of concentration. Our isolate B_{30} degraded phenanthrene efficiently at a concentration of (500ppm) in contrast to *Sphingomonas sp*. at 250 ppm concentration [Zhao *et al.* 2008]. Thus, this study showed that our isolate is a better phenanthrene degrader at comparatively high concentration in contrast to previous reports [Abdul-Ghani 2008; Janbandhu & Fulekar, 2011]. Our isolate B_{30} proved to be an efficient phenanthrene degrader both in terms of time and concentration.

The unidentified peaks, with retention time of 0.571 are previously reported as pyrene metabolite when it is degraded through phenanthrene pathway. Peak with 1.495 and 2.005 are the reported metabolites of anthracene [Neelofur *et al.* 2014]. While, peak with retention time of 4.275 was identified as salicylaldehyde with authentic standard. These cultures were further studied for 10 days and analysed by GC-MS for the investigation of end product (Annexure E, Fig. IV). Formation of various unidentified peaks was observed which can be further subjected for identification to FT-IR.

HPLC results with sharp peaks of 0.374, 0.571, 1.495, 2.005 and 4.275 are shown in Fig.6.9.

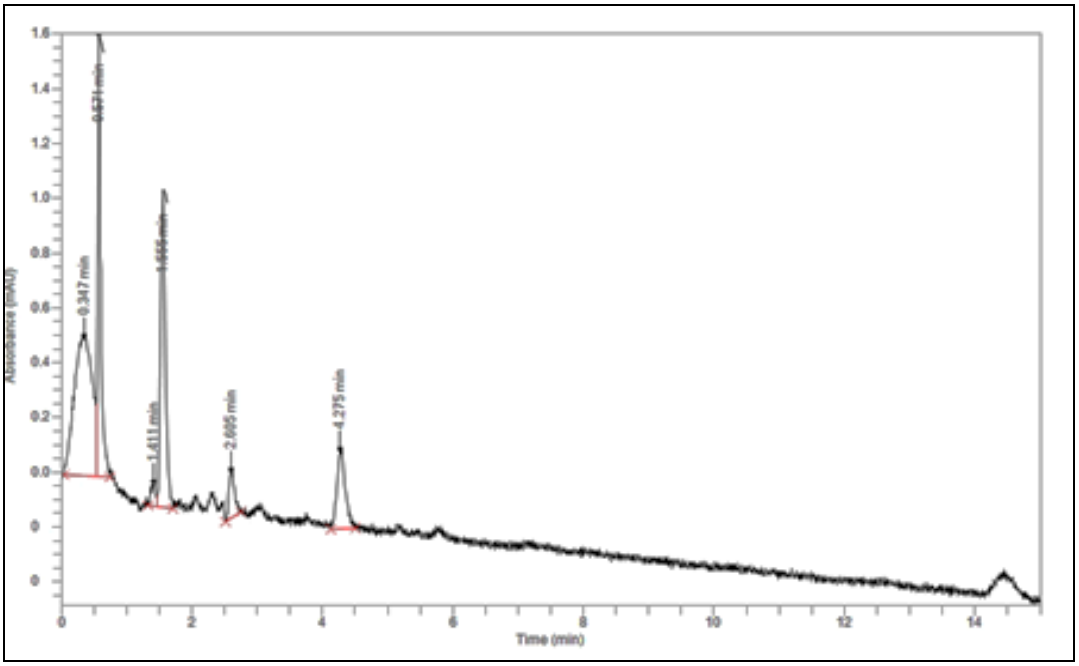

Fig. 6.9: HPLC chromatogram for phenanthrene degradation by isolate B_{30} after 120 hrs

6.3.4.2 Biodegradation of phenanthrene by isolate S_{21}

The growth rate was slower between 48-96 hours and only a fractional increase was observed i.e. 0.415, 0.460, 0.479 second, third and fourth day respectively. The degradation rate was faster than growth as about half amount of phenanthrene was removed between 48-72 hours with an increase in OD value of 0.369-0.415. About 21.29 % of phenanthrene was degraded with growth (OD) 0.369 in just 24 hours. There was slight increase in growth when isolate was incubated for further 24 hours and the percentage degradation was 80.28% on 5^{th} day of incubation.

Results of cell viability we also carried out the bacterial count by measuring $CFUmL^{-1}$ (Table II Annexure, A). There was a four-fold increase in viable cell $CFUmL^{-1}$ count (1.5×10^{-5}-1.8×10^{-21}) during incubation of 0-72 hours. In further 48 hours the increase viability increase (1.5×10^{-23}-1.8×10^{-27}) which is the may be due to bacterial isolates previously exposed to these class of pollutants. During degradation experiments on phenanthrene it is a normal phenomenon as this compound has an

outstanding ability of re-crystallization from the media. Bacterial cell usually saturates with organic carbon which reduce their ability of degradation of certain compound as observed during this study [Boopathy, 2000]. The degradation rate was of 34.86 % and bacterial growth was slow between 48 to 96 hours of incubation. 68.52% phenanthrene was degraded after 72 hours. 96.60% phenanthrene was degraded by isolates S_{21} as shown in Fig.6.10.

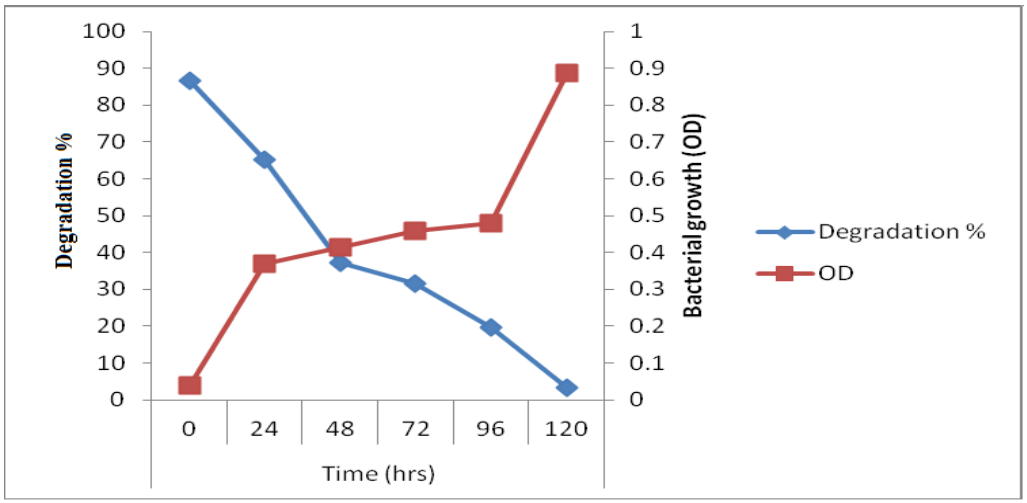

Fig. 6.10: S_{21} growth and degradation on phenanthrene

HPLC analysis revealed that there were three sharp peaks of retention time (0.333, 0.574 & 4.20) as shown in Fig. 6.11.

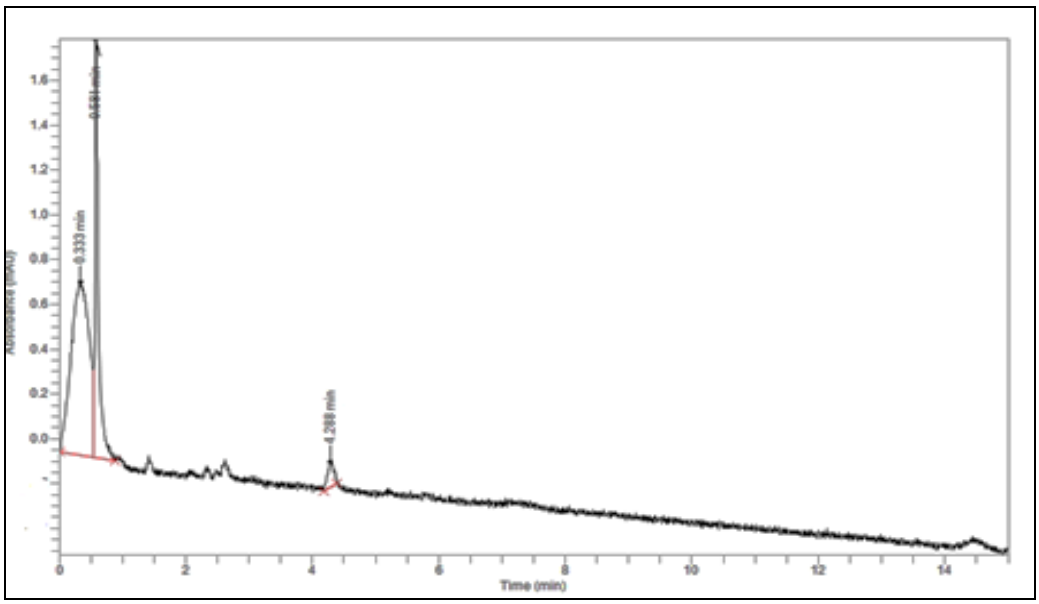

Fig.6.11: HPLC chromatogram for phenanthrene degradation by bacterial isolates S_{21} after 120 hours

Peak with 4.20 retention time on comparison with authentic standard found to be salicylic acid. These results are in line with the previously reported metabolites of pyrene through phenanthrene degradation pathway. This isolate degraded phenanthrene more efficiently than B_{30} probably because previous exposure to the PAHs components of petroleum compound in filling station as compared to that isolated from an agricultural land. Comparing the efficacy of isolates from contaminated and uncontaminated areas shows that the previously adapted isolates were most efficient degraders. 90.11% phenanthrene was degraded at 100ppm while S_{21} isolated in this study degraded 96.6% at 500ppm which is for better than previously reported [Zhao *et al.* 2008].

More than 90.11 and 82.15% degradation, at comparatively low concentration of 100mgL^{-1} and 350mgL^{-1} in previously reported study and 78.82% at 500mgL^{-1} in liquid cultures in this study. Our isolate by S_{21} shows versatility of pH range and the order of degradation rate at different pH was pH 9.0 > pH 6.0 > pH 7.0 > pH 8.0. As previously reported for pyrene degradation by isolate *K. pneumoniae* was in order of pH 7.0 >pH 8.0 >pH 10.0 > pH 6.0 >pH 5.0 while [Ping *et al.* 2014].

Biodegradation of PAHs in soil is of major and regulatory concern with regard to the bioremediation of contaminated land, with much of the published research focussing on the removal of individual compounds, in particular the LMW PAHs **[**Macleod & Semple, 2006]. Isolate S_{21} degraded phenanthrene more efficiently than B_{30} probably because of its exposure to the said compound in contaminated area of filling station as compared to that isolated from an agricultural land. Previous studies on the growth of some bacteria like *Serratia sp*. and *Acinetobacter* sp. Furthermore, through adaptation, some isolates of *Enterobacter sp.* and *Serratia rubidaea* on pyrene metabolites can attain pyrene degradation ability [Gaskin & Bentham 2005, Techer *et al.* 2011].

Comparing to the available literature it can be concluded that such a highest biodegradation rate can be attributed to the formation of biosurfactants production by S_{21} in phenanthrene degradation [Joshi *et al.* 2008]. In this study the isolates isolated from uncontaminated areas show better growth from an agriculture field than from a

contaminated one. Degradation of phenanthrene of both the isolates is shown in Fig.6.12.

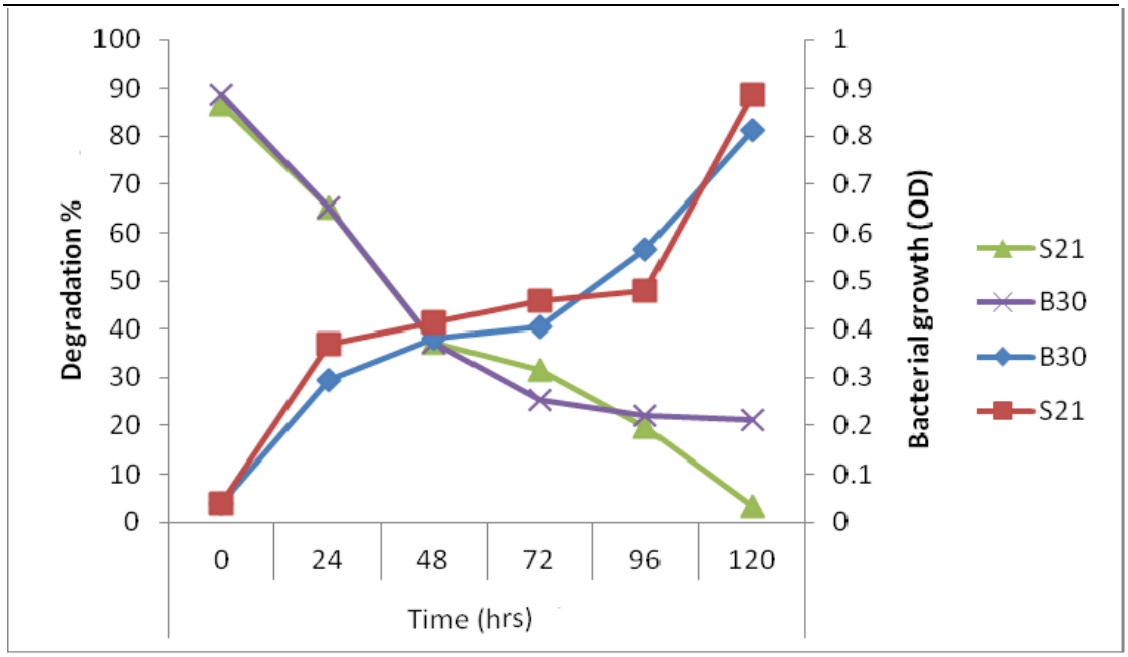

Fig. 6.12: Comparison of growth and biodegradation of two different isolates on same substrate (Phenanthrene)

There was 10% mineralization with 120 or 160 ppm in 100 h incubation while in our study acetone as solvent gave much better dissolution of phenanthrene only 34.85 and 34.86% for B_{30} and S_{21} in 24 hours. Thus, results suggest that enhanced adhesion of bacterial cells to the oil–water interface was the foremost issue accountable for improved biodegradation of phenanthrene to presumed polar metabolites [Abbasnezhad *et al.* 2011].

A relatively slow growth rate of S_{21} can be compared to a marine bacterium isolate *Vibrio* cyclotrophicus sp. from polluted marine sediments which was adept of two and three-ring PAHs including naphthalene, 2-methylnaphthalene and phenanthrene degradation. But, gas-chromatographic reports revealed that the isolate degraded several PAHs, yet it was incapable to utilize them as sole sources of carbon and energy [Simarro *et al.* 2013].

In contrast to our isolate with 80.28% degradation occurs is about half time that reported for *Pseudomonas putida* with 68% removal in 10 days. Supply of organic content perhaps can enhance phenanthrene biodegradation which means bacteria have other carbon sources beside phenanthrene to survive. Beside organic carbon, addition of nutrient also can increase reduction of PAHs. Thus results of our experiment are more satisfactory which were carried in organic content free mineral media with only phenanthrene as carbon and energy source from phenanthrene to survive with such a highest removal rate [Gottfried *et al.* 2010; Karamalidis *et al.* 2010].

The result shows that B_{30} were less adaptable to phenanthrene as compared to S_{21} due to the decreased bacteria numbers for both isolates after day 5 in contrast to previous study in which low bacterial count were observed after 2 days. These results were supported by earlier reports which proved that soil PAHs degrader populations were mostly non-growing [Mrozik & Piotrowska-Seget, 2010; Johnsen *et al.* 2005; Kamil *et al.* 2012]. In this study, degradation rate for B_{30} and S_{21}, results shows that the latter was better as phenanthrene degrader. Probably, the enzyme produced to degrade phenanthrene by isolate S_{21} was far better than that produced by B_{30} [Yuan, 2002; Abdul-Ghani, 2008]. Isolate S_{21} was unable to grow on naphthalene as previously reported that the presence of naphthalene has been shown to inhibit the growth of some phenanthrene-degrading bacteria like an *Acidovorax* isolate [Singleton *et al.* 2005].

Subsequently, analysis of PAHs during the ten-day incubation reported that phenanthrene completely 60% anthracene, 80% pyrene, 30% fluorene and 20% fluoranthene were decomposed by soil bacteria. The results showed that increase the number of benzene rings in PAH compounds caused to decreases the rate of degradation of them. The biodegradability primarily depends on the complexity of chemical structures and physico chemical properties of PAHs. The results of present study confirmed these findings. In another reported study, 84% phenanthrene degradation by isolates of *Corynebactrium urolyticum* after two weeks of incubation and 50% pyrene degradation by isolate *Pseudomonas putida* after 6 days of incubation was reported [Shafiee *et al.* 2006; Othman *et al.* 2010; Ping *et al.* 2011].

Pseudomonas stutzeri isolate is one of PAHs degrader that can produce biosurfactant known as rhamnolipid [Celik *et al.* 2008]. Biodegradation of poorly aqueous soluble hydrocarbons such as phenanthrene can be affected by an important factor such as microbial adhesion. In a previously reported study on *Pseudomonas* sp. there was enhanced phenanthrene degradation dodecanol was use as solvent. The phenanthrene degrading genus *Sphingomonas* was isolated from oil polluted was able to completely remove phenanthrene in carbon free mineral media. The water-soluble intermediates causing a change in color of the medium from clear to yellow was detected during growth on phenanthrene in culture. The accumulation of catechol-like compounds and their meta-ring clevage products may be attributed to this color change. Like *Sphingomonas sp*. isolate B_{30} also showed a variety of adaptability in its action on polycyclic aromatic substrates. In addition to phenanthrene, it was also able to utilize naphthalene, acenaphthene, phenanthrene, alpha-naphthol and also anthracene and pyrene in the presence of additional carbon source as a growth substrate. Since yellow-orange metabolites were formed during its growth in the medium containing these compounds. Production and accumulation of meta-cleavage products may also be considerable as in the metabolism of these PAHs by different bacteria [Supakaa *et al.* 2001].

On the basis of HPLC and GC-MS analysis of phenanthrene some of the identified compounds (shown in square brackets) that match mostly with that proposed previously [Seo *et al.* 2006]. It was observed that phenanthrene may be metabolised through this pathway as we identified 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid and phthalic acid which is the degradation product of naphthalene-1, 2 diol. The formation of a salicylic acid is also reported to be the ortho clevage product of 1H2N or 2H1N. Phenanthrene degradation starts from 9, 10-dioxygenase to bear phenanthrene cis-9, 10-dihydrodiol to 9, 10-dihydroxyphenanthrene which is initiated by 3, 4-dioxygenation which precedes cis-3, 4-dihydroxy-3, 4-dihydrophenanthrene, which changes enzymatic dehydrogenation to 3, 4-dihydroxyphenanthrene. The diol is then on catabolised to naphthalene-1, 2-diol by both ortho cleavages. This diol is also subsequently catabolised to naphthalene-1, 2-diol by both ortho and meta-cleavages [Seo *et al.* 2006, Pagnout *et al.* 2007]. Phenanthrene-1, 2- and 3, 4-diols majorly undergo meta-cleavage due to the rapid

accumulation of 5, 6- and 7, 8-benzocoumarin also identified in the study [Seo *et al.* 2006; Baboshin *et al.* 2008].

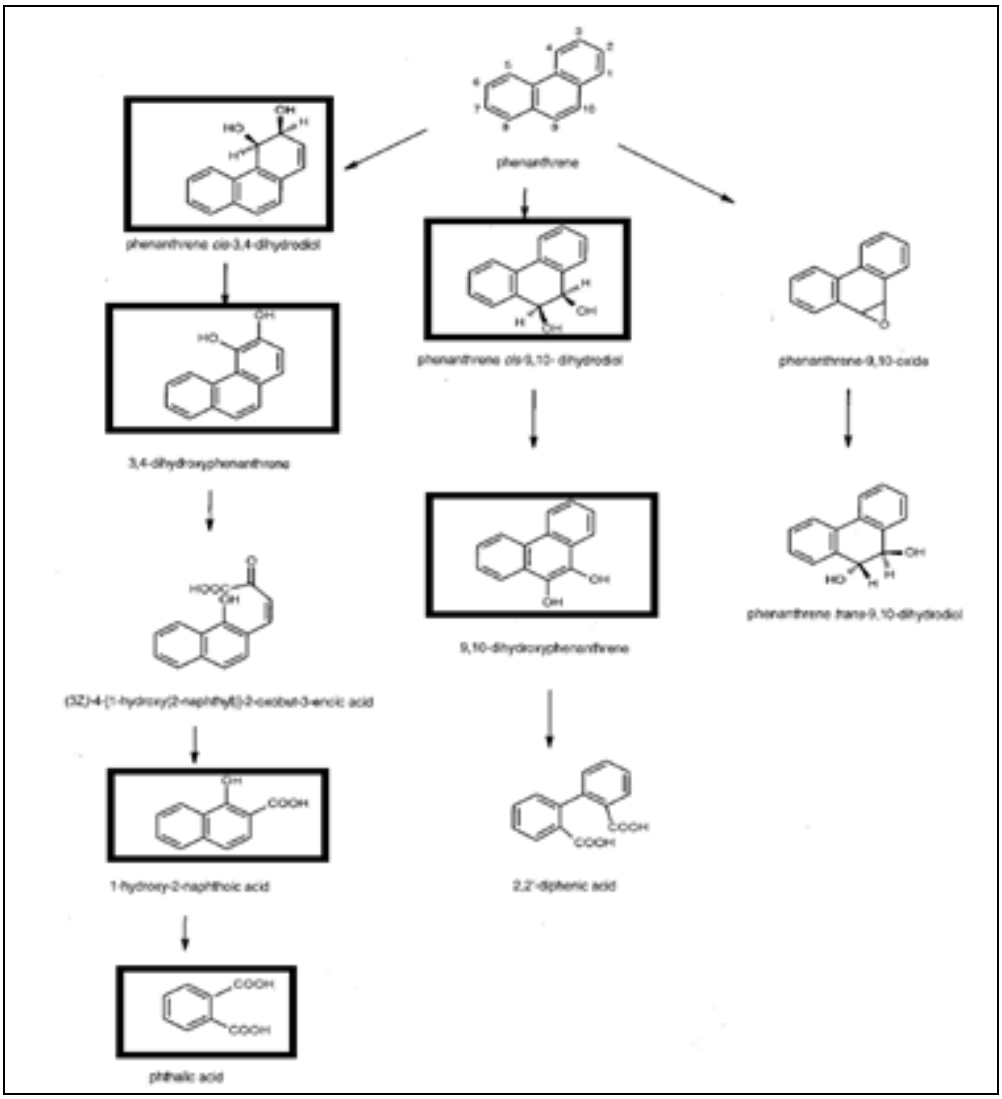

Fig. 6.13: Proposed pathway for phenanthrene degradation (Seo *et al.* 2006), the compounds in rectangles were identified in this study

6.3.5 Plasmid isolation, morphological, biochemical and phylogenetic analysis

The plasmids present in isolate S_{21} were more than one as clear from above photograph. Degradation was unaffected for isolate S_{21} but, B_{30} completely lost the ability to degrade phenanthrene after plasmid curing. It can be concluded that the

phenanthrene degradation genes were present on plasmid in isolate B_{30} and on chromosomes in S_{21}. Plasmid was present in S_{21} is shown in (Fig.6.14).

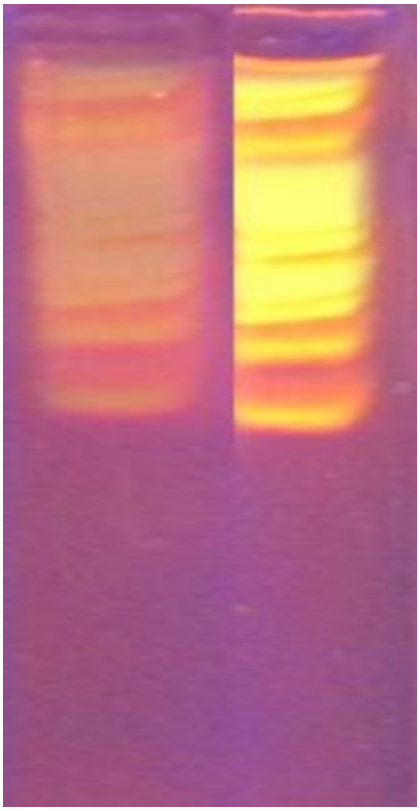

Fig. 6.14: Plasmid isolated from isolate S_{21} and ladder

Morphology shows that S_{21} diplococcus chain which were motile and spore forming as shown in Table 6.1

Table 6.1: Morphological Identification

S. No	Isolate	Gram's Test	Microscopy	Spore	Motility
1.	S_{21}	+ive	Coccobacilli diplococci	+	+

Biochemical characterization (Table 6.2), revealed that isolate S_{21} was catalase positive and lack starch hydrolyzing enzymes, casein and citrate utilization ability. It was also unable to produce indole urease with nitrate and oxidase activity.

Table 6.2: Biochemical characterization of isolate S_{21}

S. No.	Biochemical test	
1.	**Catalase**	+ive
2.	**Starch hydrolysis**	-ive
3.	**Citrate**	-ive
4.	**Urease**	+ive
5.	**Casein hydrolysis**	-ive
6.	**Gelatin liquification**	+ive
7.	**Indole production**	-ive
8.	**Nitrate**	+ive
9.	**Urease**	-ive
10.	**Oxidase**	+ive

Typing and 16S rRNA sequence analysis gave identical results strongly suggest that the microbial diversity among sampling sites could be monitored by 16SrRNA sequence analysis [Han *et al.* 2003, Becker *et al.* 2004]. Reports on the PAHs degrading *Bacillus* and *Pseudomonas* within consortium as have been provided earlier while; our isolates also have an additional capabilty of degrading these polyaromatics individually as well through co-matbolism [Zhuang *et al.* 2002; Chauhan *et al.* 2008; Luo *et al.* 2009]. This isolate has its origin from petroleum contaminate area has degradative capability of four ringed compound and can grow in a mixture of to completely mineralize PAHs to salicylic acid and catechol [Natalie *et al.* 2004].

We therefore, identify this isolate as *B. cereus* isolate 99% homologues to *Bacillus cereus* isolate D21 and 98% to *Bacillus cereus* isolate D42. This isolate was also found to have 96% identity with *Bacillus cereus* isolate RNS 01 and 96% to *Bacillus cereus isolate* M1 RH03. Both the isolates have outstanding phenanthrene degrading ability for isolate B_{30} in terms of concentration (1000ppm) and S_{21} in terms of degradation efficiency of 96.6 % at 500 ppm in just 5days in contrast to previously reported studies on *Sphingomonas* sp.

Phenathrene degrading bacteria that identified earlier are *Alcaligenes*, *Aeromonas*, *Cycloclasticus Pesudomonas*, *Comamonas*, *Flavobacterium*, *Norcardiodies*, and *Mycobacterium*, *Peudomonas* and *Flavobacterium* have been

classified as *Sphingomonas* [Han *et al.* 2003, Becker *et al.* 2004]. It is clear from this study that a number of *Bacillus* sp. that exist in the different sites of urban areas whether isolated from polluted areas or non-polluted agriculture source can have th ability to grow on PAHs. It was also observed that some *Bacillus sp*. can utilize only high molecular weight PAHs perhaps their degradative enzyme have a strong ability to make hydrophobic compound bioavailable. The phylogenetic tree for isolate S_{21} having the degradation efficiency of both LMW PAHs (phenanthrene) and HMW PAH (pyrene). Phylogenetic analysis showed that our isolate S_{21} is forming 96 bootstrap homology clad with *Bacillus cereus* as shown in Fig.6.15.

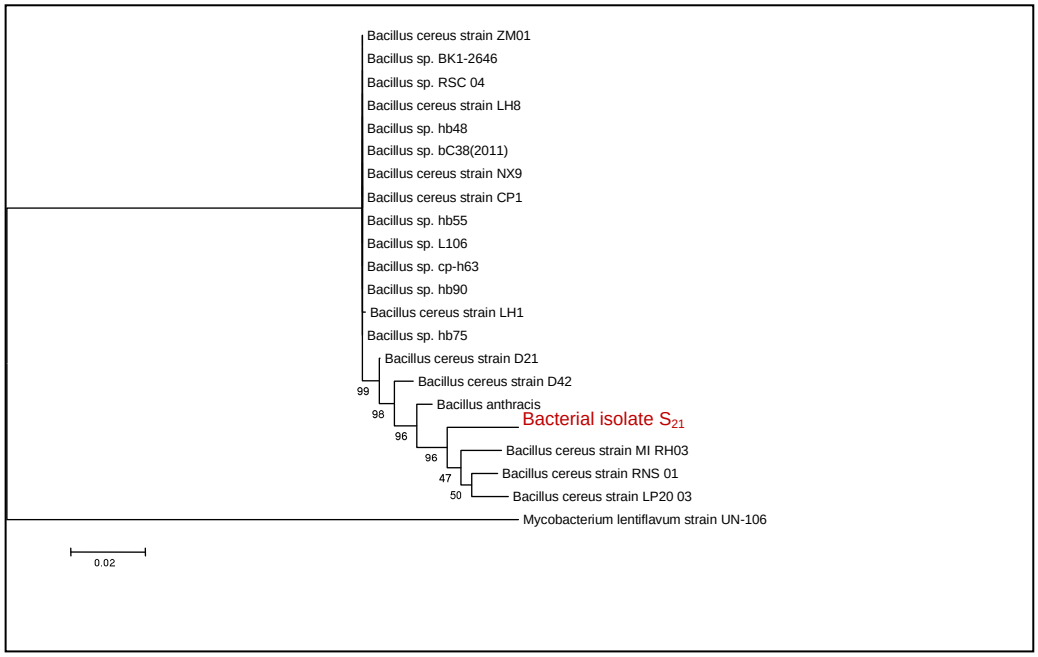

Fig. 6.15: The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [Tamura and Nei, 1993]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA6 [Felsenstein, 1985].

Plasmid isolation and morphological and phylogenetic analysis of B_{30} has been discussed in chapter-5.

BIODEGRADATION OF ACENAPHTHENE

7.1 INTRODUCTION

Acenaphthene is transported as absorbed matter on particulates suspended in air or water is degraded by microbes and readily metabolized by multicellular organisms and biodegradation may be the final route in the environment. The fate of acenaphthene in the environment can be attributed to sorption and biodegradation. Still, volatilization of acenaphthene is also an important source of its removal from soil. Accumulation degree of toxic compounds is faster than human actions and so more and more attention is paid to the effective use of natural resources in order to reduce nature pollution [Andreoni *et al.* 2007].

Acenaphthene is a natural component of crude oil and coal tar, product of combustion, natural fires associated with lightening, volcanic activity [Elliott *et al.* 1982; Gaydos *et al.* 1981]. Acenaphthene form various intermediates from a variety of products including dyes, pigments, fluorescent whiteners and pesticides (http://toxnet.nlm.nih.gov/cgi-bin/sis/search). Acenaphthene is a component of fly ash 1.8 $ngg^{-1}$of sewage waste and 2 gkg^{-1} body weight rats after daily exposure of months which cause chances in blood level. It can also cause damage to liver, kidney and bronchi [Knobloch *et al.* 1980].

Acenaphthene was found to be equally mutagenic and cause nuclear and cytological changes in microbes such as an increase in cell size and DNA content which cause disruption in mitosis proceeding polyploidy induction [Erwin, 1997]. Acenaphthene can cause nausea when engulf in huge amount [Rheinhold, 1979]. Immature fish have higher bio concentration of PAHs than adults because of a higher percentage of lipid yolk tissue. It is mainly metabolized in rats to naphthalene-1, 8-dicarboxylic acid and can bring about inhibition of the seed germination and seedling growth [Fazlurrahman 2011; Zhukov *et al.* 1971; Chang & Young, 1943; Goodwin 1976].

