ISOLATION AND CHARACTERIZATION OF NAPHTHALENE DEGRADING BACTERIA

Thesis submitted in partial fulfillment of the requirement for the award of degree of

Master of Technology
in
Environmental Sciences and Technology

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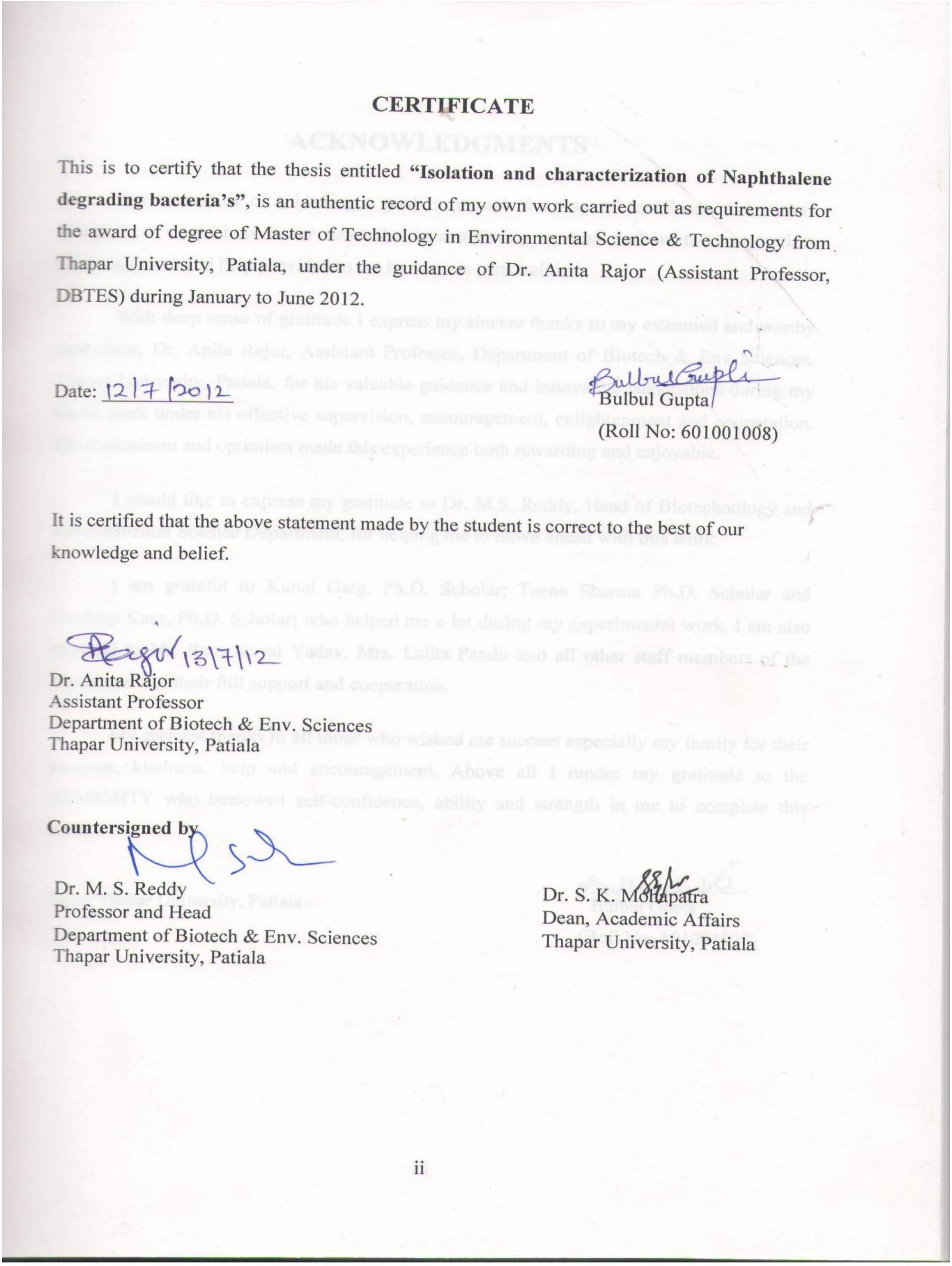

CERTIFICATE

This is to certify that the thesis entitled **"Isolation and characterization of Naphthalene degrading bacteria's"**, is an authentic record of my own work carried out as requirements for the award of degree of Master of Technology in Environmental Science & Technology from Thapar University, Patiala, under the guidance of Dr. Anita Rajor (Assistant Professor, DBTES) during January to June 2012.

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ABSTRACT

Biodegradation of organic pollutants such as polycyclic aromatic hydrocarbons using soil microorganisms where they use these compounds as carbon and energy source, is the safe, cheap and environment friendly way.

In the present study, bacterial isolates utilizing the naphthalene as sole carbon and energy were characterized taxonomically, through biochemical tests and for their degradation potential for naphthalene. Bacterial samples were isolated from crude oil contaminated soil. A total of 8 isolates were initially screened, six of these isolates were checked for growth on M9 medium containing up to 100µg/g of naphthalene. Two bacterial isolates S3 and F3 were selected on the basis of their best growth on naphthalene but S3 isolate showed the highest ability for degradation and thus was subjected to 16S rRNA sequence for identification. Phylogenetic analyses based on 16S ribosomal RNA sequences of S3 showed that it was closely related to *Pseudomonas aeruginosa* strain DSM 50071. The effects of temperature, pH and salt concentration on bacterial growth were also studied.

Biodegradation study of naphthalene was carried out by *Pseudomonas sp.*S3 and F3 at optimum pH and temperature. The optimum pH and temperature were 7 and 37ºC respectively, for bacterial growth. The biodegradation efficiency of *Pseudomonas sp.*S3 was 77.77% of naphthalene after 7 days of incubation, the detectable concentration of naphthalene (163.63µg) was decreased to 36.36µg. For F3 biodegradation efficiency was 61.11% of naphthalene after 7 days of incubation and the detectable concentration of naphthalene (163.63µg) was decreased to 63.63µg, as determined by LC/MS analysis.

Keywords: Biodegradation, LC/MS analysis, naphthalene, *Pseudomonas*.

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CHAPTER 1

INTRODUCTION

1.1 General

All substances originated into the environment either by biogenic or anthropogenic sources. Anthropogenic compounds describe synthetic compounds, and compound classes as well as elements and naturally occurring chemical entities which are mobilized by man's activities. These substances are released into the environment in amounts that are unnatural due to human activity. Anthropogenic inorganic and organic pollutants are dispersed throughout the atmosphere, hydrosphere and lithosphere, and they have tendency to transform into another compounds which may be toxic, less toxic and not toxic to flora and fauna (Figure 1.1).

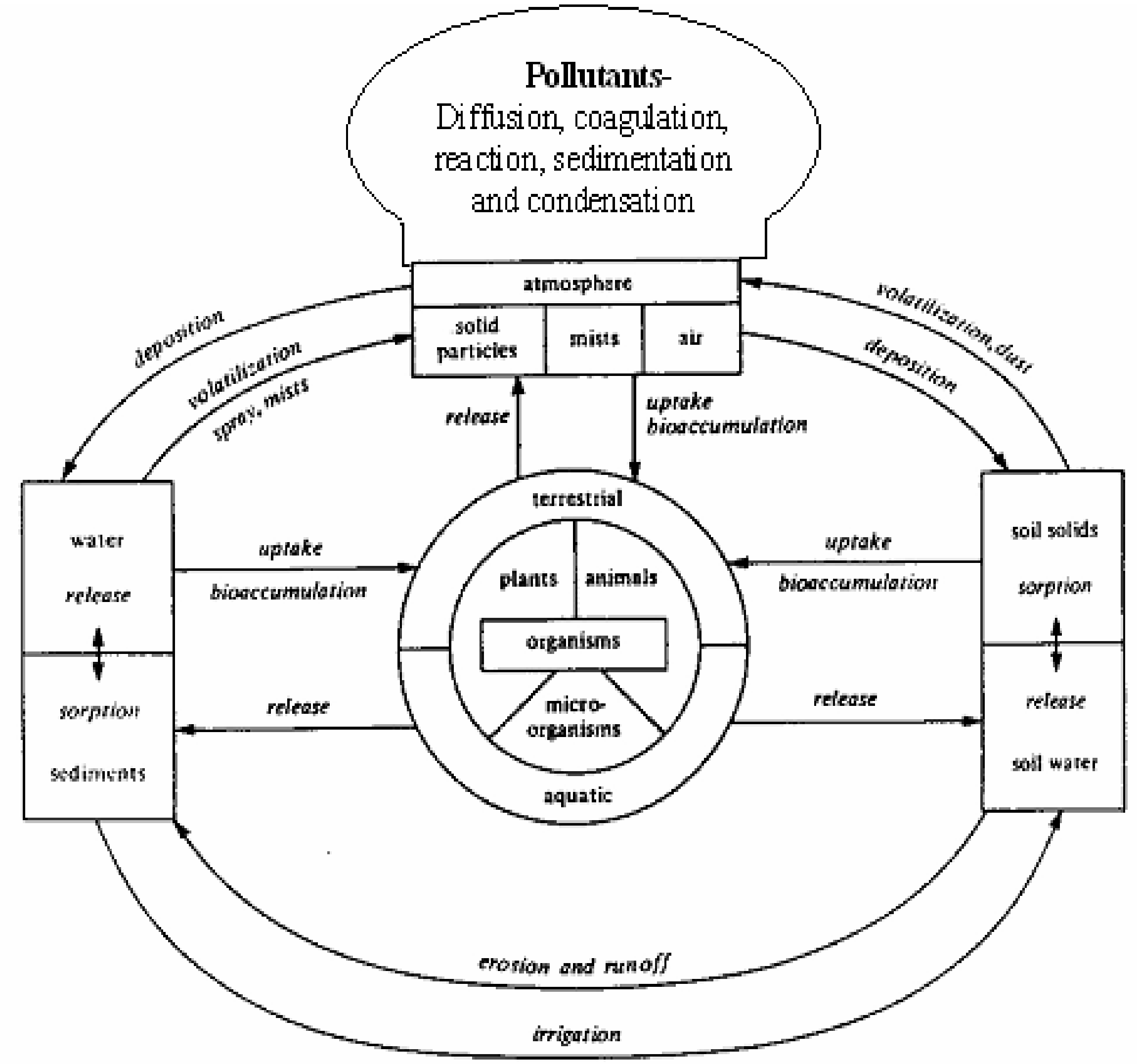


Figure 1.1: Interchange and transformation of pollutants in atmosphere, hydrosphere and lithosphere

Polycyclic aromatic hydrocarbons (PAHs) are pollutants, often carcinogenic, mutagenic, or toxic, found in most terrestrial ecosystems, that arise from industrial operations and from natural events such as forest fires. Major components of petroleum, they are continuously released into natural environments, posing a serious risk to human health (Sack et al., 1997).Polyaromatic, nitrogen and halogen containing organic compounds are recalcitrant compounds which is difficult to degrade by microorganism. Such type of compounds have higher bioaccumulation and biomagnification potency when enters into the biotic entities. Understanding the dynamic process of bioaccumulation and biomagnification is very important in protecting human beings and other organisms from the adverse effects of chemical exposure, and it has become a critical consideration in the regulation of chemicals.

1.2 Chemistry of Poly aromatic hydrocarbons (PAHs):

The simplest PAHs, as defined by the International Union of Pure and Applied Chemistry (IUPAC) (G.P Moss, IUPAC nomenclature for fused-ring systems), are phenanthrene and anthracene, which both contain three fused aromatic rings. Smaller molecules, such as benzene, are not PAHs.

PAHs may contain four-, five-, six- or seven-member rings, but those with five or six are most common. PAHs composed only of six-membered rings are called alternant PAHs. Certain alternant PAHs are called benzenoid PAHs. The name comes from benzene, an aromatic hydrocarbon with a single, six-membered ring. These can be benzene rings interconnected with each other by single carbon-carbon bonds and with no rings remaining that do not contain a complete benzene ring.

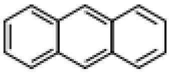
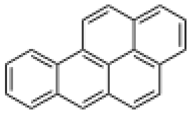
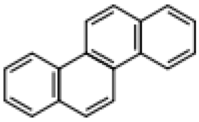
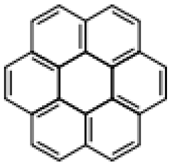
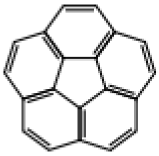
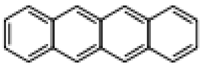
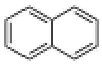
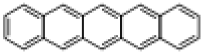
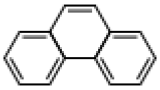
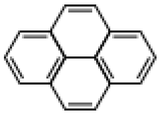
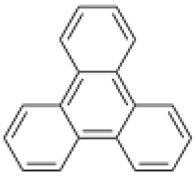
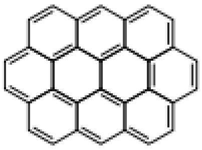
The set of alternant PAHs is closely related to a set of mathematical entities called polyhexes which are planar figures composed by conjoining regular hexagons of identical size.

PAHs containing up to six fused aromatic rings are often known as "small" PAHs, and those containing more than six aromatic rings are called "large" PAHs. Due to the availability of samples of the various small PAHs, the bulk of research on PAHs has been of those of up to six rings. The biological activity and occurrence of the large PAHs does appear to be a continuation of the small PAHs. They are found as combustion products, but at lower levels than the small PAHs due to the kinetic limitation of their production through addition of successive rings. In addition, with many more isomers possible for larger PAHs, the occurrence of specific structures is much smaller.

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

Naphthalene ($C_{10}H_8$ constituent of mothballs), consisting of two coplanar six-membered rings sharing an edge, is another aromatic hydrocarbon. By formal convention, it is not a true PAH, though is referred to as a bicyclic aromatic hydrocarbon. Aqueous solubility decreases approximately one order of magnitude for each additional ring.

1.3 Poly aromatic hydrocarbons (PAHs) :

Chemical compound		Chemical compound	
Anthracene		Benzo[*a*]pyrene	
Chrysene		Coronene	
Corannulene		Tetracene	
Naphthalene		Pentacene	
Phenanthrene		Pyrene	
Triphenylene		Ovalene	

The United States Environmental Protection Agency (EPA) has designated 32 PAH compounds as priority pollutants. The original 16 are listed. They are naphthalene, acenaphthylene,acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]flouranthene, benzo[*a*]pyrene,dibenz(ah)anthracene, benzo[*ghi*]perylene, and indeno(1,2,3-cd)pyrene. This list of the 16 EPA priority PAHs is often targeted for measurement in environmental samples.

Physical properties of Poly aromatic hydrocarbons (PAHs) :

The term PAH generally refers to hydrocarbons containing two or more fused benzene rings in linear, angular or clustered arrangements. PAHs are hydrophobic compounds and their persistence in the environment is chiefly due to their low water solubility (Cerniglia, 1992). Generally, PAH solubility decreases and hydrophobicity increases with an increase in number of fused benzene rings. In addition, volatility decreases with an increasing number of fused rings (Wilson and Jones, 1993).

1.4 Occurrence and Sources of Poly Aromatic Hydrocarbons in the environment:

The release of PAHs into the environment is widespread since these compounds are ubiquitous products of incomplete combustion. PAHs have been detected in a wide variety of environmental samples, including air, soil (Wilson and Jones, 1993), sediments, water, oils, tars and foodstuffs. Industrial activities, such as processing, combustion and disposal of fossil fuels, are usually associated with the presence of PAHs at highly contaminated sites.

Poly Aromatic Hydrocarbon in the AIR:

The main route for PAH transport is through the atmosphere. Results from ambient air monitoring programs have shown that PAH concentrations are usually of the order of a few nanograms per cubic metre of air. Motor vehicles, including spark emission and diesel automobiles, trucks and buses, also contribute to atmospheric PAH pollution through exhaust condensate and particulates, tyre particles and lubricating oils and greases.

Poly Aromatic Hydrocarbons (PAHs) in sediments:

As PAHs are characterised by low water solubility's and high octanol±water partitioning coefficients, their concentrations in water are extremely low. However, due to their hydrophobic nature, PAHs accumulate in fine grain sediments, partitioning to organic carbon-coated particles. PAH concentrations in sediments may accumulate due to a number of sources including atmospheric deposition, marine seeps of petroleum hydrocarbons, off-shore production or petroleum transportation, sewage disposal or boating.

Poly Aromatic Hydrocarbons in soils:

PAH concentrations in soils from industrialised countries have also revealed an increasing PAH burden since the mid 1800s, with a peak in the 1950/1960s. Anthropogenic combustion of fossil fuels and long range atmospheric transport of PAHs has contributed to the dispersal of PAHs throughout the environment. The concentration of PAHs in contaminated soils from industrial sites can vary depending on the industrial activity associated with the site.

