BIOREMEDIATION OF PETROLEUM HYDROCARBON POLLUTED SITES FOR THE CONSERVATION OF SOIL MICROBIAL DIVERSITY

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ABBREVIATIONS

16S rRNA	16 Svedberg ribosomal ribonucleic acid
AMO	Alkane monooxygenase
BH medium	Bushnell and Haas medium
BLASTn	Basic Local Alignment Search Tool (nucleotide)
dNTPs	Deoxynucleotides triphosphates
EDTA	Ethylene diamine tetraacetic acid
GC	Gas chromatograph
NADH	Nicotinamide adenine dinucleotide
OD	Optical density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PHC	Petroleum hydrocarbons
SN ratio	Signal-to-noise ratio
TPH	Total petroleum hydrocarbons
w/v, w/w	Weight by volume, weight by weight

ABSTRACT

Soil pollution due to crude oil is becoming a widespread environmental problem of major concern. Oil spills due to pipeline rupture, tank failures, various production and storage problems and transportation accidents are the major causes. Crude oil is a complex mixture of hydrocarbons, mainly composed of aliphatic, aromatic and asphaltene fractions along with nitrogen, sulfur and oxygen containing compounds. Diesel is one of the major constituents of crude oil and consists of nearly 75% alkanes (C_9-C_{23}) and 25 % aromatic hydrocarbons. Many studies have shown various effects of diesel especially small chain hydrocarbons (*n*-C_{10} and *n*-C_{11}) pollution on plants such as delayed/reduced germination, less plant height, lower leaf and biomass production. Diesel pollution also leads to reduction in soil microflora population. Therefore it is necessary to treat these soils for removal of petroleum pollutants. Physical and chemical means for remediation are very costly and impractical. In recent years, there has been increasing interest in developing on site and *in situ* techniques especially bioremediation for reclamation of oil-contaminated soils. Bioremediation can be achieved by natural attenuation, biopiling, bioaugmentation, phytoremediation or rhizoremediation, singly or in combination.

Present investigation was aimed at isolating and identifying potential petroleum hydrocarbons (PHC) degraders for bioremediation of diesel contaminated soils and the objectives for the study were -

- Isolation, identification and characterization of the microbial community of petroleum hydrocarbon contaminated sites.
- Identification of the pollutants present in the contaminated soils.
- Screening of the hydrocarbon degraders for their diesel degrading capability.
- Elucidating the effect of pollutants on soil communities and using bioremediation to restore the soil microbial community.

Introduction and review of literature, first chapter of the thesis presents extensive literature review on petroleum hydrocarbons, their composition, major causes of PHC contamination of soils and their effect on soil fertility and crop yield. Bioremediation of PHC polluted soils using different techniques has been discussed. The microbial diversity of PHC contaminated soils along with the biochemistry of petroleum hydrocarbon degradation has also been reviewed. The restoration of the original soil

microbial diversity through different bioremediation techniques has been emphasized. A part of this literature review has been published.

In **IInd chapter - Materials and Methods** used in present investigation are briefly described. Materials, chemicals procured from different suppliers, composition of media employed for isolation and maintenance of isolates have been listed. Brief methodology for collection of PHC contaminated soil samples, physico-chemical analysis of the soil samples, extraction of diesel range *n*-alkanes from broth and soils, isolation of the diesel degrading microorganisms from the contaminated soils and their screening have been described. Diesel range *n*-alkane profile of the soil/liquid samples was analyzed by Gas chromatography (GC). Total petroleum hydrocarbons (TPH) were analyzed gravimetrically and by GC. Different biochemical tests for bacterial and yeast isolates, identification by 16S rRNA sequencing and phylogenetic analysis of the bacterial isolates have been given. Methods for biosurfactant production and plasmid isolation are described. Enzyme assays for estimation of alkane monooxygenase activity are mentioned.

The details for detection of gene encoding alkane monooxygenase by polymerase chain reaction (PCR), and identification of alkane degradation route by GC have been explained. The effect of different diesel concentrations on plant growth, bioaugmentation of soil with selected strain, degradation of diesel followed by plant growth in treated soils and restoration of soil bacterial diversity by augmentation have been given.