7.2 Materials and Methods

7.2.1 Chemicals and Media

Analytical grade acenaphthene (99%), Salicylic acid, salicylaldehyde, catechol, gentisic acid, 1-hydroxy-2, naphthoic acid and 2-hydroxy-1, naphthoic acid, 6, 7- benzo-caumarin, 1-naphthol and were purchased from Sigma-Aldrich and Fluka (St. Louis, MO, USA). Other chemical and media components were of high purity and analytical grade from Oxide, Merck and BDH. Nutrient agar and Mineral salt medium was used for initial screening. The composition of PNR and PNRG (PNR+5mM glucose) per liter of distilled water according to a well established protocol [Pan *et al.* 2008; Ebrahimi *et al.* 2012; Khan *et al.* 2006].

7.2.2 Inoculum preparation

Inoculum was prepared following protocol of Guo *et al.* (2010).

7.2.3 Optimization of various physic-chemical conditions for PAHs degradation

Different parameters such as acenaphthene conc., temperature, pH, and substrate (alternate carbon and energy source) and agitation speed and effect of inoculum were optimized for degradation studies [Naveenkumar *et al.* 2010; Abdelhay *et al.* 2008]

7.2.4 Biodegradation Experiment

The biodegradation experiment was according to protocol [Othman *et al.* 2010].

7.2.5 Extraction of PAHs

Extraction of acenaphthene and its metabolites for HPLC and GC-MS was made according to protocol of [Shokrollahzadeh *et al.* 2010; Survery *et al.* 2009; Neelofur *et al.* 2014].

7.2.6 Molecular identification based on 16S rRNA gene and phylogenetic analysis

DNA extraction, PCR amplification was made according to protocol of (Lane 1991) and bacterial primers cloning of nearly full length 16S rRNA and sequencing were performed according to the methods described previously according to protocol of Sambrook & Russel, (2001). The 16S r RNA gene sequence of the isolates were processed manually, analysed at NCBI using BLAST tool and compared to the corresponding neighbour sequences from GenBank-NCBI database, describes in detail in chapter 4.

7.3 RESULTS AND DISCUSSION

7.3.1 Optimization of acenaphthene concentration

There was correspondingly higher growth rate of isolate with increasing levels of acenaphthene as shown in Fig.7.1. The growth (OD 600nm) of 0.8 was observed when the level of acenaphthene was (150mgL^{-1}), while the highest value of 1.21 was observed with 500mgL^{-1} acenaphthene was (B_{30}) shows an increase in growth rate with increase in concentration in growth medium [Neelofur *et al.* 2014].

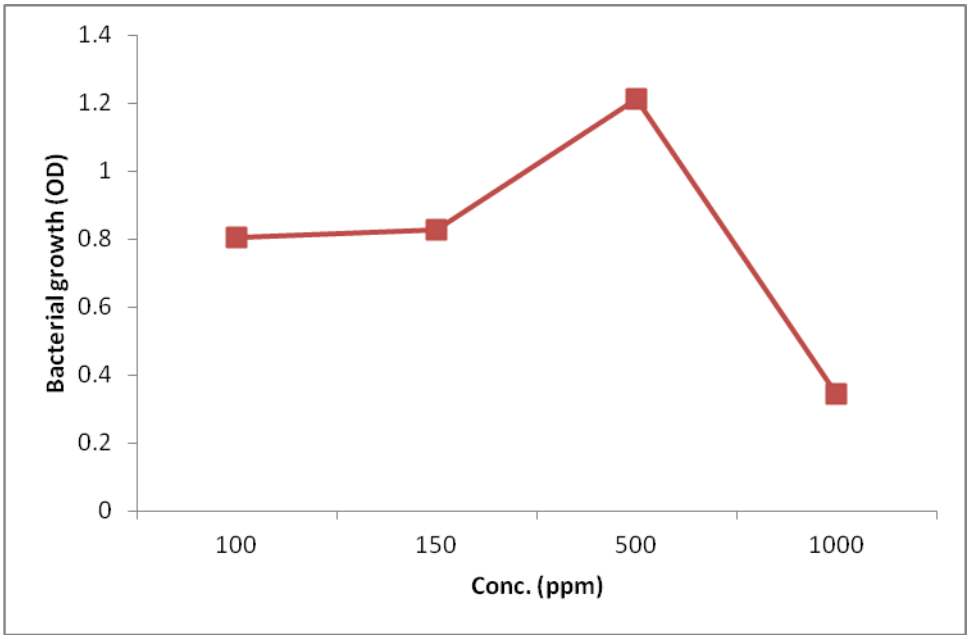

Fig. 7.1: Optimization of acenaphthene concentration

7.3.2 Optimization of temperature

Degradation of acenaphthene was optimized at 30°C as shown in Fig.7.2. The enzymes of this microorganism might be adapted to this temperature. The highest growth rate was achieved at 30ºC, while lowest growth has been observed at 40ºC and no growth at 45ºC.

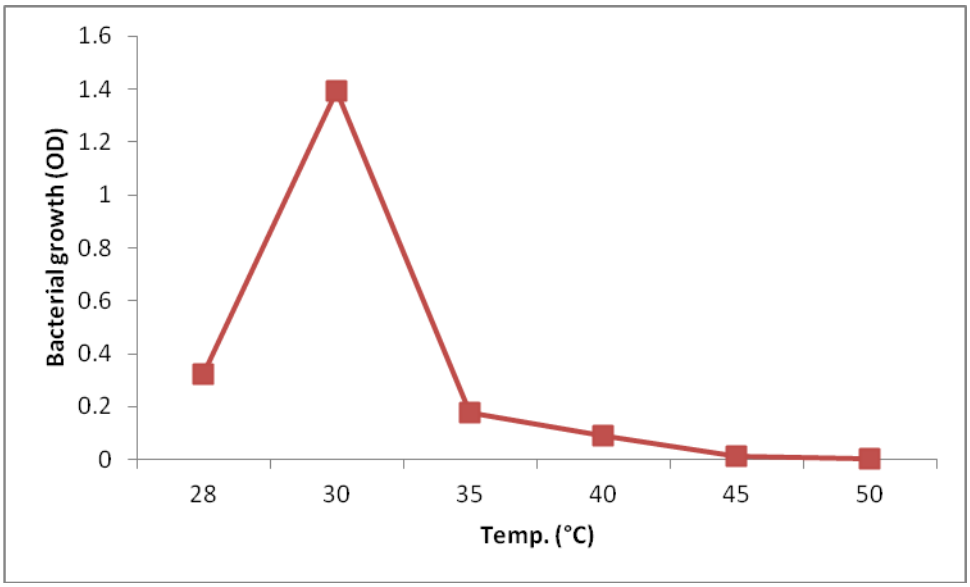

Fig. 7.2: Optimization of temperature for isolate B_{30} for acenaphthene degradation

Hence, the degradative enzymes of this isolate (B_{30}) work best at 30ºC. The biodegradation experiments were conducted at optimized temperature of 30ºC [Ahmed *et al.* 2010]. Temperature has a detrimental effect on the capability of the microorganisms to degrade PAHs. The solubility of PAHs increases with an increase in temperature with enhanced availability [Arulazhagan *et al.* 2011]. In addition, oxygen solubility decreases with increasing temperature, which reduces the metabolic activity of aerobic microorganisms [Nour *et al.* 2010].

According to our study, temperature influenced the degradation of acenaphthene and its degradation level was significant in the mesophilic range with the highest degradation occurring at 30°C.

At low temperature lower growth is an indication that this temperature is insufficient for the enzyme to speed up the degradation reaction. Enzymes being catalyst have certain optimum temperature for its maximum activity. Most of the enzymes become denatured at temperature above 40°C and decreased activity due to its inability to cleave binding in benzene, its main functional group [Mukesh *et al.* 2012].

7.3.3 Optimization of media p^H on acenaphthene degradation

Each microbe has its own range of p^H for its multiplication and metabolism as they are exposed to the harsh condition of external environment [Nnamchi *et al.* 2006]. From our study it was found that neutral p^H favoured both extensive bacterial growth and degradation of the acenaphthene (Fig.7.3). The charge imbalance inside and on the surface of the cells was prevented at p^H 7. At extremes of p^H, the proper functioning of the enzymatic machinery involved in PAH degradation might have been affected because of which the rate of degradation and bacterial growth decreased. Effect of p^H on the disappearance of anthracene was significantly inhibited when p^H varies acidic to neutral and neutral to alkaline as on phenanthrene and pyrene [Sudarat *et al.* 2000; Lakshmi *et al.* 2011].

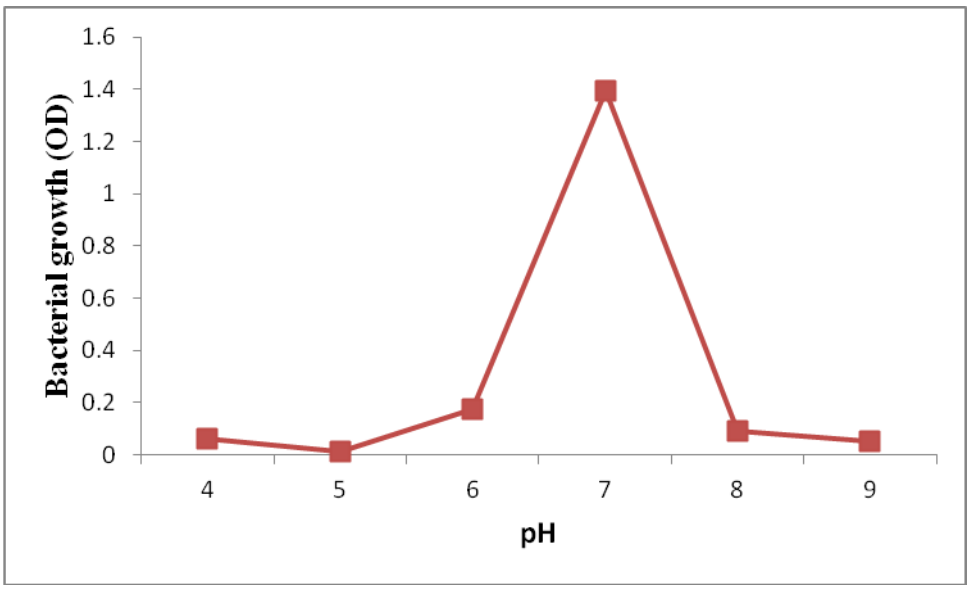

Fig.7.3 Effect of media p^H on acenaphthene degradation

7.3.4. Optimization of agitation speed on acenaphthene degradation

Agitation speed is an important factor in aerobic degradation of PAHs and should be optimized for desired bacterial growth and degradation as shown in Fig.7.4. According to the results of our study aeration considerably affect the growth of bacteria on acenaphthene [Bhattacharya *et al.* 2011]**.**

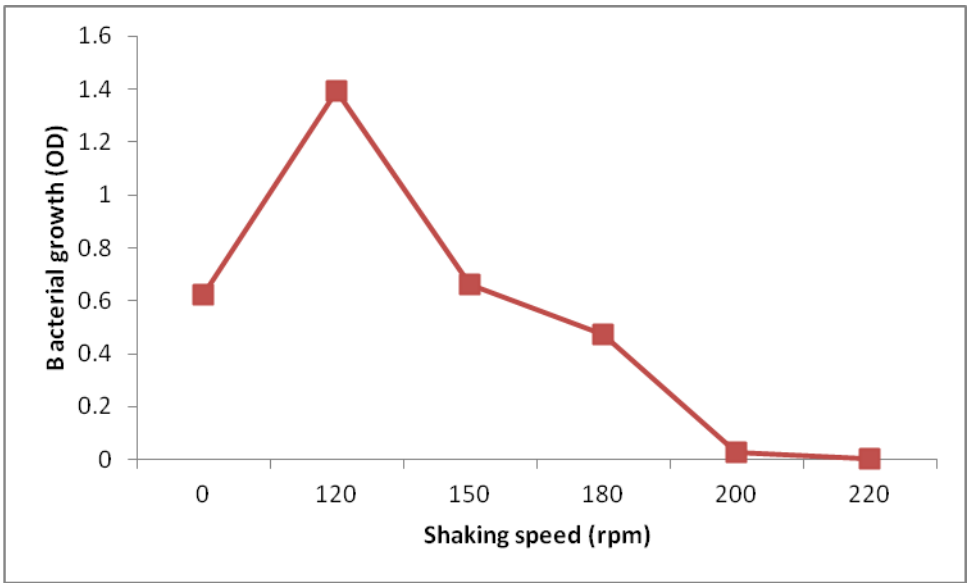

Fig. 7.4: Effect of shaking on acenaphthene degradation

Higher degradation rate can be achieved from faster agitation speed which brings about adequate oxygen supply and acenaphthene solubility in aqueous phase to make available for the microbes. Shaking speed for B_{30} was optimized at 120 rpm for acenapthene degradation. Shaking speed is important for equal distribution of nutrient to make equal supply to all the microbes. It also prevents recrystallization of compound from media and makes sure its availability to the microbes for degradation [Park *et al.* 1990; Margesin *et al.* 2001; Alias *et al.* 2011].

7.3.5 Optimization of substrate on degradative activity

Different alternate carbon sources in the form of glucose, sucrose and fructose were added to the media containing acenaphthene.

No significant effect was observed on addition of substrate on degradative activity as shown in Fig.7.5.

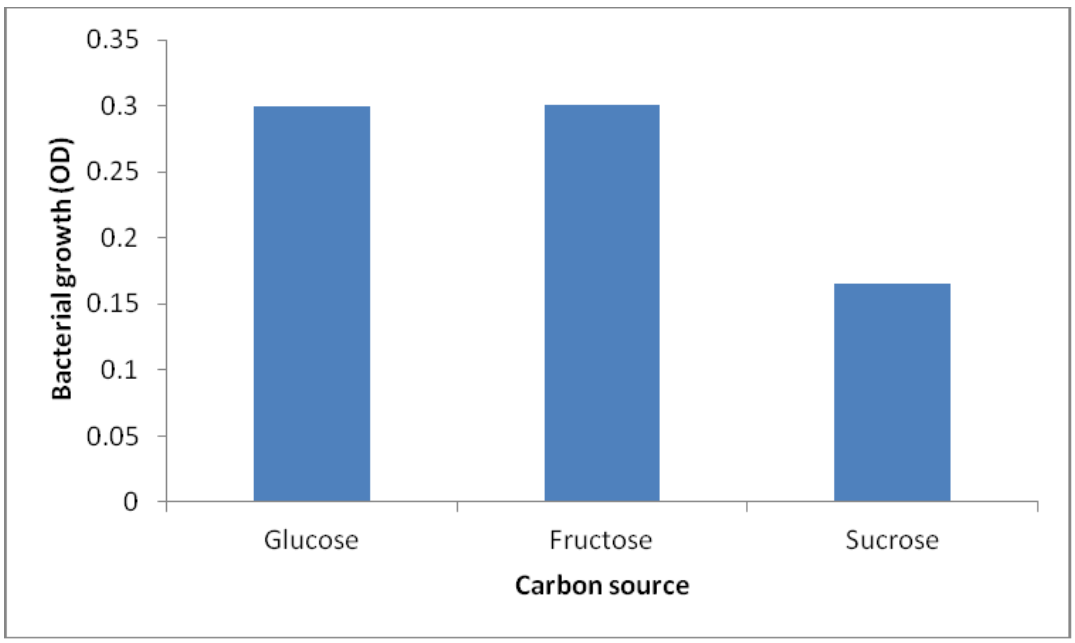

Fig.7.5: Effect of carbon source on isolate B_{30} growth on acenaphthene

Addition of different nitrogen sources, KNO_3, $NaNO_3$, $CaNO_3$ and NH_4NO_3 has no effect on growth of B_{30} as shown from OD values (Fig. 7.6)

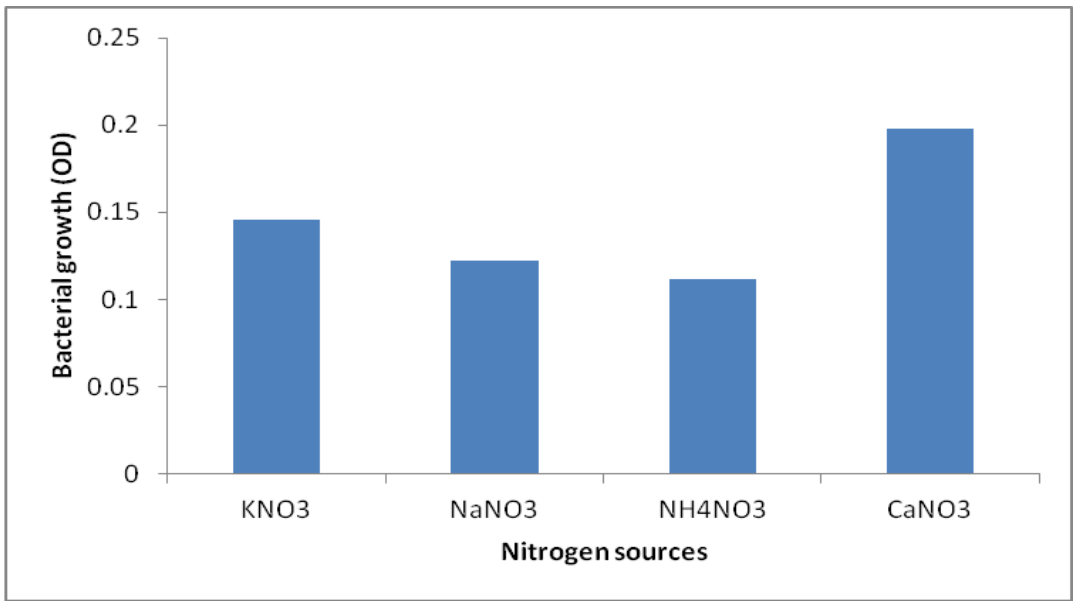

Fig.7.6: Effect of nitrogen salts on of isolate B_{30} growth acenaphthene

However, it was previously reported that the addition of fertilizer mostly containing nitrogen and potassium pose stimulatory effects on oil degradation by bacteria. Hence, it is clear from the results of our studied isolates that the soil bacteria isolated from an agriculture field with a history of exposure to natural manure were most efficient in acenaphthene degradation [Nikolopoulou & Kalogerakis, 2009].

7.3.6 Biodegradation of acenaphthene

Isolate B_{30} showed a difference in results for acenaphthene a structurally different compound unlike naphthalene and phenanthrene. The isolates show a strange increase in growth with an OD value of 0.215 to 0.742 in just 24 hours of incubation as shown in Fig. 7.7.

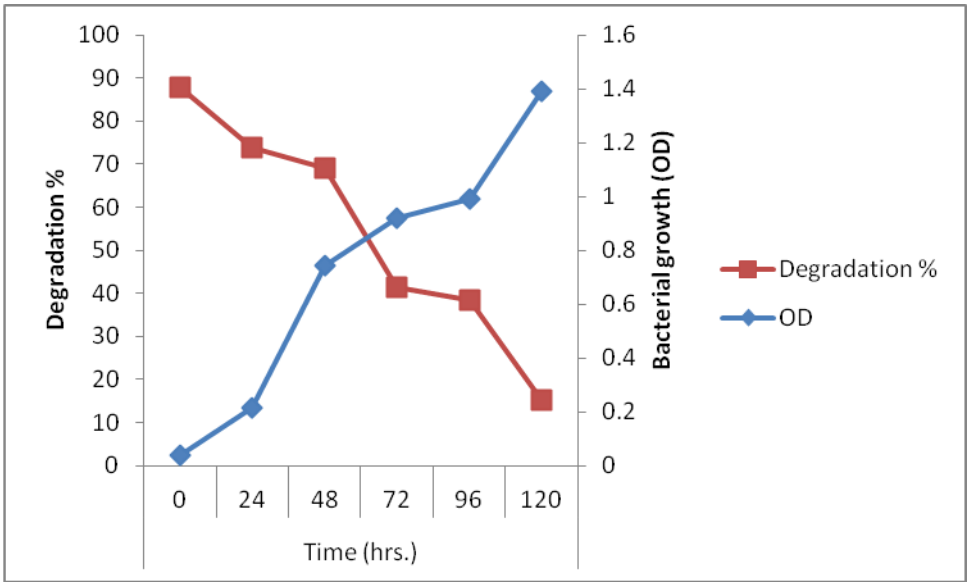

Fig. 7.7: B_{30} growth and acenaphthene degradation

The isolates show a strange increase in growth rate within 24 hours of incubation with an OD of 0.742. The growth rate was at its peak between 48 to 96, acenaphthene support growth upto120 hours of incubation with maximum OD of 1.39. About 60% acenaphthene present in the media was degraded in 72 hours in contrast to naphthalene degradation which was 66.85%. The unspent acenaphthene amount in the medium after 120 hours of incubation was 15.32% and 84.68% was degraded in five days **w**hile, the same isolate was able to remove 100% naphthalene from the media. Like for both above mentioned three ring compound the growth rate

was increasing on 48 to 96 but unlike the two compounds peaked at 120 hours with an OD of 1.39. About 58.59% of the compound was degraded in 72 hours. The unspent acenaphthene amount in the medium after 120 hours of incubation was 15.32. If we look at the viability of cell in terms of $CFUmL^{-1}$ (1.3×10^{-5}-2.7×10^{-30}) there was a six-fold increase in viable cell count from 120 hours of incubation (Table III, Annexure A).

Isolate B_{30} showed a difference in results for acenaphthene structurally different compound form naphthalene with an additional 5- carbon ring between two benzene rings. The degradation percentage of the isolate B_{30} is in order, naphthalene> acenaphthene>phenanthrene. The same isolate shows different growth pattern on utilization of different carbon source in same mineral media. The growth reaches to maximum after four days incubation when the carbon and energy source in the medium was three ring compound naphthalene or phenanthrene. While, maximum growth rate, was obtained after 120 hours when acenaphthene was provided as carbon source. The OD value was 1.60 on 96 hours for naphthalene and 1.39 on 120 hours for acenaphthene. HPLC analysis showed that three sharp unidentified peaks with 4.23 retention times as shown in Fig.7.8.

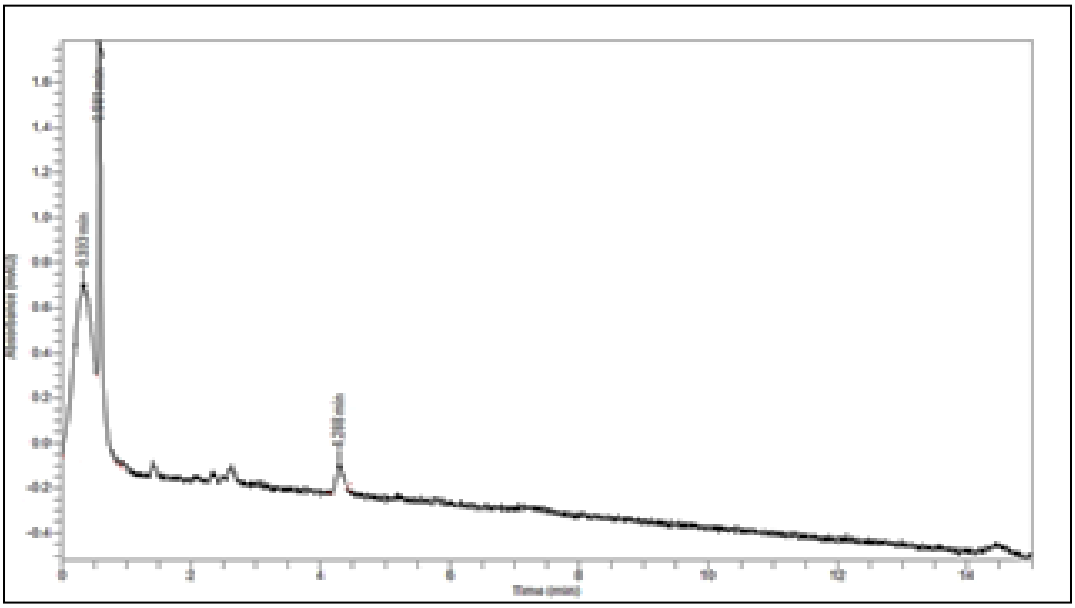

Fig. 7.8: HPLC chromatogram of acenaphthene degradation by isolate B_{30} after 48 hrs

On comparison with authentic standards identified and search on GC-MS library may be acenaphthene metabolites produced through oxidation of benzylic methylenic groups like for two ring compounds as also reported [Selifonov *et al.* 1998]. After 72 hours the peak in the same areas were observed with increase percentage which may be acenaphthene 1, 2- diol and acenaphthene 1,8-dicarboxylic acid the possible acenaphthene metabolites of acenaphthene degradation by bacteria (Fig. 7.9) from a previously reported NMR studies on acenaphthene.

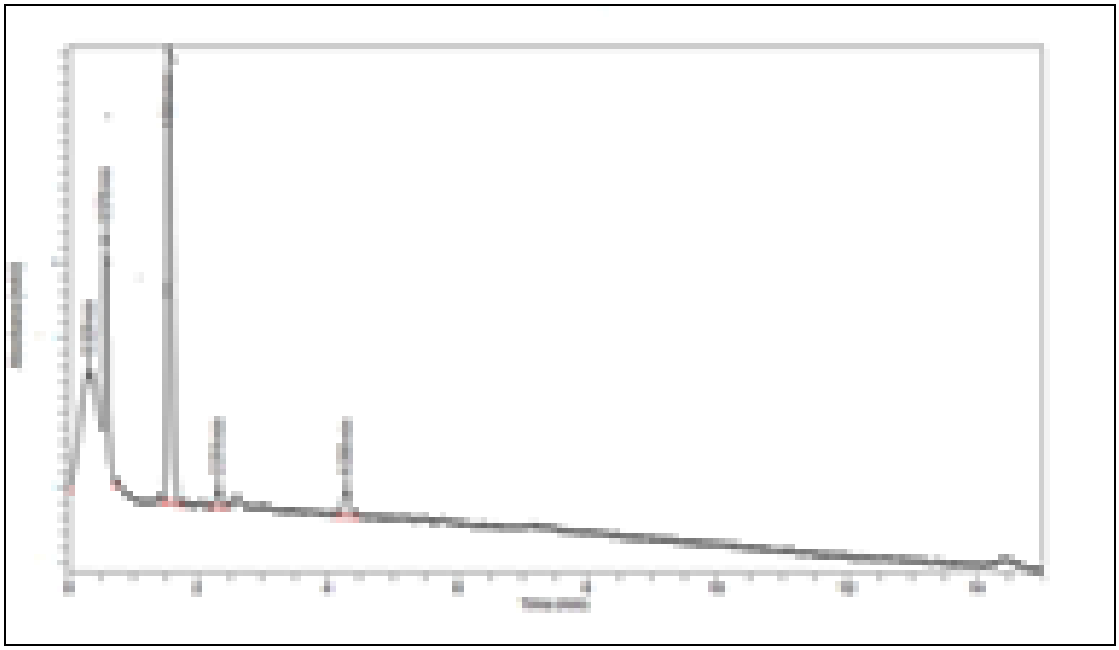

Fig. 7.9: HPLC chromatograms for acenaphthalene degradation by bacterial isolate B_{30} after 72 hrs

The results obtained demonstrate that mixed bacterial cultures, established in the laboratory by enrichments on creosote have limited biochemical diversity with respect to furnishing an efficient pathway for acenaphthene degradation and catabolism. It would appear that the enrichment technique with creosote used here does not provide favorable conditions for establishing bacterial populations which contain bacterial isolates capable of mineralizing acenaphthene. Acenaphthene-utilizing isolates, such as A2279 and the *Alcaligenes* isolates could readily be isolated from soils contaminated with coal-derived products by enrichments with acenaphthene [Selifonov *et al.* 1998].

About 34.85% of phenanthrene degradation occurs in 24 hours with bacterial growth OD of 0.295 by isolate B_{30}. 28.01 % was degraded in further 24 with a total 62.86% degradation in 48 hours. After 72 hours a total 74.68% was degraded but the growth rate of bacteria was slower as compared to that on naphthalene and acenaphthene as shown in Fig.7.10.

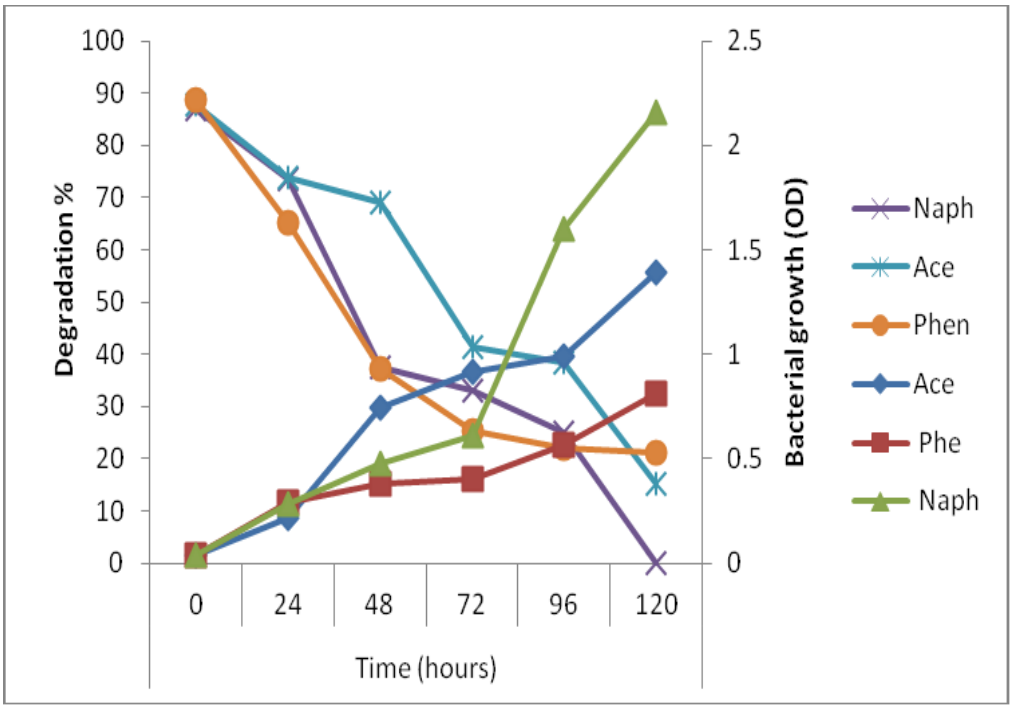

Fig. 7.10: Comparison of growth and degradation rate of B_{30} on three different PAHs

Rhodococus rhodochrous has capability to use acenapthene which also has three benzene ring but, different molecular weight of 154g. Napthalene is possible to be degraded because it has lower molecular weight 128g. The degradable component of acenapthene by *Rhodococus rhodochrous* may has increased expression of oxygenase enzyme system responsible for benzene ring with HMW. *Corynebacterium agropyri* has an ability to degrade acenapthene at the fastest rate. Subsequently, degradation of acenaphthene and naphthalene but no reduction of fluorene, phenanthrene and anthracene but in this study the B_{30} degraded acenaphthene and naphthalene as well as phenanthrene. These results demonstrate that this isolate only degrades lower molecular weight of PAHs [Othman *et al.* 2010]. The degradation rate for the isolate B_{30} was in order of naphthalene> acenaphthene>phenanthrene. The

same isolate shows different growth pattern on utilization of different carbon source in same mineral media.