Poly Aromatic Hydrocarbons in marine organisms:

In addition to air, sediment and soil, PAHs may accumulate in marine organisms. Uptake of PAHs by marine organisms is dependent on the bioavailability of the PAHs (i.e. partitioning of the compound between sediment, water and food), as well as the physiology of the organism. The organism size, ingestion rate, growth rate, membrane permeability, ventilator rate, gut residence time and osmo-regulation are biological processes that influence the organism's uptake of PAHs.

Poly Aromatic Hydrocarbons in plants:

PAHs may also accumulate in vegetation that could indirectly cause human exposure through food consumption (Wagrowski and Hites, 1997). Atmospheric deposition of PAHs onto plants occurs, and in some cases, various plants have been found to take up these compounds. A number of factors can influence the accumulation of PAHs in plants, such as the physical properties of the PAH, the plant species and structure, as well as environmental conditions including atmospheric PAH concentrations, temperature and wind conditions.

Poly Aromatic Hydrocarbons toxicity:

Many PAHs are carcinogenic and they are, therefore, of significant concern as environmental contaminants. Numerous studies have indicated that one-, two- and three-ring compounds are acutely toxic, while higher molecular weight PAHs are considered to be genotoxic.

1.5 Biodegradability of Poly Aromatic Hydrocarbons(PAHs):

Numerous genera of micro-organisms have been observed to oxidise PAHs. There is a great diversity of organisms capable of degrading the low molecular weight PAH, such as naphthalene, acenaphthene and phenanthrene. It was suggested that nocardioform bacteria (e.g. *Rhodococcus*, *Nocardia*, *Mycobacterium and Gordona*) may play a crucial role in the degradation of high molecular weight PAHs in soils. This suggestion was based on results reported by the authors for screening pyrene-degrading bacteria (from soil samples) and on the observation that there are very few reports of bacteria outside the *nocardioform actinomycetes* group capable of growing on high molecular weight PAHs.

However, some Pseudomonas species have been observed to degrade some four- and five-ring PAHs. Fungi, in particular the white-rot fungi, play an important environmental role in recycling wood and related materials, which is due primarily to the relatively non-specific processes used to initiate the degradation of the lignin fraction. Lignin degradation is carried out by mechanisms related to the production of highly reactive intermediates by enzymes, such as lignin peroxidase and manganese dependent peroxidase.

Algae and cyanobacteria have also been shown to oxidise PAHs. While the oxidation of naphthalene by a number of algae and cyanobacteria has been reported, relatively few studies have demonstrated the degradation of high molecular weight compounds by these organisms.

1.6 Degradation of aromatic hydrocarbon:

Aromatic hydrocarbons are ubiquitous in nature. The commercial, industrial, natural activities generate huge amount of aromatic hydrocarbons causes a great concern due to potential hazard to flora and fauna. A number of physiochemical processes including adsorption, incineration, and absorption can be used to treat PAH’s but the costs for chemicals and fuels as well as further treatment or disposal of secondary wastes are increasingly inhibiting adoption of these solutions. The processes leading to the eventual removal of hydrocarbon pollutants from the environment has been extensively documented and involves the trio of physical, chemical and biological alternatives. The currently accepted disposal methods of incineration or burial in secure landfills (USEPA 2001; ITOPF 2006) can become prohibitively expensive when the amounts of contaminants are large. This often results in cleanup delays while the contaminated soil continues to pollute groundwater resources if on land, and death of aquatic life if on waterways (Pye and Patrick 1983), thus necessitating speedy removal of the contaminants. The biodegradation of pollutants is not a new concept as it has been intensively studied in controlled conditions (Sugiura et al., 1997; Chaillan et al., 2004) and in open field experiments (Chaıneau et al., 2003; Gogoi et al., 2003), but it has acquired a new significance as an increasingly effective and potentially inexpensive cleanup technology.

PolyAromaticHydrocarbons + micro-organism ——► CO2 + H2O

Rate of Biodegradation depends on pH, temperature, oxygen, microbial population, degree of acclimation, accessibility of nutrients, chemical structure of the compound, cellular transport properties, and chemical partitioning in growth medium. A number of bacterial species are known to degrade PAHs and most of them are isolated from contaminated soil or sediments. *Pseudomonas aeruginosa, Pseudomons fluoresens, Mycobacterium spp., Haemophilus spp., Rhodococcus spp., Paenibacillus spp.* are some of the commonly studied PAH-degrading bacteria. Lignolytic fungi too have the property of PAH degradation. *Phanerochaete chrysosporium, Bjerkandera adusta, and Pleurotus ostreatus* are the common PAH-degrading fungi. The biodegradation of PAHs has been observed under both aerobic and anaerobic conditions and the rate can be enhanced by physical/chemical pretreatment of contaminated soil. Addition of biosurfactant-producing bacteria and light oils can increase the bioavailability of PAHs and metabolic potential of the bacterial community. The supplementation of contaminated soils with compost materials can also enhance biodegradation without long-term accumulation of extractable polar and more available intermediates. Its potential contribution as a countermeasure biotechnology for decontamination of oil polluted systems could be enormous. The initial interest was in the fate and persistence of pesticides in soils (Prince 1993). However, the field has expanded in recent years to encompass a wide variety of chemicals and a broad array of issues. Some technologies are being developed that markedly enhance microbial destruction or degradation of organic pollutants that otherwise would have persisted at the cleanup of many polluted groundwater and soils using the orthodox physical and chemical methods (Alexander 1994).

Bioremediation, which employs the biodegradative potentials of organisms or their attributes, is an effective technology that can be used to accomplish both effective detoxification and volume reduction. It is useful in the recovery of sites contaminated with oil and hazardous wastes (Caplan 1993). Besides, bioremediation technology is believed to be non-invasive and relatively cost effective (April et al., 2000). In some cases it may not require more than the addition of some degradation enhancers to the polluted system. It could end up being the most reliable and probably least expensive option for exploitation in solving some chemical pollution problems (Mesarch et al., 2000). Sprague (1982) noted that no single microbial species has the enzymatic ability to metabolise more than two or three classes of compounds typically found in crude oil. A consortium composed of many different bacterial species is thus required to degrade crude oil significantly. The use of a bacterial consortium provides certain advantages over biostimulation in cases where pollutant is having high toxicity or a lack of appropriate microorganisms (both quantity and quality) is important. Determination of the potential success of application of bacterial consortium requires an understanding of the survival and activity of the added microorganism(s) or their genetic materials, and the general environmental conditions that control the degradation rates such as the peculiarity of the contaminated site, for example, water or soil systems (Vogel 1996). These factors may very well vary from place to place and from organism to organism. The success of the bioremediation efforts in the cleanup of the oil tanker *Exxon Valdez* oil spill of 1989 (Atlas and Bartha, 1998) in Prince William Sound and the Gulf of Alaska has created tremendous interest in the potential of biodegradation and bioremediation technology. Many countries are currently convinced of the full potential of this technology and are, therefore, encouraging research into improving and optimising the procedures.

CHAPTER 2

Literature Review

2.1 NATIONAL STATUS

Environmental pollution is increasing with the increase in the industrial development all over the world; increment of this pollution caused many hazards for all organisms, even for humans such as carcinogenicity and toxicity. Also there has been increasing pollution with hydrocarbon compounds, many of these hydrocarbons considered to be a potential health hazard (Hafez et al, 2008). Some of the hydrocarbon compound pollutants are polycyclic aromatic hydrocarbons (PAHs). Polycyclic aromatic hydrocarbons (PAHs) are large group of organic compounds with two or more fused aromatic rings in linear, angular or cluster arrangements.

2.1.1 Characterization of Poly aromatic hydrocarbons (PAHs) degrading microbial consortium:

In developing countries like India, there are many industrial areas discharging effluent containing large amount of polyaromatic hydrocarbon (PAH) which causes hazardous effect on the soil-water environment. The biodegradation of phenanthrene using adapted microbial consortium isolated from petrochemical contaminated environment (Janbhandu & Fulekar, 2011) was conducted, where microbial consortium from 3 decade old petrochemical refinery field located in Nagpur, Maharashtra with history of PAH disposal was collected. Based on biochemical tests and 16S rDNA gene sequence analysis the consortium was identified as *Sphingobacterium* sp., *Bacillus cereus* and a novel bacterium *Achromobacter insolitus* with effective phenanthrene-degrading ability. The biodegradation data of phenanthrene indicates about 100%, 56.9% and 25.8% degradation at the concentration of 100 mg/l, 250 mg/l and 500 mg/l respectively within 14 days. GC–MS analysis of phenanthrene degradation confirmed biodegradation by detection of intermediates like salicylaldehyde, salicylic acid and catechol. All the results indicated that the microbial consortium have a promising application in bioremediation of petrochemical contaminated environments and could be potentially useful for the study of PAH degradation and for bioremediation purpose.

Further in 2010 several naphthalene and anthracene degrading bacteria were isolated from rhizosphere of *Populus deltoides*, which were grown in non-contaminated soil.

Among these, four isolates, i.e. *Kurthia* sp., *Micrococcus varians*, *Deinococcus radiodurans* and *Bacillus circulans* utilized chrysene, benzene, toluene and xylene, in addition to anthracene and naphthalene. *Kurthia* sp and *B. Circulans* also showed positive chemotactic response for naphthalene and anthracene (Bisht *et al, 2010).* The mean growth rate constant (K) of isolates were found to increase with successive increase in substrate concentration (0.5 to 1.0 mg/50ml). *B. Circulans* SBA12 and *Kurthia* SBA4 degraded 87.5% and 86.6% of anthracene while, *Kurthia* sp. SBA4, *B. circulans* SBA12, and *M. varians* SBA8 degraded 85.3 %, 95.8 % and 86.8 % of naphthalene respectively after 6 days of incubation as determined by HPLC analysis.

Biodegradation of Pyrene by *Pleurotus ostreatus* HP – 1 and its correlation with Extracellular Ligninolytic Enzyme Production (Hardik et al, 2010) was also studied in which a fungal isolate *Pleurotus ostreatus* HP – 1 (GenBank Accession no. EU420068) was obtained by successive transfer from rotted wood sample, collected from forest locality in Gujarat, India. The biodegradation of four ring PAH, pyrene was studied in submerged cultures of these fungi. Maximum degradation of 38.21% was observed on 10th day of incubation period after addition of pyrene to 6 days grown culture. The PAH biodegradation potential of the bassidiomycetes fungi positively correlated with their potential to produce ligninolytic enzymes and fungal biomass. Apart from this, kinetics of phenanthrene degradation by *Staphylococcus* sp. strain PN/Y involving 2-hydroxy-1-naphthoic acid in a novel metabolic pathway was studied(Mallick & Dutta, 2008) where *staphylococcus* sp. strain PN/Y isolated from creosote-contaminated soil was studied which was previously reported to degrade phenanthrene as sole source of carbon and energy. Unlike other phenanthrene degraders, *Staphylococcus* sp. PN/Y degraded phenanthrene by a novel pathway involving 2-hydroxy-1-naphthoic acid (2H1NA), which was further metabolized by unique *meta*-cleavage dioxygenase, ultimately leading to TCA cycle intermediates. At a concentration of 1 g l^{-1} of phenanthrene, the maximum accumulation of 2H1NA was found to be 323 mg l^{-1}. In addition, 2H1NA utilized by *Staphylococcus* sp. PN/Y as sole carbon source, the growth yield at 96 h was 445, 557, 560, 480, 419, 334, 212 and 66 mg of protein (g 2H1NA)$^{-1}$ when cells were grown on 50, 100, 200, 400, 500, 650, 800 and 1000 mg l^{-1} of 2H1NA, respectively. Growth was found to be inhibited at initial higher concentration of 2H1NA, which may attributed due to concentration-dependent toxicity of 2H1NA and/or the toxicity of its decarboxylated metabolite, 2-naphthol.

Naphthalene biodegradation was also studied (Zafar et al, 2009) using the bacterial strain *Pseudomonas putida* S2. Three medium variables out of seven medium components were selected under Plakett-Burman (PB) design as having significant response on naphthalene biodegradation. These variables were citric acid (additional carbon sources), ammonium sulfate and sodium chloride. The levels of these three variables were optimized by the application of genetic algorithm (GA) based response surface methodology (RSM) in terms of maximum biodegradation efficiency. The maximum biodegradation efficiency of 55.51% was observed at concentrations of 1.0 g l^{-1}, 1.0 gl^{-1}, and 0.7gl^{-1} for citric acid, ammonium sulfate and sodium chloride, respectively. In addition, the interactive effects of significant medium variables were analyzed using three dimensional surface plots simulated by network output in terms of maximum fitness function.

2.1.2 Strains isolated from oil contaminated soil:

A bacterial strain, PS4040, capable of degrading polycyclic aromatic hydrocarbons for use as the sole carbon source was isolated from oily-sludge-contaminated soil. The 16S rRNA gene showed 98.8% homology to that of *Leclercia adecarboxylata*. Comparative molecular typing with the clinical strain of *L. Adecarboxylata* revealed that there were few comigrating and few distinct amplimers among them (Sarma et al, 2004). Another *Pseudomonas putida* strain was isolated from oil fields of Gujarat, could use naphthalene as sole source of carbon and energy and mineralized naphthalene via salicylate. This strain was also chemotactic towards these compounds. An 83 kb mega plasmid was found to contain all the genes responsible for degradation of naphthalene and salicylate in this strain67. The plasmid was transferred into a plasmid-free strain of *P. putida* KT2442 which resulted in the acquisition of chemotaxis and degradation properties by this strain, thus establishing the role of this plasmid in chemotaxis associated with complete mineralization of these two compounds68.

One more organism, *Arthrobacter sulfureus* (Jain et al, 2005) which was also isolated from Gujarat oil fields, could utilize phenanthrene as the sole source of carbon and energy. The pathway for degradation of this compound was determined in this strain along with that in three other strains, *Acidovorax delafieldi* P4-1, *Brevibacterium* sp. HL4 and *Pseudomonas* sp. DLC-P11. Novel pathways for phenanthrene degradation in *Brevibacterium* HL4 and *Pseudomonas* DLC-P11 were proposed. These organisms could utilize several other PAHs such as acenaphthene, anthracene, fluorine, fluoranthene, naphthalene and pyrene, making them potentially useful in cleaning up petroleum contaminated soils containing a mixture of different PAHs69. *Pseudomonas putida* is a good candidate for metabolic engineering and genetic manipulation applications for expression of genes encoding several degradative enzymes.

Therefore, a *P. putida* strain was engineered to increase the efficiency of degradation of naphthalene and salicylate70. In this strain, utilization of glucose (a simple carbon source) was blocked through metabolic engineering making it primarily dependent on the more complex carbon source, i.e. the organic compound in question (naphthalene/salicylate) thus degrading it more efficiently.

In another investigation (Roy et al., 2007), decomposition of polyaromatic hydrocarbons was studied using mixed culture. Mixed strains were isolated from soil of petrol stations of different Indian cities and the best performing strains from these sources were used for subsequent bioprocess study using simulated mixture of anthracene and naphthalene as carbon source in methanol solution. The cell growth curve and substrate depletion time history curve obtained from batch fermentative process show that the reaction engineering behaviour of the systems under study can well be represented by classical substrate uninhibited Monod's model and in a separate attempt the intrinsic kinetic parameters μmax and KS were evaluated following differential analysis of experimental data.

2.1.3 Sources & Distribution of Poly Aromatic Hydrocarbons (PAHs):

Research on Sundarban mangrove wetland in India (Domínguez et al, 2010) was conducted where the distribution and potential sources of 16 polycyclic aromatic hydrocarbons (PAHs) in sediment cores (<63 microm particle size) of the Sundarban mangrove wetland, northeastern coast of Bay of Bengal (India), were investigated by gas chromatography coupled to mass spectrometry. The total concentrations of 16 PAHs summation operator(16)PAHs) ranged from 132 to 2938 ng/g, with a mean of 634 ng/g, and the sum of 10 out of 16 priority PAHs summation operator(10 PAH) varied from 123 to 2441 ng/g, with a mean of 555 ng/g, and the 5 carcinogenic PAHs (benzobfluoranthene, benzokfluoranthene, benzoapyrene, indeno1,2,3-cdpyrene, and dibenza,hanthracene) accounted for 68-73% of the priority PAHs. Maximum concentrations of the sediment core were obtained at subsoil depth of 12-16 cm. The prevalence of four to six aromatic ring PAHs and cross-plots of specific isomer ratios such as phenanthrene/anthracene, fluoranthene/pyrene, and suggested the predominance of wood and coal combustion sources, the atmospheric deposition, and surface runoff to be the major transport pathways.