Findings of the present investigation are given in **Chapter III – Results and Discussion** and can be summarized as follows:

Seven petroleum contaminated soil samples were collected from service stations and motor garages from Maharashtra, Andhra Pradesh and West Bengal, India. Analysis of the soil samples showed that all the samples were contaminated with TPH. The soil samples were enriched with diesel as a sole source of carbon. Total 70 bacterial (I1-I70) and 3 yeast (Y1-Y3) isolates were obtained. Biochemical tests and 16S rRNA sequencing of bacterial isolates revealed that they were from 5 different groups: Actinobacteria, α-Proteobacteria, β- Proteobacteria, γ-Proteobacteria and Firmicutes. The isolates were members of various genera such as *Acinetobacter*, *Achromobacter*,

Bacillus, *Brevibacillus*, *Cellulomonas*, *Enterobacter*, *Exiguobacterium*, *Janibacter, Kocuria*, *Lysinibacillus*, *Microbacterium, Micrococcus, Novosphingobium*, *Ochrobactrum*, *Pseudomonas, Skermanella* and *Staphylococcus.* Based on the morphology and biochemical characteristics, the three yeast isolates were identified as belonging to the genera *Pichia*, *Rodotorula* and *Yarrowia.*

All the 73 isolates were primarily screened for their growth in liquid medium containing diesel as a sole source of carbon and based on OD_{600nm} measurements nine isolates - *Bacillus amyloliquefaciens* (I7), *Bacillus* sp. (I34), *Bacillus subtilis* (I26), *Staphylococcus haemolyticus* (I57), *Microbacterium testaceum* (I38), *Pseudomonas aeruginosa* (I41), *Exiguobacterium antarticum* (I43), *Acinetobacter baumannii* (I58) and *Yarrowia* sp. (Y2) were selected for further studies. The nine isolates were then tested for their alkane monooxygenase (AMO) activity. Isolate I58 showed the highest AMO activity (8.6±1.1 U/mg protein) followed by Y2, I38, I41 and I26. GC analysis indicated that these five isolates could utilize diesel range *n*-alkanes, however, the efficiency of utilization varied among them with isolate *A. baumannii* (I58) being the best (51.1±1.3% degradation within 7 days). Also *A. baumannii* showed complete degradation of toxic short chain *n*-alkanes C_{10} and C_{11} and long chain *n*-alkanes C_{20} – C_{25} after 1 month. Polymerase chain reaction using three primer sets a) ALK 1F plus ALK 1R b) ALK 2F plus ALK 2R and c) ALK 3F plus ALK 3R showed amplification with ALK 2F plus ALK 2R primer set indicating the presence of functional catabolic gene for alkane monooxygenases in all the five selected isolates. Fragment of expected size (271 bp) was amplified. Absence of plasmid in *A. baumannii* indicated that the required genes for hydrocarbon degradation activity are located on the bacterial chromosome. With cultures grown on 1% *n*-hexadecane, presence of 2-hexadecanol indicated that isolates Y2, I38, I41, I26 and I58 follows the sub-terminal oxidation pathway for alkane degradation.

Among the 9 isolates, *A. baumannii* and *P. aeruginosa* showed the highest emulsification index (E24) of ~60% in BH medium with 1% diesel as sole source of carbon. The cell surface hydrophobicity was also determined for these isolates and found to be in the range of 2.6±0.3-26.2±2.3%. Based on the results and individual alkane degradation profile, *A. baumannii* was selected for the bioaugmentation studies.

With varying concentrations of diesel, GC analysis indicated that *A. baumannii* could degrade maximum of *n*-alkanes (50.8±1.8%) when the concentration of diesel was 1 % (w/v), while it could degrade 22.5±2.8 and 8.9±0.9% of *n*-alkanes when the concentration was 5 and 10 % respectively. Time course of *n*-alkane degradation pattern by *A. baumannii* in presence of 1% (w/v) diesel showed that degradation increased from 24h (11.7±2.4%) to 7d (51.1±1.3%). The % degradation for individual *n*-alkanes was also increased with time and in the range of 43.5 - 53.9% on 7^{th} d. Initial inoculum of $1x10^7$ CFU/ml reached to $8.6x10^9$ after 7 d. To study the biodegradation potential of *A. baumannii* in soil, microcosms were set up with varying concentrations of diesel, viz., 0.5, 1, 2, 5% (w/w). The results of the experiment showed that *A. baumannii* could degrade up to 30.2±3.2, 26.0±1.5; 17.9±2.0 and 11.3±1.0% of TPH from 0.5, 1, 2, 5 % (w/w) of diesel respectively within 7d. While, *n*-alkanes degradation was in the range of 50.5±2.1 to 11.9±2.4 % for 0.5 to 5% diesel (w/w). Degradation of C_{10} and C_{11} *n*-alkanes which are reported to be toxic to seed germination was observed at different concentrations of diesel.