From GC-MS analysis results it was clear that acenaphthene degradation was slightly differ than that of naphthalene and phenanthrene with the formation of some unidentified peaks (Annexure E, Fig. III). The compounds identified in this study were similar with that proposed by previously reported work and are graphically represented as below Fig.7.11. The proposed pathway described for acenaphthene is initiated by the formation of naphthalene-1, 8 dicarboxylic acids. It is further metabolized to 1-acenaphthenol, 1, 2-acenaphthenediol, acenaphthenequinone that cleaves to anhydrous 1, 8-naphthoic acid and carboxylic acid which are inter-convertible [Selifonov *et al.* 1998; Schocken & Gibson, 1984; Kuzuona *et al.* 2006].

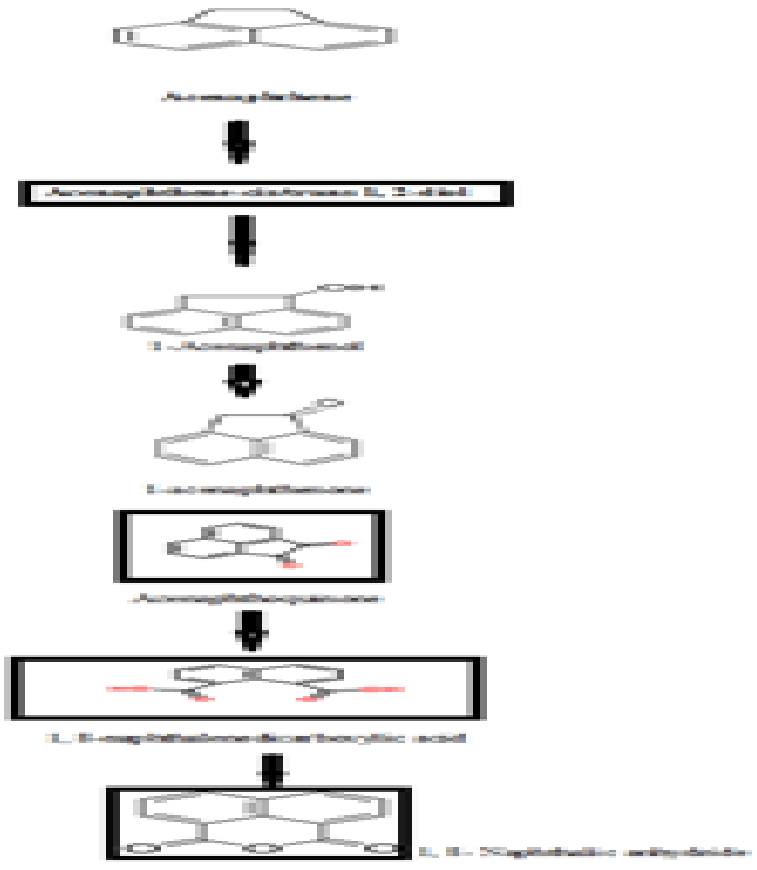

Fig.7.11: Proposed pathway for acenaphthene biodegradation (Selifonov *et al.* 1998), compounds in rectangles were identified during this study

BIODEGRADATION OF PYRENE

8.1 INTRODUCTION

Pyrene is HMW PAH with four fused rings released as partial burning practices. It is carcinogenic and can cause nephron and neuron level diseases in animal and is a tumer inducer and changes in blood level. Low birth defects and immune system damage is also observed after exposure benzo (a) pyrene) containing pyrene [ATSDR 1990]. Microbial degradation is supposed to be one of the principal mechanisms of PAH removal from soils and is affected largely by contaminant availability and metabolism by microbes [Macleod & Semple, 2006; Doick *et al.* 2005; Li *et al.* 2008]. Microorganisms play a significant role in the degradation of PAHs in terrestrial and aquatic ecosystems, and microbial degradation is the main process in natural decontamination. Microbial degradation of PAHs in soil is restricted by various factors that often result in lower-than-expected bioremediation efficiency. One of these factors is the low bioavailability of the compounds. Most widely studied bacterial isolates that are able to degrade pyrene are *Rhodococcus sp., Bacillus cereus, Burkholderia cepacia* and *Mycobacterium* [Vinas *et al.* 2005].

8.2 Materials and methods

8.2.1 Chemicals and Media

Analytical grade pyrene (99%), Salicylic acid, salicylaldehyde, catechol, gentisic acid, 1-hydroxy-2, naphthoic acid and 2-hydroxy-1, naphthoic acid, 6, 7-benzo-caumarin, 1-naphthol and were purchased from Sigma-Aldrich and Fluka (St. Louis, MO, USA). Other chemical and media components were of high purity and analytical grade from Oxide, Merck and BDH. Nutrient agar and Mineral salt medium was used for initial screening. The composition of PNR and PNRG (PNR + 5 mM glucose) per liter of distilled water [Pan *et al.* 2008; Ebrahimi *et al.* 2012; Khan *et al.* 2006] is as; PN (20x) 50 mL used as 50 mLL^{-1}: KH_2PO_4 13.6% (wv^{-1}), $(NH_4)2SO_4$ 2.4% (wv^{-1}), NaOH 2.5% (w/v) and R salts used as $7mlL^{-1}$ $MgSO_4.7H_2O$ 8% (wv^{-1}), $FeSO_4.7H_2O$ 0.2% (wv^{-1}), HCl 0.4% (wv^{-1}), Agar (2%) was used as solidifying agent.

8.2.2 Inoculum preparation

Inoculum was prepared according to protocol of Guo *et al.* (2010).

8.2.3 Optimization of various physic-chemical conditions for PAHs degradation

Different parameters such as acenaphthene conc., temperature, pH, and substrate (alternate carbon and energy source) and agitation speed and effect of inoculum were optimized for degradation studies [Naveenkumar *et al.* 2010; Abdelhay *et al.* 2008]

8.2.4 Biodegradation experiment

The biodegradation experiment was performed in 100ml PNR media, 10% of bacterial inoculum and 1000mgL^{-1} acenaphthene dissolved in acetone at pH of 7.0 at 30°C and 180rpm [Othman *et al.* 2010].

8.2.5 Extraction of PAHs for GC-MS analysis

Extraction of acenaphthene and its metabolites for HPLC and GC-MS was made according to protocol of [Shokrollahzadeh *et al.* 2010; Survery *et al.* 2009; Neelofur *et al.* 2014].

8.2.6 Plasmid isolation and agarose gel electrophoresis

The plasmid was isolated according to manufacturer instructions of Invitrogen mini prep kit and photographed according to protocol of **[**Survery *et al.* 2009**]**.

8.2.7 Morphological and biochemical characterization of bacterial isolates

The isolates were identified morphologically by Gram's staining and microscopy while biochemically by Bergey's manual of systematic bacteriology.

8.2.8 Molecular identification based on 16S rRNA gene and phylogenetic analysis

DNA extraction, PCR amplification was made according to protocol of (Lane, 1991) and bacterial primers cloning of nearly full length 16S rRNA and sequencing were performed according to the methods described previously according to protocol

of Sambrook & Russel, (2001). The 16S rRNA gene sequence of the isolates were processed manually, analysed at NCBI (National Centre for Biotechnology Information) using BLAST tool and compared to the corresponding neighbour sequences from GenBank-NCBI database, describes in detail in chapter 4.

8.3 RESULTS AND DISCUSSION

8.3.1 Optimization of different parameters

Various physico-chemical factors like concentration of PAHs, p^H of the media, temperature and different nutrients on pyrene degradation and growth of the selected isolates were studied. Results are given below:

8.3.2 Optimization of Concentration

There was a concentration dependent growth of all the four isolates and optimized concentration was 1000mgL^{-1} (Fig. 8.1). As reported previously, that the isolates that have previous exposure to pollutants present in soil or sediments are more resistant. Hence, these microbes show enhance growth rate with increasing concentration of these pollutants under laboratory conditions [Rosmarie, 1993; Ekunwe *et al.* 1993; Rice *et al.* 1984].

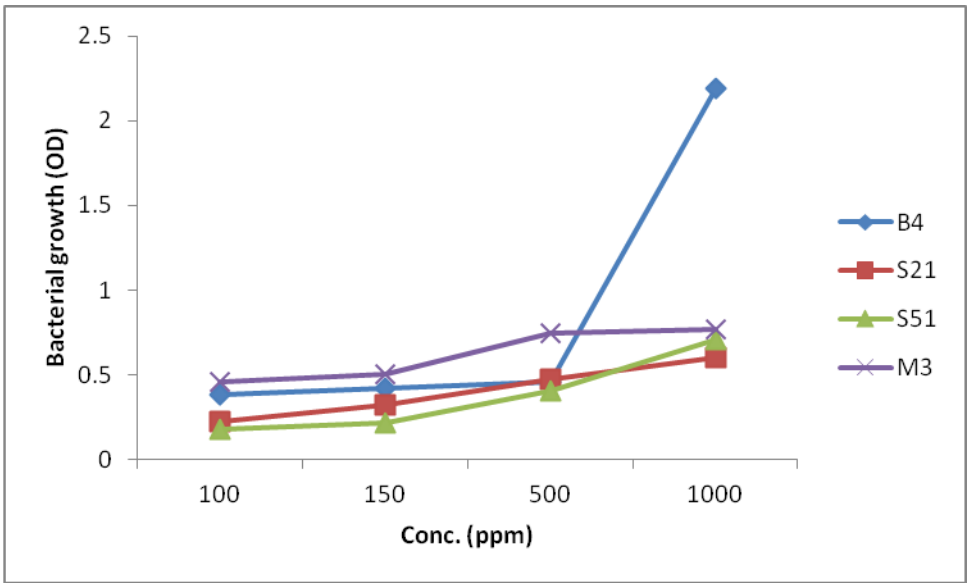

Fig. 8.1: Optimization of pyrene concentration

8.3.3 Optimization of temperature

Optimized temperature for isolates B_4, S_{21} and S_{51} for their maximum activity was 30ºC while, M_3 was 35°C as shown in Fig.8.2. The enzymes of these microorganisms might be adapted to this temperature [Ahmed *et al.* 2010]. The capability of the microorganisms to degrade PAHs is highly affected by the temperature of surrounding environment. The solubility of PAHs increases with an increase in temperature which increases the affinity of compound with reduced availability [Arulazhagan *et al.* 2011].

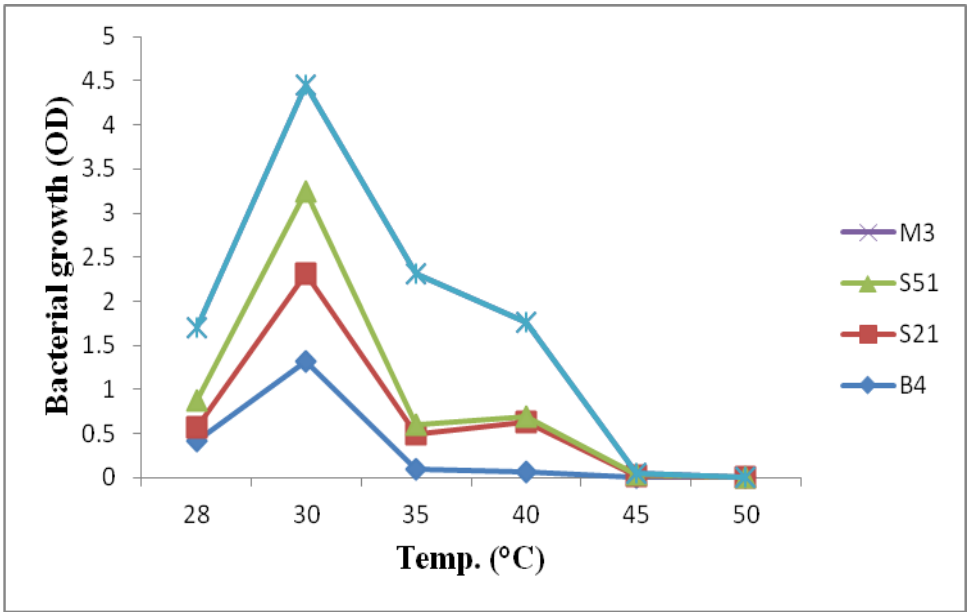

Fig. 8.2: Optimization of temperature for pyrene degradation

In addition, oxygen solubility decreases with increasing temperature, which reduces the metabolic activity of aerobic microorganisms [Nour *et al.* 2010]. Biodegradation of PAHs can occur over a wide temperature. Most studies have focus on mesophilic temperatures rather than the efficiency of transformations at very low or high temperatures. According to our study, temperature influenced the degradation of pyrene and its degradation level was significant in the mesophilic range with the highest degradation occurring at 30°C [Mukesh *et al.* 2012]. Isolates M_3 showed growth at 35 and 40°C is an indication that this isolate has enzyme adaptation at high temperature. Enzymes being catalyst have certain optimum temperature for its maximum activity [Deeb *et al.* 1999].

Low temperature also affects microbial growth, propagation and subsequently the rate of degradation. It cause reduce enzymatic activity which is essential for degradation, the optimum temperature for hydrocarbon degradation has been reported in range of 30-40°C. At temperature above this range, the enzymatic activity is inhibited due to denaturation of enzymatic protein [Leahy & Clowell, 1990].

8.3.4 Optimization of media p^H on pyrene degradation

It was found in this study that neutral p^H favoured both extensive bacterial growth at the expense of pyrene (Fig.8.3). Microbial system has no mechanism for adjusting their internal p^H is considerably affected by the p^H of their immediate environment because [Nnamchi *et al.* 2006]. The charge imbalance inside and on the surface of the cells was prevented at p^H 7. Effect of p^H on the disappearance of phenanthrene and pyrene was significantly inhibited when p^H varies acidic to neutral and neutral to alkaline [Ukiwe *et al.* 2012]. Pyrene removal was effected greatly the p^H of the growth media, with the degradation of PAHs significantly inhibited at p^H 4 and 5, relative to p^H 7 [Lakshmi *et al.* 2011].

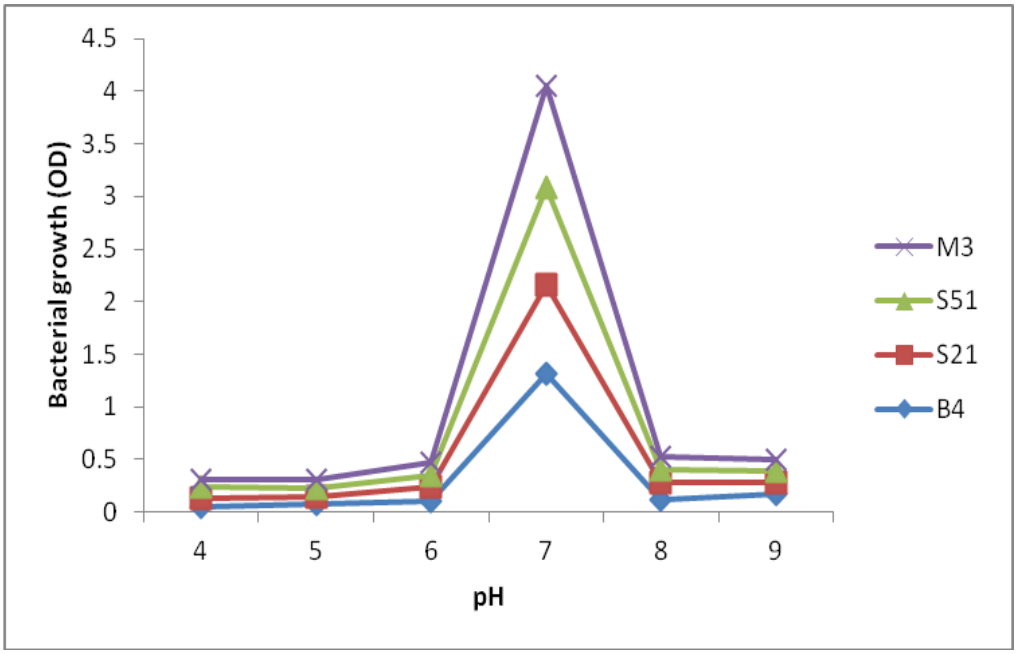

Fig. 8.3: Optimization of media p^H for pyrene degradation

8.3.5 Optimization of agitation speed on pyrene degradation

Shaking speed was optimized at 120 rpm for all the strains as shown in Fig. 8.4. Aeration is a limiting factor for degradation involving aerobic microorganisms as ample supply of oxygen is required for their growth and incorporation of molecular oxygen into aromatic structure of PAHs. So, agitation speed should be optimized for desired bacterial growth and degradation for an aerobic degradation of PAHs

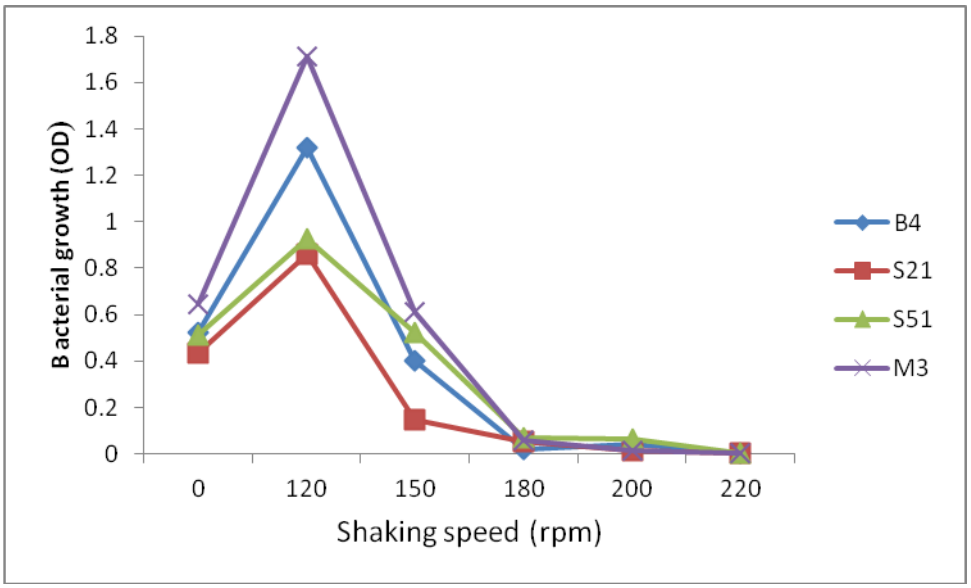

Fig. 8.4: Optimization of shaking speed for pyrene degradation

Better aeration results in adequate supply of oxygen, an even distribution of nutrients and low oxygen tension and temperature. Consequently, adequate availability of oxygen could enzymatically incorporate atmospheric oxygen more efficiently in the aromatic ring of the hydrophobic compounds like, pyrene [Alias *et al.* 2011; Bhattacharya *et al.* 2011; Park *et al.* 1990].

8.3.6 Effect of different substrates on pyrene degradation

Except for S_{21} on glucose and S_{51} on all the tested carbon sources with slightly enhanced the growth rate. These results show that all the four isolates were able to use pyrene as its sole carbon source [Hadibrata & Kristanti, 2012].

Effect of glucose on bacterial growth and pyrene degradation as an additional nutrient for has effect on bacterial multiplication (Fig.8.5).

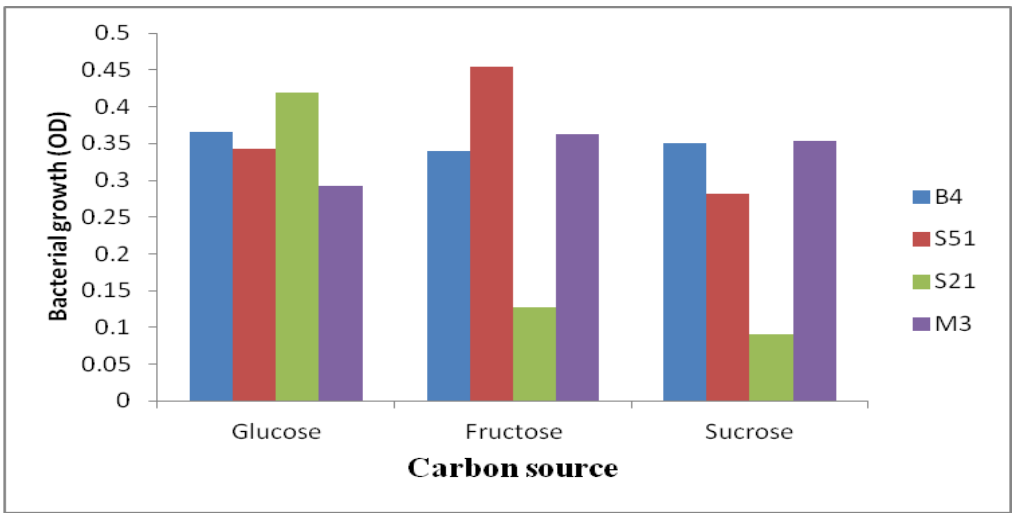

Fig. 8.5: Optimization of carbon source on pyrene degradation

However, addition of different nitrogen sources has no significant effect on growth of bacteria and degradation activity of pyrene as shown in Fig. 8.6. So, both carbon and nitrogen sources used in this experiment may act as competitive substrate in pyrene degradation as compared to earlier reported work where the addition of nitrogen and potassium increase the rate of crude oil degradation. It can be concluded, that the isolates isolated in our study were proved to be most efficient degraders of pyrene [Wick *et al.* 2003; Raza *et al.* 2010].

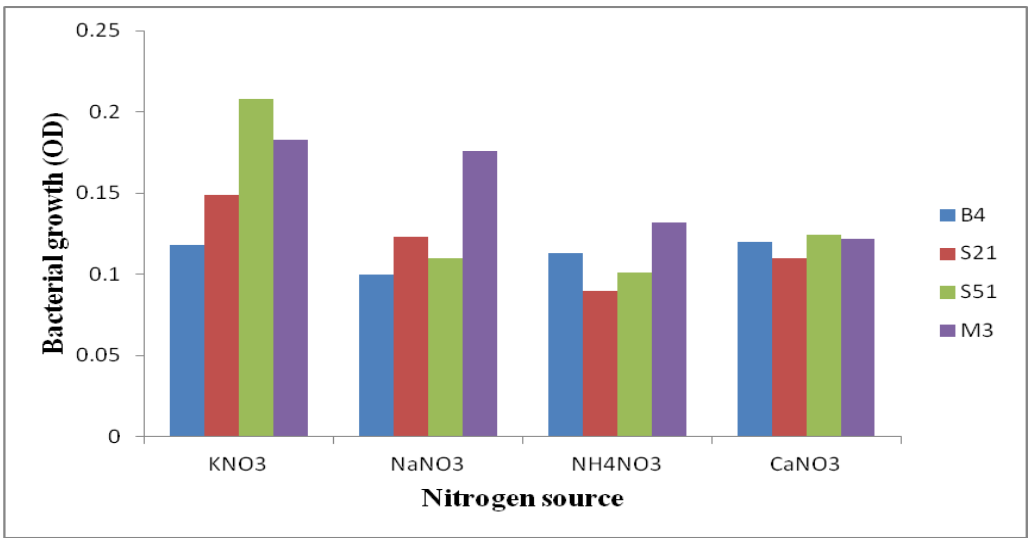

Fig. 8.6: Effect of nitrogen source on pyrene degradation

8.3.7 Optimization of inoculum concentration for pyrene degradation

The optimized inoculum concentration percentage was 10 for all the isolates for pyrene degradation as shown in Fig.8.7.

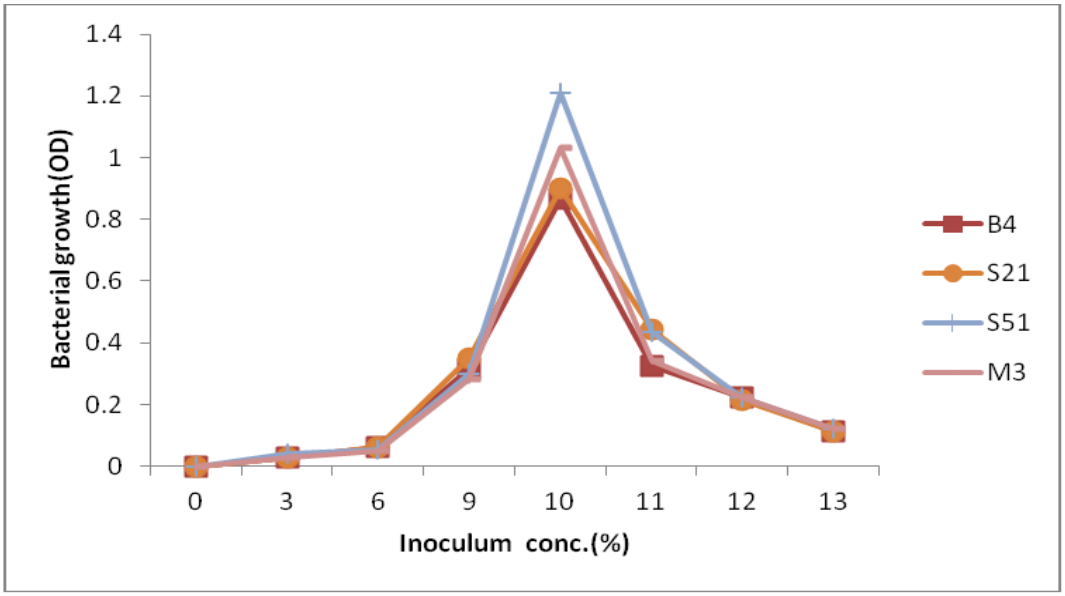

Fig. 8.7: Optimization of inoculum concentration (%)

8.4 Biodegradation

The rate of bioremediation a pollutant depends on the number and type of degrading organisms, environmental conditions, chemical and nature structure of the compound to be degraded. Some microorganisms can consume PAHs as a source of carbon and energy [Johnsen *et al.* 2005]. Algae, fungi and bacteria have PAHs degrading capabilities and involve the breakdown of organic compounds through biotransformation into less complex substances [Herwijnen *et al.* 2003]. Several species were observed growing on plates inoculated with pyrene and salicyclate medium in the enrichment technique [Gaskin & Bentham, 2005]. However, it cannot be clear whether genetic induction or degradation by co-metabolism only occurred among those bacterial populations. Besides, their varying pyrene degrading abilities would lead to the assumption that some of the bacterial genera like *Ralstonia* sp. could have developed only by the assimilation of salicylate and pyrene intermediate metabolites [Chauhan et al. 2008].

8.4.1 Biodegradation of HMW PAHs by isolates from contaminated area

The microbial population of contaminated areas has an extra ordinary ability to degrade HMW PAHs**.** However, the impact of the presence of other contaminants on microbial degradation is important in terms of biodegradation and bioremediation, since single contaminants are rarely found at contaminated sites. Bacteria become adapted to contaminants by exchange of gene located on catabolic machinery through horizontal gene transfer or through process of mutation. The degree of adaptation also depends on soil condition and contact time of microbes with pollutant. As contaminated environment has a number of PAHs and microbes has the choice to change metabolism to a non-substrate in case of limited nutrient availability [Ye *et al.* 2011**]**.

8.4.1.1 Biodegradation of pyrene by isolate S_{21}

S_{21} growth and degradation on pyrene is shown in Fig. 8.8. The growth rate was 0.929 OD after 120 hours incubation with 76.52% degradation and cell viability of 2.7×10^{-28} (Annexure A, Table IV).

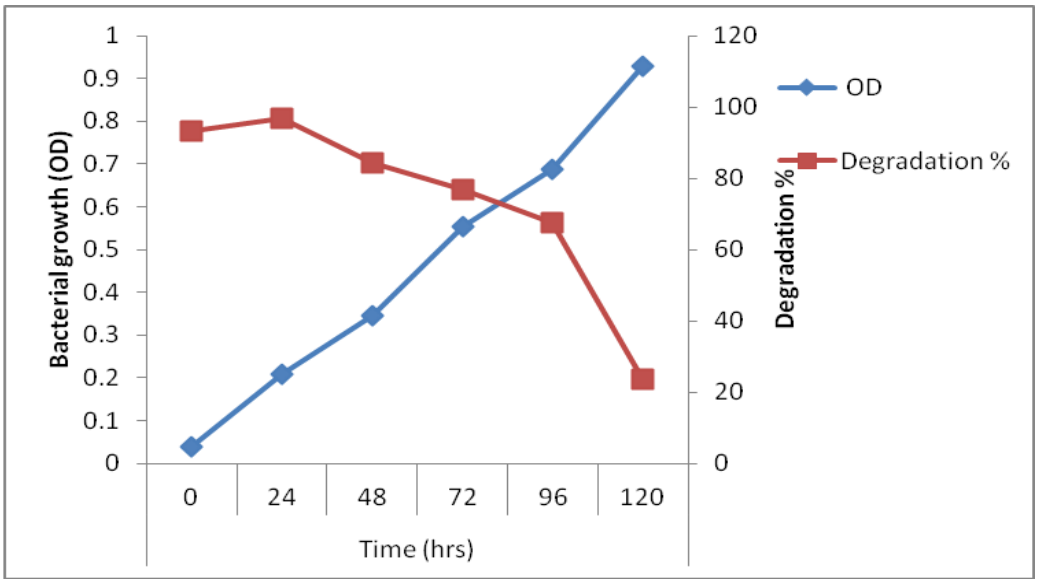

Fig. 8.8: Growth of isolate S_{21} at the expense of pyrene

The isolate S_{21} degraded 76.52 % of phenanthrene in 120 hours. There was a slight change in OD in first 24 hours of incubation due to slow growth rate. Although

the growth rate was slow in first 24 hours of incubation with only 3.11% degradation. There was a fourfold increase in growth of the isolates 48 hours and after 96 hours of incubation it was doubled and only 32.28 % degradation. The isolate shows same growth pattern on phenanthrene and pyrene with growth maxima between 48-96 hours. 44.24% pyrene was degraded during incubation period of 96 to 120 hours but bacterial growth rate was slow. HPLC analysis revealed that there was a single sharp peak and on comparison with authentic standard it was identified to be phathalic acid (Fig.8.9). A second peak with retention time of 1.539 in phenanthrene peak region is an indication of pyrene degradation through phenanthrene pathway previously reported [Haritash & Kaushik, 2009]

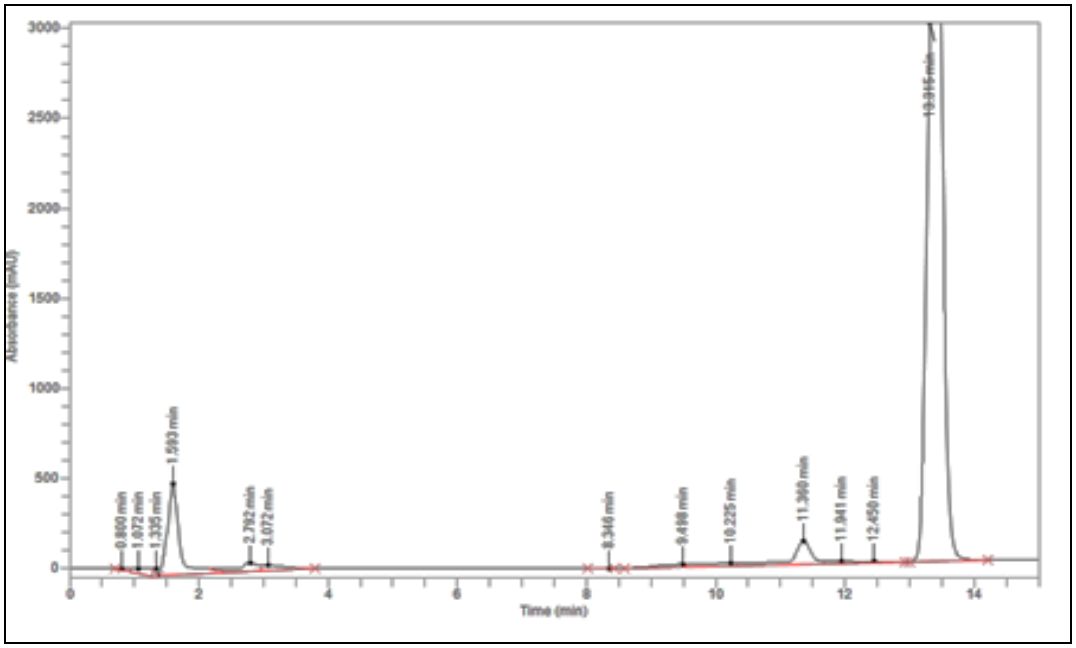

Fig. 8.9: HPLC chromatogram for pyrene degradation by bacterial isolate S_{21} after 120 hrs

From GC-MS analysis of 10 days culture some unidentified peaks were observed (Annexure E, Fig. VIII). The low solubility and hydrophobicity of many hydrocarbon compounds make them unavailable to microorganisms [Joner & Leyval. 2003]. The degradation efficiency was greater for three ring phenanthrene than four ringed pyrene.