Distribution and biodegradation of polycyclic aromatic hydrocarbons in contaminated sites of Hisar (Bishnoi et al,2009) was also reported where fifty two soil samples were collected from various location of the Hisar city. These samples were analysed for six PAHs (naphthalene, acenaphthene, phenanthrene, anthracene, fluoranthene and pyrene). Total mean concentration of six PAHs varied from 51.79 to 148.82 mg /kg dry weight of the soil. PAH concentration was higher in soil samples from local auto market while lower concentration was recorded in agricultural soil. Effect of pH (5.0 to 9.0), temperature (20° to 40°C), and concentration of PAHs (5 to 20 mg/kg) on biodegradation were optimized. Biodegradation of phenanthrene (3-ring) and pyrene (4-ring) was also evaluated using two acclimatized microbial strains *Pseudomonas putida and Pseudomonas paucimobilis.* Biodegradation was maximum in sterilized artificial spiked soil with phenanthrene (5 mg/kg) and pyrene (5 mg/kg) pH 7.0 and at 30°C (optimized conditions) than the native unsterilized contaminated soil (without optimized conditions) in 42 days of incubation period with *Pseudomonas putida and Pseudomonas paucimobilis.* Phenanthrene was completely disappeared after 28 days with *Pseudomonas putida* and 35 days with *Pseudomonas paucimobilis* whereas Pyrene was disappeared upto 97.4% with P.*putida* and 95.5% with *Pseudomonas paucimobilis* after 42 days incubation period at optimum conditions. Another research reflecting the threat of PAHs from petrogenic sources showed that PAH deposition has increased in recent years in the Kumaun Himalaya, where deposition of polycyclic aromatic hydrocarbons (PAHs) was investigated in dated sediment cores from Lakes Nainital and Bhimtal in the Kumaun Himalaya and is associated with an increase in anthropogenic activity. The lake sediments dominantly contain low molecular weight (LMW) PAHs, derived mainly from petrogenic sources. Concentrations of individual and total PAHs were categorized on the basis of sediment quality criteria. According to this, the pollutants pose a potential threat to aquatic life. (Choudhary & Routh, 2010).

Apart from these investigations a study during autumn and winter season was conducted in developing city environment of northern India to ascertain the contamination levels and their distribution behaviour in soil along the roadsides (Kumar et al., 2011). The concentration of polycyclic aromatic hydrocarbons was determined at ten locations of Jalandhar city, Punjab in India at 1, 2 and 3 m distances from roadside in soil covering all the major traffic intercepts within city. The samples were extracted in acetone and dichloromethane (1:1) using soxhlet extraction. The total average concentration (city average) was found to be 4.04µg/g and 16.38µg/g during winter and autumn respectively.

DiBenzo (ah) Anthracene and Benzo (a) Pyrene were the individual polycyclic aromatic hydrocarbons found in highest concentration at all the intercepts ranging between 0.008 to 28.4µg/g during winter and 0.01 to 252.55µg/g during autumn. Average concentration of non-carcinogenic and carcinogenic polycyclic aromatic hydrocarbons during winter and autumn was found to be 2.1 and 6.4 and 4.74 and 35.08 µg/g respectively. Total carcinogenic polycyclic aromatic hydrocarbons concentration was found quite high (80 %) in comparison to non-carcinogenic polycyclic aromatic hydrocarbons (20 %) at most of the intercepts.

2.1.4 Hazards of Poly Aromatic Hydrocarbons (PAHs):

A research indicating the hazard of PAHs to human health in India shows the presence of polycyclic aromatic hydrocarbons in placenta, maternal blood, umbilical cord blood and milk of Indian women. (Madhavan & Naidu, 1995) Samples from mothers were analysed for the presence of selected polycyclic aromatic hydrocarbons (PAHs). Benzo(a)pyrene (B(a)P), dibenzo(a,c)anthracene (DBA) and chrysene (Chy) were collected and PAHs were detected in all the four types of sample. Levels of dibenzo(a,c)anthracene were higher in the above samples compared with the other two PAHs. Umbilical cord blood and breast milk samples showed relatively high concentrations of all the three PAHs and thus demonstrated that the developing foetus/new born were exposed to these carcinogenic environmental contaminants. Apart from this, a survey from Central Pollution Control Board indicates PAHs as toxic air pollutants and people exposed to it at sufficient concentrations and durations may have an increased chance of getting cancer or experiencing other serious health effects. These health effects can include damage to the immune system, as well as neurological, reproductive (e.g., reduced fertility), developmental, respiratory and other health problems. In addition to exposure from breathing air toxics, some toxic air pollutants such as mercury can deposit onto soils or surface waters, where they are taken up by plants and ingested by animals and are eventually magnified up through the food chain. Like humans, animals may experience health problems if exposed to sufficient quantities of air toxics over time.

2.2 INTERNATIONAL STATUS

2.2.1 Parameters affecting biodegradation of Poly Aromatic Hydrocarbons (PAHs):

Parameter affecting the fate of PAHs in the environment is the extent of loss due to mechanisms of biodegradation. The ability of aerobic microorganisms to degrade naphthalene, phenanthrene, and other low-molecular-weight PAHs is well known (Cerniglia et al, 1984). However, subsurface organic-impacted sediments are commonly anaerobic. Although PAHs typically had been thought to be recalcitrant to biodegradation without oxygen (Evans & Fucks, 1988), recent studies have demonstrated PAH degradation under sulfate-reducing (Coates et al, 1997) and nitrate-reducing (Karl et al, 2000) conditions. A report on anaerobic degradation of Naphthalene by microbial pure cultures under nitrate-reducing conditions indicated the ability of isolates to grow anaerobically in liquid culture with naphthalene as the sole source of carbon and energy in the presence of nitrate. Three naphthalene-degrading pure cultures were obtained, designated NAP-3-1, NAP-3-2, and NAP-4. Isolate NAP-3-1 tested positive for denitrification using a standard denitrification assay. Isolates NAP-4 and NAP-3-1 transformed 70 to 90% of added naphthalene, and the transformation was nitrate dependent. No significant removal of naphthalene occurred under nitrate-limited conditions or in cell-free controls. Nitrate consumption, along with the results from the ^{14}C radiolabel study, are consistent with the oxidation of naphthalene coupled to denitrification for NAP-3-1 and nitrate reduction to nitrite for NAP-4. Phylogenetic analyses based on 16S ribosomal DNA sequences of NAP-3-1 showed that it was closely related to *Pseudomonas stutzeri* and that NAP-4 was closely related to *Vibrio pelagius*. This report demonstrated the nitrate-dependent anaerobic degradation and mineralization of naphthalene by pure cultures. (Karl et al, 2000).

Besides that aerobic biodegradation of benzene, toluene and naphthalene was studied (Zhang & Bouwer, 1997) in pre-equilibrated soil-water slurry microcosms. The experiments were designed to simulate biodegradation at waste sites where sorption reaches equilibrium before biodegradation becomes important. Rates of biodegradation were reduced by the presence of soil. The rate of biodegradation was observed to decrease with increasing organic compound hydrophobicity, soil/water ratio, soil particle size, and soil organic carbon content. These results clearly indicate that the rate of biodegradation is affected by both the extent and rate of sorption. Further anaerobic transformation processes for aromatic compounds, particularly polycyclic aromatic hydrocarbons (PAHs) in soil microorganism were studied by (Karthikeyan & Bhandari, 2001). Industrial activities introduce a wide variety of xenobiotic aromatic compounds into the biosphere. These aromatic and polycyclic aromatic compounds find their way into anaerobic marine and freshwater sediments, saturated aquifers, and waterlogged soils where neither their impact on carbon flow nor their propensity to persist is clearly understood. Although some halogenated aromatic compounds are degraded via anaerobic pathways, anaerobic transformation of aromatics remains limited to a small range of chemicals. A simplified pathway of PAH biotransformation and a conceptual pathway of PAH degradation under different redox-conditions was presented in this report.

In addition to this, the degradation potential of five polycyclic aromatic hydrocarbons (PAHs) by aerobic mixed bacterial cultures was investigated. Microorganisms were isolated from hydrocarbon contaminated soils of Shadegan wetland located in southwest of Iran. The degradation experiments were conducted in liquid cultures. PAH or PAHs concentration was 100 mg/L at the beginning of degradation experiments. After ten days incubation, the mixed culture was capable of degrading phenanthrene completely and anthracene 80%, pyrene 60%, fluorene 30%, and fluoranthene 20%, individually. Optimal temperature and pH were as 30 °C and 7.0 respectively. Results showed that in samples containing a mixture of the five PAHs, fluoranthene could degrade co metabolically and the rate of degradation increased from 20 to 44%. It was observed also that PAHs degradation could be enhanced by the individual addition of yeast extract or glucose. The isolated mixed culture is valuable in bioremediation of PAH-contaminated environments (Shafiee et al, 2006).

A laboratory study (Gomare & Lahane, 2011) was also undertaken to access optimal conditions for biodegradation of hydrocarbon. Among 21 hydrocarbons degrading bacterial cultures isolated from contaminated soil sample collected from petrol pump stations of different places of Latur, Udgir and Solapur. *Bacillus sp, Acinetobacter spp, Clostridium spp, and Pseudomonas spp* were selected for the study based on the efficiency of hydrocarbon i.e. naphthalene/anthracene in methanol utilization. Phenotypic examination of the recovered bacteria revealed that they belong mainly to the genus *Pseudomonas spp.* and *Bacillus spp.* Biochemical test were used as an indication for the ability of these bacteria to grow on petrol.

2.2.2 Bacterial strains for Poly Aromatic Hydrocarbons (PAHs)-degradation:

The contamination of soils and groundwater with petroleum compounds is among the most prevalent problems in the environmental area today, thus a need exist to develop new technologies, such as bioremediation that uses microorganisms, to detoxify environmental pollutants and transform them into simpler, less toxic compounds. Soiled contaminated by petroleum hydrocarbons may be treated using various means and applications. There are reports of physical treatment via thermal or chemical process (Piskonen and Itavaara, 2004). However, these treatments are not only unsound economically; they are also prone to prolonged cycle time (Leahy and Colwell, 1990). The next choice of treatment would be to involve microbiological applications as according to, “biodegradation can be an effective and inexpensive approach to remediating soils which contain PAHs and other hydrocarbon compounds”.

Microbial bioremediation of the PAHs:

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

1. **The choice of microbial consortium.**

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

2. **Factors affecting the biodegradation mechanism.**

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

i. ***Biosurfactant.*** According to Leahy and Colwell (1990), biosurfactants are important agents in the effective uptake of PAHs by bacteria and fungi. The formation of emulsions in the presence of biosurfactants is reported to be in 96% of hydrocarbon metabolizing freshwater bacteria.

ii. ***pH.*** Most important PAHs degrading heterotrophic bacteria and fungi perform best when pH is neutral. At pH 7, the mineralization of oily sludge in soil is also improved, thus, enhancing the overall biodegradation process (van Hamme et al., 2003).

iii. ***Nutrients.*** van Hamme et al (2003) also reported that nitrogen and phosphorus contents greatly affect the microbial degradation of hydrocarbons. He further stated that adjustment of the ratios of these two elements ratios by the addition of nitrogen and phosphorus in the form of slow releasing fertilizers stimulated the biodegradation of crude oil and individual PAHs.

iv. ***Salinity.*** Studies have shown that there are generally positive correlations between salinity and rates of mineralization of PAHs such as phenanthrene and naphthalene as reported by Leahy and Colwell (1990). However, hyper salinity will result in the decrease in microbial metabolic rates.

v. ***Oxygen.*** Aerobic biodegradation is the most effective pathway for bioremediation, which means the presence and concentration of oxygen is the rate-limiting parameter in the biodegradation and catabolism of cyclic and aromatic hydrocarbons by bacteria and fungi (van Hamme et al., 2003). This is because PAHs break-down processes involve the utilization by oxygenases, for which molecular oxygen is required. Although anaerobic degradation of PAHs by microorganisms has been shown to occur, the rates are somewhat negligible and limited to halogenated aromatic compounds such as the halobenzoates, chlorophenols and alkyl-substituted aromatics.

vi. ***Temperature.*** Temperature is another important variable that influences petroleum biodegradation. Optimum temperature dictates the rate of PAHs metabolism by microorganisms and also the pattern of the microbial community. Temperature also has direct effect on the physical nature and chemical composition of the PAHs constituents. When temperatures are low, PAHs tend to be more viscous and their water solubility is greatly reduced (Leahy and Colwell, 1990).The optimum temperature is typically in the range of 30 to 40°C. At temperature above this norm, enzymatic activities are inhibited as proteins denature (Leahy and Colwell, 1990).

vii. ***Genetic enhancement/mechanisms.*** Genetic compatibility and readiness is probably one of the most important determining factors in the success of microbial catabolism of PAHs. Bacterial species with either chromosomal or plasmid-borne genes capable of PAHs of hydrocarbon metabolisms are well documented. The most extensively characterized gene is encoded by the *Pseudomonas putida Gpo1* (van Hamme et al, 2003). The role of plasmid is also well documented in the bacteria communities, especially in the *Pseudomonads*. The metabolic pathways for compounds such as naphthalene, salicylate, camphor, octane, xylene, and toluene have been shown to be encoded on plasmids in *Pseudomonas spp*.

2.2.3 Isolation of Poly Aromatic Hydrocarbons (PAHs) degrading bacteria’s:

Diversity of naphthalene – degrading bacteria from a petroleum contaminated soil in Italy had been studied in 2005 and the 6 different communities of microorganism were isolated for their ability to degrade naphthalene, the main pollutant present at the site. The analysis lighted the ability of selected bacteria to metabolise hydrocarbon through the formation of gentisic acid or catechol as intermediates (Alquati et al., 2005).Bacterial strains were also isolated from different contaminated sites in middle delta, Egypt and screened for PAHs degradation. Enrich media was used to isolate the anthracene and phenanthrene degrading bacteria. Fourteen bacterial isolates showed high degradation for both anthracene and phenantherene. For genotyping, these isolates were subjected to RAPD-PCR using four different primers. The data showed that the fourteen isolates were not related to each other. Only four isolates showed the highest ability for degradation was subjected to 16S rDNA sequence for identification (Hassan et al., 2009).

Another bacterial strain for degrading phenanthrene, anthracene and pyrene was isolated from the polluted soil in the Jinan Oil Refinery Factory, Shandong Province of China (Ling et al., 2011). The isolate was identified as Bacillus vallismortis with respect to its 16S rDNA sequence, DNA-DNA relatedness and fatty acid profiles, as well as various physiological characteristics. The strain was Gram-positive, motile, endospore forming, aerobic, oxidase and catalase-positive. Bacillus vallismortis strain JY3A could utilize naphthalene, phenanthrene, anthracene, pyrene, fluorene, benzene, toluene, phenol, methanol, ethanol, Tween 80, cyclohexane or catechol as sole carbon source. The strain alone removed 90.5% of pyrene at an initial concentration of 150ppm in 15days in the presence of 0.5% (w/w) Tween 80. However, in co-culture with Phanerochaete chrysosporium, JY3A reduced the concentration of pyrene by nearly 55.4% after 7days of incubation.

A pyrene-degrading bacterial strain ZQ5 was also isolated from the oil-contaminated soil in Shenfu Irrigation Area (Zhong et al., 2010) by using selective enrichment culture with pyrene as the sole carbon source. The strain was identified as Stenotrophomonas sp., based on its morphological, physiological, and biochemical characteristics, and similarity identification of 16S rDNA sequence. The pyrene-degrading characteristics and the effects of culture condition on the degrading efficiency of the strain were investigated in shaking flask culture. After shaking culture with the initial concentration of pyrene being 100mgl^{-1} at 30 degrees C for 10 days, the degradation rate of pyrene was 91.2%. Adding 100 mg x l^{-1} of salicylic acid into culture medium could enhance the degrading efficiency of the strain and for the degradation of pyrene by ZQ5, the optimal medium pH reported was 7-8, and the optimal salt concentration was lower than 2%.

Four strains with high phenanthrene-degrading ability were also isolated from petroleum badly polluted soil. The strain *Pseudomonas* sp. ZJF08 demonstrated the highest rate of degradation (138. 1 mg·L^{-1}·day^{-1}) among them and degraded 97.1% of the phenanthrene in one week. The activities of two key enzymes of ZJF08, polycyclic aromatic hydrocarbon dioxygenase and catechol-2, 3-oxygenase (C23O), were also assayed during the degradation of phenanthrene. Both of them reached their maximums on the 2nd day of degradation. The C23O gene (*C7*) of *Pseudomonas* sp. ZJF08 was cloned and expressed in *Escherichia coli*, and its gene product was purified by a Ni-NTA-agarose column. The optimum temperature for the purified C23O was 40°C at pH 7.5 and the C23O activity could be still detected when the temperature reached 70°C. The results showed that the C23O from *Pseudomonas* sp. strain ZJF08 exhibited better thermostability than its homologs reported (Zhou et al., 2007).

More research on the microbial degradation of polycyclic aromatic hydrocarbons (PAHs) has resulted in the isolation of numerous genera of bacteria, fungi and algae capable of degrading low molecular weight PAHs (compounds containing three or less fused benzene rings). High molecular weight PAHs (compounds containing four or more fused benzene rings) are generally recalcitrant to microbial attack, although 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 utilise four-ring PAHs as sole carbon and energy sources while co metabolism of five-ring compounds has been reported. The focus of this review is on the high molecular weight PAH benzo[a]pyrene (BaP). There is concern about the presence of BaP in the environment because of its carcinogenicity, teratogenicity and toxicity. BaP has been observed to accumulate in marine organisms and plants which could indirectly cause human exposure through food consumption. This review by (Juhasz et al, 2000) provides an outline of the occurrence of BaP in the environment and the ability of bacteria, fungi and algae to degrade the compound, including pathways for BaP degradation by these organisms.