Optimization of parameters for diesel range *n*-alkane degradation by *A. baumannii* in liquid medium was done by Taguchi design of experiments and optimum conditions obtained were - shaking, diesel 1%, NH_4NO_3 concentration 0.5%, KH_2PO_4:K_2HPO_4 0.05%, pH 8.0, temperature 37ºC, inoculum size 10^8 CFU/ml and incubation for 9 d. At the optimum conditions, diesel range *n*-alkane degradation by *A. baumannii* was 58.75±2.3%.

Bioaugmentation studies using *A. baumannii* were performed to check its potential to degrade diesel in spiked soil microocosms and reclaimation of polluted soil in terms of restoration of the orignal bacterial flora and plant growth. Soil microcosms spiked with different concentrations of diesel (0.5%, 1%, 2% and 5% w/w) were set up and augmented with *A. baumannii*. Two uninoculated controls were run in parallel where one was with diesel while the second was without diesel. After two weeks, *Arachis hypogaea* (groundnut) seeds were sown in the soils. The TPH degradation was analyzed after every 7d up to 30d and was seen to reduce in the bioaugmented soil microcosm. The changes in bacterial flora in soil microcosms were observed. The original count of bacteria in the control devoid of diesel was $4.6\pm0.52\times10^8$ CFU/g and it remained in the range of $2.1\text{-}8.2\pm0.33\text{-}0.81\times10^8$ CFU/g over the period of 30 d. In the uninoculated control the bacterial count was drastically

reduced indicating a negative effect of diesel on the soil bacterial flora. In the soils bioaugmented with *A. baumannii* containing 0.5 and 1% diesel (w/w), the count of bacteria increased up to 10^{11} CFU/g at 14d and 21 d which then reduced to 10^{9} CFU/g on 30 d. The bacterial count reduced to 10^{7} and 10^{5} in bioaugmented soil microcosms with 2 and 5% diesel respectively which may be due to the toxic effect of diesel at such higher concentrations. *A. hypogaea* showed improved growth performance in bioaugmented soils as compared to uninoculated control soils. It was observed that seed germination doubled as compared to uninoculated control in 0.5, 1 and 2% diesel containing soils. An increase in the growth parameters of the plants like total plant height, shoot and root length and plant fresh weight was seen in the presence of 0.5 to 2% diesel, indicating successful bioaugmentation.

To confirm the role of *A. baumannii* in restoration of soil bacterial flora, *B. subtilis* subsp. *subtilis* str. SC-8 92_1 (B2) isolated from agriculture soil was used as a model organism. Sterile soil was spiked with 1% (w/w) diesel and B2 were inoculated in 3 (A, B, C) sets. In set A, inoculated with *A. baumannii* on 1st d, the count of B2 increased after 7 d, whereas in other sets the count was reduced. *A. baumannii* was then inoculated in set B on 7 d. Increase in the count of B2 after 14 d in set A and B as compared to the count on 7th d and decreased count in set C confirmed the role of *A. baumannii* in restoration of B2 in soil with 1% (w/w) diesel.

Conservation of the original soil microbial flora and growth of *A. hypogea* in bioaugmented soils indicated that *A. baumannii* could be effectively used for the bioremediation PHC contaminated soils.