8.4.1.2 Biodegradation of pyrene by isolate S_{51}

The growth of the isolate S_{51} after 24, 72 and 96 hours of incubation, the growth OD value was 0.415, 0.530 and 0.843 respectively as shown in Fig. 8.10.

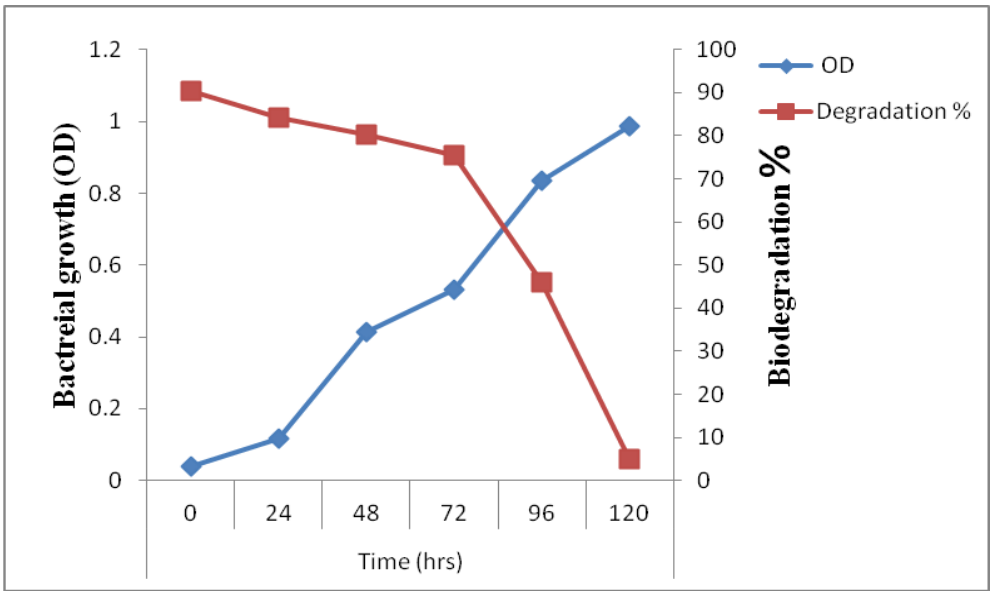

Fig. 8.10: S_{51} growth and pyrene degradation

It is clear from the OD values that a two-fold increase in growth rate on pyrene as carbon substrate. Cell viability for isolate S_{51} was 1.3×10^{-5}-2.4×10^{-24} in 0-96 hours incubation and 2.7×10^{-29} $CFUmL^{-1}$ after 120 hours (Annexure A, Table IV). During this period the amount of pyrene 44.34% with 23.48% unspent amount. The growth pattern was same i.e., maximum after 96 hours of incubation for both the chemicals whether phenanthrene and pyrene. The degradation efficiency was greater for three ring phenanthrene than four ringed pyrene. This isolate produce dark yellow color during this on solid media plate as well as liquid media. The exact mechanism of this yellow color formation is still a mysterious phenomenon. But, it has already been reported to be due to the formation of muconic semi-aldehyde which is the meta-clevage product of catechol. So, it can be concluded that this isolate exhibit astonishing ability for the complete mineralization of high molecular weight PAHs to simpler one [Haritash & Kaushik, 2009].

HPLC analysis of isolate S_{51} revealed that there was a single sharp peak with retention time of 1.539 on comparison with authentic standard it was identified to be

catechol (Fig.8.11). From GC-MS analysis of 10 days culture some unidentified peaks were observed ((Annexure E, Fig. IX)

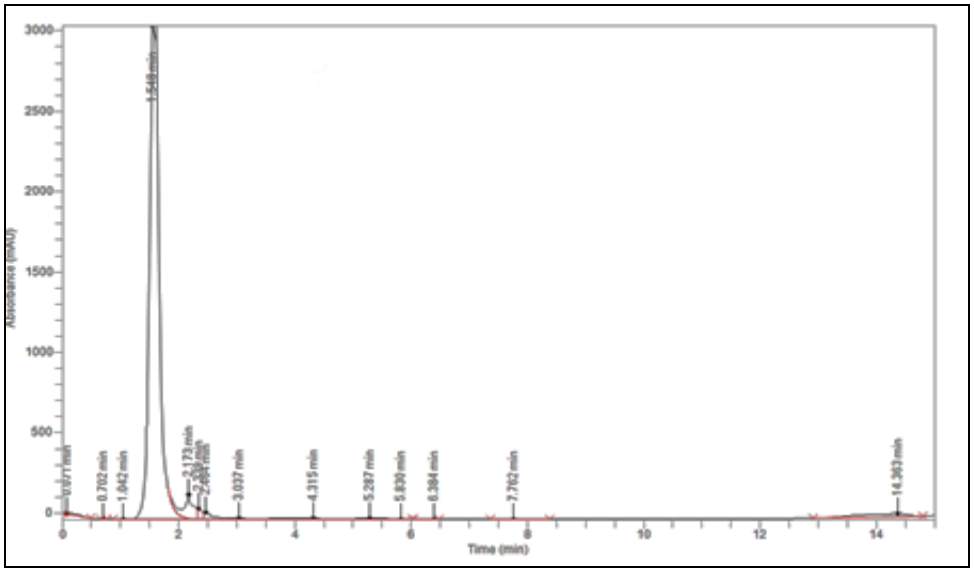

Fig. 8.11: HPLC chromatogram of pyrene degradation by isolate S_{51} after 120 hrs

8.4.1.3 Biodegradation of pyrene isolate M_3

Isolate M_3 degraded 76.31% pyrene in five days (Fig.8.12). In first 24 hours the degradation rate was slower and about 9.08% with a slower growth rate of bacteria.

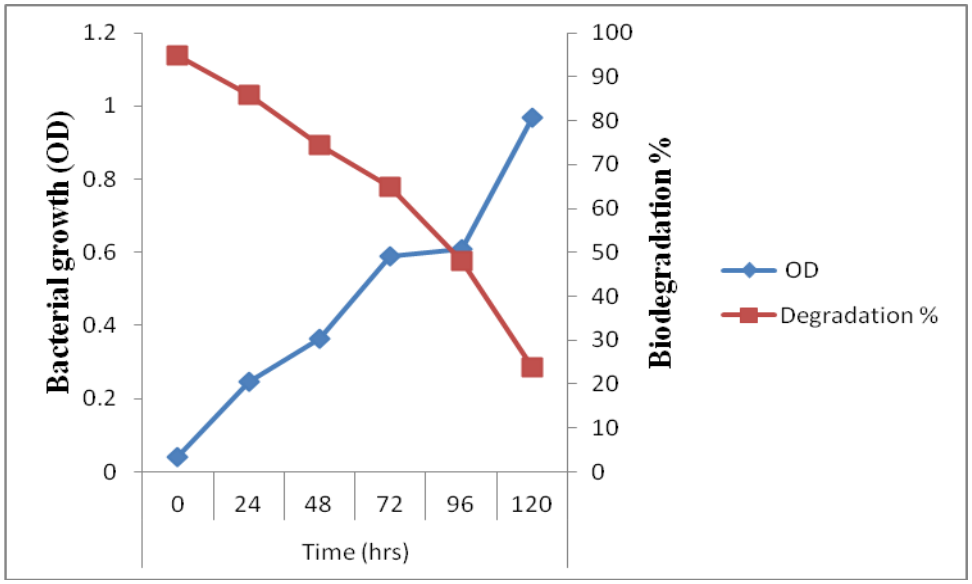

Fig. 8.12: Growth of M_3 at the expense of pyrene

11.20% was degraded on further incubation for 24 hours. While about 50% of pyrene was disappeared in 96 hours with bacterial growth rate (OD value) of 0.609. The growth rate with OD value of 0.97 was observed after 120 hours with about 23% degradation between day 4th and 5th. Cell viability for isolate M_3 was 1.3×10^{-6}-2.4×10^{-26} in 0-96 hours incubation and 2.7×10^{-31} $CFUmL^{-1}$ after 120 hours (Annexure A, Table IV). HPLC chromatogram after 24 hours (Fig.8.13) showed that there was a sharp single peak with retention time of 2.943 which disappeared after 48 hours and two other peaks with 4.269 and 4.609 starts to appear.

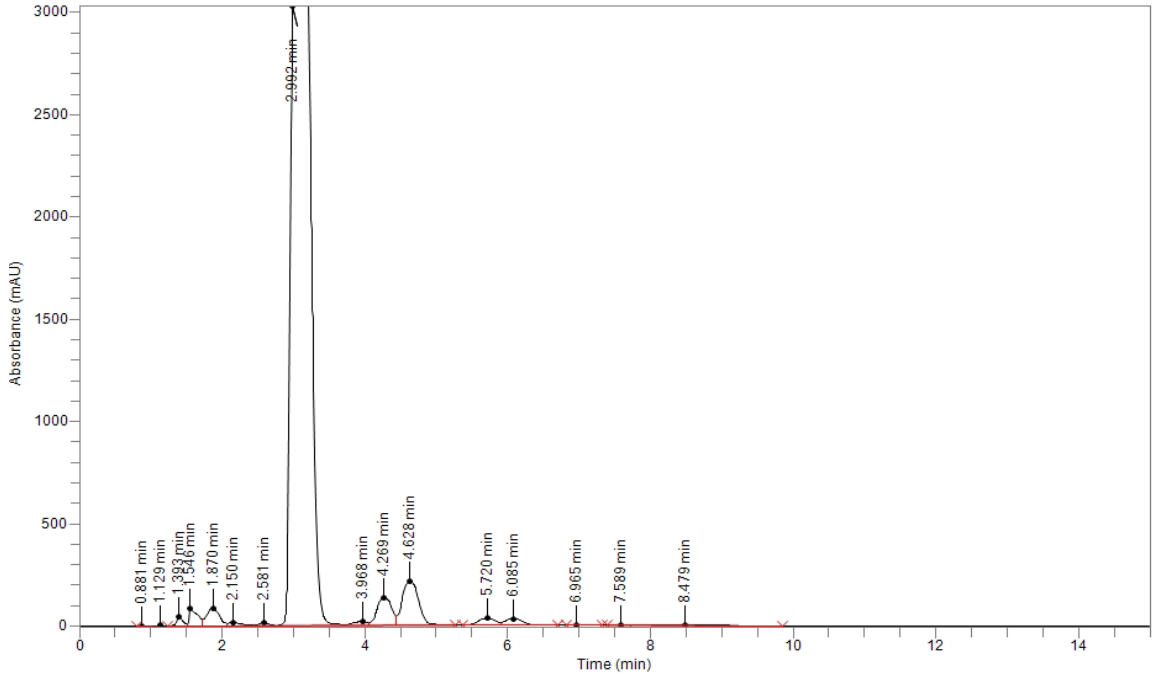

Fig. 8.13: HPLC chromatogram of pyrene degradation by isolate M_3 after 24 hrs

It can be concluded from HPLC and GC-MS analysis through comparison with authentic standards and GC-MS library that the peaks with retention time in range of 4.2-4.6 may be phenanthrene formed as metabolite of pyrene. Which, on further degradation for 24 hours converted to compounds with retention time of 9.006 and 9.25. Peaks in this range of retention time (9.0-10.0) have close resemblance with that formed by catechol. While, peaks formed in the range of 8.0-8.5 retention time are mostly formed by salicyalaldehyde and salicylic acid.

After 72 hours two sharp peaks with retention time of 9.25 and 10.08 were observed (Fig.8.14).

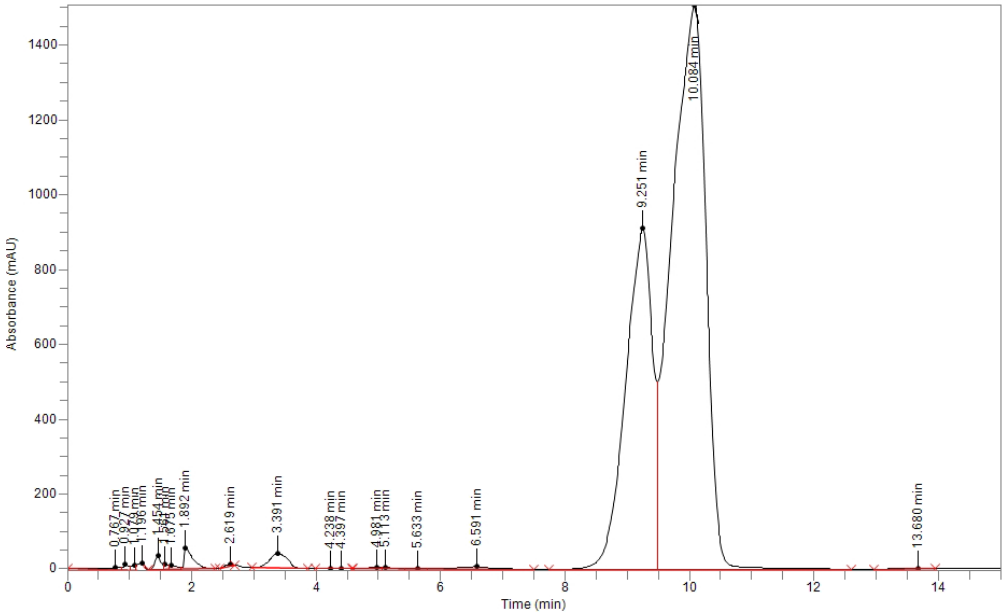

Fig. 8.14: HPLC chromatogram of pyrene degradation by isolate M_3 after 72 hrs

Between 4th and 5th two other peaks of 8.002 and 8.55 which remained unaffected even after 48 hours of incubation (Fig.8.15).

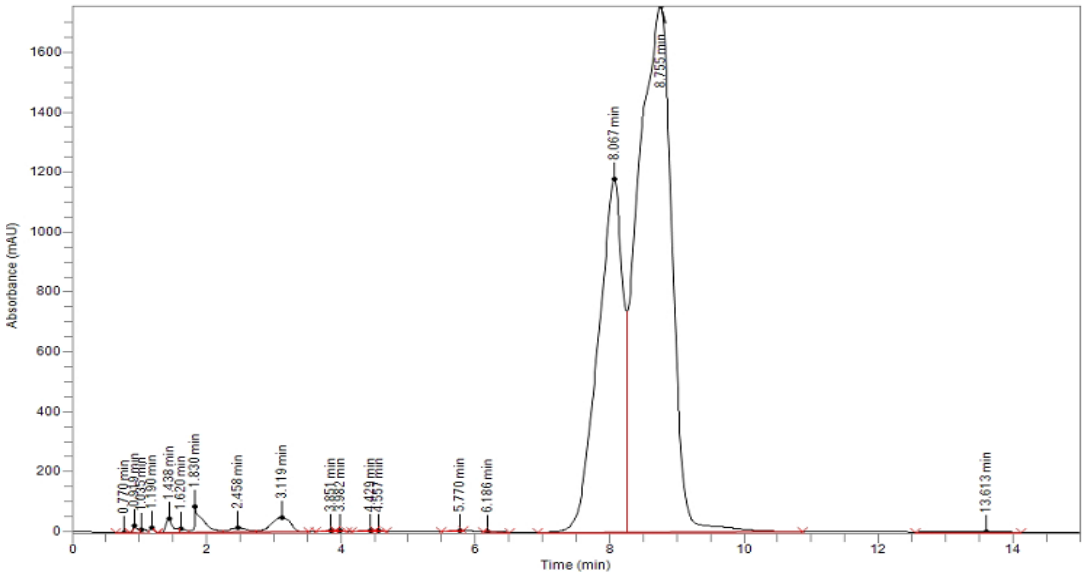

Fig. 8.15: HPLC chromatogram of pyrene degradation by isolate M_3 after 120 hrs

8.4.2 Biodegradation of HMW PAHs by isolates from un-contaminated area

Adaptation processes, allow the development of microbial populations with the ability to degrade PAHs and its mechanism is still unclear. These processes are controlled by the characteristics of contaminants and various soil factors. Adaptation of isolates depends on the concentration of contaminant and contact times of bacteria and contaminants. The continuous exposure, of microbes to contaminants induces adaptation to make recalcitrant compound easily accessible for degradation [Macleod & Semple, 2006, Ye *et al.* 2011; Kastner *et al* 1998; Johnsen *et al.* 2005].

8.4.2.1 Biodegradation of pyrene by isolate B_4

B_4 isolates show a different degradation pattern of pyrene with a maximum growth of 1.32 OD value after 120 hours incubation as shown in Fig. 8.16. B_4 isolate shows a different degradation pattern of pyrene with a maximum growth of 1.32 OD value after 120 hours incubation as shown in Fig. 8.17. In the first 24 hours there was a sudden increase in growth rate up to 0.395 and 0.522 on further 48 hours incubation with the utilization of only 3.60 % pyrene.

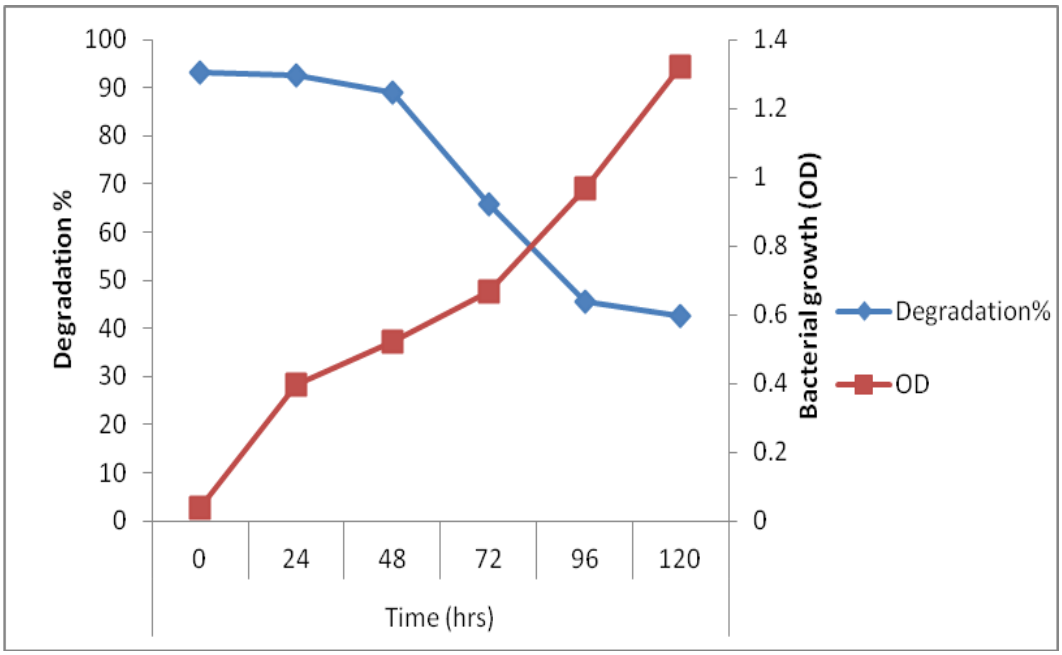

Fig. 8.16: B_4 growth and pyrene degradation

In the first 24 hours there was a sudden increase in turbidity up to 0.395 and 0.522 on further 24 hours incubation with the utilization of only 3.60 % pyrene. The utilization of pyrene was 23.23% for increase in bacterial growth with an OD value from 0.522 to 0.667. The increase in turbidity was 0.667 to 0.966 and 1.32 with utilization of 20.20% and 3.07% respectively. The remaining quantity of pyrene in the medium was 91.88 %, which may be due to less availability in the medium or recrystallization from the medium can probably be due to due to hydrophobic nature. Cell viability for isolate B_4 was 1.3×10^{-6}-2.4×10^{-20} in 0-96 hours incubation and 2.7×10^{-23} $CFUmL^{-1}$ after 120 hours as shown in Annexure table IV.

B_4 after 48 hours only 9% of pyrene was transformed with % reduction from 98% to 89%. The utilization of pyrene was slower 10.91% after 48 hours for increase in bacterial growth with an OD value of 0.667. The increase growth rate was 0.966 and 1.32 with degradation 57.41 % from 96-120 hours of incubation. The remaining quantity of pyrene in the medium was 42.59%. It can be concluded from previous studies PAHs degrading bacteria isolated with a strong ability to degrade phenanthrene and pyrene at different degradation rates. The enhanced growth was observed when three PAHs compounds were mixed with an individual isolate in the medium [Khalid *et al.* 2004].

HPLC analysis show that a sharp peak with retention time 14.5 min an peak area % of 11.7 which can be compared with authentic standards and identified as 1,8-pyrenequinone as reported by Paul *et al* (1998). Certain metabolites in peak area of 2.58, 5.39, 0.81 and 0.45 with retention time of 14.3, 2.23, 4.3 and 5.2 respectively. Pyrene oxidation may probably result in formation of certain metabolites with retention time of 0.237 with peak area of 9.39%, another peak with peak area 2.139 with area percentage 20.56, peaks with retention time of 3.7, 4.334 and 5.2, with peak area of 5.94%, 6.68% and 1.79%. About 50.56% pyrene was transformed to different metabolites.

8.4.2.2 Comparison of phenanthrene and pyrene biodegradation by isolates S_{21}

Isolate S_{21} degraded phenanthrene 500mgL^{-1} and pyrene 1000mgL^{-1} with 96.60 and 76.52% are closely related to the isolate capable to degrade 50.0% of the pyrene at 28°C within 5 days (Fig.8.8).

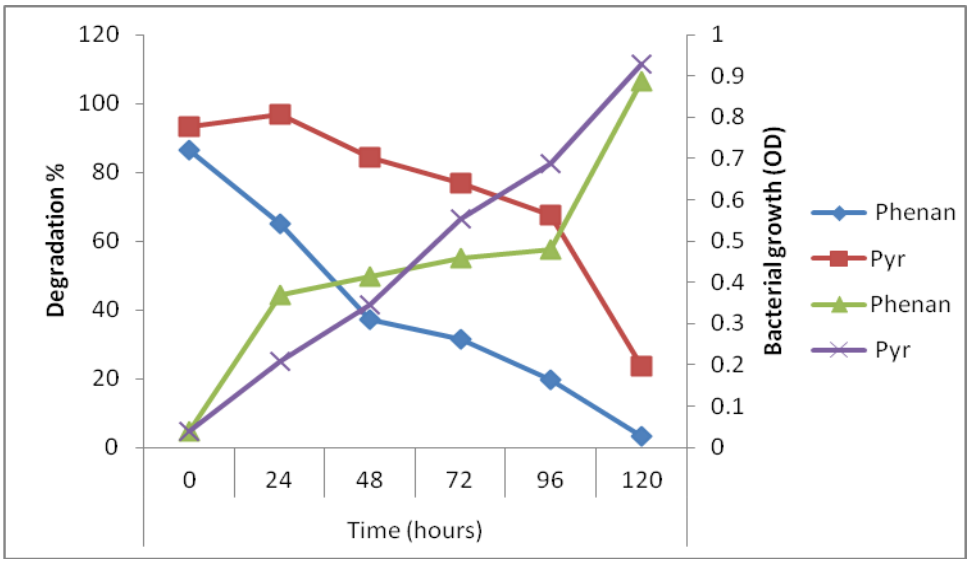

Fig. 8.17: Comparison of growth and degradation of S_{21} on LMW and HMW PAHs

Another species S_{51} from the petroleum contaminated area, degraded pyrene with growth maxima after 120 hours of incubation, with bacterial growth OD value 0.929 with 71.99 % degradation. In the first three days there was only 24.60 % was degraded with 0.554 OD value of bacterial growth. The enhanced growth rate was observed after 96 hours was 54% and 71.99 with an OD 0.689 and 0.969 after 120 hours respectively. The degradation rate was slower than isolate S_{21} was 76.52 % and is also capable of phenanthrene degradation. Isolate S_{51} degraded 71.99 % pyrene in five days, are in line with the recently reported study on degradation of *Pseudomonas stutzeri* CET 930 of wastes containing pyrene in one week [Moscoso *et al.* 2014].

8.4.2.3 Comparison of growth and degradation of different strains on pyrene

Cell viability for all the three isolates were calculated after five days was M_3 (2.5×10^{-31}) and S_{21} (2.3×10^{-29}) and S_{51} (2.8×10^{-28}) (Annexure A, Table IV). The results demonstrate that the degradation capability on pyrene from soil bacteria from

contaminated sites has comparatively more potential to degrade HMW PAHs (pyrene) and can be applied in an efficient bioremediation process [Ping *et al.* 2014].

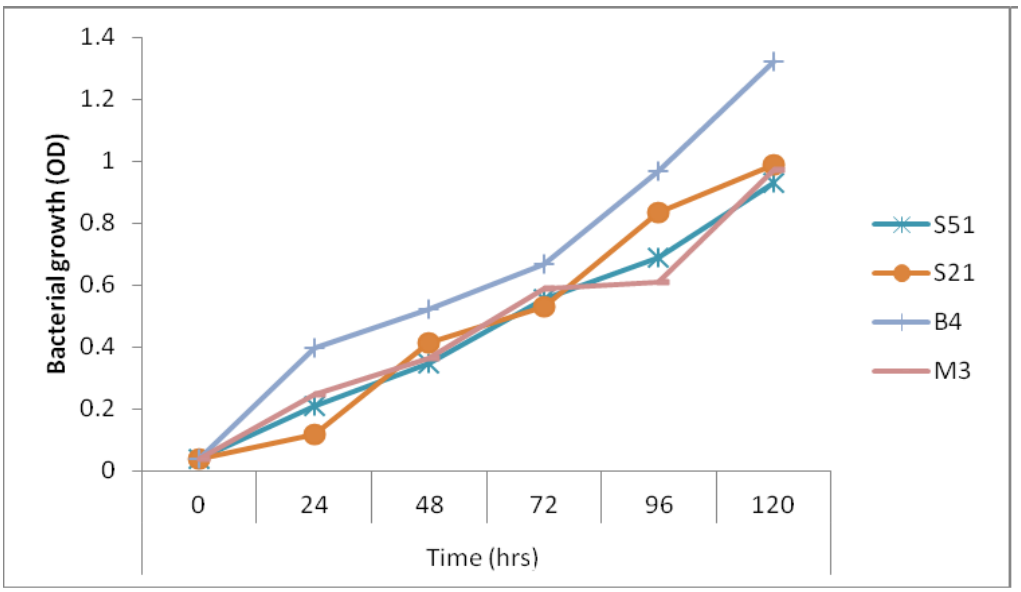

Fig. 8.18: Comparison of growth of different isolates on pyrene

These isolates are capable of pyrene degrading capability at 1000mgL^{-1}, efficiency in 5 days i.e., B_4 (57.41%), S_{21} (76.52%), S_{51} (71.99%) and M_3 (76.31%) as shown in Fig.8.19. These results are in contrast to previously reported work for *Klebsiella pneumoniae* that degraded only 63.4 % of 20mgL^{-1} in 10 days.

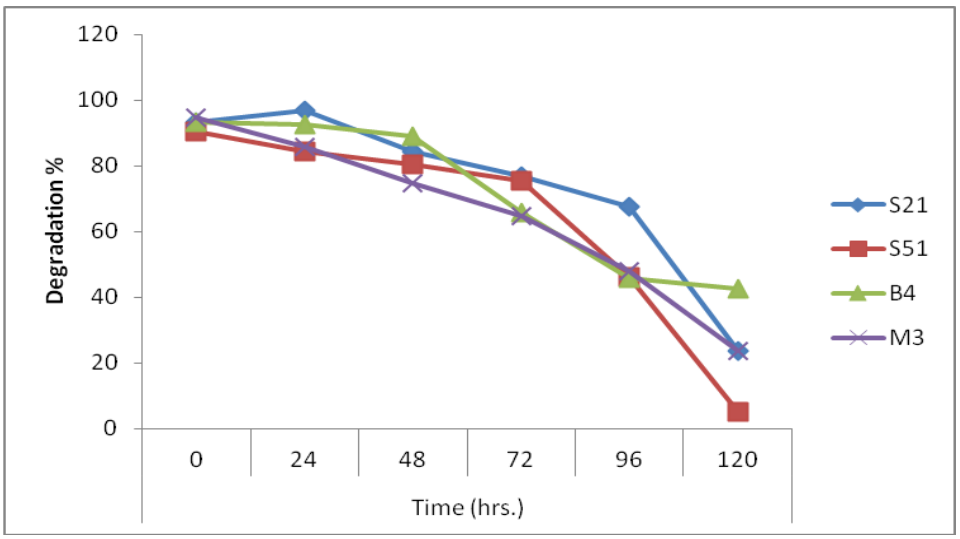

Fig. 8.19: Comparison of degradation of pyrene by different isolates

In an already reported study on two bacterial isolates, *Enterobacter hormaechei* and *Pseudomonas pseudoalcaligenes* were isolated from different petroleum contaminated sites. Pyrene was degraded by these isolates with efficiency 77.7 and 83.7% within 15 days of incubation, respectively. The second phenomenon was inhibition occurred with ASU-016 which completely retarded pyrene degradation. The formation of coloured products during microbial degradation of hydrocarbons causes colouring of the media [Hesham *et al.* 2014; Michael Stieber 1994]. Microbes that can grow on salycilate develop enhanced degradation of those PAHs which degrade these compounds through salycilate pathway [Gaskin & Bentham 2005].

Pyrene degrading isolates isolated belongs to *Rhodococcus sp., Bacillus cereus, Burkholderia cepacia* Mycobacterium [Juhasz *et al.* 1997], *Cycloclasticus* sp. P1, *Pseudomonas fluorescens, Pseudomonas stutzeri, Sphingomonas sp., Sphingomonas paucimobilis,* and *Stenotrophomonas maltophilia*. Pyrene is metabolised by bacteria into intermediated such as pyrene-cis/trans-4, 5dihydrodiol and pyrenol. Further, ring fission produce 4-phenanthroic acid, phthalic acid [Heitkamp *et al.* 1988]. About 27 enzymes necessary identified for constructing a complete pathway for pyrene degradation from gene and protein studies has been reported. 9, 10-hydroxylation of anthracene to produce anthracene-9, 10-dihydrodiol, which is converted to 9, 10-dihydroxyanthracene and spontaneously oxidized to 9, 10-anthraquinone [Vila *et al.* 2001; Moody *et al.* 2001; Swaathy *et al.* 2014]. The compounds in rectangles are identified in this study by comparison with authentic standards, GC-MS library were most match with the pathway proposed previously [Liang *et al.* 2006; Kim & Freeman, 2005, Kim *et al.* 2007; Rehmann *et al.* 1998].