Further, a report (Brian et al, 2001) shows the isolation of marine bacterium (Strain P-2P44^T) from creosote-contaminated marine sediments by using a most-probable number procedure in which phenanthrene was the sole carbon and energy source. Growth experiments showed that P-2P44T utilized several two- and three-ring polycyclic aromatic hydrocarbons (PAHs) as substrates, including naphthalene, 2-methylnaphthalene and phenanthrene. Additionally, gas-chromatography experiments showed that P-2P44T degraded several other PAHs, though it was unable to use them as sole sources of carbon and energy. Phylogenetic analyses confirmed that strain P-2P44T is a member of the genus *Vibrio*, most closely related to *Vibrio splendidus*. However, strain P-2P44T shared only 98<3% 16S rDNA identity and 35% DNA–DNA reassociation with the type strain of *V. splendidus*. Strain P-2P44T differed phenotypically from *V. splendidus*. Together, these differences indicated that strain P-2P44T represents a novel species in the genus *Vibrio*, for which the name *Vibrio cyclotrophicus* sp. nov. is proposed; the type strain is P-2P44T (=ATCC 700982^T=PICC 106644^T).

2.2.4 **Effect of high sediment content on biodegradation of Poly Aromatic Hydrocarbons (PAHs):**

The contamination of polycyclic aromatic hydrocarbons (PAHs) has become one of the major problems in the Yellow River of China. As the Yellow River is the most turbid large river in the world, it remains unknown to which extent the high suspended sediment content in the river may affect the fate and effect of PAHs. Here is a report on the effect of sediment on biodegradation of chrysene, benzo(a)pyrene and benzo(g,h,i) perylene with phenanthrene as a co-metabolism substrate in natural waters from the Yellow River. Biodegradation kinetics of the PAHs in the river water with various levels of sediment contents were studied in the laboratory by fitting with a biodegradation kinetics model for organic compounds not supporting growth. The results indicated that the biodegradation rates of PAHs increased with the sediment content in the water. When the sediment contents were 0, 4 and 10 g/l, the biodegradation rate constants of chrysene with the initial concentration of 3.80 g/l were 0.053, 0.084 and 0.111 d^{-1}, respectively (Xia et al., 2006).

2.2.5 **Role of plasmids in degradation of polycyclic aromatic hydrocarbons:**

Polycyclic aromatic compounds are a group of highly recalcitrant organic pollutants. The initial steps in the degradation of polycyclic aromatic hydrocarbon (PAHs) involve the dihydroxylation of the aromatic ring, a step catalysed by dioxygenase enzymes. The degradation of many xenobiotic and hydrocarbon compounds is known to be mediated by plasmid encoded enzymes. In this review, an insight is given into the role of plasmid in degradation of PAHs, acquisition of degradative ability by these organisms via horizontal transfer and clustering, resulting from tranposon-mediated recombination. There is preponderance of information showing high level of plasmid involvement in the degradation of naphthalene and other 2- and 3-ring PAHs. Information on higher molecular weight PAHs is however scanty. Recent studies suggest possible involvement of plasmid in HMWPAH degradation than was previously thought. Many plasmids involved in PAH-degradation are megaplasmids, of linear configuration, encoding part or the whole genes for the complete pathways. In recent times, validation of propositions on degradative gene acquisition by horizontal gene transfer (HGT) has been obtained from field studies. HGT and transposition seems to be more chronologically linked and less fortuitously directed than previously thought. Improvement on the methods used in isolation of degraders and study of these is important, towards making a significant stride in elucidating plasmid involvement in PAH degradation (Obayori & Salam, 2010).

2.2.6 Fungi for Poly Aromatic Hydrocarbons (PAHs) degradation:

Due to the hydrophobicity of PAHs they tends to accumulate on the soil organic matter and thus, their desorption from soil limits their availability for the microorganisms to biodegrade these pollutants. In a report the extracellular enzymes produced by white rot basidiomycetes (Eibes,2006) believed to have the ability of degrading PAHs and other organopollutants were studied but the high hydrophobicity of polycyclic aromatic hydrocarbons (PAHs) greatly hamper their degradation in liquid media, so an organic solvent was used to assist the degradative action of ligninolytic enzymes from white rot fungi. The enzymatic action of the enzyme manganese peroxidase (MnP) in media containing a miscible organic solvent, acetone (36% v/v), was evaluated as a feasible system for the in vitro degradation of three PAHs: anthracene, dibenzothiophene and pyrene. These compounds were degraded to a large extent after a short period of time (7, 24 and 24 h, respectively), at conditions maximizing the MnP-oxidative system. The order of degradability, in terms of degradation rates was as follows: anthracene > dibenzothiophene > pyrene. Anthracene was degraded to phthalic acid. A ring cleavage product of the oxidation of dibenzothiophene, 4-methoxybenzoic acid, was also observed.

Further the wood-rotting fungi *Pleurotus ostreatus* and *Antrodia vaillantii* was inoculated in the soil sample artificially contaminated with fluorene, phenanthrene, pyrene, and benz[*a*]anthracene. During 12 weeks of incubation, polycyclic aromatic hydrocarbon (PAH) degradation and the formation of persistent degradation products were monitored by chemical analysis. In addition, the effect on the indigenous soil bacteria was studied by plate count techniques and by measuring the concentration of bacteria-specific phospholipid fatty acids (PLFAs). In both soils inoculated with fungi, the PAH degradation was enhanced compared to the control soil without fungi. The white-rot fungus *P. ostreatus* accelerated the degradation rate radically the first weeks, while the effect of the brown-rot fungus was more pronounced at later stages during the 12-week study. In a soil with no amendments, the final degradation result was similar to that in the soil with added fungi, although the degradation pattern for the individual PAHs was different (Andersson et al, 2009).

CHAPTER 3

MATERIALS AND METHODS

This chapter discusses the materials used and methodology adopted during the study.

3.1 CHEMICALS

Analytical grade chemicals were used for the experiments.

3.2 SELECTION OF NAPHTHALENE DEGRADING BACTERIA

3.2.1 Culture media

Nutrient agar and Minimal medium (M9) was used for initial screening.

Composition of M9 media:

Table 3.1: M9 media Composition

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

Media was sterilized in an autoclave at 121 ^{o}C and 15 psi for 15 minutes.

3.2.2 COLLECTION OF SAMPLE

Soil samples were collected from crude oil polluted soil near fuel filling stations, Chandigarh.

3.2.2.1 Screening of Naphthalene degrading bacteria

The screening of the naphthalene degrading bacteria was done by dissolving 1 g of contaminated soil sample in the nutrient broth (NB) and incubated for 21 days at 37 ^{o} C on rotary shaker at 150 rpm. After growth of consortia in NB, serial dilutions were made and 100µl of the liquid was surface spreaded on nutrient agar plates and plates were incubated at 37 ^{0}C for 72h.On the basis of morphology and colour the microbial colonies were selected from nutrient agar plates containing naphthalene (100 ppm). Each colony was picked and streaked on nutrient agar plates with increased concentration of naphthalene. Out of these the best grown isolates were selected and were maintained in M9 medium and stored at 4 ^{0}C for further work.

3.2.3 Identification of selected bacterial isolates

3.2.3.1 Morphological and biochemical characterization

Selected isolates were characterized by colony morphology on nutrient agar, gram staining and morphological characteristics according to Cappuccino and Sherman (2010). Additional biochemical test were performed according to Aneja (2008) for taxonomic characterization which included gelatine liquefaction, lipase screening, casein hydrolysis, H_2S production, lipase test, xylanase test, pectinase screening, methyl red-voges proskauer (MR-VP), citrate utilization, carbohydrate utilization and amino acids utilization.

a) Biochemical Tests

i. Gelatin Agar Medium

Table 3.2: Composition of Gelatin Agar Medium

Composition	Concentration(g/l)
Gelatin	40
Tryptic Soy broth	30
Distilled water	1000 ml

Procedure:

Stab a small inoculum of the bacterium about ¾ of the way to the bottom of a tube of deep agar with the inoculating needle. Repeated with separate stab tubes for each of bacteria and incubated at 37°C for 24-48 h. Placed the incubated inoculated stab and the uninoculated control into the refrigerator,for approximately 30 minutes. Compared the inoculated stab with the control by tapping the tubes gently.

ii. H_2S Production

Table 3.3: Media Composition for test of H_2S Production

Composition	Concentration(g/l)
Peptone	30
Beef extract	3
FAS	0.2
Sodium thiosulphate	0.02
Agar	15
Distilled Water	1000 ml

Procedure:

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

iii. Starch Hydrolysis

Table 3.4: Media Composition for Starch Hydrolysis Test

Composition	Concentration(g/l)
Starch	2.0
Peptone	0.5
Beef extract	0.3
Agar	1.5
Distilled Water	100ml

Procedure:

Inoculated the plates of starch agar with the assigned bacteria's and incubated at 37°C for 24-48 hours. Dripped a small amount of Gram's iodine on the plate around the inoculated area and a small amount in an uninoculated area away from the inoculum. A clear zone was observed around the inoculum. Compared the inoculate area with the uninoculated area, and recorded the results.

iv. Citrate Utilization

Table 3.5: Media Composition for Citrate Utilization Test

Composition	**Concentration (g/l)**
$(NH_4)_2HPO_4$	0.1
K_2HPO_4	1
NaCl	5
Sodium Citrate	2
$MgSO_4$	0.2
Bromothymol blue	0.08
Agar	2
Distilled Water	1000 ml

Procedure:

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

v. Methyl Red-Voges Proskauer test (MR-VP broth)

Table 3.6: Media Composition for MR-VP Test

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

Procedure:

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

vi. Carbohydrate Utilization

Table 3.7: Media Composition for Carbohydrate Utilization Test

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

Sugar: Glucose, Fructose, Sucrose, Mannitol, Maltose.

Procedure:

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

vii. Pectinase Screening

Table 3.8: Media Composition for Pectinase Screening Test

Composition	**Concentration (g/l)**
Ammonium Sulphate	1
K_2HPO_4	6
KH_2PO_4	3
Pectin	5
Yeast extract	1
Distilled Water	1000ml

Procedure:

The autoclaved medium, cooled to 45-50 ^{0}C was poured into sterile petri plates and allowed to solidify. Aseptically inoculated the plates and incubated at 37 ^{0}C for 24-48 h. After growth flooded with CTAB buffer (1% solution) for 10 min and clear zone was observed around the growth on the media.

viii. Lipase Screening

Table 3.9: Media Composition for Lipase Screening Test

Composition:	**Concentration(g/l)**
Nutrient Broth	13
$CaCl_2.2H_2O$	0.1
Tween 80	10 ml
Agar	20
Distilled water	1000 ml

Procedure:

Medium was sterilized by autoclaving at 121 ^{0}C for 15 mins. Tween 80 was autoclaved separately and added to medium. Incubated the inoculated plates at 37 ^{0}C in an inverted position for 24-48 h. Observed the plates for the formation of opaque zone around the growth.

ix. Xylanase Screening

Table 3.10: Media Composition for Xylanase Screening Test

Composition	**Concentration(g/l)**
Yeast extract	1
Xylan powder	5
Agar	20
Distilled water	1000 ml

Procedure:

Inoculated the plates with the bacteria's and incubated at 37^{0}C in an inverted position for 24-48 hours. After growth occur, flooded the plates with Congo red solution (1% solution) for 30 minutes, then washed with 1 M NaCl. Observed the plates for the formation of a clear zone around the growth.

x. Cellulase Screening

Table 3.11: Media Composition for Cellulase Screening Test

Composition	**Concentration g/l**
Yeast extract	1
Carboxtmethylcellulose sodium salt	5
Agar	20
Distilled water	1000 ml
Congo red solution	10

Procedure:

Incubated the inoculated plates at 37^0C in an inverted position for 24-48 h and flooded the plates with Congo red solution (1% solution) after the growth occurred. Observed the plates for the formation of a clear zone around the growth.

xi. Amino acid utilization test

Table 3.12: Media Composition for Amino acid utilization Test

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

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

Procedure:

Transferred 0.1 ml of inoculum of each of the bacteria's into broths (tyrosine, valine, glycine, arginine, alanine, leucine and threonine) and incubated the inoculated broths at 37^oC for 24-72 h in a rotary shaker at 150rpm. Results were observed for each broth and compared to the uninoculated controls.

b) PHYSIOLOGICAL TESTING

The optimum temperature, pH and salinity concentration for the growth of the strains was determined.

i. Effect of Incubation Temperatures on growth.

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

Procedure:

Inoculated the flasks containing M9 media with the bacterial culture's and incubated the inoculated flasks and the control flasks at different temperatures (10^{o}C, 20^{o}C, 28^{o}C, 35^{o}C and 45^{o}C) for 24 to 48 hours in a rotary shaker at 150 rpm. Examined the cultures after 2 days of incubation for the presence or absence of growth. The degree of growth was determined by using spectrophotometer at wavelength 600 nm.

ii. Effect of pH on growth.

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

Procedure:

Inoculated each of a series of the tubes with bacterial cultures by adding 0.1 ml of the culture. Incubated the bacterial inoculated tubes with different pH (6-10) for 24-48 h at 37^{o}C in a rotary shaker at 150 rpm. Examined the growth by using spectrophotometer at wavelength 600 nm.

iii. Effect of salt concentrations on growth.

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

Procedure:

Different salt concentrations (4%,8%,12%,16% and 20% of NaCl) was added to the M9 medium tubes. Inoculated each of a series of the tubes with bacterial cultures by adding 0.1 ml of the culture to each tube and incubated the bacterial inoculated tubes for 24-48 hours at 37^oC in a rotary shaker at 150 rpm. Growth was examined after 2 days with mass spectrophotometer at maximum wavelength of 600 nm.

c) Morphological studies

i. Gram staining:

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

ii. Spore staining:

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

3.2.3.2 Molecular characterization based on 16S rRNA gene.

The 16S r RNA gene sequencing study was performed by Xcelris Labs Limited, Ahmedabad (India). DNA was isolated from bacterial strain S3 by using QIAamp DNA Purification Kit (Qiagen). The 16S r RNA gene was amplified by using PCR method with universal bacterial primers 8F (5’-AGAGTTTGATCMTGGCTCAG) and 1492R (5’-GGYTACCTTGTTACGACTT-3’). Amplified PCR product was purified using Qiagen Mini elute Gel extraction kit according to the manufactures protocol. Sequencing of the purified 16S rRNA gene was performed using BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) as recommended by manufacturer. The purified sequencing reaction mixure were electrophoresed automatically using ABI 3730*1 Genetic Analyser (Applied Biosystems, USA). The 16S r RNA gene sequence of the strain S3 was processed manually, analysed at NCBI (National Centre for Biotechnolgy Information) using BLAST tool and compared to the corresponding neighbour sequences from GenBank-NCBI database.

Consensus sequence (~1402 bp) was imported into the Multalin program and multiple alignment was performed with related Pseudomonas species (GenBank-NCBI database). The results obtained were further imported into MEGA software for the construction of a phylogenetic tree using Bootstrap analysis with 1000 replicates, the substitution method used was Kimura 2-Parameter model and the statistical method used was Neighbor-joining.

BIODEGRADATION STUDIES OF NAPHTHALENE

3.3.1 Biodegradation of Naphthalene in Soil.

Biodegradation of naphthalene using F3 and S3 was evaluated in soil. Clean uncontaminated soil was collected, dried, sieved and sterilized by autoclaving. 100 ppm and 200 ppm of naphthalene dissolved in methanol was added to the clean soil and mixed thoroughly. Clean soil (5 gram) and contaminated soil (5 gram, collected from the fuel filling stations) was placed in 150 ml conical flasks into duplicate vials. The samples were inoculated with 1 O.D (at 600 nm) of cultures in 50 ml of M9 medium. All vials were incubated at 37 °C in a rotary shaker at 150 rpm. Whole sample contained in the individual vials were extracted at 0 time, one week and two weeks for the purpose of measuring residual concentration (Bishnoi et al., 2009).