Chapter 1

Introduction and Review of Literature

CHAPTER I

1.1 Petroleum or crude oil

Petroleum or crude oil is a naturally occurring flammable liquid that is found in geological formations beneath the Earth's surface. A fossil fuel is formed when huge quantities of dead organisms and organic matter get buried underneath sedimentary rock and subjected to intense pressure and heat. The components of petroleum and petroleum products number in thousands. They range in molecular weight from methane to very large uncharacterized components with molecular weight in the thousands. The toxicity of the components varies immensely. Overall, the components of these mixtures have only two common properties such as they are derived from petroleum and they contain hydrocarbons i.e. hydrocarbon functional groups (C-H). The constituent hydrocarbon compounds are present in various proportions resulting in great variability in crude oils from different sources. The relative proportions of these fractions are dependent on many factors such as the source, geological history and age of crude oil (Balba *et al.*, 1998). Although there is a considerable variation between the ratios of organic molecules, the elemental composition of petroleum is well-defined - Carbon - 83 to 87%, Hydrogen - 10 to 14%, Nitrogen - 0.1 to 2%, Oxygen - 0.05 to 1.5%, Sulfur - 0.05 to 6.0%, Metals - < 0.1%.

1.2 Petroleum hydrocarbons (PHC)

Crude oil is thus a complex mixture of hydrocarbons, composed of paraffins (15-60%), naphthenes (30-60%), aromatics (3-30%) and asphaltics (remainder) fractions along with nitrogen, oxygen and sulfur containing compounds. The aliphatic fraction (paraffins and naphthenes) includes linear or branched-chain alkanes and cycloalkanes. The aromatic fraction contains mono- , di - , and poly-nuclear/cyclic aromatic hydrocarbons (PAH) containing alkyl side chains and/or fused cycloalkanes. The resins and asphaltenes contain more polar compounds, consisting of heterocyclic, oxygenated hydrocarbons and high molecular weight aggregates (Fig. 1.1). Crude oil is not used directly as a fuel but as a feedstuff for the petrochemical factories to produce commercial fuels (petrol, diesel, kerosene etc.), plastics, synthetic rubbers, and other chemicals (Harayama *et al.*, 1999). These products have more defined

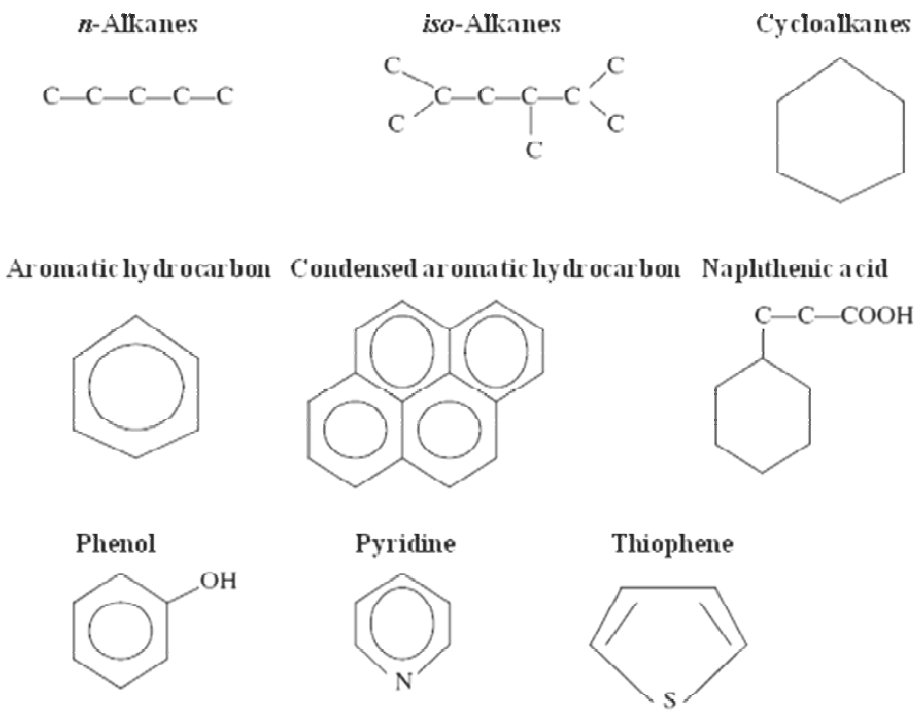


Fig. 1.1 Structural classification of some crude oil components (Okoh 2006)

chemical composition and properties. Composition of diesel is described briefly in following section.