In a previously reported data obtained from GC-MS analysis system of ethyl acetate extracted of pyrene cultures inoculated with *Corynebacterium variabilis* sp. suggest the proposed pyrene biodegradation which also involve phenanthrene, naphthalene and o-phethalate degradation pathways. More research on the microbial degradation of PAHs has resulted in the isolation of numerous genera of bacteria, fungi and algae capable of degrading low molecular weight and high molecular weight PAHs. The latter are generally recalcitrant to microbial attack, even though some fungi and algae are capable of transforming these compounds. Until recently,

only a few genera of bacteria have been isolated with the ability to utilize four-ring PAHs as sole carbon and energy sources while co metabolism of five-ring compounds has been reported [Simarro *et al.* 2013; Khan *et al*. 2015].

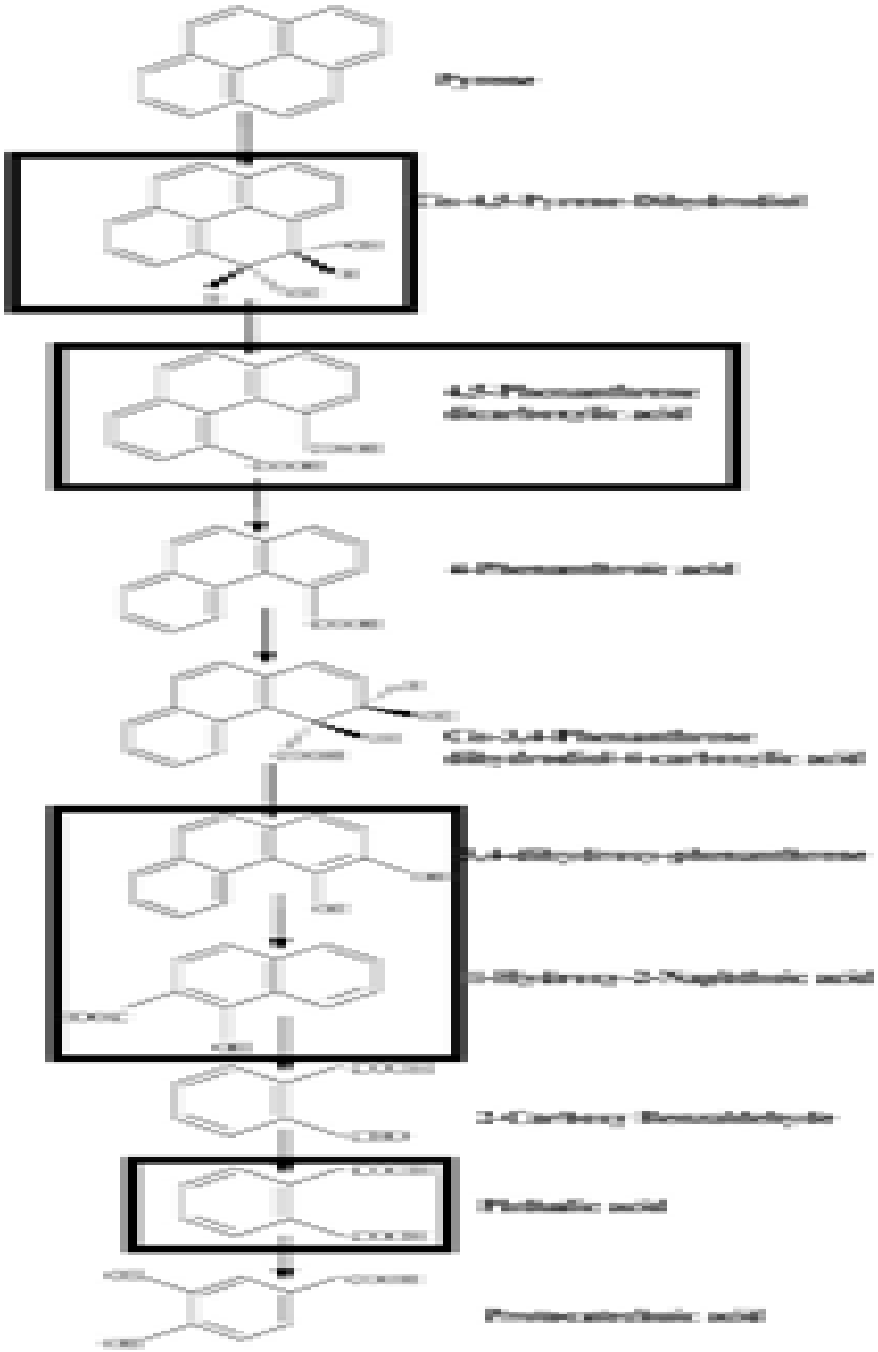

Fig. 8.20: Proposed pathway for pyrene degradation, compounds in rectangles were identified in this study

8.5 Plasmids isolation and gel electrophoresis

The plasmid isolated from the three isolates capable of pyrene degradation. The size of plasmid from S_{51} and M_3 were seemed to be a bit larger than ladder (1Kb) as seen on gel electrophoresis as clear figure 8.21.

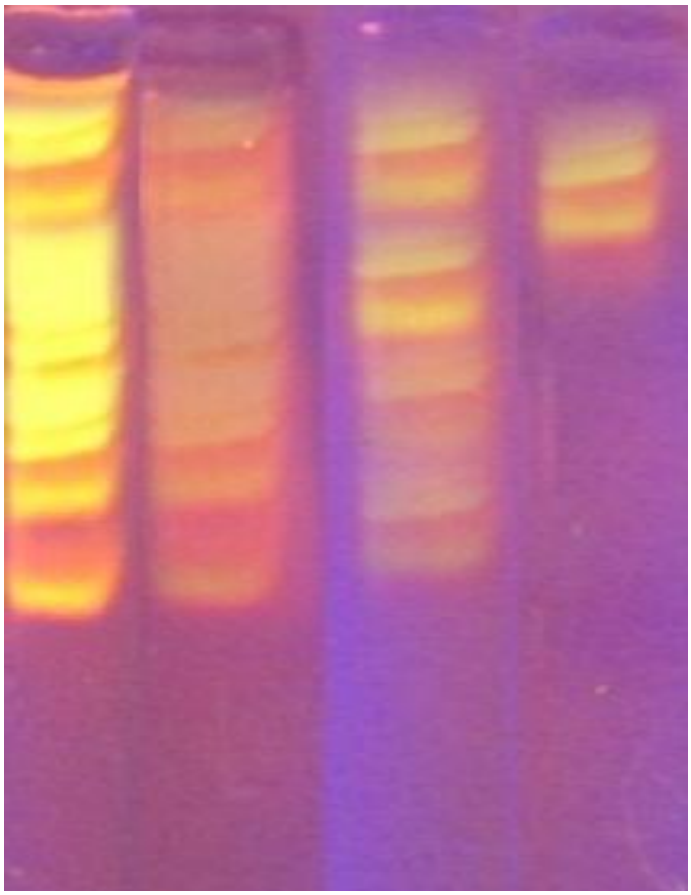

Fig. 8.21: 1Kb ladder and isolated Plasmids

Isolate B_4 showed reduction in pyrene degradation rate after plasmid curing. This can be may be a possible explanation that some of the pyrene degradation genes may be located on plasmid while, others on chromosomes. The degradation of pyrene by the isolates B_4, S_{51} and M_3 was not plasmid mediated rather it was chromosomally controlled.

One of the most important factor that aid to degradative efficiency of microorganism is the presence of plasmid which play a pivotal role in obtaining novel catabolic pathways in microbial communities. Like previously reported studies, some interesting findings form isolated a *Mycobacterium* sp. from a petroleum contaminated area was capable to grow on HMW PAHs possesses a large plasmid. But, this plasmid was later confirmed to have no role in degradation. The first reported pyrene degrader and from the studies of a large number of isolates it was found that genes for pyrene degradation are localized on chromosomes [Khan *et al.* 2001; Krivobok *et al.* 2003; Kim *et al.* 2007].

Likewise, the presences of plasmid have no effect on pyrene degradation as clear from our studies. The isolates were able to degrade pyrene after curing plasmid from these isolates. It is not unlikely that plasmids are involved in the degradation of HMW PAH than previously thought [Miller *et al.* 2007]. The isolation of plasmid from of two isolates of *B. cereus* and *B. megaterium* having pyrene degrading capability in consortium has been carried out [Lin & Cai, 2008; Obayori *et al.* 2008].

8.6 Morphological and Biochemical characterization of bacterial Isolates

The isolates were identified morphologically by Gram's staining and microscopy while biochemically by Bergey's manual of systematic bacteriology. All the isolates were gram positive except S_{51}which was gram negative, and all were spore forming as shown in Table 8.1.

Table 8.1: Morphological Identification

S. No	Isolate	Gram's Test	Microscopy	Spore	Motility
1.	B_4	+ive	Rods	+	+
2.	S_{21}	+ive	Coccobacilli diplococci	+	+
3.	S_{51}	–ive	Cocci chains	+	-
4.	M_3	+ive	Cocci in chain	+	-

B_4, B_{30} and M_3 were endosporous. Morphologically, B_4 was rod –shaped, S_{21} diplococcus chain, S_{51} was circular, raised convex and coccus, and M_3 appeared large opaque. Biochemical tests revealed that all the isolates showed gelatin liquification

and catalase activity, and the starch hydrolysis, casein hydrolysis and nitrate reduction were positive for all the isolates except S_{21} as shown in Table 8.2.

Table 8.2: Biochemical characterization

S. No.	Biochemical test	Bacterial isolate			
		B_4	**S_{21}**	**S_{51}**	**M_3**
1.	**Catalase**	+ive	+ive	-ive	+ive
2.	**Starch hydrolysis**	+ive	-ive	-ive	-ive
3.	**Citrate**	-ive	-ive	+ive	+ive
4.	**Urease**	+ive	+ive	+ive	-ive
5.	**Casein hydrolysis**	+ive	-ive	+ive	+ive
6.	**Gelatin liquification**	+ive	+ive	+ive	-ive
7.	**Indole production**	-ive	-ive	-ive	-ive
8.	**Nitrate**	+ive	+ive	+ive	+ive
9.	**Urease**	-ive	-ive	+ive	+ive
10.	**Oxidase**	+ive	+ive	+ive	+ive

Indole production was negative for all the isolates, while urease test were negative for all isolates except, S_{51} and M_3. Simon citrate test was negative for all the isolates except M_3. All the isolates showed oxidase activity. Sugar fermentation tests showed that all the isolates were unable to ferment lactose and fructose. In our study isolate B_4 has resemblance biochemical characters with that, of *Leclercia decarboxylata* a member of Enterobacteriaceae which also possess the pyrene degrading ability [Sarma *et al.* 2004]. Our isolate S_{51} having irregular edges identified as *Clostridium* like previously reported studies on soil collected from petrol pump. Hydrocarbons degrading bacterial cultures isolated from contaminated soil sample collected from petrol pump. *Bacillus sp, Acinetobacter, Clostridium and Pseudomonas* sp. was the efficient naphthalene and anthracene degraders respectively. M_3 was characterized as *Bacillus licheniformis* on the basis of morphology and biochemical characterization and growth temperature. Final identification will be done on the basis of molecular and phylogenetic analysis [Gomare *et al.* 2011].

8.7 Molecular identification and phylogenetic analysis

Isolate B_4 was similar to *Bacillus sp*. having 99% homologous sequences on BLAST having 87% similarity with *Bacillus cereus isolates* DZ4, *Bacillus cereus isolates* 165PP and *Bacillus cereus isolates* as shown in Fig. 8.22.

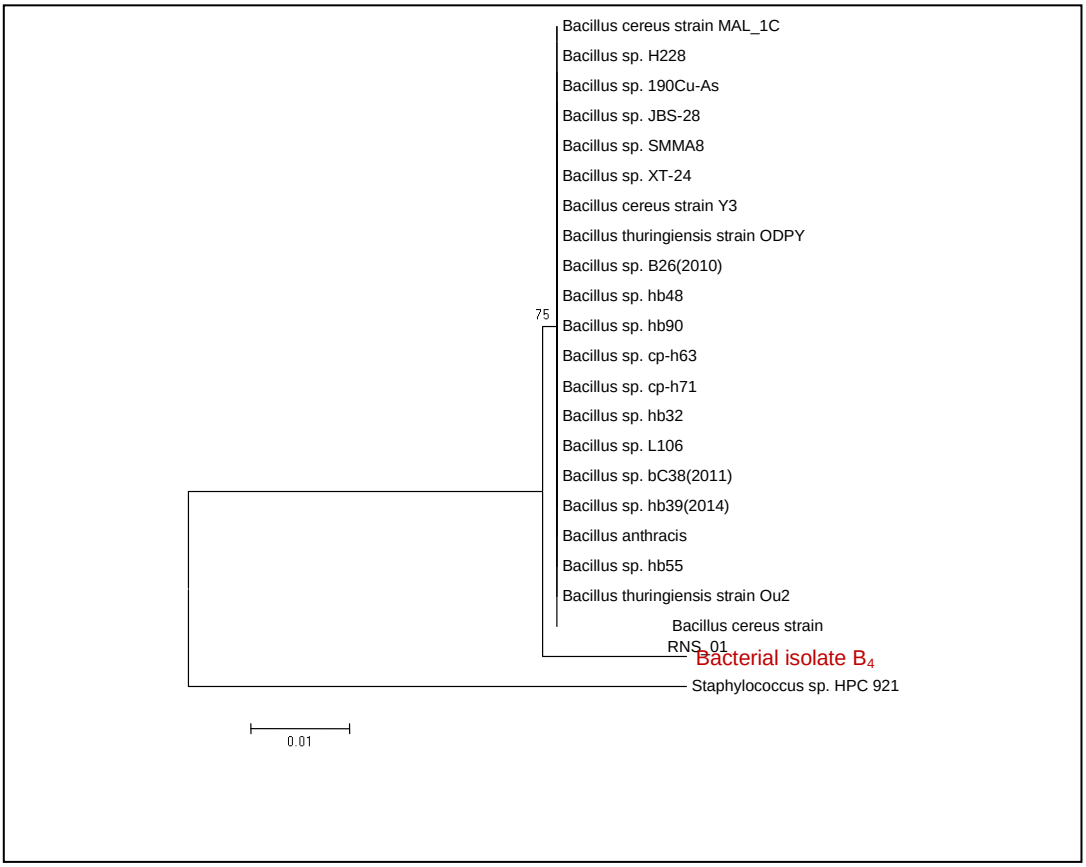

Fig. 8.22: The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [Tamura and Nei, 1993]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA6 [Felsenstein, 1985].

Since our isolated formed 75% bootstrap clad with all *Bacillus* sequences downloaded, we identify it as an isolate *Bacillus* sp. This isolate was able to utilize

only HMW PAH (pyrene) but, was unable to grow on two and three LMW PAHs like napthalene and anthracene or pyrene [Natalie *et al.* 2004].

Isolate S_{51} which was categorized as *Bacillus* and this isolate formed distant clad with different *Bacillus* sequences 63% homologus to *Bacillus* sp. L106, *Bacillus* sp. cp-h24, *Bacillus* sp. *3-cpa-15*, *Bacillus* isolate BC-2 and *Desulfovibrio vulgaris* isolate RL as in Fig.8.23.

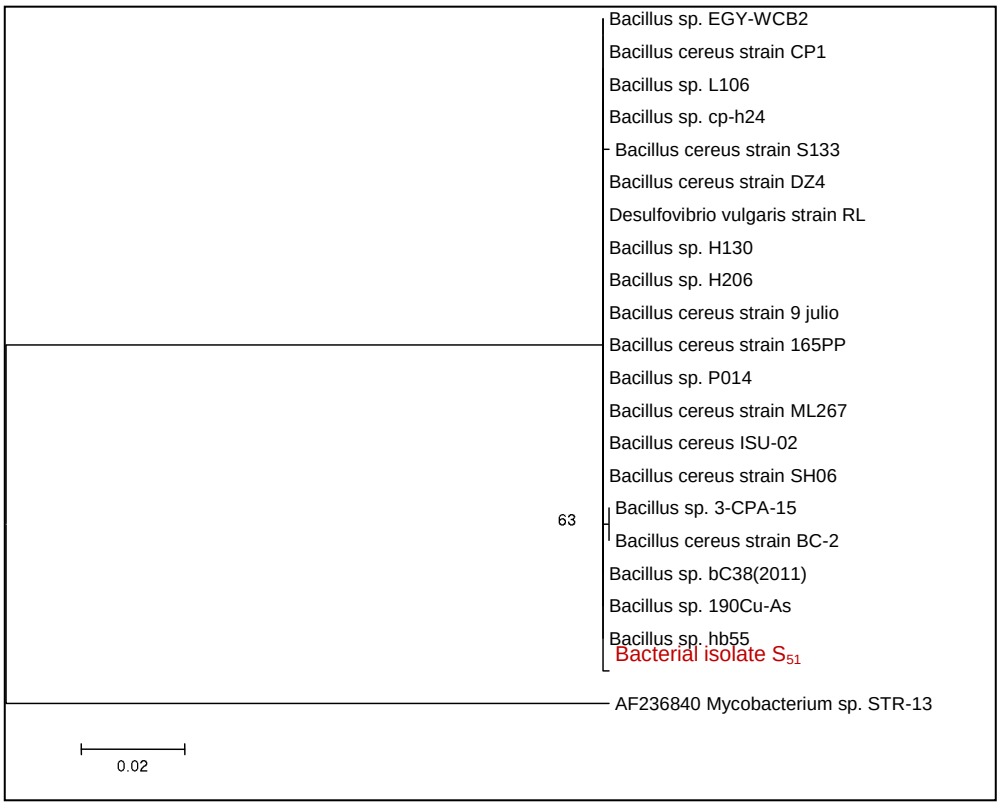

Fig. 8.23: The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [Tamura and Nei, 1993]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA6 [Felsenstein, 1985].

8.8 Differential phylogenetic tree of bacterial isolates

The phylogenetic analysis as revealed from the evolutionary trees shown in respective chapter for our studied isolates was shown to belong to different species of *Bacillus*. So in order to check whether our isolates are different we construct a combine tree as shown in Fig. 8.24. As clear from the above figure that our isolate B_{36} and B_{30} were 12 % different from one another while, isolate S_{51} and B_4 were 14 % distinct. The latter shows 100% similarity with isolate S_{21} and S_{13} which are 18% unrelated.

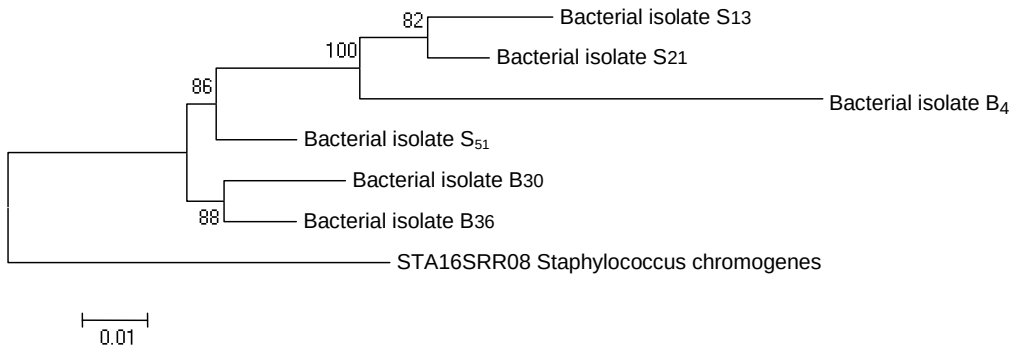

Fig.8.24: Differential phylogenetic tree of bacterial isolates

The isolate B_4 shows 100% relatedness with S_{21} and S_{51} but the clad distance is comparatively larger than other isolates in this tree is a indication that it may doesn't belong to *Bacillus cereus sp*. It is also supported by biochemical characterization which showed that B_4 belongs to some species of *Bacillus thurengensis*.

CO-METABOLISM

9.1 INTRODUCTION

PAHs are universally distribution in environment due to their stability and persistence is depends upon their chemical and physical properties [Samanta *et al.* 2002]. It is therefore important to look into and understand biodegradation of PAHs mixture for successful implementation of bioremediation technology. Biodegradation of individual and mixture of PAHs bacteria have been reported by several researchers [Yu *et al.* 2005, Desai *et al.* 2008]. Thus, these studies have not investigated in detail the interaction among PAHs during biodegradation [Gaskin & Bentham 2005].

The desired microorganisms can be obtained by providing a target substrate as the sole carbon source [Mesdaghinia *et al.* 2005]. The breakdown of a contaminant by an enzyme or cofactor of another compound is the process of co-metabolism. PAHs with two and three rings, such as naphthalene and phenanthrene, acenaphthene and acenaphthylene and fluorene are somewhat easy to degrade [Fuchs 2008, Hazen 2010].

Co-metabolic bioremediation has been used on a variety of contaminants, including PAHs, explosives, dioxane, polychlorinated biphenyls, pesticides and halogenated aliphatic hydrocarbons. The bacteria involved in cometabolic degradation do not receive a carbon or energy benefit from the contaminants. In addition, intermediate products that act as inhibitors of microbial metabolism can be produced during cometabolic bioremediation [Hazen 2010, Rui *et al.* 2004, Semprini *et al.* 2005; Powell *et al.* 2011]. It is the transformation non-growth substrate in the presence of growth substrate to extend the range and extent of PAHs degradation. This is techniques that make possible the degradation of xenobiotics normally unavailable to degradation [Herwijnen *et al.* 2003; Zhong *et al.* 2007].

Oxidation of phenanthrene and anthracene to the corresponding hydroxynaphthoic acids occurred quantitatively. The isolate converted phenanthrene; anthracene, fluoranthene and carbazole of coal-tar-pitch extract [Baboshin *et al.* 2008]. *Sphingomonas* VKM B-2434 has the ability to transform substances naphthalene, acenaphthene, phenanthrene and anthracene separately and PAH

mixtures as a sole source of carbon and energy. The isolate is capable of carrying cometabolic oxidization of pyrene [Liu *et al.* 2013]. Co-metabolism experiment was carried out according to protocol of [Zhong *et al.* 2007; Supaka *et al.* 2001]. The degradation of five PAHs in mixture and individually supplemented with glucose and yeast extract. The mixed bacterial culture degraded phenanthrene, completely, anthracene to 60%, pyrene to 80%, fluorene to 30%, and fluoranthene to 20%. The results show that as the number of benzene rings in PAH compounds increases the rate of degradation decreases [Shafee *et al.* 2006; Lily *et al.* 2013].

9.2 Material and methods

Chemicals of analytical grade and media were prepared in details in chapter 3 and 4.

9.2.1 Inoculums preparation

All the isolates were grown in Nutrient Broth and incubated at 150 rpm in an orbital shaker at 28±2°C. After reaching late exponential phase bacterial cells were harvested at 8000×g at 4°C for 10 minutes, washed twice in phosphate buffer (pH 7.2) and resuspended in BSM to obtain bacterial suspension with an OD 600nm less than 0.4. The suspension was used as the inoculums in all optimization, biodegradation and co-metabolism experiments [Guo *et al.* 2010].

9.2.2 Co-metabolism experiment

Experiment for cometabolic degradation of mixed PAH, and metabolites were studied on the basis of retention time comparison with available authentic standards and literature values. Experiment for cometabolic degradation of five selected compounds was prepared according to protocol of Zhong *et al.* (2007) with some amendments. Before bacterial inoculation, the added PAH was evenly mixed with the liquid medium, and the flasks were shaken on a rotary shaker (150 rpm) at 30°C overnight. The added acetone was evaporated, and the residual acetone was so small that it did not have any toxic effect to microorganisms according to our previous experience. The isolate was grown in NB for 3 days, centrifuged at 5,000×g for 10 min, washed twice, and resuspended in 0.85% NaCl to obtain the cell suspension with an optical density at 600 nm (OD 600) of 1.0. The 500-µl aliquot of the cell

suspension previously grown in NB was used as an inoculum and was added to the respective growth medium. A sterilized PAH flask without any bacterial inoculation was used as the abiotic control. Each treatment and control was prepared in triplicate. All cultures were started with cell density at an OD_{600nm} of 0.02 and were incubated at 30°C on a rotary shaker (150 rpm) in the dark. The growth in terms of OD_{600nm} of the culture was measured at days 0, 1, 3, 5, and 7.

9.2.3 Extraction of selected PAHs from sample

The samples were then agitated in an incubated shaker at 150rpm for two weeks. Samples were periodically collected from reactor flasks for day 1, 2, 5, 7 for the` purpose of measuring residual concentration of PAHs. PAHs were extracted by protocol of Shokrollahzadeh *et al* with some modifications [Shokrollahzadeh *et al.* 2010]. The growth medium was extracted twice by dichloromethane (DCM) and acetone 10mL each by shaking at 30°C for 24 hours. The extraction phase was stripped of water droplets by pipetting the upper layer after centrifugation at 12,000xg. The remaining moisture was removed by addition of 4gm anhydrous sodium sulphate (Na_2SO_4) and evaporated to dryness in a rotary evaporator and concentrated in a rotary evaporator to around 2ml. The samples were then filtered, using 0.45 µm syringe filters.

9.2.4 High performance liquid chromatography (HPLC) analysis

Followed by the extraction with ethyl acetate, the samples were filtered through 0.2mm syringe filter and analyzed in a high performance liquid chromatography. HPLC (PerkinElmer) with the C18 reverse phase column was used to analyze anthracene under isocratic condition using acetonitrile: water (80:20) (vv^{-1}) as mobile phase and detection wavelength –254 nm. The flow rate of the mobile phase (acetonitrile) was maintained at $1.2mLmin^{-1}$. The samples volume was 2mL in each vial of HPLC tray. Metabolites were studied on the basis of retention time comparison with available authentic standards and literature values.

9.3 RESULTS AND DISCUSSION

High molecular weight PAHs those with four or more aromatic rings have been studied through co-metabolism. Only few studies have observed the improved degradation of recalcitrant PAHs when more labile PAHs are present. Generally, the biodegradation efficiency for all tested PAHs compounds follow the same trend in either single or mixed substrate cultures which can be ranked in the following decreasing order; Nap > Ace > Phe > Pyr > Ant. This observation is in agreement with other reports where low molecular weights PAHs are more biodegradable than high molecular weights PAHs. The molecular weight, water solubility and lipophobicity of a compound would affect its bioaccumulation and degradation by microorganisms. PAHs with low molecular weights such as 2-rings, naphthalene and 3- rings, phenanthrene and anthracene are more liable to bacterial degradation and more extensive than high molecular weights PAHs having more than 3-rings, pyrene [Supakaa *et al.* 2001; Hong *et al.* 2008; Yu *et al.* 2005].

9.4 Co-metabolism of high molecular weight PAHs

The growth of isolates B_4, B_{30} and B_{36} growth OD values are shown in Fig. 9.1.

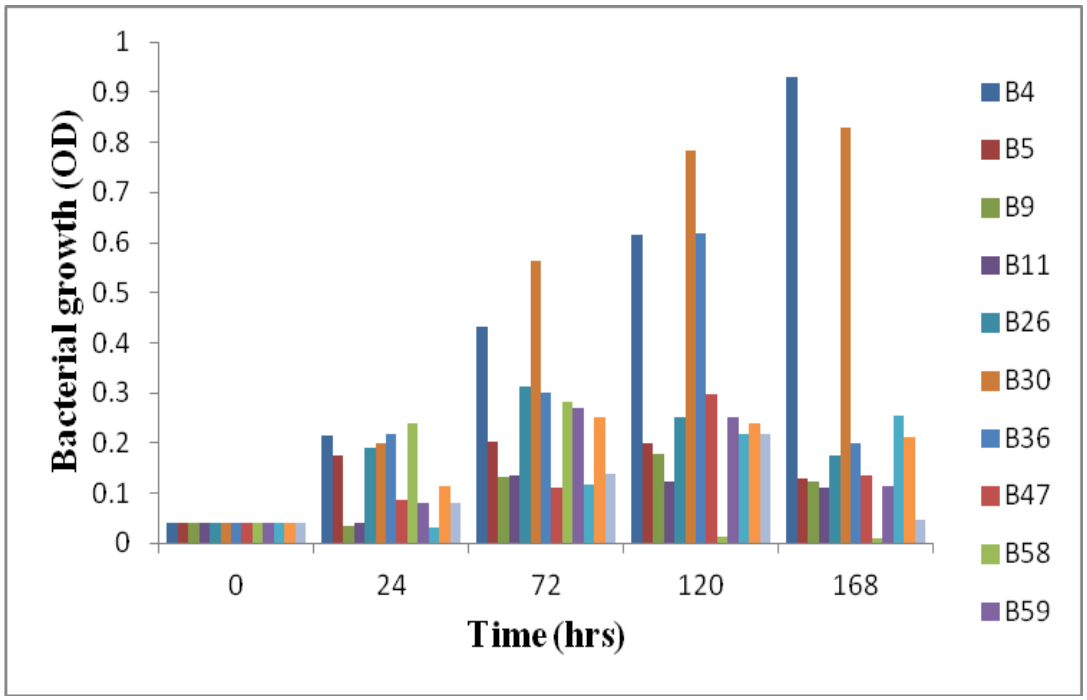

Fig. 9.1: Co-metabolic degradation of mixture of five PAHs by B-isolates

The growth of S-isolates on mixed PAHs as shown in Fig.9.2. Three isolates S_{13}, S_{21} and S_{51} were best with highest OD values.

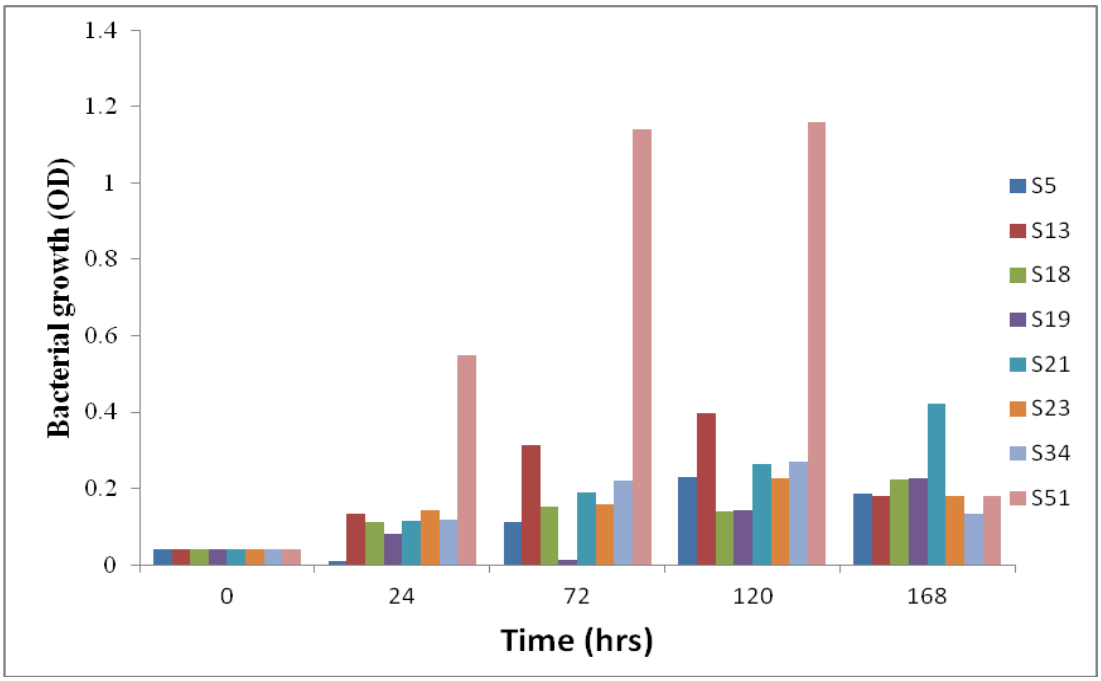

Fig. 9.2: Co-metabolic degradation of mixture of five PAHs by S-isolates

Results of growth of four isolates from mulberry are shown in Fig. 9.4. M_3 was selected for further studies.