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

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

3.2.3 Analytical method

3.3.2.1 Liquid Chromatography/ Mass Spectrophotometer Analysis:

Aliquots (5 ml) were centrifuged at 10,000 rpm for 10 minutes and supernatant was filtered by 0.2 µm filter and kept for analysis. Analysis was performed by LC, the analytical column C18, 150* 4.6 mm, 5 µm was used. A gradient elution was used. Solvent A (deionised water) and solvent B (acetonitrile) were used as the mobile phase. Gradient elution (0min, 50%B; 0-20min, linear change from 50 to 100% B; 20-25 min, 100% B; 25-26 min, 50% B; 26-30 min, 50% B; 30.10 min, stop; run time, 30 min) was performed with a 0.7 ml/min at constant flow rate. The column oven temperature was 40^{0}C, and the injection volume was 20µl, for all standards and samples (Smoker et al., 2010). The mass spectrometer was operated in positive mode with the PhotoSpray ESI source. The effluent from the LC column was directly introduced into the source. The concentration of naphthalene was determined at λ254 nm by comparison to standard curve. Concentration of PAHs was calculated by comparing peak areas of sample chromatogram with the peak area of standard chromatogram.

Concentration of PAHs in sample (µg/g) =

$$\frac{\textbf{Peak area of chromatogram of sample}}{\textbf{Peak area of chromatogram of standard PAHs compounds}} \times \textbf{Concentration of standard PAH compounds}$$

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 SELECTION OF NAPHTHALENE DEGRADING BACTERIA

A total of eight bacterial isolates were screened for their growth on naphthalene from oil contaminated sites. Six strains showed the best growth on nutrient agar plates with naphthalene (100ppm). These strains when tested for their growth in Minimal medium (M9) with naphthalene as sole source of carbon and energy, different levels of growth were observed. Majority of the isolates showed growth up to 100 ppm concentration of the naphthalene tested. Strain F3 and S3 showed rich growth up to 200 ppm naphthalene. So these two strains were selected as naphthalene degrading bacteria for further study.

4.1.1 Identification of selected bacterial isolates

4.1.1.1 Morphological and biochemical characterization

a) Morphological characterization:

The two bacterial isolates F3 and S3 were analyzed taxonomically. The colony morphology of F3 was off white in colour with irregular margins and rough surface whereas the colony morphology of S3 was greenish in colour with irregular margins and smooth surface. Both the isolates were gram negative and all were rod shape. Spore staining revealed the presence of spores in F3 as well as in S3. (Table 4.1)

b) Biochemical characterization:

Biochemical tests revealed that both the isolates showed positive results for gelatin liquefaction, amino acid utilization and carbohydrate utilization indicating that these can be used as a nutrient source (complex protein) and bacterium has the enzyme necessary to perform the hydrolysis, bacterium can hydrolyze lactose (into glucose and galactose and then ferment either of the monomers released, usually only the glucose), and can hydrolyze sucrose (into glucose and fructose) while for starch hydrolysis and cellulase activity results were negative for both the isolated bacterium indicating that bacterium cannot hydrolyze starch (a polysaccharide) into maltose and glucose and cannot generate the enzyme necessary for the hydrolysis of cellulose.

For the Methyl red test, yellow colour was recorded as a end point for both the strains, indicating that little or no acid remains in the medium (has likely been converted into non-acidic or neutral end products that can often be detected by the VP test) but for Voges proskauer test result was positive for S3 while negative reaction was showed by F3 strain, as deep "rose" colour appears as end point for S3 culture indicating the presence of non-acidic end product while for F3 deep rose color doesn’t appear indicates the presence of acid in the medium.

Further for Xylanase activity and Hydrogen sulphide production negative results was showed by both the strains reflecting the inability of the bacterium to release enzyme necessary for hydrolysis of xylan and inability to reduce a sulphur metabolite in the medium and to produce H_2S (a colourless gas that generates a black colour in the agar medium). S3 showed negative result for lipase activity while F3 showed positive result for the test reflecting that F3 can carry out the hydrolysis of lipase by releasing the enzymes while S3 cannot. Pectinase screening test was positive for both the strains showing the release of necessary enzymes by both the strains. Citrate utilization and catalysis test was positive for F3 while negative result was showed by S3 that determines F3 can use citrate as the sole source of carbon whereas S3 cannot. (Table 4.2- 4.5)

Table 4.1: Morphological characteristics of bacterial isolates

STRAIN	GRAM STRAIN	SHAPE	SPORE STAIN
S3	Negative	ROD	+
F3	Negative	ROD	+

Table 4.2: Biochemical characterization of selected bacterial isolates

Strain	Pectinase Screening	Lipase Screening	Xylanase Screening	Starch Hydrolysis	Cellulase Activity	Gelatin Liquification
F3	+	+	--	--	--	+
S3	+	--	--	--	--	+

Table 4.3: Biochemical characterization of selected bacterial isolates

Strain	Hydrogen sulphide Production	Citrate Utilization	Citrate Catalyses	Methyl Red test	Voges Proskauer
F3	--	+	+	--	--
S3	--	--	--	--	+

Table 4.4: Amino acid utilization test of the bacterial isolates:

Amino acids » Strain ↓	Tyrosine	Valine	Glycine	Arginine	Alanine	Leucine	Threonine
F3	+	+	+	+	+	+	+
S3	+	+	+	+	+	+	+

Table 4.5: Carbohydrate utilization test of the bacterial isolates:

Carbohydrate » Strain ↓	Sucrose	Glucose	Lactose	Mannitol
F3	+	+	+	+
S3	+	+	+	+

4.1.1.2 Physiological characterization.

The optimum temperature, pH and salt concentration in M9 medium for the growth of both the strains was determined.

i) Effect of temperature:

a) The effect of temperature on the growth of F3 was evaluated in the M9 medium. The results showed that the maximum growth was observed at 37ºC with $O.D_{600}$ of 0.186 after 48 hours, and growth decreased at both 28 and 44ºC (OD_{600}'s of 0.132 and 0.18), respectively (Figure 4.1), indicating the bacteria to be mesophillic in nature.

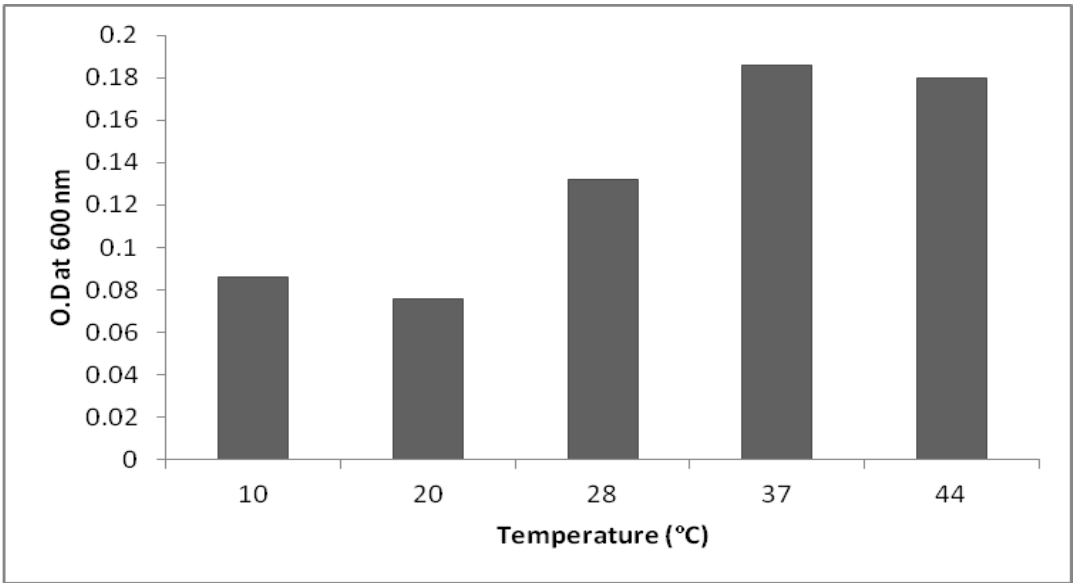


Figure 4.1: Growth of F3 in M9 medium with 100 ppm naphthalene at different temperatures.

b) The effect of temperature on the growth of S3 was also evaluated in the M9 medium. The results showed that the maximum growth was observed at 37ºC with $O.D_{600}$ of 0.298 after 48 hours, and growth decreased at both 28 and 44ºC (OD_{600}'s of 0.208 and 0.148), respectively (Figure 4.2), indicating the bacteria to be mesophillic in nature.

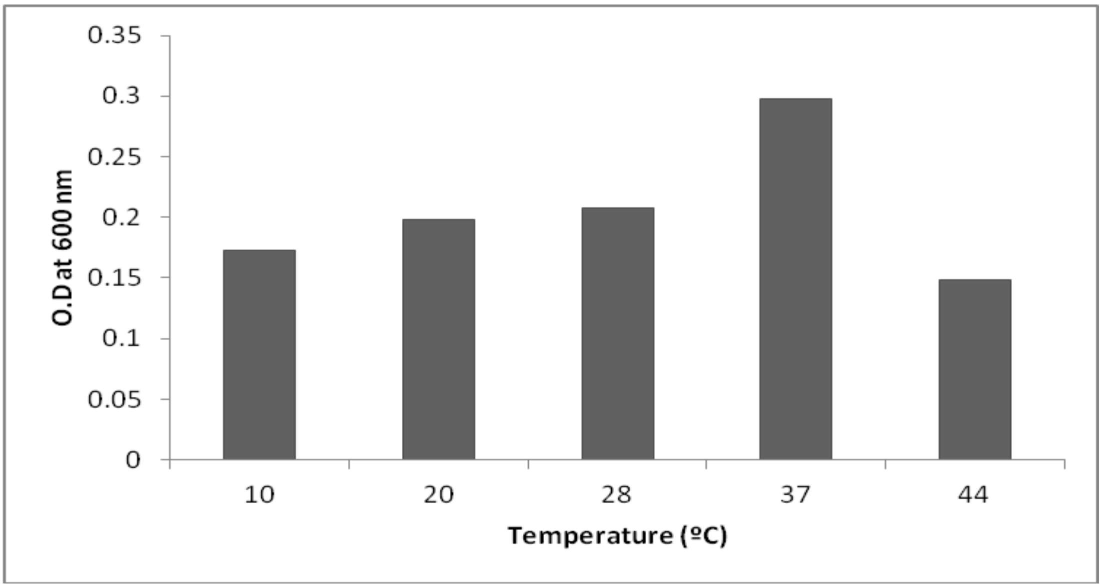


Figure 4.2: Growth of S3 in M9 medium with 100 ppm naphthalene at different temperatures.

ii) Effect of pH:

a) The effect of pH on growth of F3 in M9 medium was evaluated. The maximum growth was observed at pH 7 with an $O.D_{600}$ of 0.981 after 48 hours. Good growth was also observed at pH 8 with a maximum OD_{600} around 0.952. There was less growth at pH 10 (OD_{600} of 0.624), respectively. Figure (4.3)

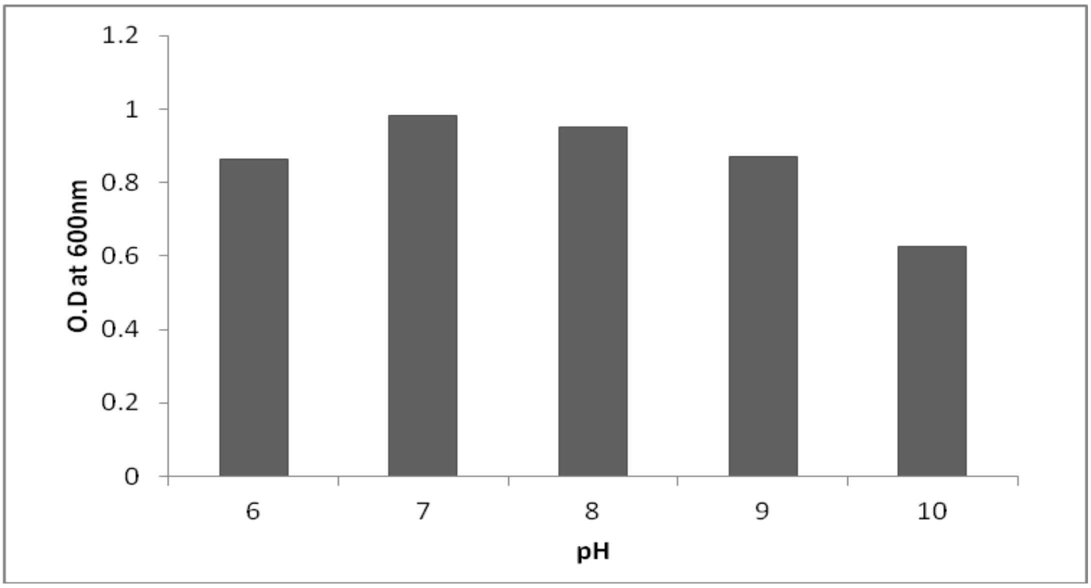


Figure 4.3: Growth of F3 in M9 medium with 100 ppm naphthalene at different pH.

b) The effect of pH on growth of S3 in M9 medium was evaluated. The maximum growth was observed at pH 7 with an $O.D_{600}$ of 1.220 after 48 hours. Good growth was also observed at pH 8 with a maximum OD_{600} around 1.179. There was less growth at pH 9 and 10 ($OD_{600's}$ of 0.905 and 0.404), respectively. Figure (4.4).

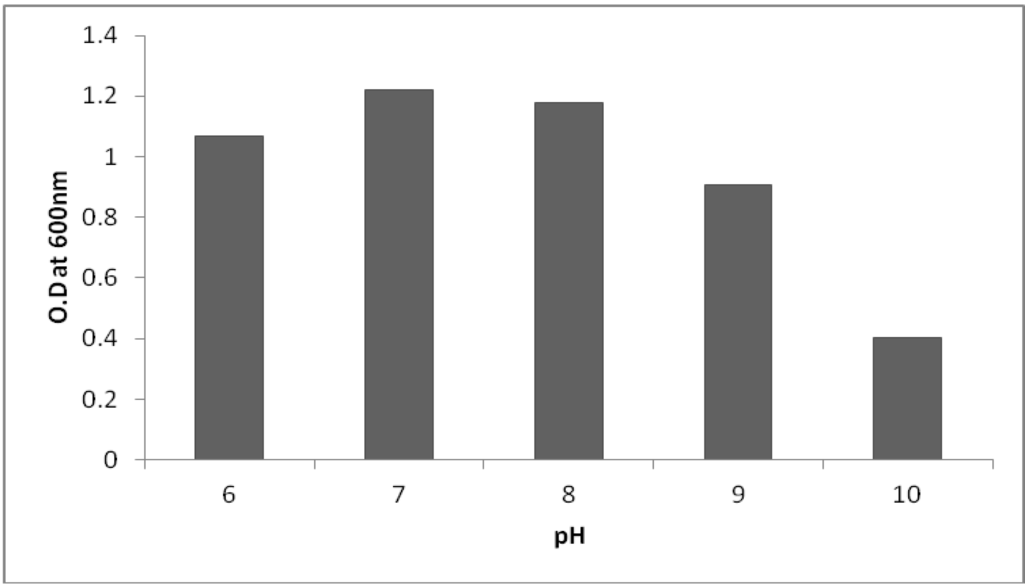


Figure 4.4: Growth of S3 in M9 medium with 100 ppm naphthalene at different pH.

iii) Effect of salt concentration:

a) For F3 culture maximum bacterial growth occurred at 12% of the salt (NaCl) concentration with $O.D_{600}$ (0.067) whereas minimum occurred at 20% $O.D_{600}$ (0.032) and the optimum range for the growth was considered to be between 8-16% as the figure (4.5) shows, indicating the bacteria to be moderately halophilic in nature.

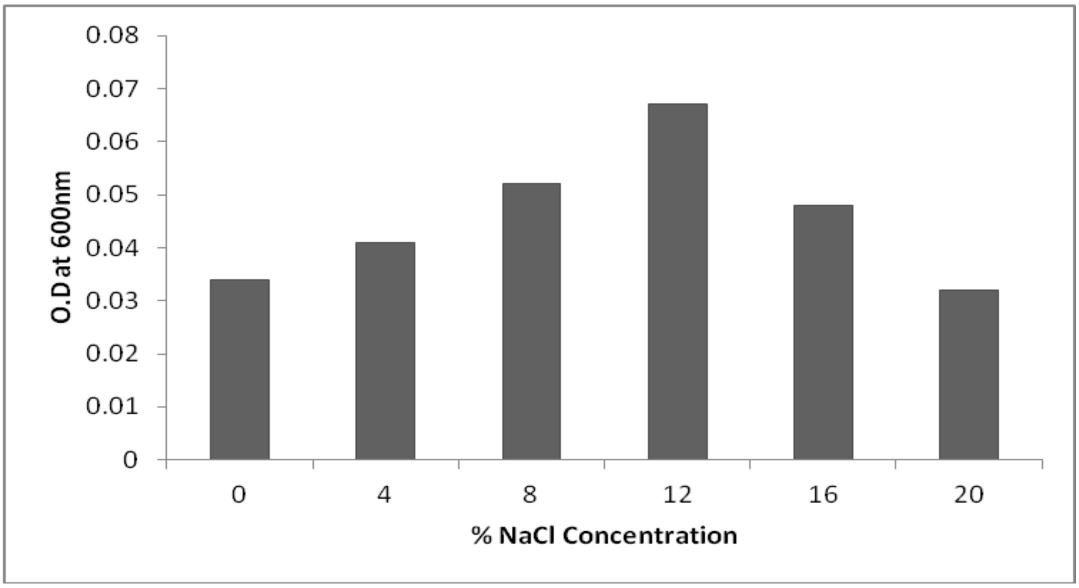


Figure 4.5: Growth of F3 in M9 medium with 100 ppm naphthalene at different salt (NaCl) concentration.

b) For S3 culture maximum bacterial growth occurred at 20% of the salt (NaCl), concentration $O.D_{600}$ (0.078) whereas minimum occurred at 0% $O.D_{600}$ (0.026) and the optimum range for the growth was considered to be between 16 to 20% as the figure (4.6) shows, indicating the bacteria to be extremely halophilic in nature.

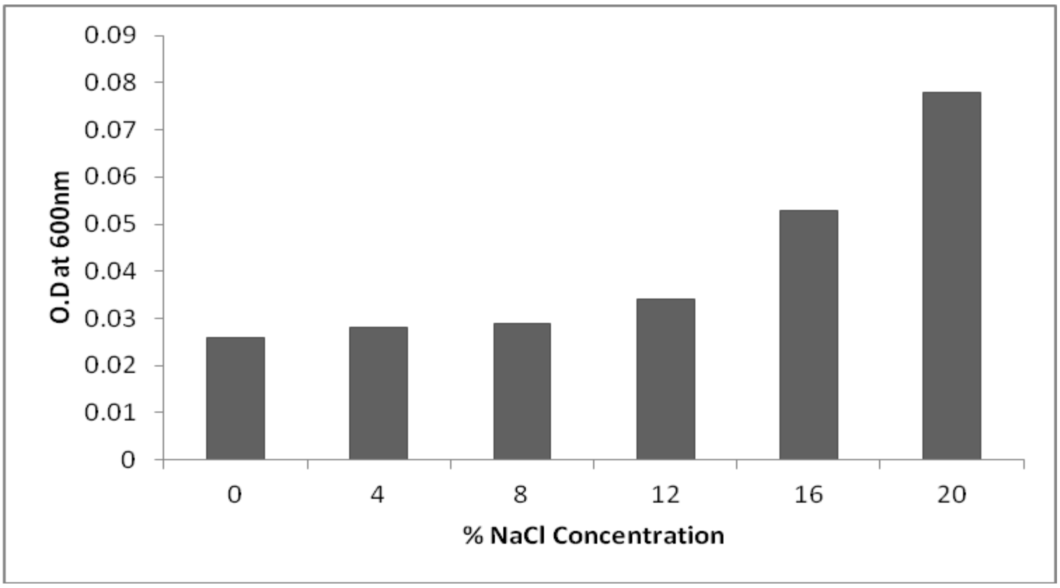


Figure 4.6: Growth of S3 in M9 medium with 100 ppm naphthalene at different salt (NaCl) concentration.

4.1.1.3 Molecular characterization based on 16S rRNA gene

The S3 bacterial isolate was further identified on the basis of sequence analysis of 16S rRNA. The obtained sequence was submitted to the BLAST in order to find a homology with other 16S rRNA sequences. Comparing the sequence of the 16S rRNA gene of the isolates with the sequences in GenBank revealed that the isolate S3 showed 98 % homology in 16S rRNA sequence to *Pseudomonas aeruginosa* strain DSM 50071 and phylogenetic analyses based on 16S ribosomal RNA sequences of S3 also showed that it was closely related to *Pseudomonas aeruginosa* strain DSM 50071.Figure (4.7).

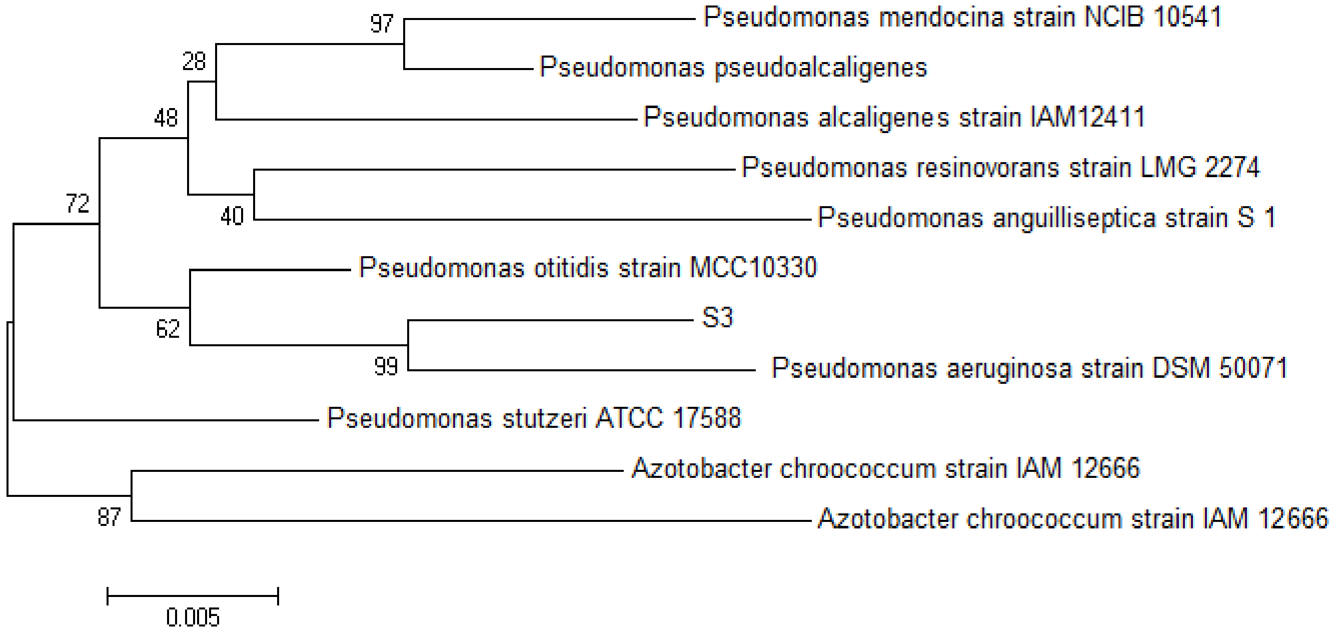


Figure 4.7: Neighbour-joining tree showing strain S3 and related *Pseudomonas* strains. Numbers below tree nodes represent the percentage bootstrap support for 1000 replicates, respectively. Bar, approximately 0.005% nucleotide divergence.

Biodegradation studies of Naphthalene

4.2.1 Biodegradation of naphthalene in soil:

Biodegradation study was conducted with naphthalene adapted bacterial strains F3 and S3 at optimised conditions. Biodegradation was done in M9 medium using standard (100ppm) naphthalene soil samples and contaminated soil samples. The samples were extracted at 0day, after 7 days and 15 days of incubation. The peak area of the different chromatograms was calculated for calculating the biodegradation efficiency of both the strains.

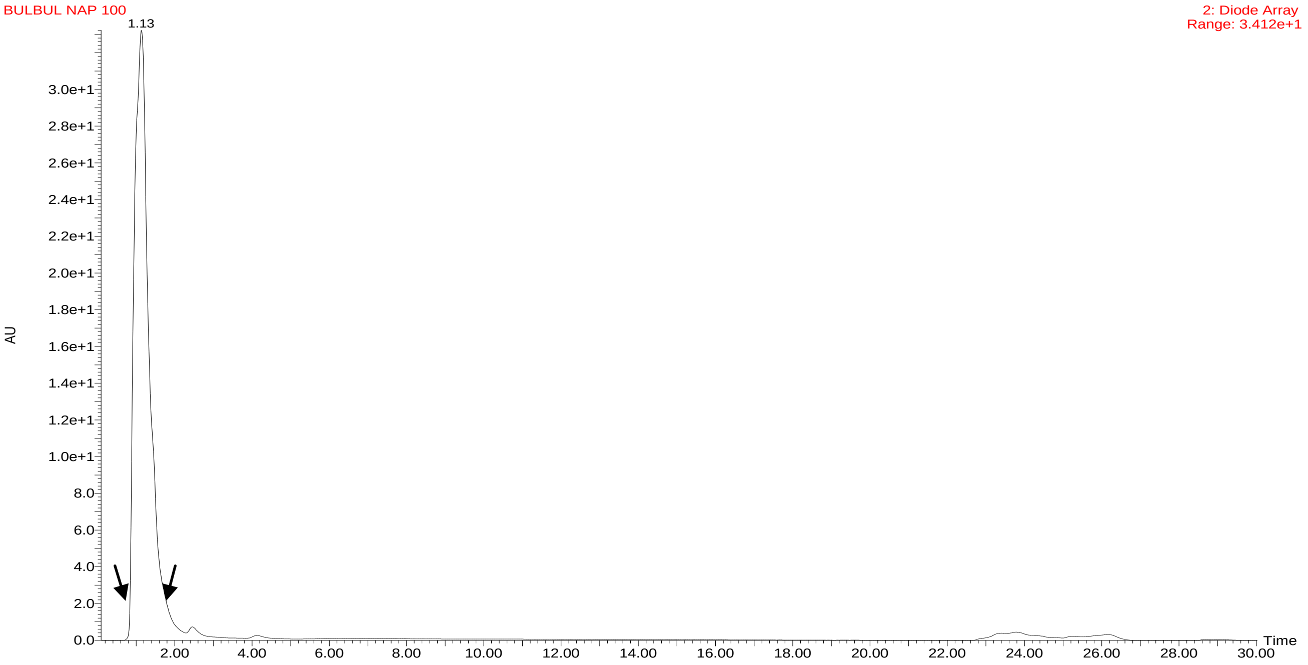


Figure 4.8: Chromatogram of Naphthalene standard (100µg/g) at 0 day of incubation.

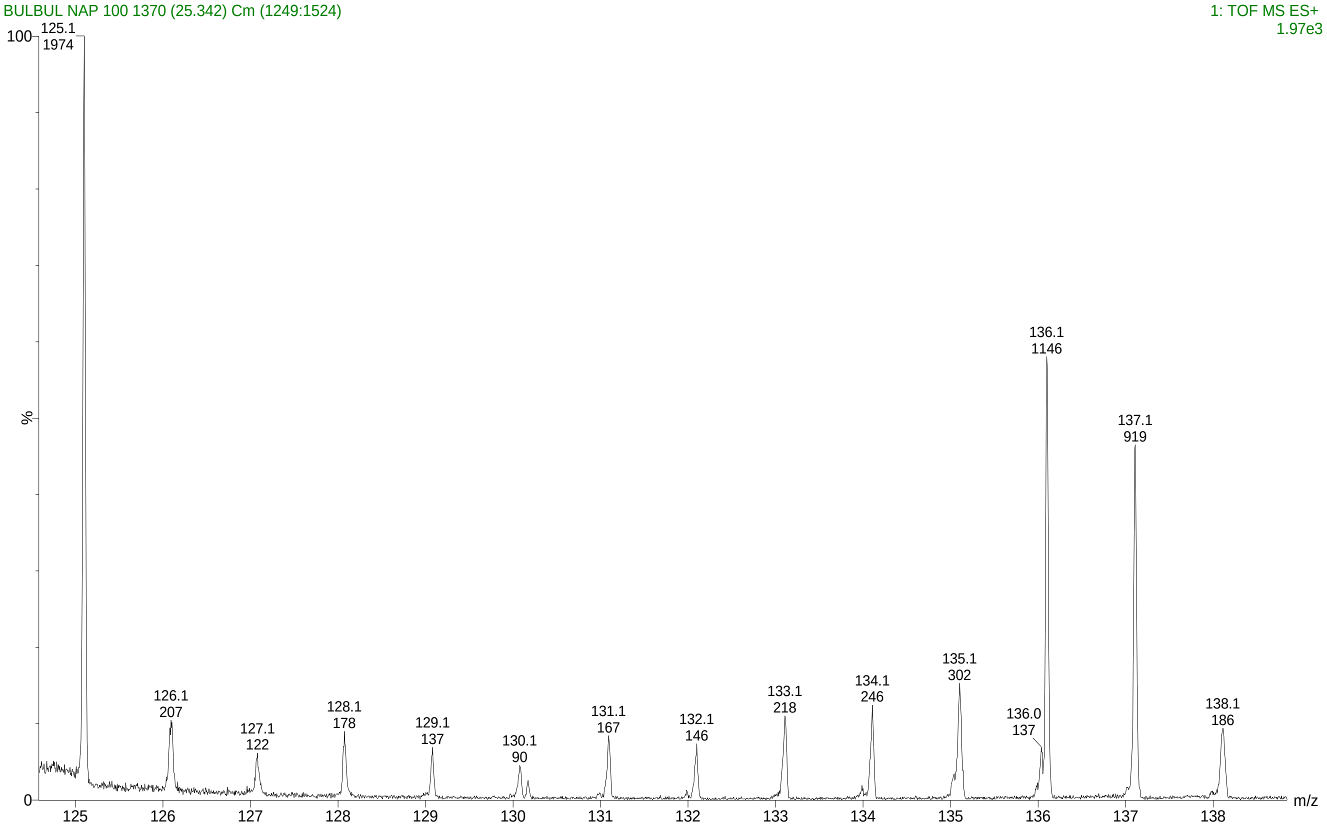


Figure 4.9: Mass spectra obtained by LC/MS of standard (100µg/g) of Naphthalene at 0 day of incubation.

Peak area of chromatogram of 100µg/g standard of Naphthalene at 0 day of incubation = 1.1cm^2.

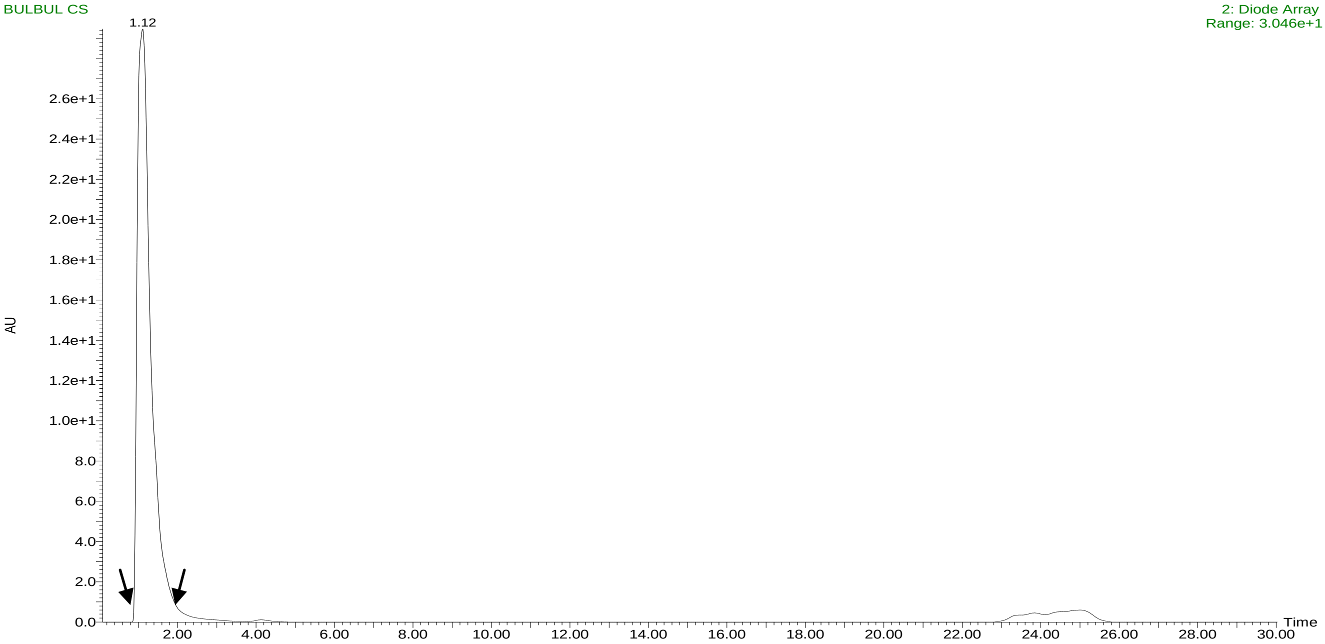


Figure 4.10: Chromatogram of Contaminated Soil at 0 day of incubation.