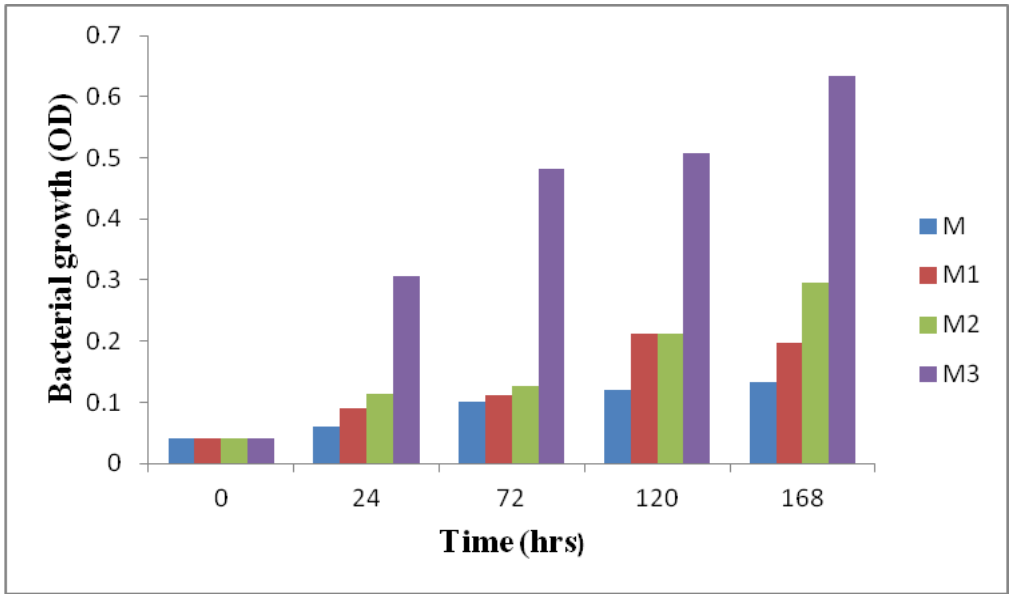

Fig.9.3: Co-metabolic degradation of mixture of five PAHs by M-isolates

Previously reported studies on naphthalene, was100% degraded to about after one week of incubation in a mixed culture. Where, biodegradation % for each substrate in a mixture is lower than that in single substrate culture except for naphthalene which shows no significant difference. The average biodegradation % recorded nearly 90% for PAHs in single culture after 4 weeks, respectively [Wei *et al.* 2009]. Studies usually have shown that compound to degrade at faster rates than those with high molecular weights. The biodegradability primarily depends on the complexity of chemical structures and physico-chemical properties of PAHs [Bamforth & Singleton, 2005]. When mixture of five PAHs was present in aqueous solution, the degradation rates were delayed for phenanthrene, and decreased for anthracene and pyrene [Shafee *et al.* 2006; Lily *et al.* 2013].

Isolates from sewage water were tested against PAHs mixture is shown in Fig.9.4. W_2 was studied further for co-metabolism.

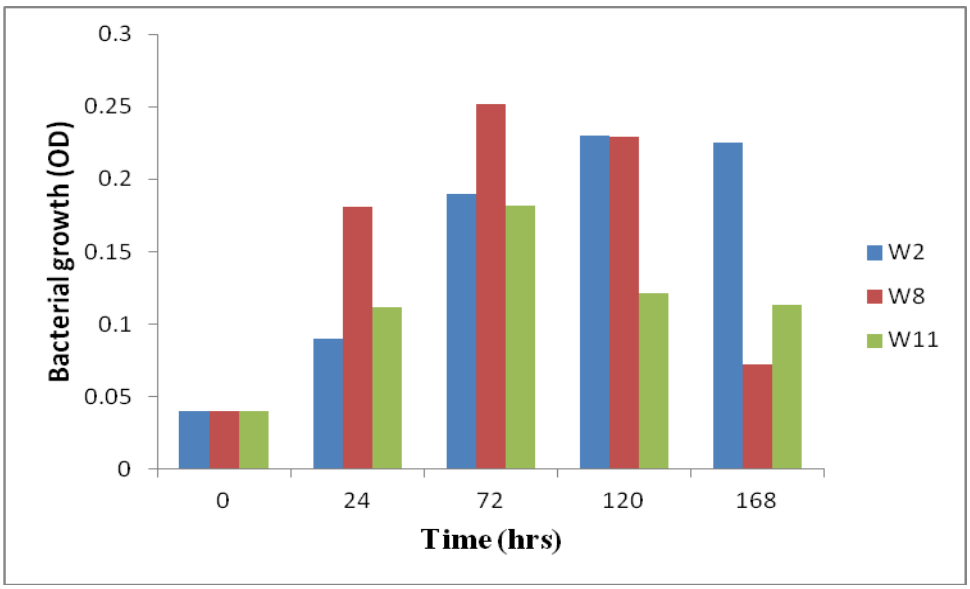

Fig. 9.4: Co-metabolic degradation of mixture of five PAHs by W-isolates

9.4.1 Co-metabolic degradation by isolates isolated from an uncontaminated soil

Results of co-metabolic biodegradation of PAHs in mixture (naphthalene, acenaphthene, phenanthrene, anthracene and pyrene) by bacterial isolates B_4, B_{30} and B_{36} from agriculture field are given as under:

9.4.1.1 Co-metabolism metabolites of B_4

Growth of isolate B_4, when, exposed to a PAHs mixture there was alinear increase in bacterial growth as shown in Fig. 9.5. After 72 hours of incubation 76.70% phenanthrene was disappeared and two sharp peaks with 31.42 % and 65.01% were observed. The visible yellow-orange metabolites obtained indicate the accumulation of certain metabolites from naphthalene, dibenzofuran, fluorene, and anthracene while no activities were observed in case of fluoranthene and pyrene [Supakaa *et al.* 2001].

Naphthalene and acenaphthene were completely disappeared in 24 hours and degraded a sharp peak of 81.40% salicylic acid was identified. These results are in line with that reported for *Bacillus sp. on* naphthalene, phenanthrene, anthracene and pyrene [Simarro *et al.* 2013].

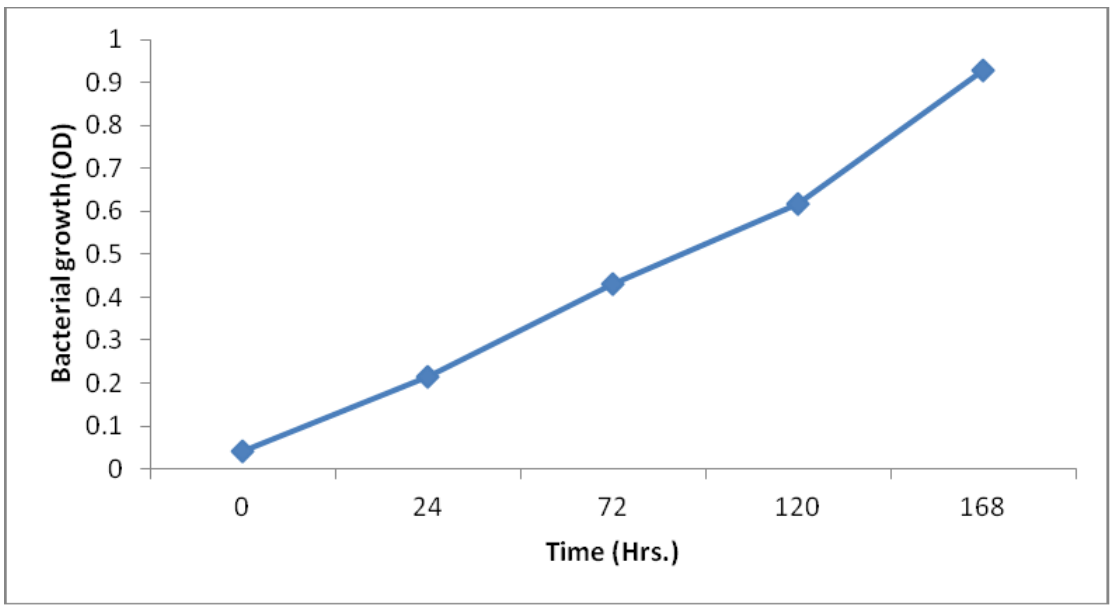

Fig. 9.5: Growth of B_4 on mixture of five PAHs

Six PAHs napthalene, acenapthylene, acenapthene, fluorene, phenanthrene and anthracene in mixtures using PAHs degradative bacteria isolated from single PAHs as substrate [Othman *et al.* 2010]. When mixture of five PAHs was present in aqueous solution, the degradation rates were delayed for phenanthrene, and decreased for anthracene, fluorene, and pyrene [Shafee *et al.* 2006, Lily *et al.* 2013]. On comparison with authentic standards and available literature identified these peaks as phthalic acid and catechol as shown in Table.9.1.

Table 9.1: HPLC metabolites formed during co-metabolism by isolate B_4

Time in Hours	HPLC Metabolites
24	Salicylic acid 81.40%
72	Phthalic acid 631.42 % and catechol 65.01%
120	Benzocaumarin 57.64% and Salicylaldehyde 32.54%
168	Catechol 86.81 % and 18.90% unidentified anthracene and pyrene metabolites

After 120 hours two other peaks, with different retention time and degradation % of 57.64 and 32.54 was observed and identified as 6, 7-benzo-caumarine and salicylaldehyde. When the culture was extracted after 144 hours of incubation an increased amount of catechol 86.81 % and 18.90% anthracene and previously reported anthracene and pyrene metabolites (Fig.9.6).

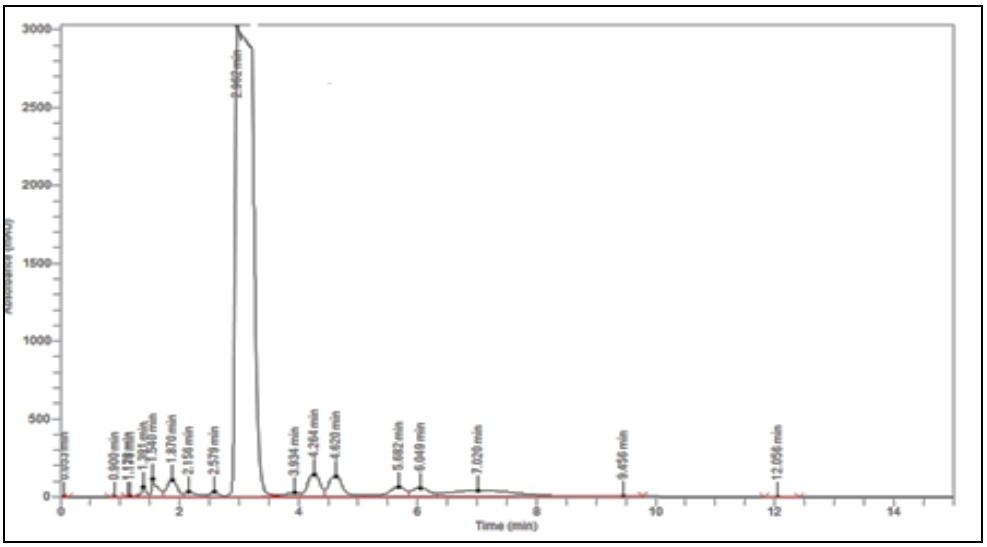

Fig. 9.6: HPLC chromatogram of co-metabolism by isolate B_4 after 144 hrs

It can be comparable with the previous work that phenanthrene when bio transformed through 2-hydroxybenzoic acid to salicylic acid and then to catechol [Mallick *et al.* 2007].

9.4.1.2 Co-metabolic degradation by isolate B_{30}

Growth pattern of isolate B_{30} on PAHs mixture is shown in Fig.9.7.

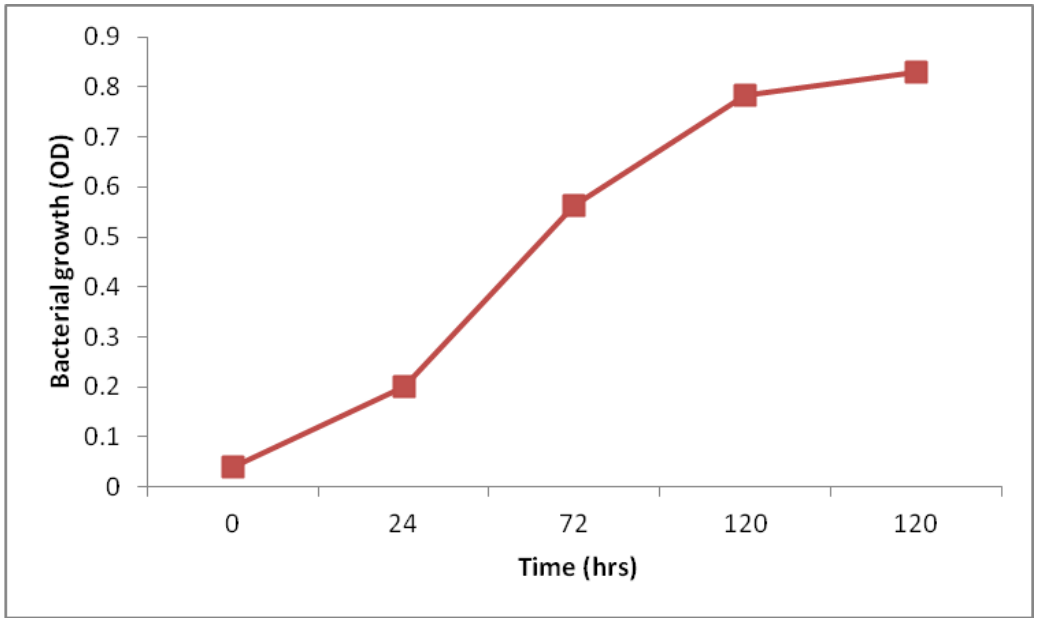

Fig. 9.7: Growth of B_{30} on mixture of five PAHs

The metabolites formed as shown in Table.9.2. After 24 hours a single sharp peak with 92.04% was observed identified as salicylic acid. After 72 hours there was decrease in salicylic acid (85.46%) and formation of phthalic acid (30.86%), catechol (64.51%) and traces of 1-methoxy-2 hydroxyanthracene and anthracene metabolites.

Table 9.2: HPLC metabolites formed during co-metabolism by isolate B_{30}

Time in Hours	HPLC Metabolites
24	Salicylic acid 92.04%
72	Phthalic acid 30.86% and catechol 65.51%, traces of 1-methoxy-2 hydroxyanthracene and anthracene metabolites
120	Salicylaldehyde 31.35%
168	63.93% catechol, 24.69% pyrene metabolite, gentisic acid 8.92%

After 120 hours 31.35% salicylaldehyde was formed and decrease in catechol percentage. Phthalic is reported metabolite of naphthalene [Arulazhagn *et al.* 2010]. On 7th day 63.93% catechol, 24.69% and pyrene metabolite of 8.92% and trace amount of gentisic acid was recovered (Fig.9.8).

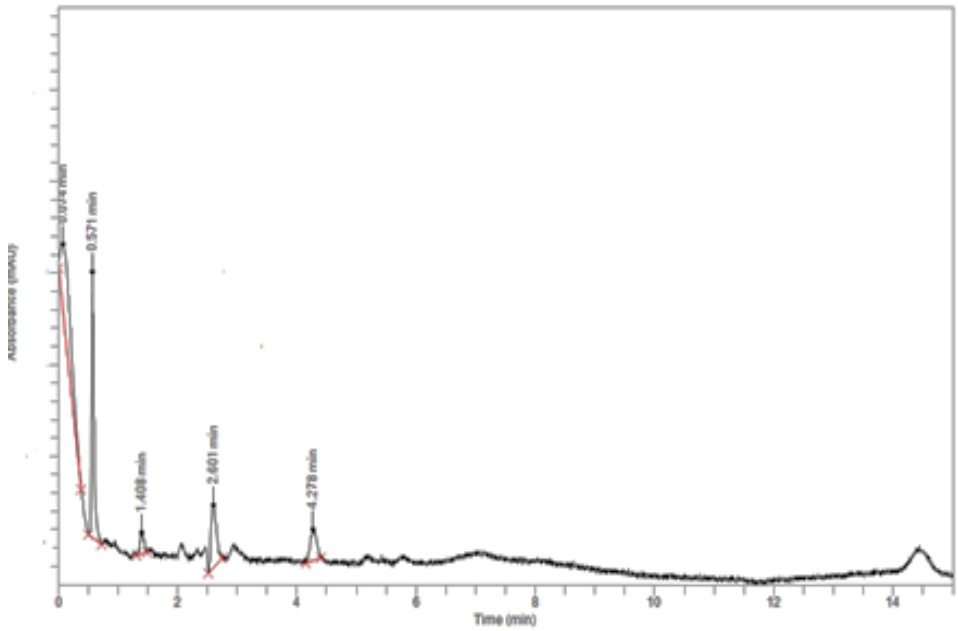

Fig. 9.8: HPLC chromatogram of co-metabolism by isolate B_{30} after 168 hrs

The latter is a metabolite of naphthalene degradation via salicylic acid and of phenanthrene when degraded through naphthalene pathway. This isolate was unable to degrade anthracene and pyrene individually like that of the ability of previously reported in degrading four-ring PAHs, fluoranthene and pyrene, to serve as sole carbon and energy source for growth was examined. Neither anthracene nor pyrene could be used by the bacterium as no growth was observed.

To induce its ability to degrade these compounds, naphthalene, acenaphthene and phenanthrene was added to anthracene and pyrene-containing media. Significant concentrations of fluoranthene and pyrene were decreased over seven days of incubation. It was also observed that degradation of the four-ring PAHs took place rapidly when phenanthrene was present in the culture medium but ceased immediately when phenanthrene was completely degraded [Othman *et al.* 2010]. Bacteria can grow on one aromatic substrate to transform other aromatic substrates as well cause competitive inhibition of phenanthrene degradation by naphthalene-1-

methylnaphthalene, 2-methylnaphthalene, and fluorene [Stringfellow & Aitken, 1995].

9.4.1.3 Co-metabolic degradation by isolate B_{36}

Isolate B_{36} growth was faster between 72 and 168 hours of incubation as shown in Fig.9.9.

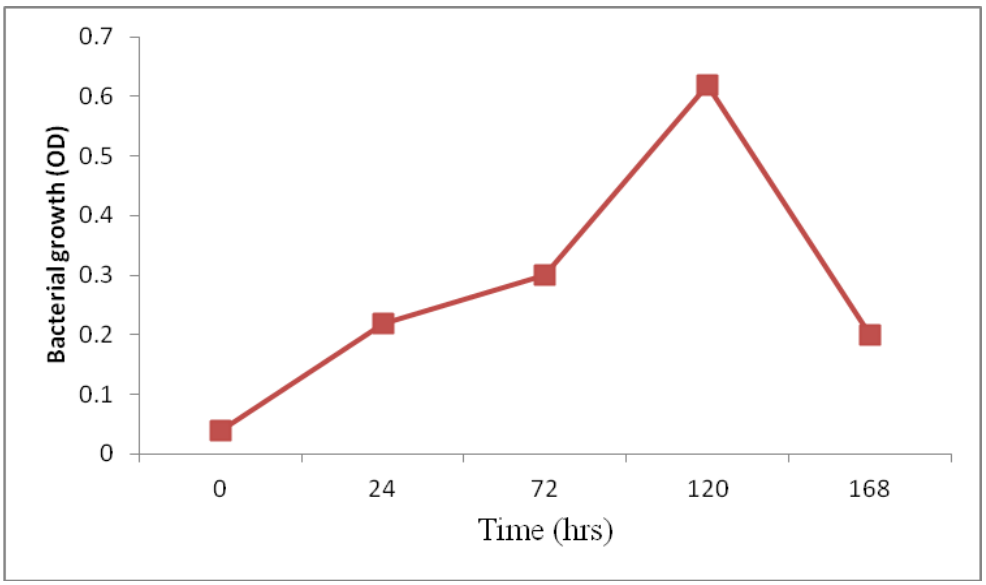

Fig. 9.9: Growth of B_{36} on mixture of five PAHs

B_{36} Isolate co-mtabolically degraded PAHs mixture, results are shown in Table.9.3.

Table 9.3: HPLC metabolites formed during co-metabolism by isolate B_{36}

Time in Hours	HPLC Metabolites
24	Salicylic acid 87.61%
72	phenanthrene carboxylic acid, 25.01% 1-methoxy-2 hydroxyanthracene, 64.43%
120	Catechol 31.13%, 1-methoxy-2 hydroxyanthracene 64.86%, Phenanthrene 31.33% and traces of naphthalene
168	6, 7 benzo-caumarine 63.71%, Salicylaldehyde 31.18% and catechol 61.93%

After 24 hours of incubation the formation of salicylic acid with a peak area of 87.61% was observed. Subsequently, 72 hours metabolites were identified to be phenanthrene carboxylic acid, 1-methoxy-2 hydroxyanthracene, (25.01%) and 64.43% pyrene was recovered. 31.13% catechol and 1-methoxy-2 hydroxyanthracene (64.86%) and traces of naphthalene was recovered. 31.33% of phenanthrene and 6,7 benzo-caumarine 63.71% was obtained, after 120 hours [Mallick *et al.* 2007; Roy *et al.* 2013]. Salicylaldehyde (31.18%) and catechol (61.93%) were observed after seven days of incubation as shown in Fig. 9.10.

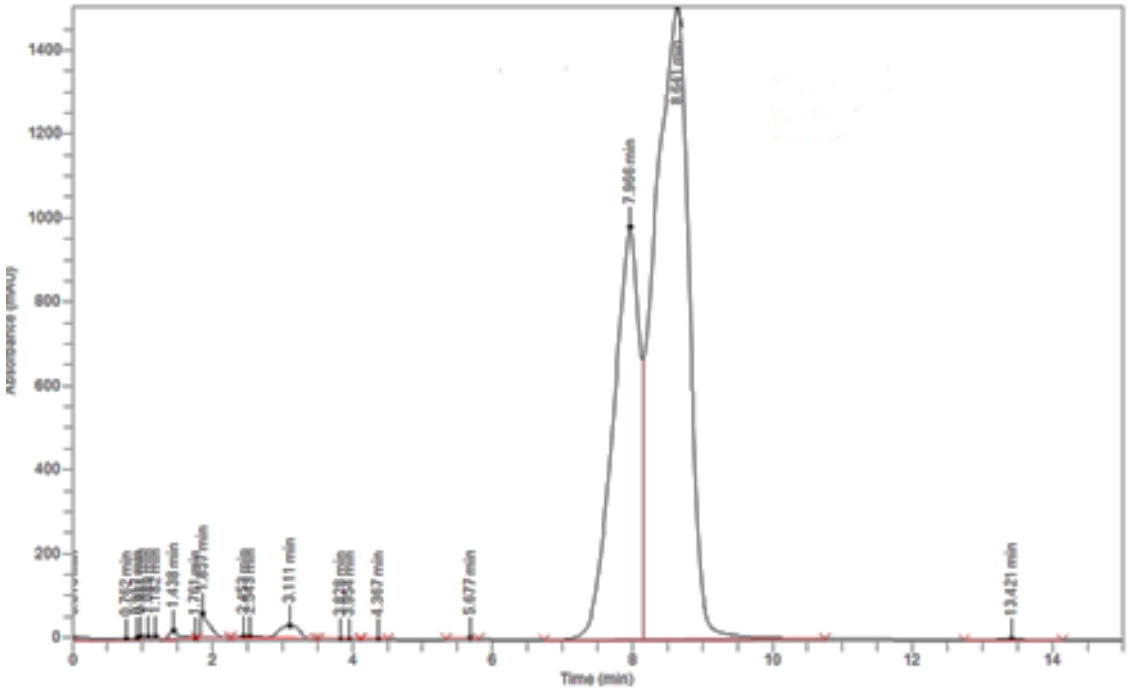

Fig. 9.10: HPLC chromatogram of co-metabolism by isolate B_{36} after 168 hrs

Combined treatment was repeated for individual anthracene and for the PAH mixture with *Sphingomonas* sp. on the PAH mixtures produced 9, 10-anthracenedione. Biodegradation of PAHs was enhanced in artificial mixtures containing 9, 10-anthracenedione [Lehto *et al.* 2003].

9.4.2 Co-metabolic degradation by isolates isolated from contaminated soil and sewage water

The microbes of contaminated areas are more adapted to biodegradation due to previous exposure. This can be attributed to the presence of mobile genetic elements that constantly gone through mutation and bring resistant genes through horizontal gene transfer [Nnamchi *et al.* 2006; Al-Thani *et al.* 2009; Sho *et al.* 2004, Obayori & Salam, 2010; Li *et al.* 2007].

9.4.2.1 Co-metabolic degradation by isolate S_{13}

S_{13} growth was faster between 4th and 5th day of incubation as shown in Fig. 9.11. After a few hour of incubation with PAHs mixture was S_{13} transformed anthracene to its metabolite 85.75% as reported by Neelofur *et al.* (2014) and salicylaldehyde by comparison with standard peak.

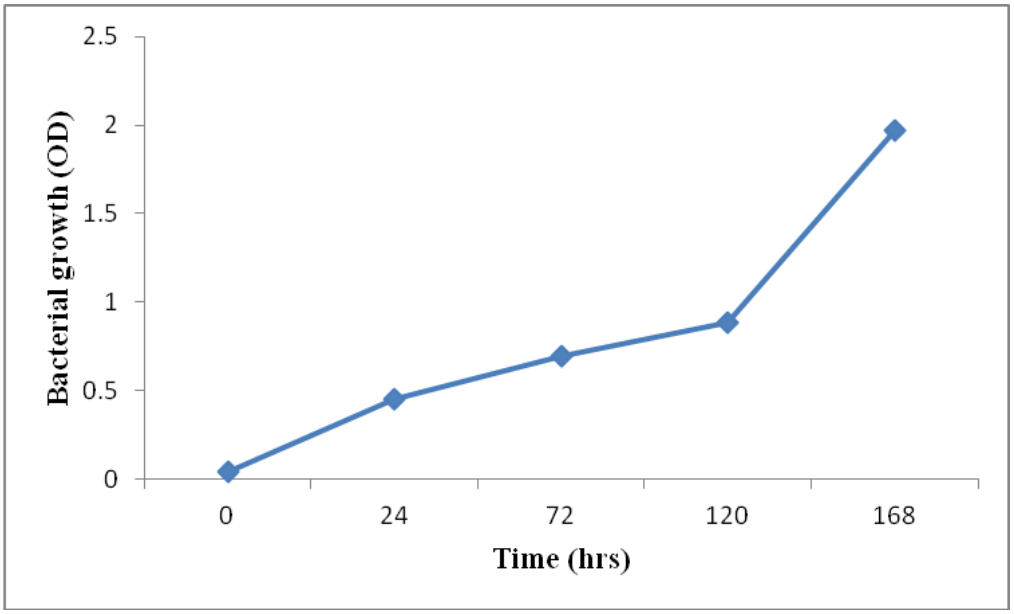

Fig. 9.11: Growth of S_{13} on mixture of five PAHs

After 24 hours salicylaldehyde 86.47% and phenanthrene dicarboxylic acid (5.17%) were extracted. After 72 hours catechols (30.85%) and 6, 7 benzo-caumarin was identified. Further incubation for 48 hours have no effect on the catechol and phthalic acid. This pathway was similar to that proposed by Liang *et al.* (2006) for biodegradation of Pyrene by *Mycobacterium* sp. Oxidation of 4-phenanthroic acid by

ring-hydroxylating dioxygenase produces 3, 4-phenanthrene dihydrodiol-4-carboxylic acid, which is further transformed to 3, 4-dihydroxyphenanthrene by dehydrogenase/decarboxylase. Once 3, 4-dihydroxyphenanthrene is formed, it enters the phenanthrene degradation pathway [Krivobok *et al.* 2003].

After 120 hours phthalic acid 32.16% and catechols 64.34% were obtained as shown in Table.9.4.

Table 9.4: HPLC metabolites formed during co-metabolism by isolate S_{13}

Time in Hours	HPLC Metabolites
24	Salicylaldehyde 86.47% and phenanthrene dicarboxylic acid 5.17%
72	Catechol 30.85% and 6, 7 benzo-caumarin
120	Phthalic acid 32.16% and catechol 64.34%
168	Phthalic acid 32.16% and catechol 64.34%

1-Hydroxy-2-naphthoic acid and then mineralized 1, 2-dihydroxy naphthalene, which is metabolized via salicylic acid and phthalic acid? The quinone, pyrene-4, 5-dione can be also formed following the non-enzymatic autoxidation of 4, 5-dihydroxypyrene [Kim *et al.* 2004; Liang *et al.* 2006; Nour *et al.* 2010]. After 72 hours of incubation the compound identified were 6, 7-benzocaumarin and 1-methoxy-2-hydroxyanthracene of 31.34% and 65.55% after 120 hours 31.07% phthalic acid and 63.05% catechol which on further degradation of 48 hours transformed to salicylaldehyde. The results of our study are in line with that of *B. thermoleovorans* which produce, phthalic acid, a second transformation product with 2-carboxy functionality was identified. When, anthracene degradation occurs through phthalic acid which may arise from degradation of 2-carboxycinnamic acid. A subsequent decarboxylation reaction would provide benzoic acid [Annweiler *et al.* 2000].

9.4.2.2 Co-metabolic degradation by isolate S_{21}

Isolate S_{21} growth was slower in first 24 hours and increase between 48 and 72 hours of incubation and a very sharp increase between 6th and 7th day as shown in Fig. 9.12. S_{21} after 24 hours of incubation salicylic acid (81.04%), salicylaldehyde (1.77%) and acenaphthene (4.28%) were extracted, salicylic acid (90.18%) and traces of anthracene metabolites were observed after 96 hours.

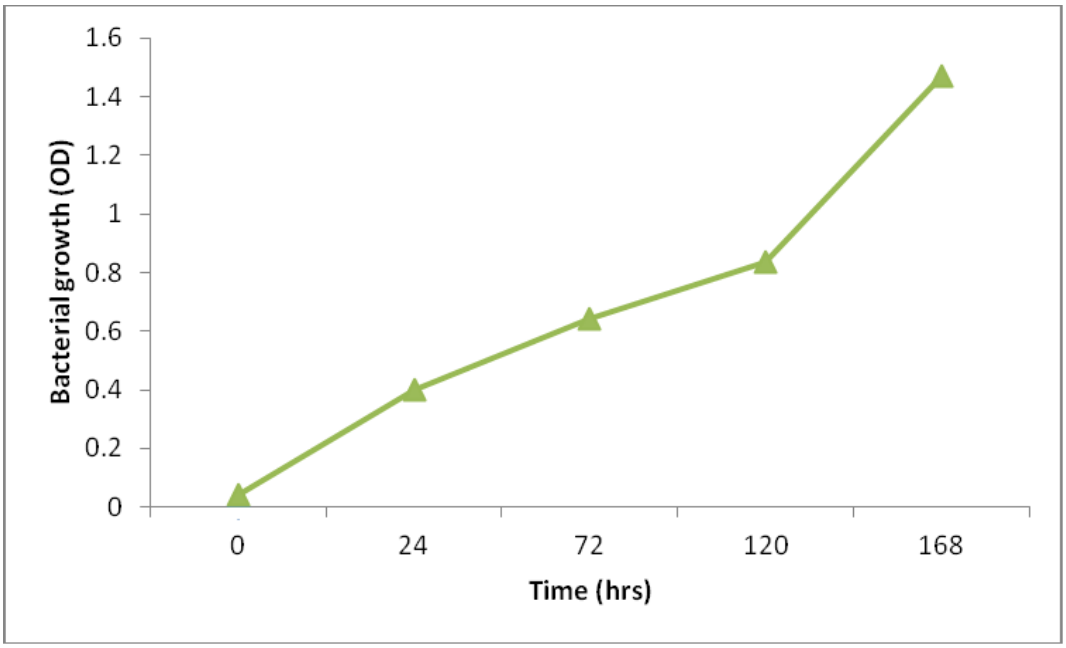

Fig. 9.12: Growth of S_{21} on mixture of five PAHs

The latter two compounds remain unchanged on further incubation for 48 hours as shown in Table.9.5.