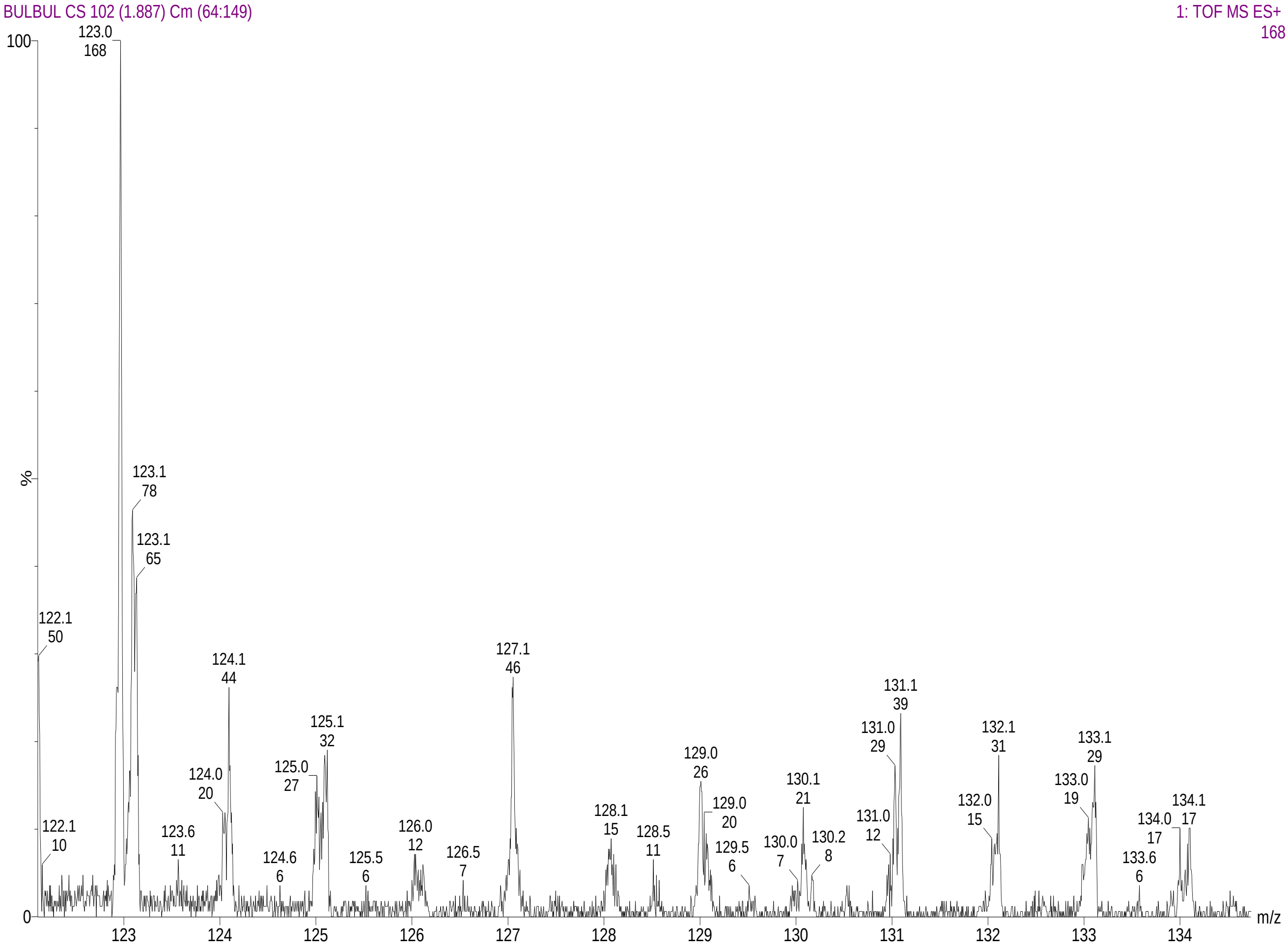


Figure 4.11 Mass spectra obtained by LC/MS of Contaminated Soil at 0 day of incubation.

Peak area of chromatogram of Naphthalene in Contaminated Soil at 0 day of incubation = 1.8 cm^2

Concentration of Naphthalene in sample (µg/g) at 0 day of incubation = (1.8/1.1) * 100
= 163.63µg/g

4.1.1.2 Biodegradation study of Naphthalene using S3 strain after 7 days of incubation.

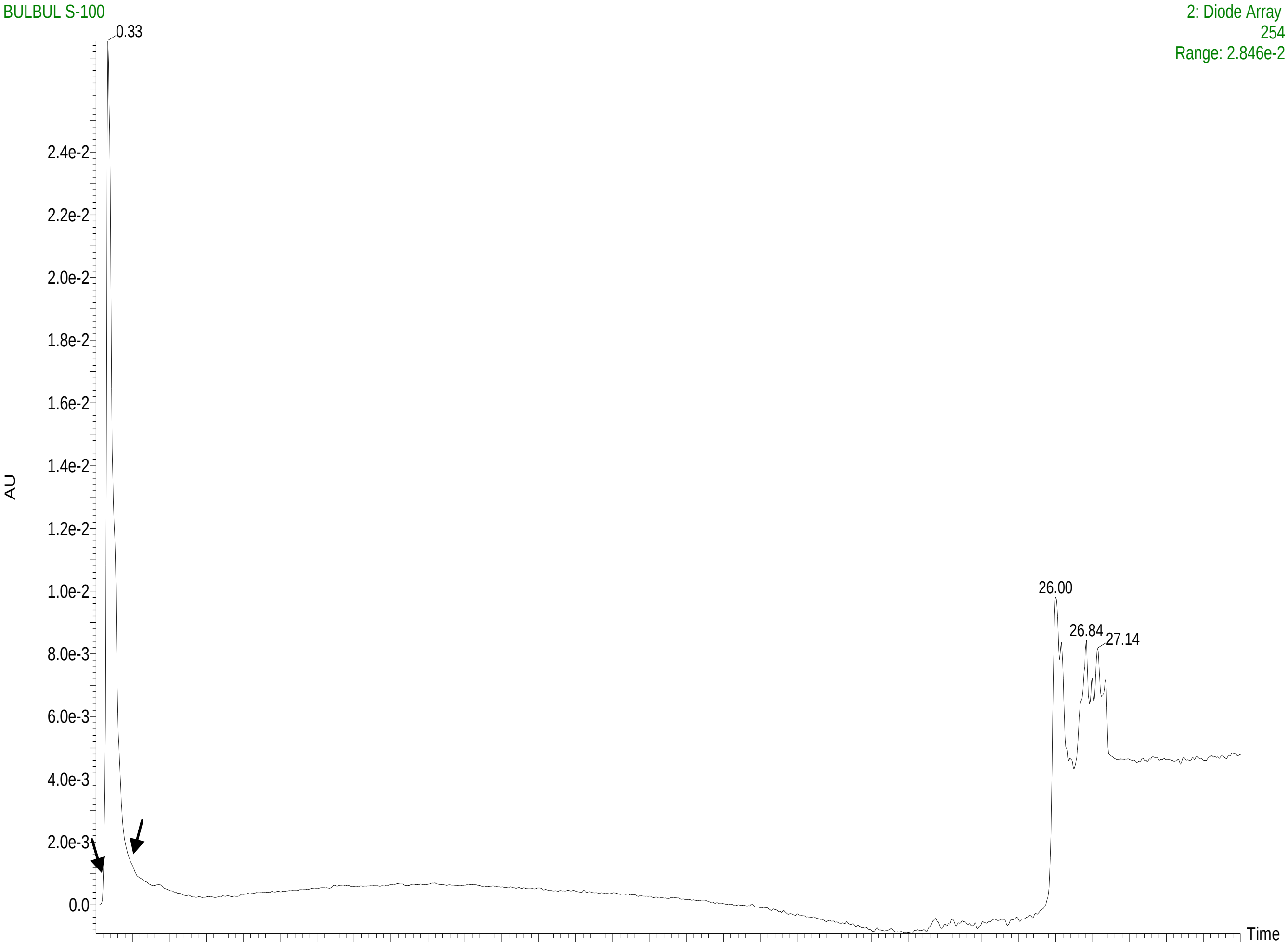


Figure 4.12: Chromatogram of Naphthalene standard (100µg/g) after 7 day of incubation with S3.

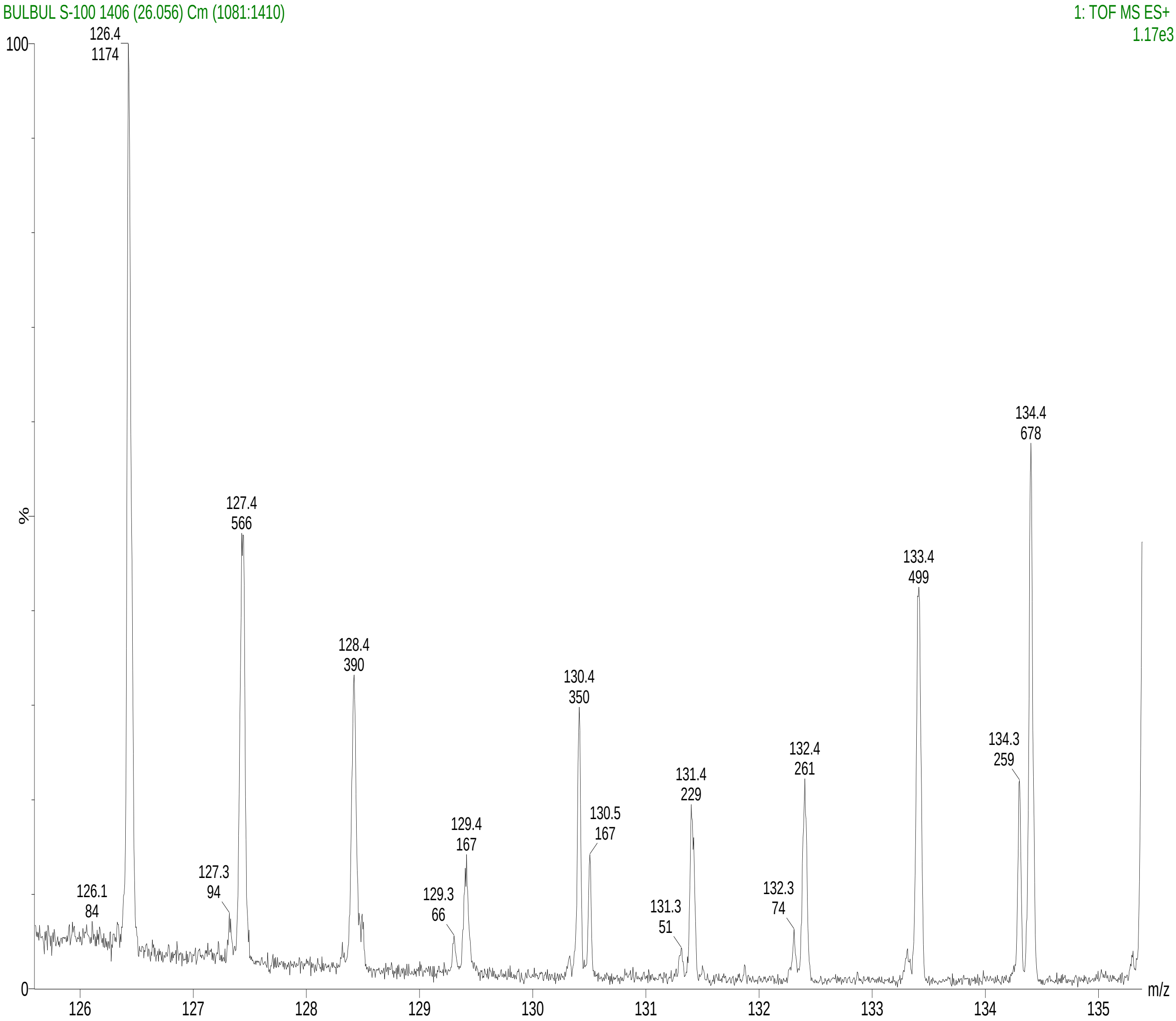


Figure 4.13: Mass spectra obtained by LC/MS of standard (100µg/g) of Naphthalene after 7 days of incubation with S3.

Peak area of chromatogram of standard (100µg/g) of Naphthalene after 7 days of incubation with S3 = 1.1cm^2

Figure 4.14: Chromatogram of Contaminated Soil after 7 days of incubation with S3 strain.

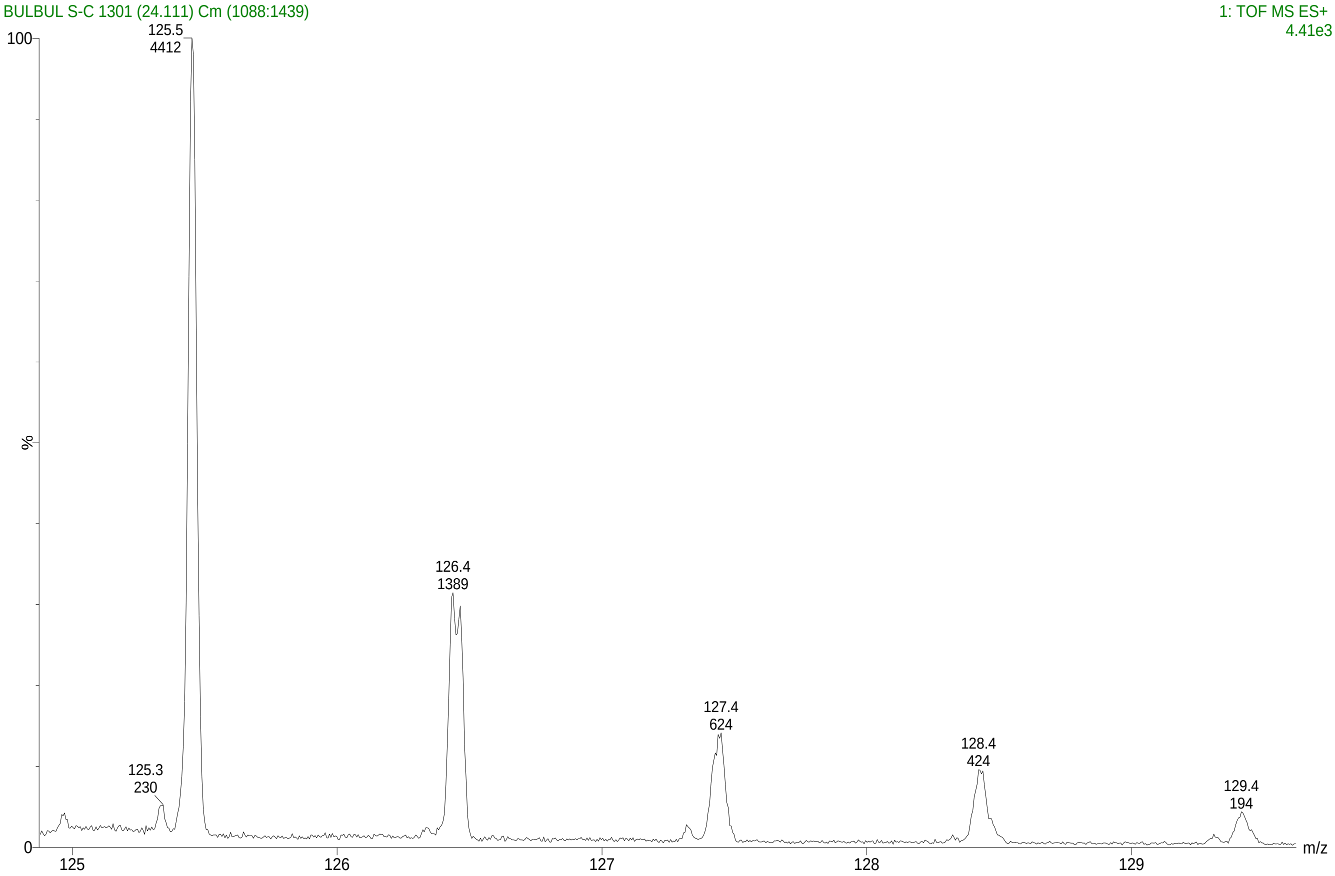


Figure 4.15: Mass spectra obtained by LC/MS of Contaminated Soil after 7 days of incubation with S3 strain.

Peak area of chromatogram of Naphthalene in Contaminated Soil after 7 days of incubation with S3 = 0.4 cm^2

Concentration of Naphthalene in sample (µg/g) after 7 days of incubation with S3

= (0.4/1.1) * 100

= 36.36µg/g

Biodegradation efficiency (%) of S3 = [(163.63-36.36)/(163.63)] × 100

= 77.77 % .... (i)

4.2.1.2: Biodegradation study of Naphthalene using F3 strain after 7 days of incubation.