Table 9.5: HPLC metabolites formed during co-metabolism by isolate S_{21}

Time in Hours	HPLC Metabolites
24	Salicylic acid 81.04%, salicylaldehyde 1.77% and acenaphthene 4.28%
72	salicylic acid 90.18% and traces of anthracene metabolites
120	1, 2- dihydroxy-anthracene 29.75%, pyrenequinone 65.63%
168	Catechol 64.37% and salicylaldehyde 31.70%

After 120 hours 1, 2-dihydroxy-anthracene (29.75%), pyrenequinone (65.63%), catechols (64.37%) and (31.70%) salicylaldehyde were formed (Fig. 9.13).

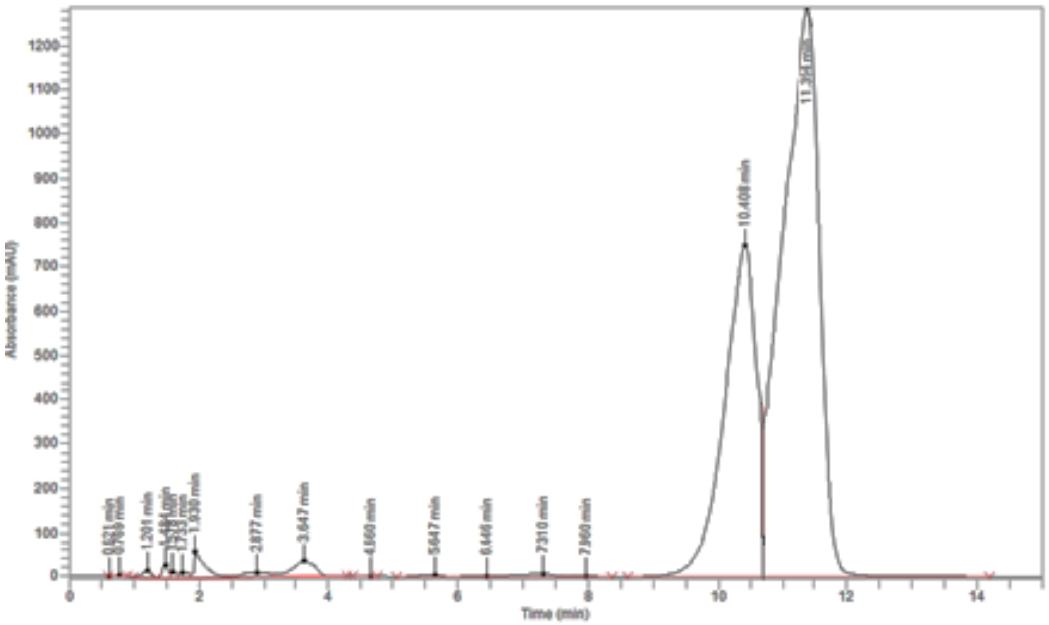

Fig. 9.13: HPLC chromatogram of co-metabolism bacterial isolate S_{21} after 168 hours

9.4.2.3 Co-metabolic degradation by isolate S_{51}

Growth of isolates S_{51} was faster after 24 hours and was increasing upto 7th day of incubation as shown in Fig. 9.14.

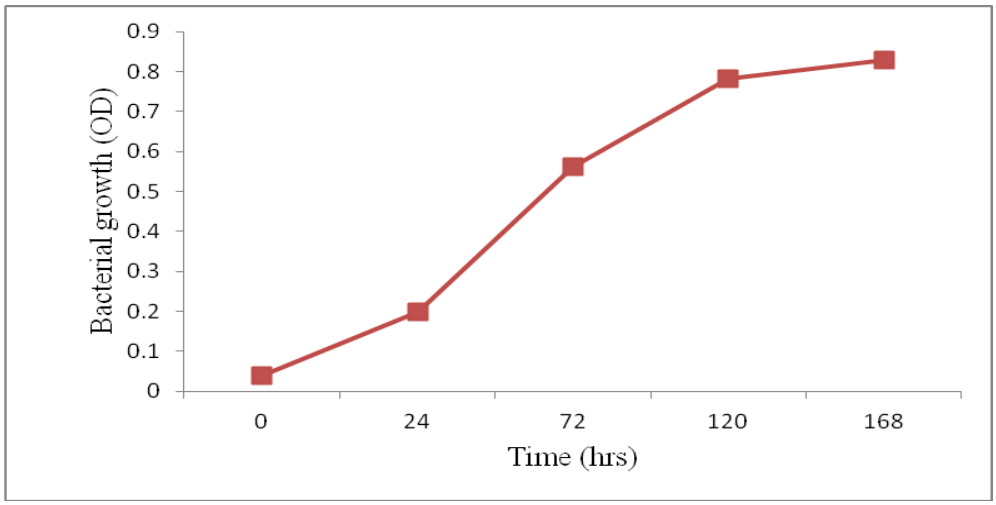

Fig. 9.14: Growth of S_{51} on mixture of five PAHs

Isolate S_{51} formed two sharp peaks (Fig. 9.15) identified on comparison to be salicylaldehyde and catechol.

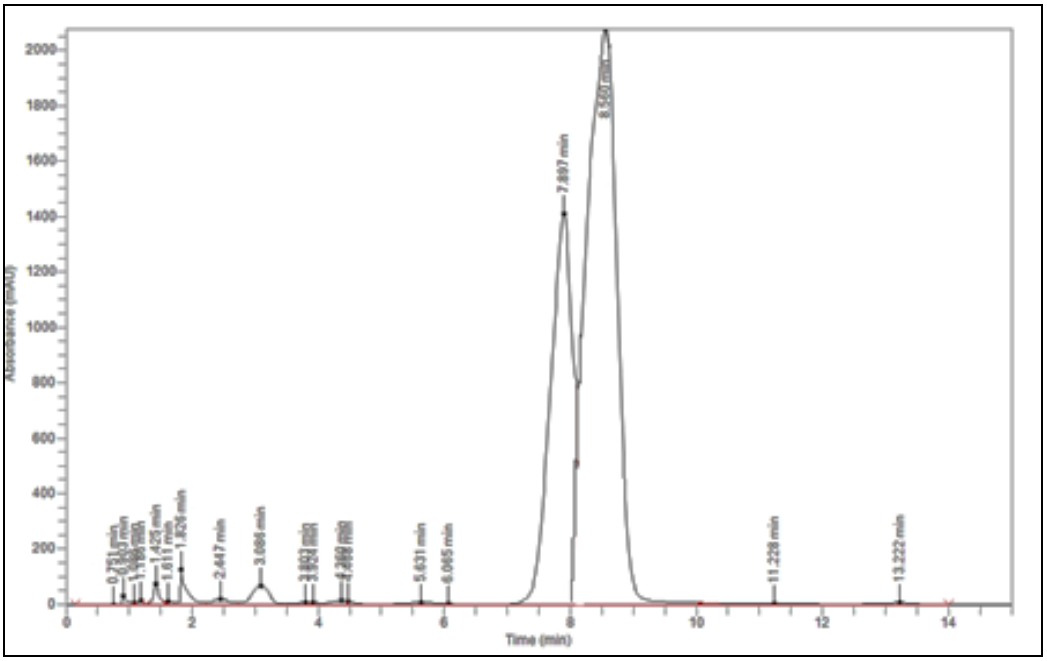

Fig. 9.15: HPLC chromatogram of co-metabolism by isolate S_{51} after 168 hrs

Catechols (63.94%) and (31.43%) salicylaldehyde and anthracene traces after 168 hours as shown in Table.9.6.

Table 9.6: HPLC metabolites formed during co-metabolism by isolate S_{51}

Time in Hours	HPLC Metabolites
24	Salicylic acid 81.04%
72	Salicylic acid 90.18%
120	Catechol 63.94% and salicylaldehyde 31.43% and anthracene traces
168	2, hydroxylmuconic semialdehyde

As reported earlier the orange colour is due to formation of 2, hydroxylmuconic semialdehyde, meta-cleavage of catechols. Oxidation of phenanthrene and anthracene to the corresponding hydroxynaphthoic acids occurred

quantitatively. The isolate converted phenanthrene; anthracene, fluoranthene and carbazole of coal tar pitch extract [Baboshin *et al.* 2008].

9.4.2.4 Co-metabolic degradation by isolate M_3

Growth and co-metabolic degradation products of isolate M_3 is shown in Fig.9.16.

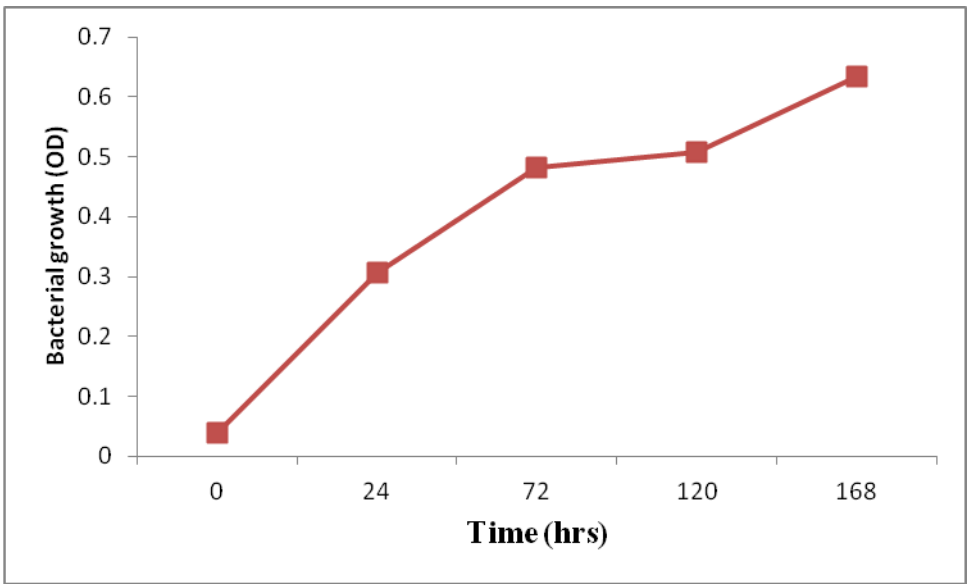

Fig. 9.16: Growth of M_3 on mixture of five PAHs

The metabolites formed during co-metabolism by isolate M3 are listed in Table.9.7.

Table 9.7: HPLC metabolites formed during co-metabolism by isolate M3

Time in Hours	HPLC Metabolites
24	Salicylic acid 87.01%, Salicylaldehyde 1.43%, Phenanthrene-1,8 dicarboxylic acid 3.92%
72	6, 7-benzocaumarin 31.34% 1-methoxy-2-hydroxyanthracene 65.55%
120	Phthalic acid 32.03%, catechol 63.77%
168	Benzocaumarin 93.13%

After 24 hours of incubation a mixture of salicylic acid salicylaldehyde and phenanthrene dicarboxylic acid was observed with 87.01, 1.43 and 3.92% respectively.

9.4.2.5 Co-metabolic degradation by isolate W_2

Isolate W_2 growth was slow in first 72 hours of incubation and an increased time dependent growth pattern as shown in Fig.9.17.

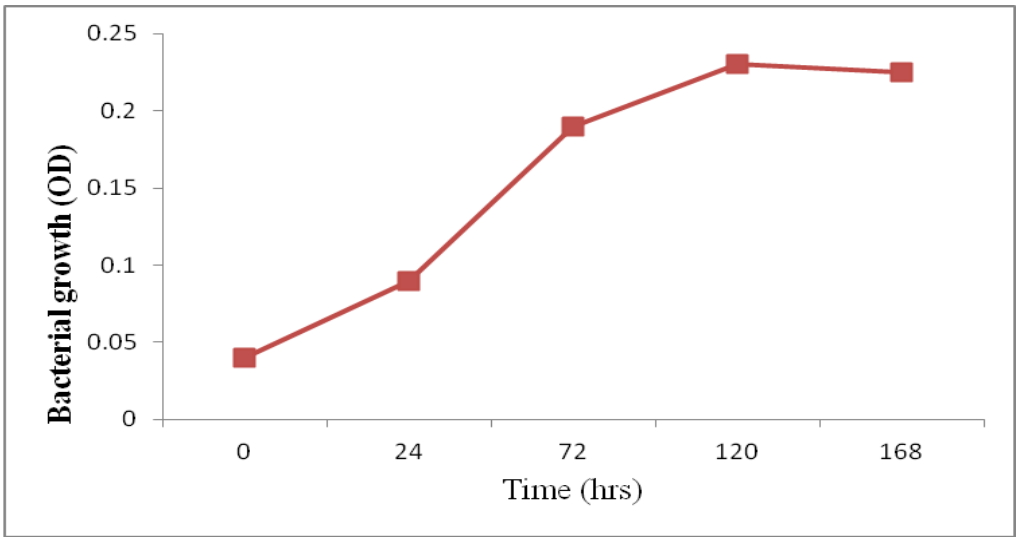

Fig. 9.17: Growth of W_2 on mixture of five PAHs

The list of metabolites formed by isolate W_2 is summarized in Table.9.8. This isolate, formed pyrene-dihydrodiol (65.91%) and 1-methoxy-2, hydroxyanthracene (29.73%), after 48 hours of incubation as previously reported metabolite of anthracene [Neelofur *et al.* 2014].

Table 9.8: HPLC metabolites formed during co-metabolism by isolate W_2

Time in Hours	HPLC Metabolites
24	Pyrenedihydrodiol 65.91% and 1-methoxy-2,hydroxyanthracene 29.73%
72	Catechol 64.05% and phthalic acids 31.44%
120	Benzocaumarin 32.89% and 1-hydroxy2-naphthoic acid 63.37% Phthalic acid 32.03% and catechol 63.77%
168	Benzocaumarin 93.13% and traces of pyrene metabolites

Previously reported work, on phenanthrene and naphthalene degradation was carried out completely in 72 hours. After 120 hours 1, hydroxy-2-napthoic acid, 1, 2-di-hydroxyanthracene, pyrenequinone which are the oxidation products of the LMW PAHs characteristics of *Bacillus* sp. After 96 hours catechols and phthalic acids were formed (64.05%) and (31.44%). after 120 hours benzocaumarin (32.89%) and 1-hydroxy2- naphthoic acid (63.37%) were formed. Phthalic acid (32.03%) and catechol (63.77%) was obtained after 120 hours (Fig.9.18).

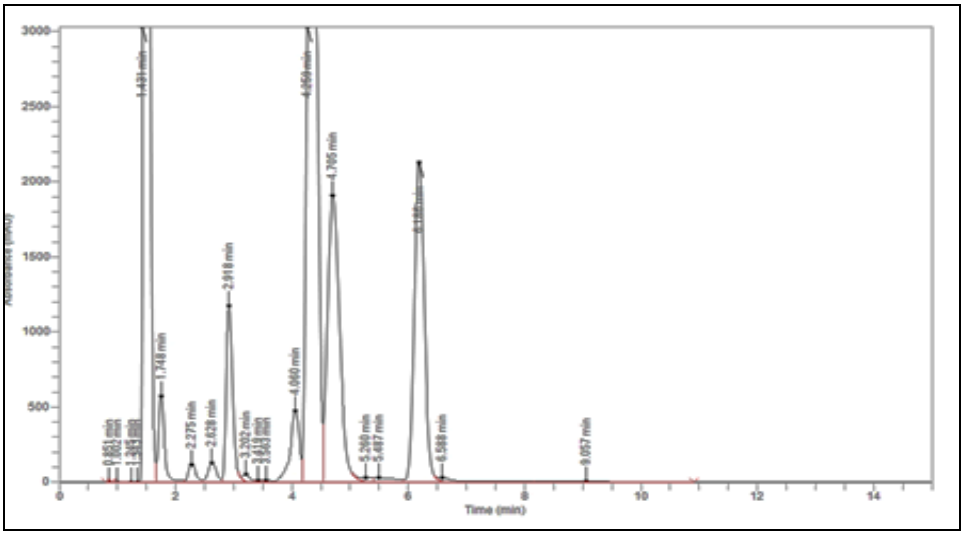

Fig. 9.18: HPLC chromatogram of co-metabolism by isolate W_2 after 24 hrs

Pseudomonas from municipal wastes capable of degrading phenanthrene as sole sources of carbon and energy, was characterized as *Sphingobium* sp., and the metabolic pathways involved in the degradation of phenanthrene was reported earlier [Joner & Leyval, 2003]. During the degradation of the PAHs mixture the compounds identified were pyrene dihydrodiol and methoxy-2-hydroxyanthracene. Phthalic acid and catechol which is oxidation product of phenanthrene and anthracene and naphthalene were identified. This shows that the further incubation products benzocaumarin, salicylaldehyde and phathalic acid and catechols recovered may be produced from pyrene after 168 hours [Roy *et al.* 2013]. In this study the production of benzocaumarin (93.13%) and traces of pyrene metabolites were detected which are already reported from a no of studies on co-metabolism [Fu-min *et al.* 1993; Mallick *et al.* 2007; Garcia *et al.* 2004; Rodrigo *et al.* 2005; Herwijnen *et al.* 2003].

CONCLUSION

It can be concluded that isolation through simple serial dilution results in obtaining more efficient wild PAHs degrader than those isolated through classical enrichment process

The bacterial isolates isolated from previously exposed sites to PAHs were more adopted to degrade these under lab conditions

Isolates were able to assimilate PAHs on solid media and produce yellow coloration which is an indication of the meta-clevage of catechol (oxidation product of PAHs)

Isolates from petroleum contaminated sites were more adapted to degradation under both weak acidic and basic condition

Some isolates of *Bacillus* can only degrade HMW PAHs like pyrene while unable to degrade the simplest LMW such as naphthalene

The isolates which fail to degrade some PAHs were degraded more efficiently during co-metabolic degradation

Some isolates after physical mutation lose the ability to degrade a certain compound like Anthracene degrading ability was lost by B_{30} after exposure to UV-light

It can be concluded that some isolates found in temperate regions also have the ability to grow at temperature range that fall in psychrophile range (B_{36}) growth at 4°C and thermophilic range (M_3) at 40°C as well.

FUTURE PROSPECTS

1. PAH contaminated environments like soil and sediments can be exploited for other bacterial isolates capable of degrading PAHs.
2. These isolates can also be checked for their ability to biodegrade other organic contaminants like pesticides, TNTs and PCBs etc.
3. These bacterial isolates can be applied in fields for bioremediation of PAHs and other organic compounds.
4. Identification of metabolites from PAHs and to elucidate the complete pathways by selected isolates.
5. An advanced molecular level study to elucidate novel biodegrading enzyme and investigation of genes involved in biodegradation of PAHs.

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ANNEXURE "A"

Table I: CFUmL^{-1} for anthracene biodegradation

Time (Hrs).		0	24	48	72	96	120
CFUmL^{-1}	S_{13}	1.5×10^{-6}	2.9×10^{-11}	3.8×10^{-14}	1.8×10^{-17}	3.2×10^{-20}	1.4×10^{-23}
	B_{36}	1.3×10^{-4}	1.6×10^{-7}	1.8×10^{-10}	2.1×10^{-13}	2.4×10^{-16}	2.7×10^{-18}

Table II: CFUmL^{-1} for phenanthrene biodegradation

Time (Hrs).		0	24	48	72	96	120
CFUmL^{-1}	S_{21}	1.5×10^{-5}	2.9×10^{-13}	3.8×10^{-18}	1.8×10^{-21}	3.2×10^{-23}	1.4×10^{-27}
	B_{30}	1.3×10^{-5}	1.6×10^{-12}	1.8×10^{-15}	2.1×10^{-17}	2.4×10^{-20}	2.7×10^{-23}

Table III: CFUmL^{-1} for naphthalene and acenaphthene degradation

Time (Hrs).		0	24	48	72	96	120
CFUmL^{-1}	B_{30} Naph	1.5×10^{-5}	2.9×10^{-14}	3.8×10^{-20}	1.8×10^{-24}	3.2×10^{-27}	1.4×10^{-32}
	B_{30} Ace	1.3×10^{-5}	1.6×10^{-13}	1.8×10^{-17}	2.1×10^{-22}	2.4×10^{-26}	2.7×10^{-30}

Table IV: CFUmL^{-1} for pyrene biodegradation

Time (Hrs).		0	24	48	72	96	120
CFUmL^{-1}	B_4	1.5×10^{-6}	2.9×10^{-11}	3.8×10^{-14}	1.8×10^{-17}	3.2×10^{-20}	1.4×10^{-23}
	S_{21}	1.3×10^{-4}	1.6×10^{-13}	1.8×10^{-16}	2.1×10^{-18}	2.4×10^{-21}	2.7×10^{-28}
	S_{51}	1.3×10^{-5}	1.6×10^{-12}	1.8×10^{-17}	2.1×10^{-19}	2.4×10^{-24}	2.7×10^{-29}
	M_3	1.3×10^{-6}	1.6×10^{-11}	1.8×10^{-18}	2.1×10^{-23}	2.4×10^{-26}	2.7×10^{-31}

ANNEXURE "B"

Microscopy

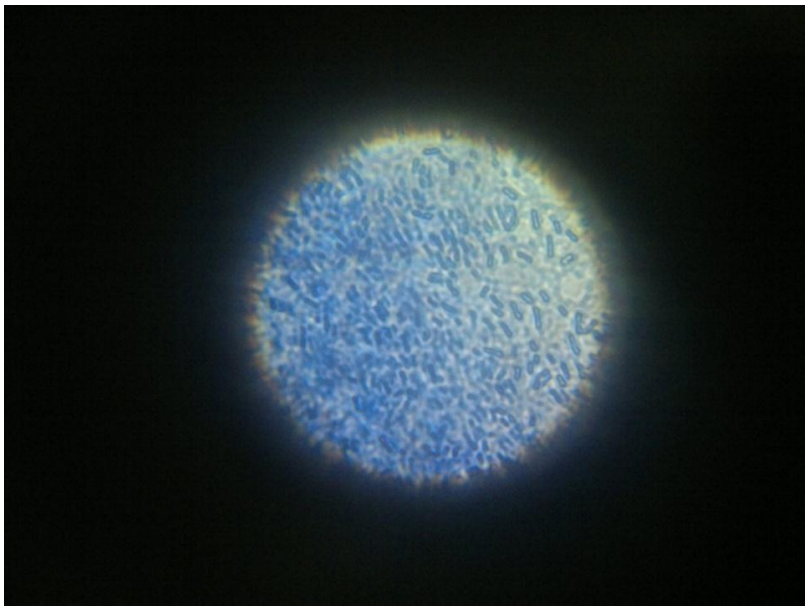

Fig. I: Bacterial isolate S_{13} as seen under oil immersion lens after Gram staining

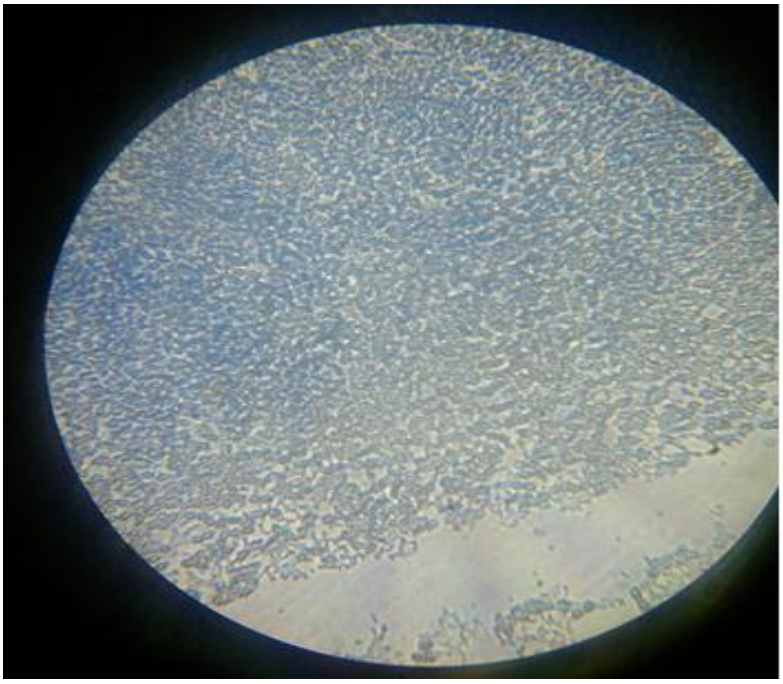

Fig. II: Bacterial isolate B_{36} as seen under oil immersion lens after Gram staining

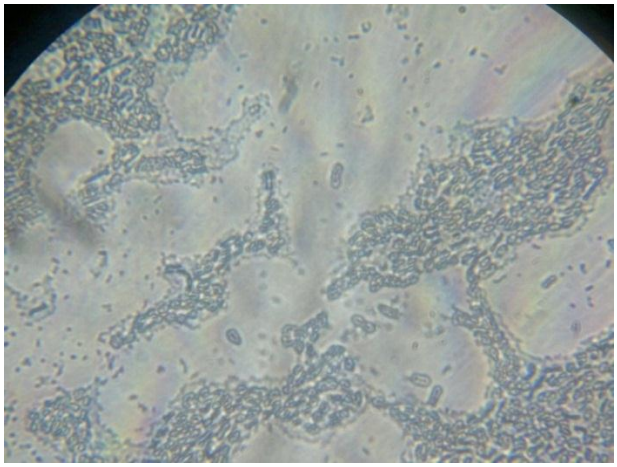

Fig. III: Bacterial isolate B_{30} as seen under oil immersion lens after Gram staining

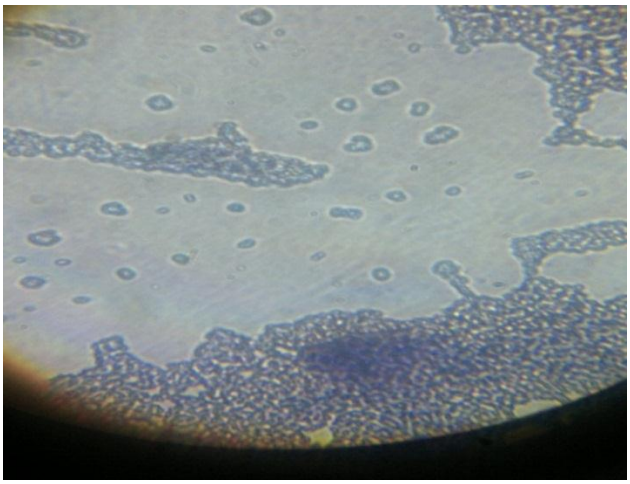

Fig. IV: Bacterial isolate S_{21} as seen under oil immersion lens after Gram staining

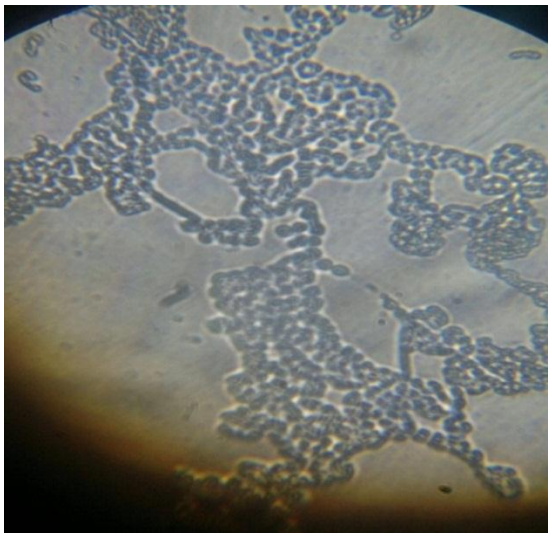

Fig. V: Bacterial isolate M_3 as seen under oil immersion lens after Gram staining

ANNEXURE "C"

Biodegradation experiments

Fig. I: Flasks containing anthracene for biodegradation by isolate S_{13}

Fig. II: Flasks containing naphthalene for biodegradation by isolate B_{30} (5^{th} day)

Fig. III: Flasks containing naphthalene for biodegradation by isolate B_{30} (10^{th} day)

Fig. IV: Flask containing phenanthrene for biodegradation by S_{21} at pH 6, 8 & 9 & B_{30} at pH8 (4^{th} day)

Fig. V: Flask containing phenanthrene for biodegradation by S_{21} at pH 6, 8 9 & B_{30} on phenanthrene at pH 8 on day 3rd day

Fig. V: Flask containing phenanthrene for biodegradation by S_{21} at pH6, 9 (10th Day)

Fig. VI: Flask containing phenanthrene &pyrene for biodegradation by S_{21}

Fig. VII: Flask containing Pyrene for biodegradation by S_{21}, B_4 (10^{th} Day)

Fig. VIII: Extraction of culture filtrate from bacterial isolate S_{51} and B_4 (pyrene) for HPLC analysis

ANNEXURE "D"

Table I: List of Coloured Compounds Produced in Growth Media (PNR liquid media amended with selected PAHs)

Isolate	Substrate	Colour compound
B_{30}	Acenaphthene	creamy appearance
B_{36}	Anthracene	light yellow
S_{13}	Anthracene	creamy in appearance
B_4	Pyrene	light yellow
S_{21}	Pyrene	creamy appearance
S_{51}	pyrene	Orange colour
M_3	Pyrene	creamy appearance

ANNEXURE "E"

GC-MS Analysis of metabolites after 10 days of incubation

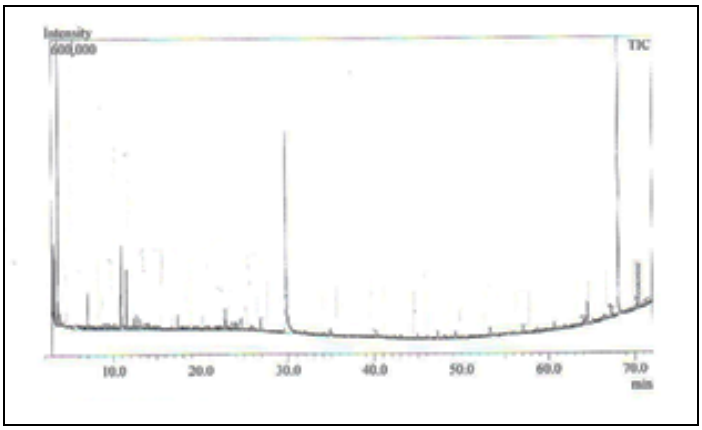

Fig. I: GC-MS spectra for biodegradation of Pyrene by bacterial isolate B_4 after 10 days of incubation

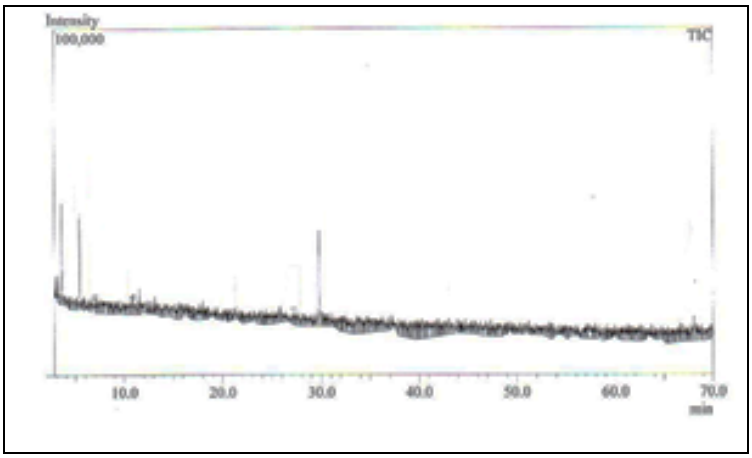

Fig. II: GC-MS spectra for biodegradation of acenaphthene by bacterial isolate B_{30} after 10 days of incubation

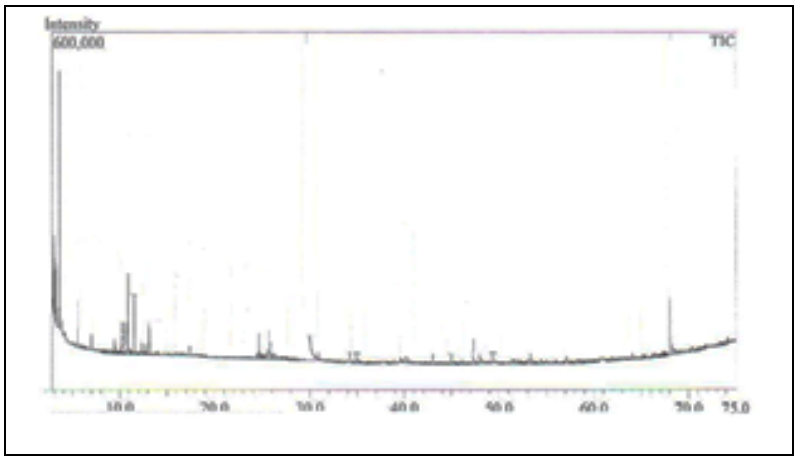

Fig. III: GC-MS spectra for biodegradation of naphthalene by bacterial isolate B_{30} after 10 days of incubation

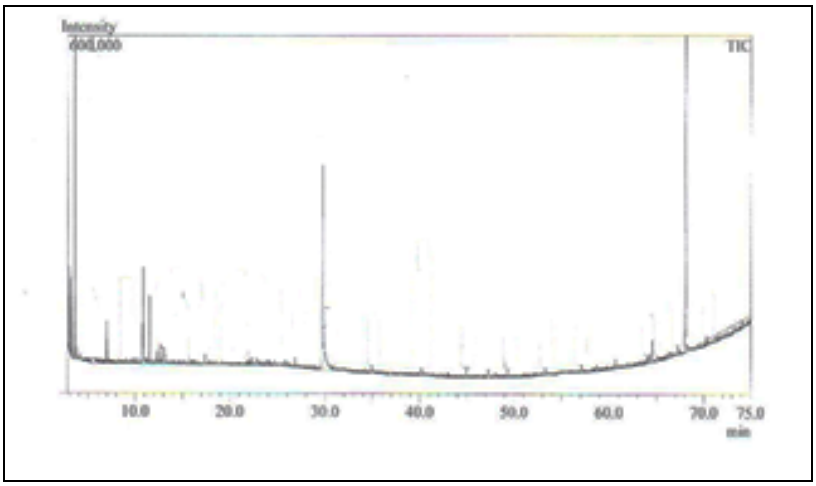

Fig. IV: GC-MS spectra for biodegradation of phenathrene by bacterial isolate B_{30} after 10 days of incubation

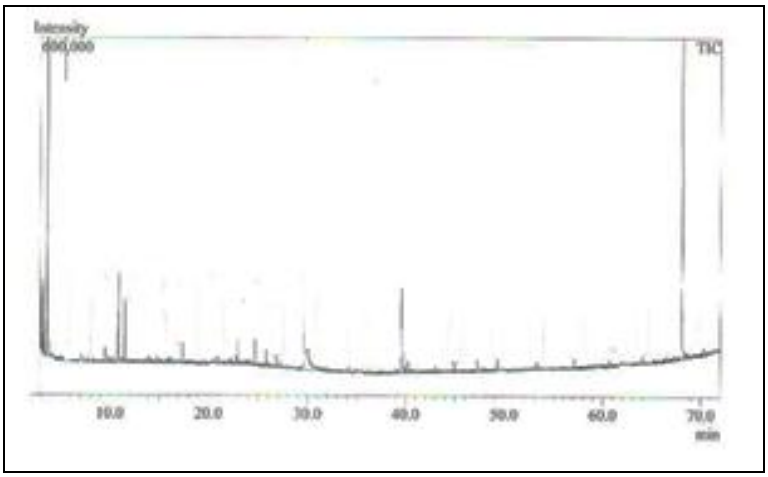

Fig. V: GC-MS spectra for biodegradation of phenanthrene bacterial isolate S_{21} after 10 days of incubation

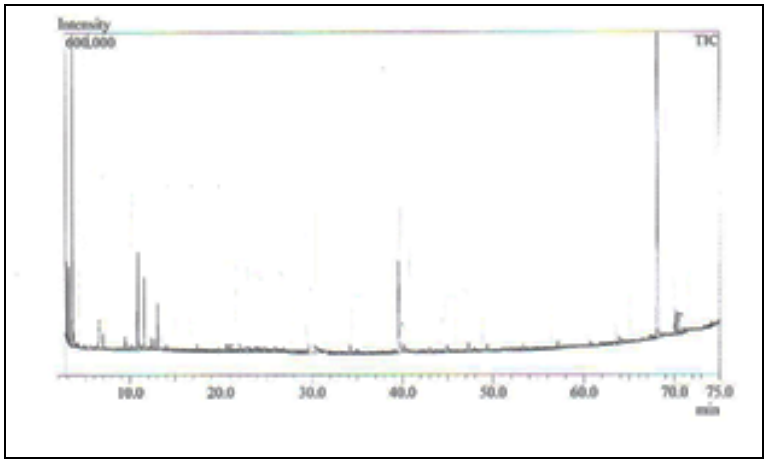

Fig. VI: GC-MS spectra for biodegradation of anthracene by bacterial isolate B_{36} after 10 days of incubation

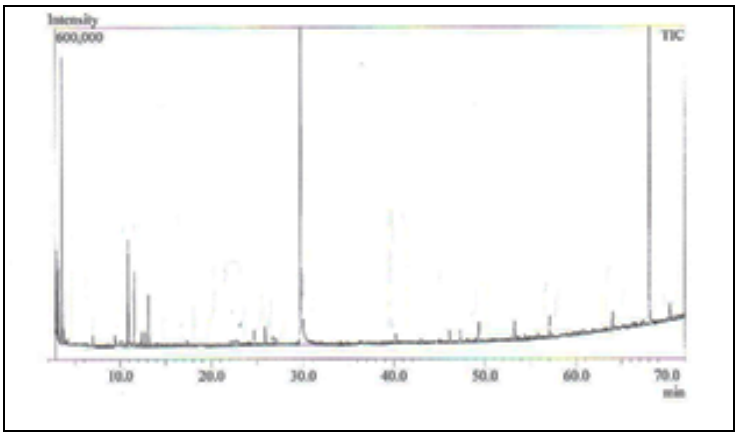

Fig. VII: **GC-MS spectra for biodegradation of pyrene by bacterial isolate M_3 after 10 days of incubation**

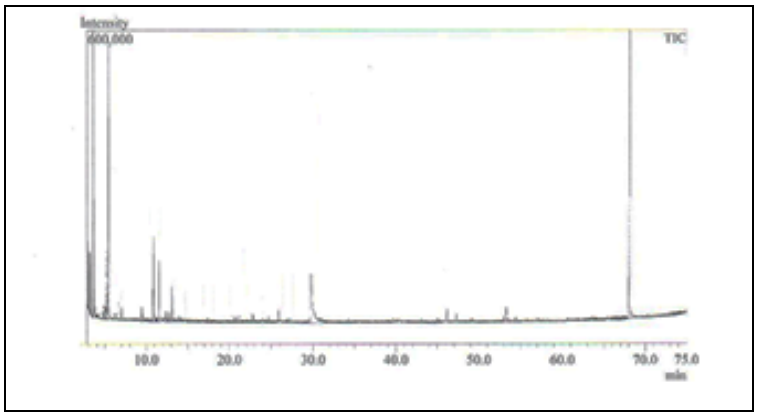

Fig. VIII: **GC-MS spectra for biodegradation of pyrene by bacterial isolate S_{21} after 10 days of incubation**

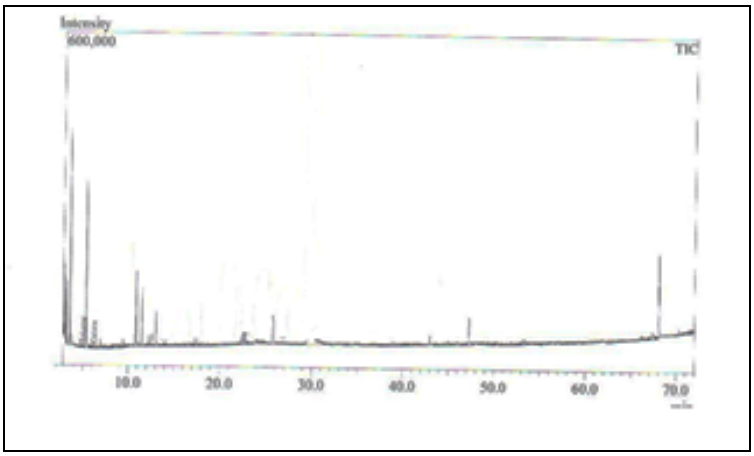

Fig. IX: GC-MS spectra for biodegradation of pyrene by bacterial isolate S_{51} after 10 days of incubation.

ANNEXURE "F"

Statistical analysis

The mean and standard deviation of three replicateswere calculated. The mean values were comparing by parametric one-way ANOVA tests at the level of $p \leq 0.05$. All the statistical analysis was performed using software called Statistical Package for Social Sciences (SPSS for Window, SPSS Inc, USA).

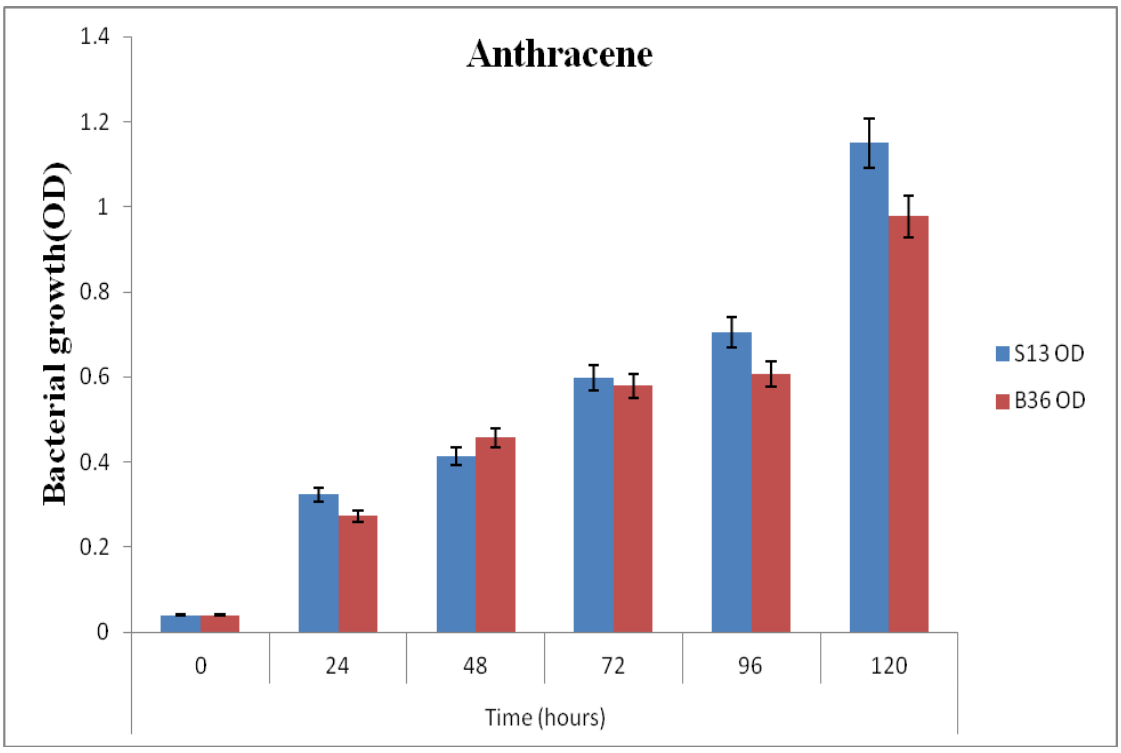

Fig. I: Growth of isolate S_{13} & B_{36} on anthracene showing error bars with percentages.

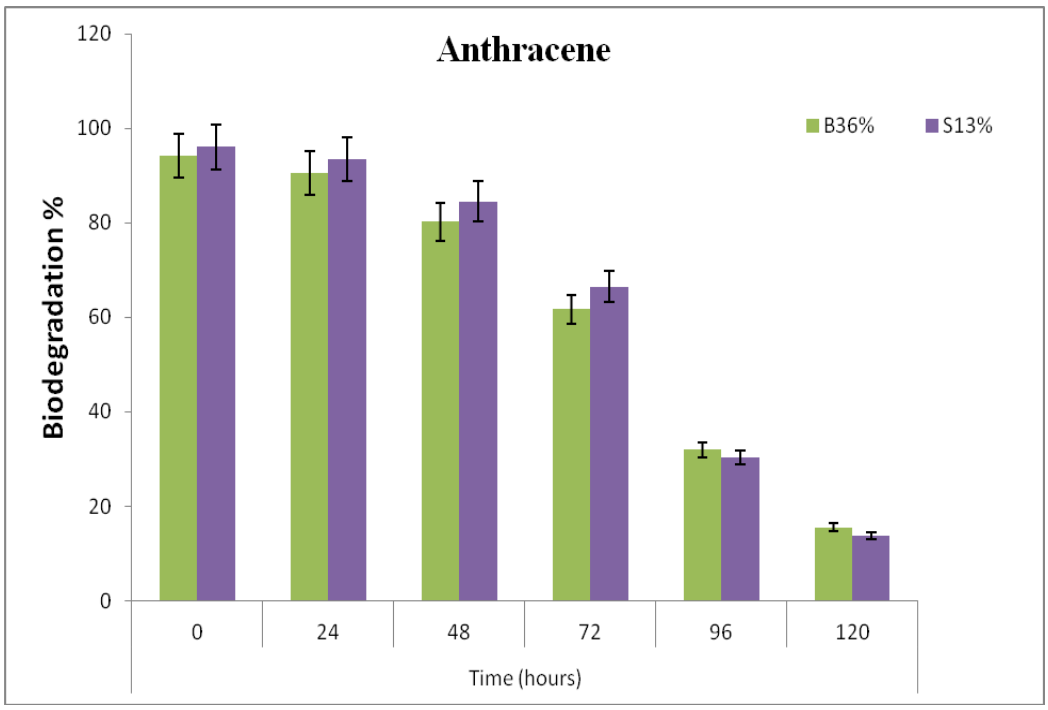

Fig. II: Biodgeradation of anthracene by isolate S_{13} & B_{36} showing error bars with percentages.

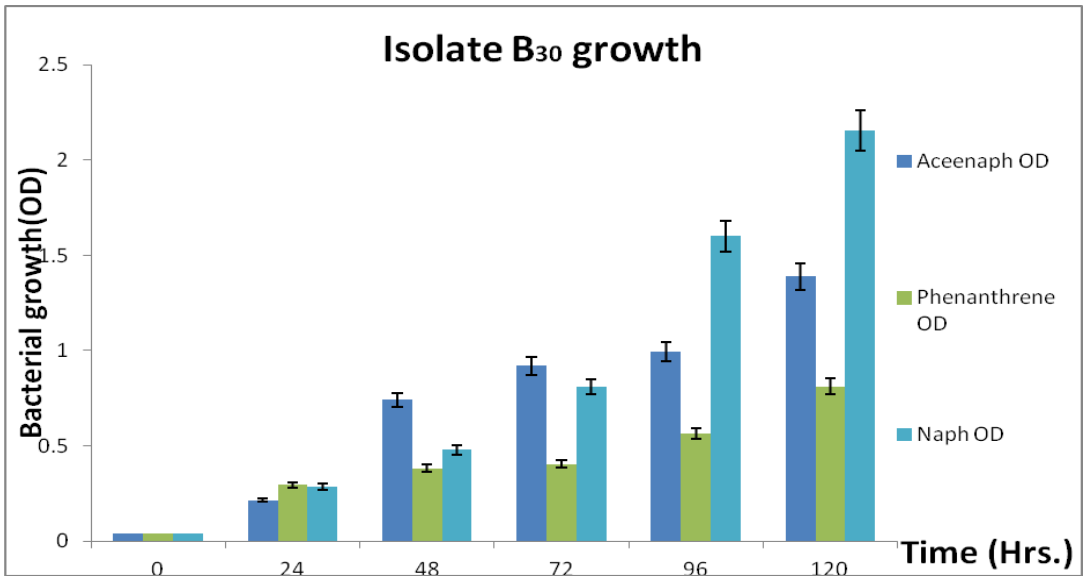

Fig. III: Growth of isolate B_{30} on anphthalene, acenaphthene & phenanthrene showing error bars with percentages

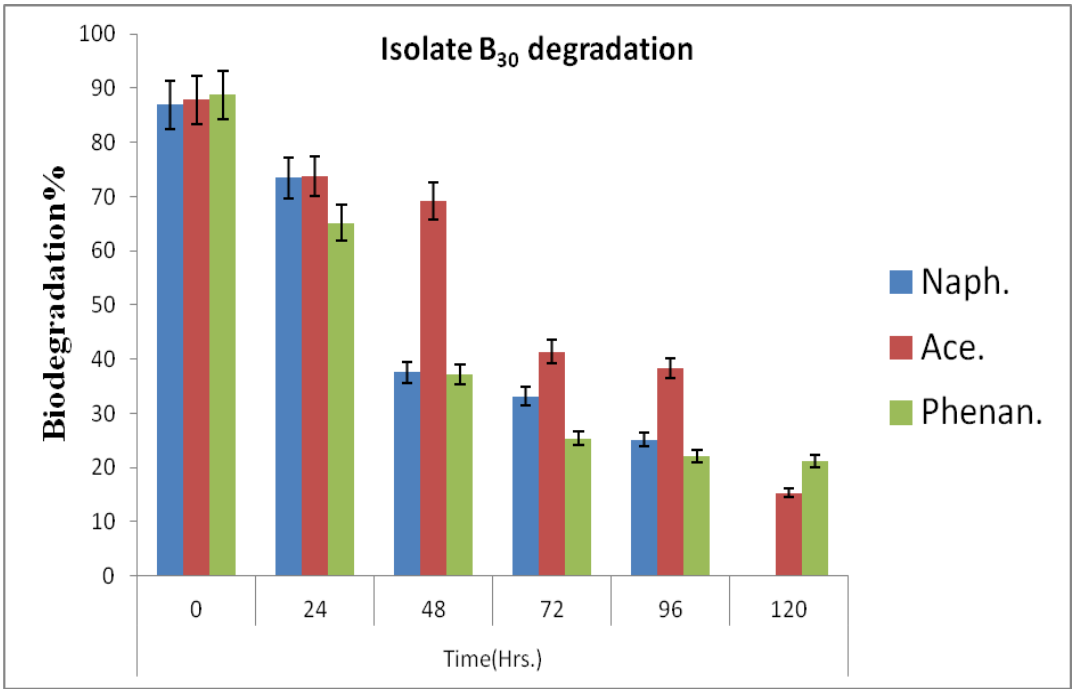

Fig. IV: Degradation anphthalene, acenaphthene & phenanthrene by isolate B_{30} showing error bars with percentages.

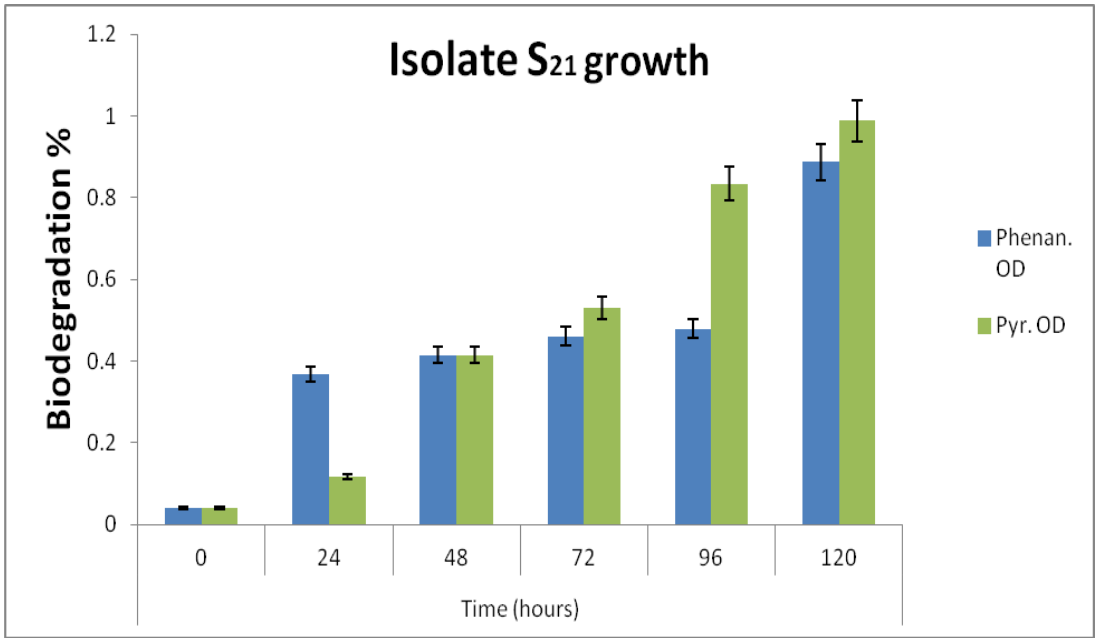

Fig. V: Growth of isolate S_{21} on phenanthrene & pyrene showing error bars with percentages.

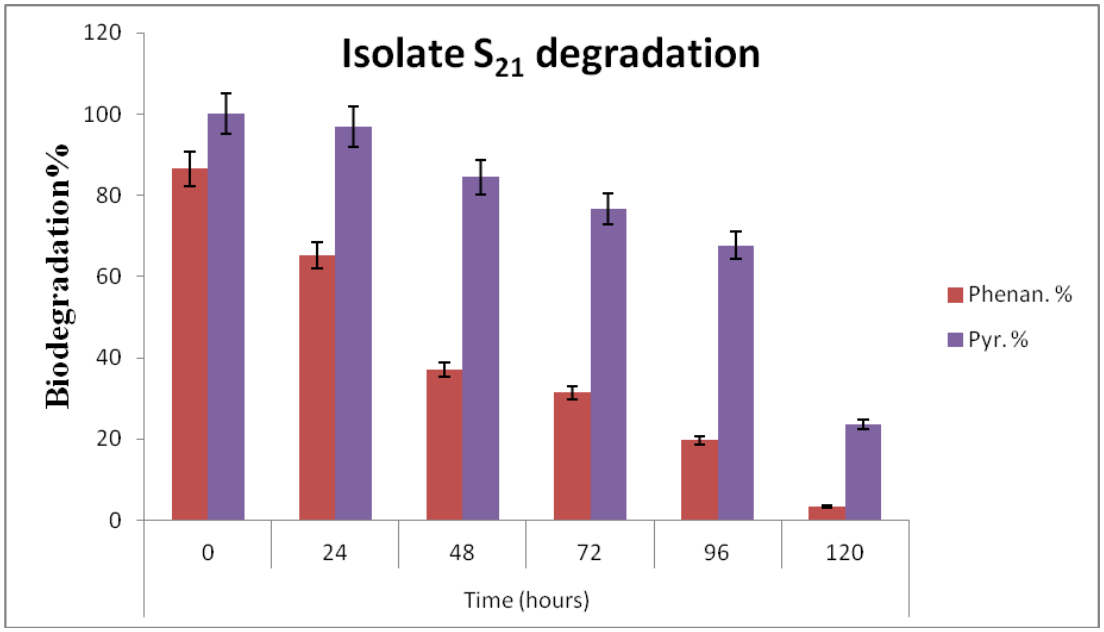

Fig.VI: Degradation of phenanthrene & pyrene by isolate S_{21} showing error bars with percentages.

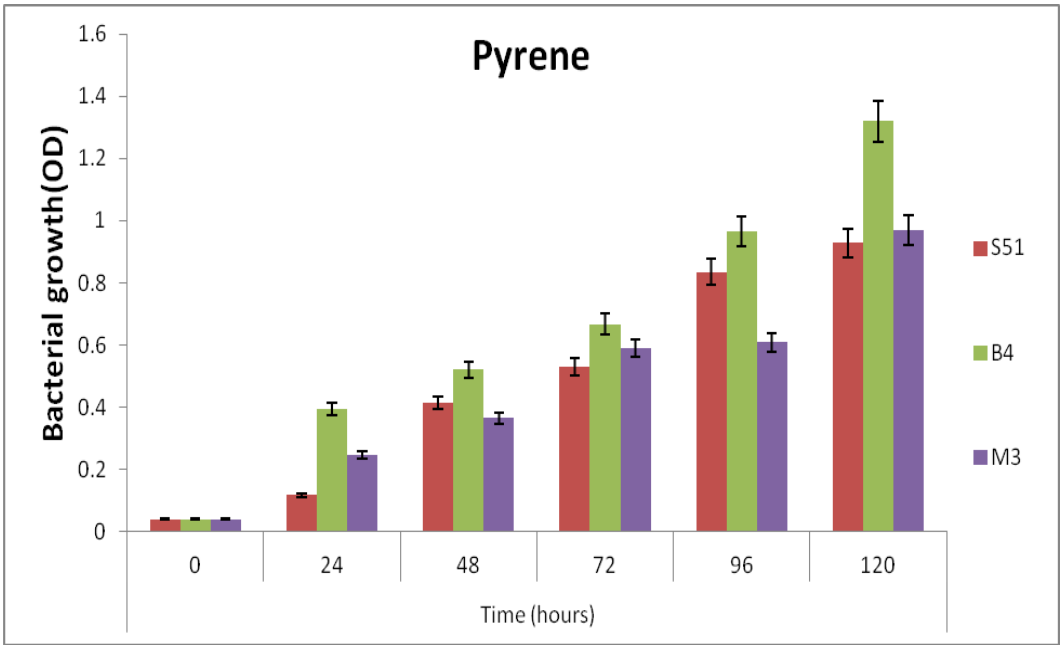

Fig. VII: Growth of isolates S_{51}, B_4 & M_3 on pyrene showing error bars with percentages.

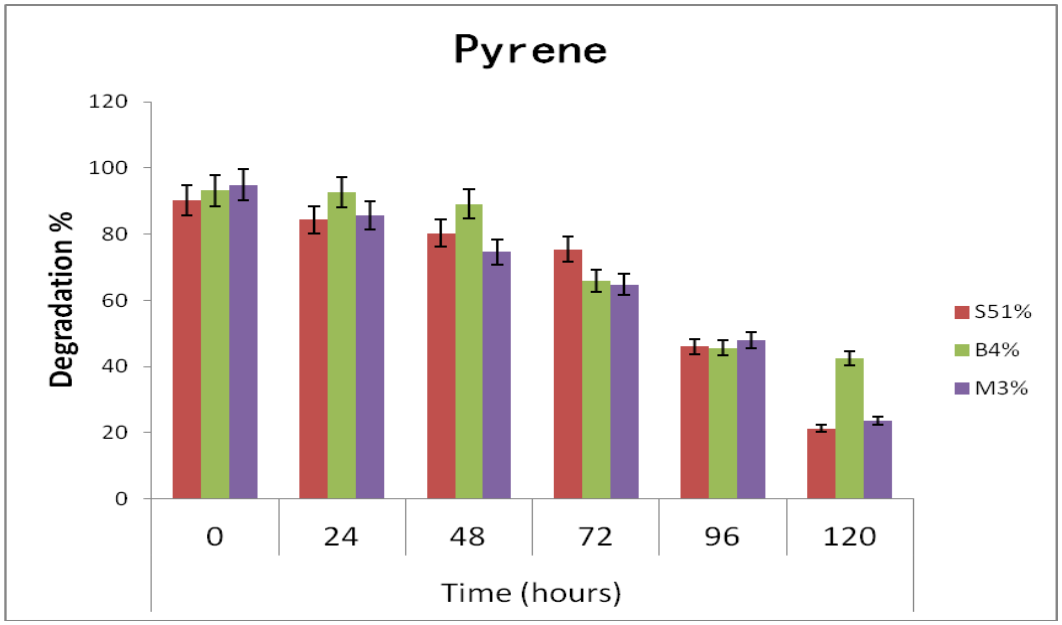

Fig. VIII: Degradation of pyrene by isolates S_{51}, B_4 & M_3 showing error bars with percentages.

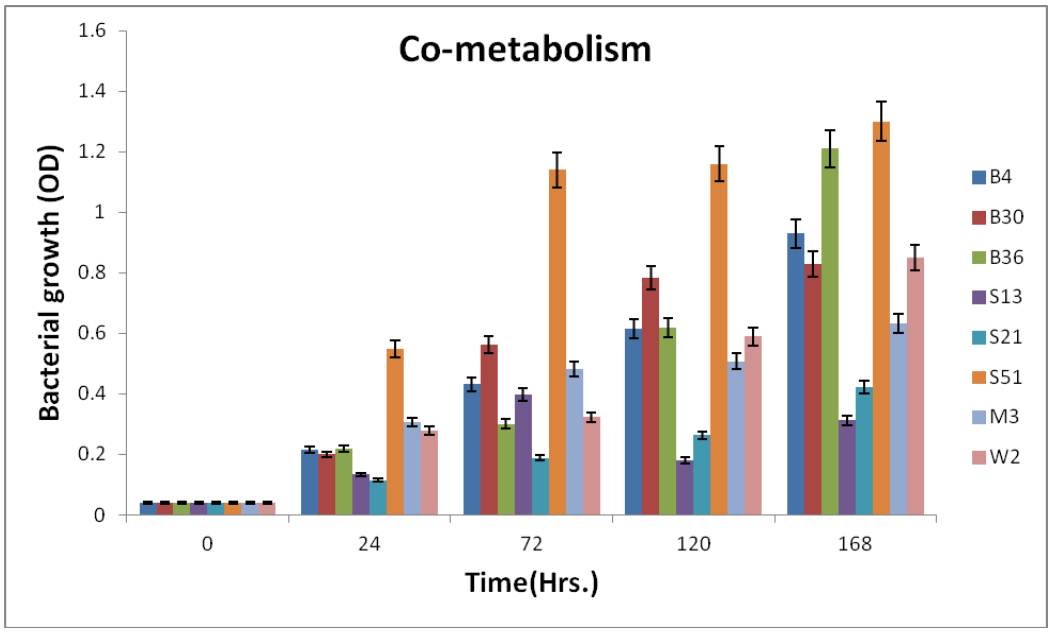

Fig. IX: Growth of isolates on PAHs mixture (naphthalene, acenaphthene, anthracene, phenanthrene& pyrene) in co-metabolism experiment showing error bars with percentages.