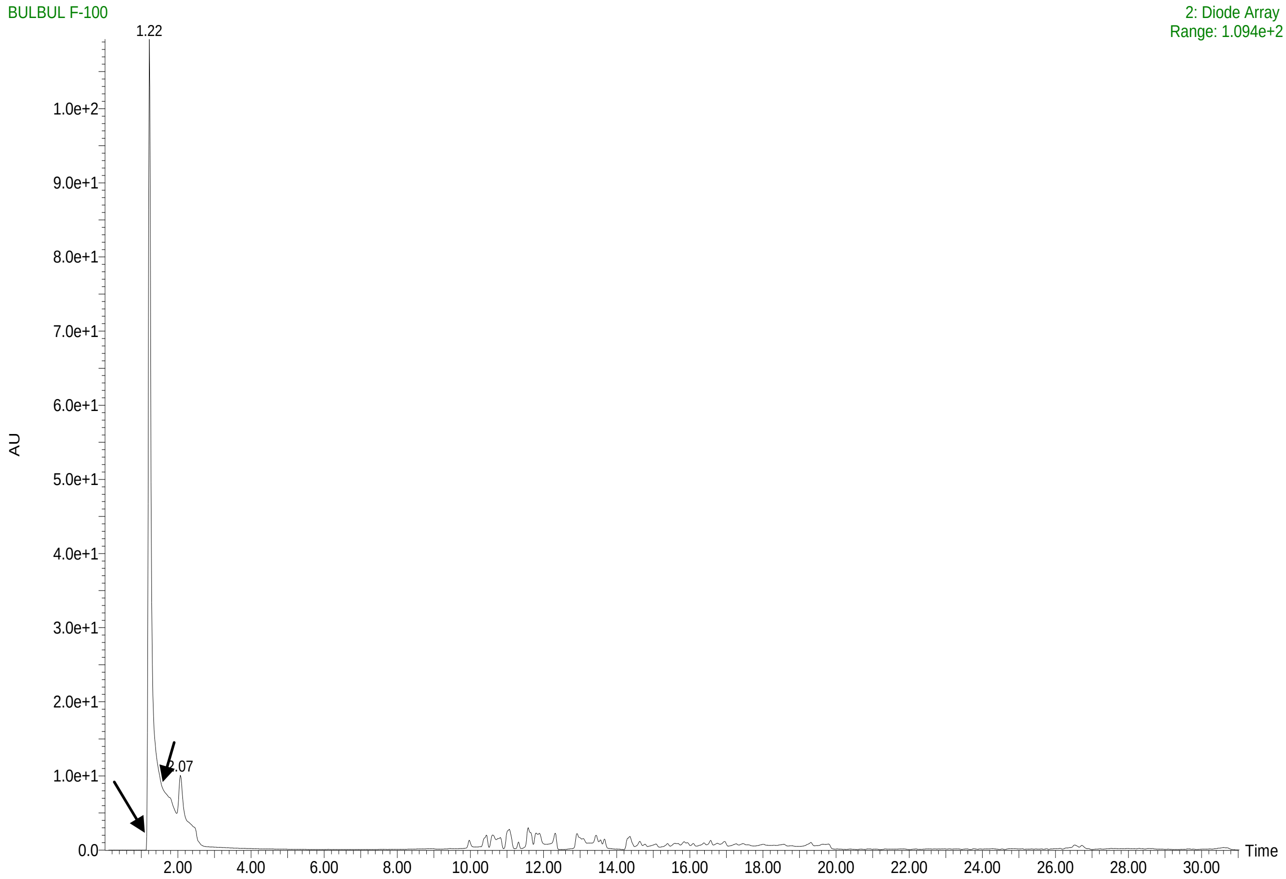


Figure 4.16: Chromatogram of Naphthalene standard (100µg/g) after 7 day of incubation with F3.

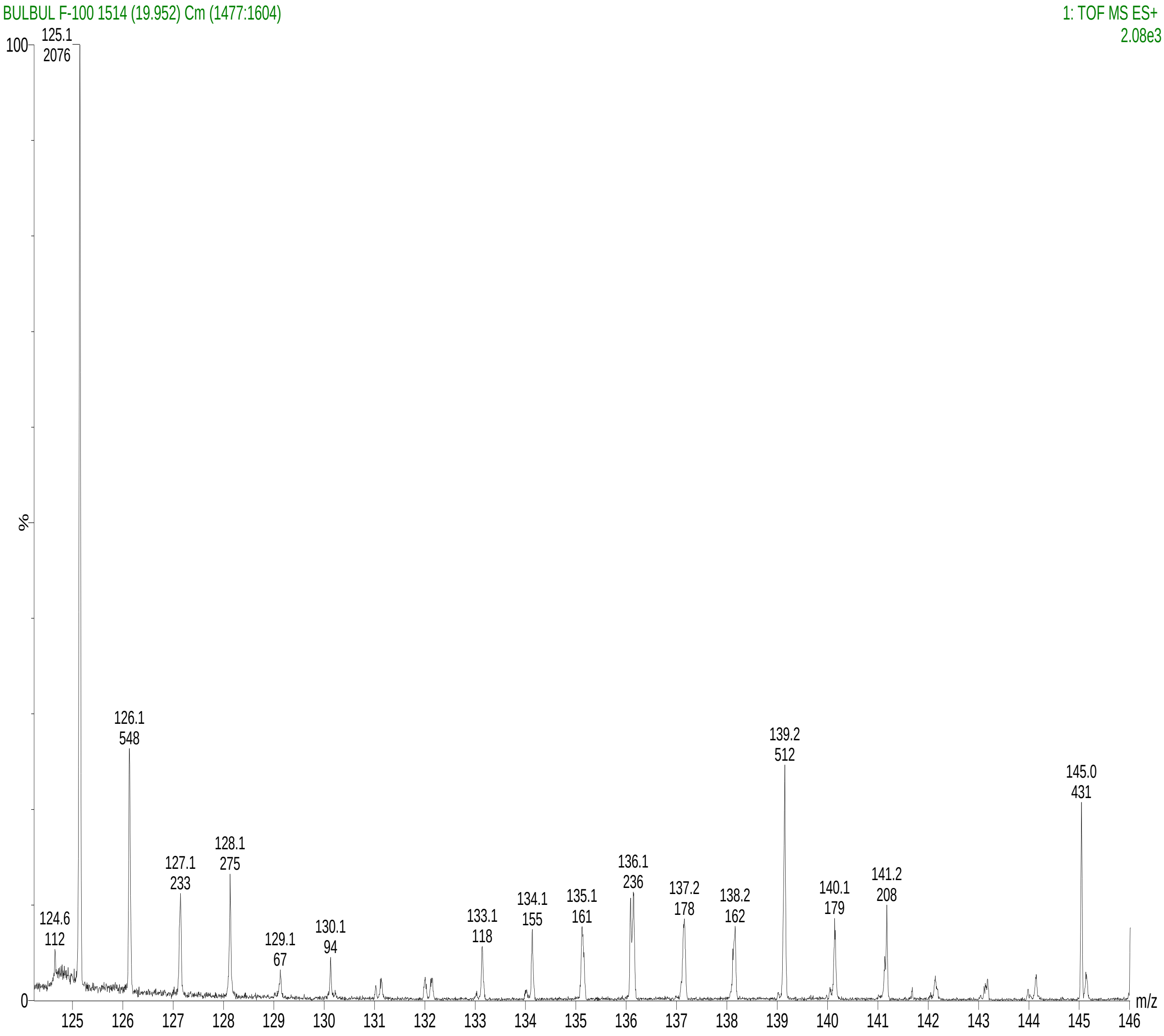


Figure 4.17: Mass spectra obtained by LC/MS of standard (100µg/g) of Naphthalene after 7 days of incubation with F3.

Peak area of chromatogram of standard (100µg/g) of Naphthalene after 7 days of incubation with F3 = 1.1 cm^2

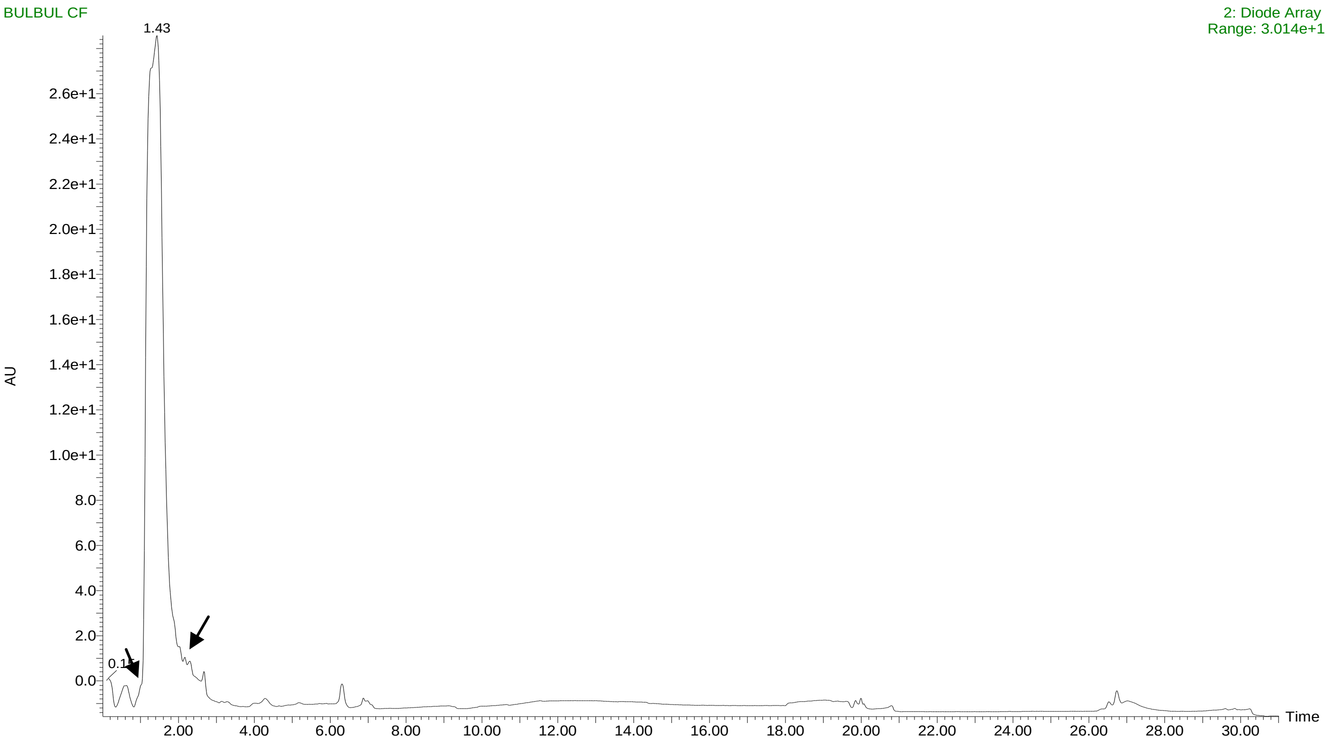


Figure 4.18: Chromatogram of Contaminated Soil after 7 days of incubation with F3 strain.

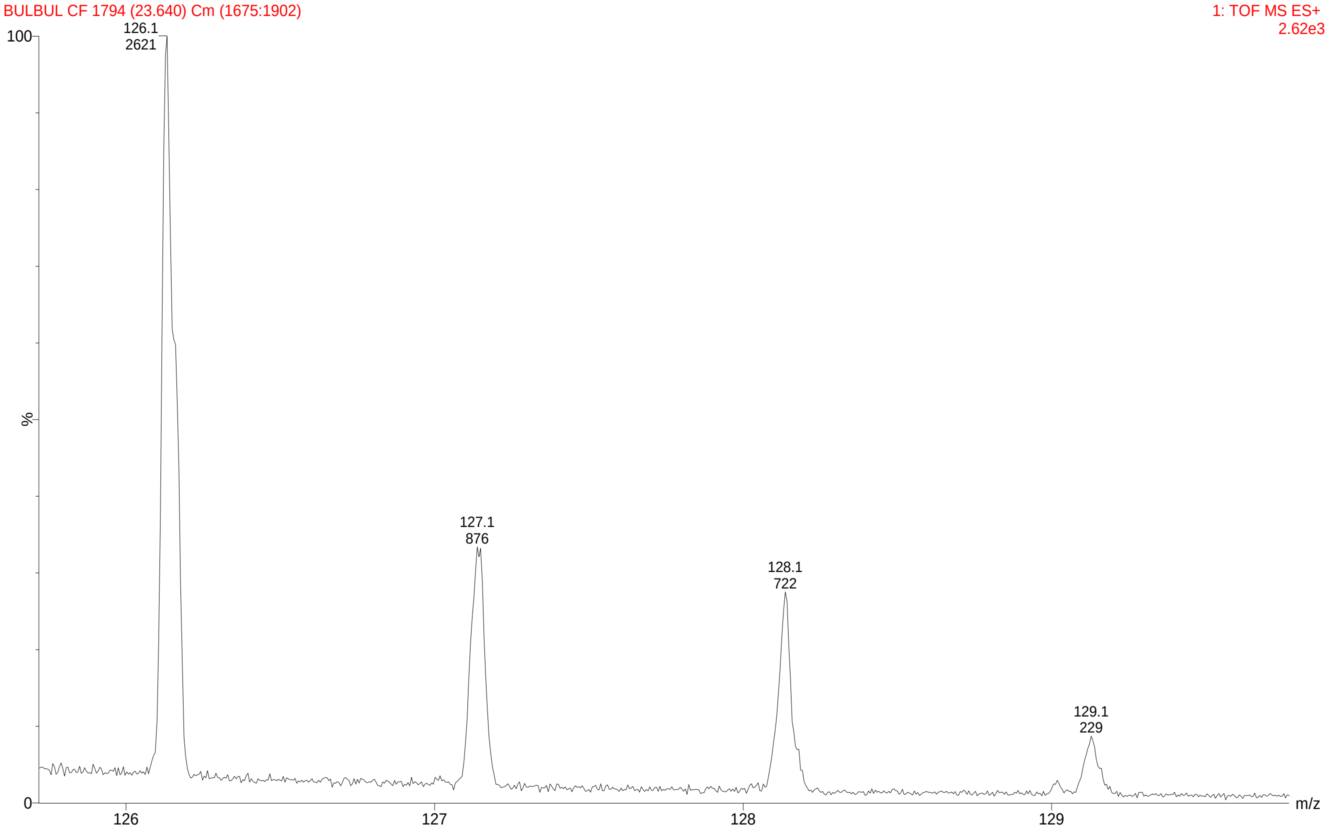


Figure 4.19: Mass spectra obtained by LC/MS of Contaminated Soil after 7 day of incubation with F3.

Peak area of chromatogram of Naphthalene in Contaminated Soil after 7 days of incubation with F3 = 0.7 cm^2

Concentration of Naphthalene in sample (µg/g) after 7 days of incubation with F3

= (0.7/1.1) * 100

= 63.63µg/g

Biodegradation efficiency (%) of F3 = [(163.63-63.63)/(163.63)] × 100

= 61.11 % .... (ii)

Equation (i) and (ii) shows the biodegradation efficiency of both the strains after 7 days of incubation. It showed more efficiency of strain S3 (*Pseudomonas sp.S3)* to degrade naphthalene(163.63 µg/g), reducing the detectable concentration to 36.36 µg/g as compared to strain F3 whose biodegradation efficiency was less than strain S3 (*Pseudomonas sp.*S3*)* reducing the detectable concentration to 63.63 µg/g. Thus from this study it is summarize, that at optimum conditions strain S3 (*Pseudomonas sp.*S3*)* showed better result for degradation of naphthalene after seven days of incubation. *Pseudomonas stutzeri* P-16 and *P. saccharophila* P-15 isolates from creosote contaminated soil were also reported to degrade naphthalene, 2-methylnaphthalene and 1-methylnaphthalene (Stringfellow and Aitken, 1995). In addition to microbial potential, the right ecological conditions are also very important for microbial activity for biodegradation. A report showed maximum degradation of naphthalene by *Pseudomonas* sp. HOB1 was achieved in the pH and temperature ranges of 7.5-8.5 and 35-37ºC, respectively (Pathak et al., 2009). Therefore the use of these isolates alone or in combination could result in effective degradation of PAHs and related compounds in the bioremediation process in the contaminated environment.

CHAPTER 5

5.1 CONCLUSION

- Soils contaminated with hydrocarbon are good sources for the isolation of PAHs degrading bacteria which can then be used for the removal of such compounds from the environment.

- The isolation of bacterial strains in the present study from oil contaminated soil suggested that these isolates may be well adapted for use because of pre-exposure and acclimatization to the contaminants and can be used effectively in remediating the contaminated sites.

- Screening of the isolates (S3 and F3) at different temperature and pH indicate the optimum pH and temperature as 7 and 37^0C.

- The biodegradation efficiency of S3 was 77.77% whereas for F3 the biodegradation efficiency was 61.11%, respectively, after seven days of incubation.

- Phylogenetic analyses based on 16S ribosomal RNA sequences of S3 showed that it is closely related to *Pseudomonas aeruginosa* strain DSM 50071.

- From the present study, it is concluded that soil is the rich source of microbes having the degradative capability of pollutants like PAHs. These microbes have the ability to degrade the pollutants efficiently in pure or in mixed culture.

5.2 FUTURE PROSPECTS

- PAH contaminated environments like soil and sediments can be exploited for other bacterial strains capable of degrading PAHs.

- These isolates can also be checked for their ability to degrade other organic contaminants like pesticides, TNTs and PCBs etc.

- These bacterial isolates can be applied in fields for bioremediation of PAHs and other organic compounds.

- Identification of metabolites from PAHs biotransformation to elucidate the complete pathways by selected isolates.

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