1.2.1 Diesel

Petroleum diesel is produced by the fractional distillation of crude oil between 200 °C and 350 °C at atmospheric pressure, resulting in a mixture of carbon chains that typically contain between 8 and 21 carbon atoms per molecule. Diesel is composed of about 75% saturated hydrocarbons (primarily paraffins including *n*-, *iso*-, and cyclo-paraffins), and 25% aromatic hydrocarbons (including naphthalenes and alkylbenzenes). The average chemical formula for common diesel fuel is $C_{12}H_{23}$, ranging approximately from $C_{10}H_{20}$ to $C_{15}H_{28}$. The density of petroleum diesel is about 0.832 kg/l. About 86.1% of the fuel mass is carbon, and when burned, it offers a net heating value of 43.1 MJ/kg (Demirel, 2012).

1.3 Pollution of (PHC)

PHC represent one of the major environmental pollutants (Okoh, 2006). Sinking of super tankers like Torney Canyon, Exon Valdez, etc. and subsequent damage to the

environment led to PHC pollution being recognized as the most significant contamination problem on the continent (Snape *et al.*, 2001).

1.3.1 Sources

The development of petroleum industry into new frontiers, the apparent inevitable spillages that occur during routine operations, and records of acute accidents during transportation are main sources of PHC pollution. Other causes of oil contamination include seepage from natural deposits, leakage of storage tanks and pipelines, land disposal of petroleum wastes, transportation and accidental spills (Balba *et al.*, 1998). The most visible source of petroleum pollution is the oil-tanker spills. The accidents with tankers, pipelines, and oil wells release huge quantities of petroleum in to the land and marine eco-systems. The largest oil spills in history by volume were in the range of 140-800 thousand tons and most of them were due to tanker accidents (www.aspenpublishers.com/environment.asp). Among petroleum products, diesel has been widely used in various industries. Due to its relatively high mobility, the possibility of contamination of surface waters and groundwaters as well as soils is high (Gallego *et al.* 2001).

1.3.2 Indian scenario

India is a significant consumer of energy. One of the major sources of energy in India after coal is crude oil. Oil constitutes over 35% of the primary energy consumption in India. The estimated reserves of crude oil in India are 757 million metric tons. Crude oil production during 2009-2010 was 33.69 million metric tons which has increased by 11.91% to 37.71 million metric tons in 2010-2011. The consumption of petroleum products during 2009-2010 was 138.19 million metric tons which increased to 141.18 million metric tons during 2010-2011(Garg, 2012). Thus demand for petroleum products exceeds their production for which crude oil has to be imported from other countries. The production of automobiles has greatly increased in the last decade in India due to which the demand for petroleum products is expected to rise to more than 240 million metric tons by 2021-22 which will further increase by 51.61% in 2031-32 (Garg, 2012). Thus the refining capacity in the country has gradually increased over the years by setting up of new refineries in the country as well as by expanding the refining capacity of the existing refineries to meet the growing demand of petroleum products. There are a total of 21 refineries in the country processing approximately

193 million metric tons of crude oil. Thus, India is a major petroleum consuming country thereby predisposing the environment to the drastic effects associated with its exploration and exploitation. Recently, in August 2010 two ships collided off the Mumbai coast leaking >2000 tons of oil into the sea. The large oil spill in the open ocean may do less harm to marine ecosystem than the relatively small spill near the shore. The consequences of these oil spills include widespread, long-term, and serious damage to human health, natural resources, marine ecosystems and terrestrial life.

1.3.3 Effect of PHC pollution in soils

PHCs that enter the soil may be adsorbed on the surface of mineral and organic soil constituents, fixed within the soil pores and fissures, found in mobile form or may form a continuous cover on the soil surface (Trofimov and Rozanova, 2003). Crude oil contamination of land affects certain soil parameters such as the mineral and organic matter content, the cation exchange capacity, redox properties and pH value. Due to hydrophobic nature of PHCs, contaminated soils are characterized by lower water holding capacity, moisture content and hydraulic conductivity as compared to unpolluted soils (Nwaoguikpe, 2011). Crude oil creates anaerobic condition in the soil, coupled to water logging and acidic metabolites; the result is high accumulation of aluminum and manganese ions, which are toxic to plant growth. Oil pollution leads to substantial increase in the organic carbon content in the soil, which is due to the carbon of oil products. Under the impact of oil pollution, soil humus becomes enriched in humic acids, whereas the degree of humification of soil organic matter decreases (Trofimov and Rozanova, 2003).

These changes in soil properties greatly affect or cause toxicity to the biotic components of the ecosystem.

1.3.4 Toxicity of PHC

The toxic effects of the petroleum hydrocarbon spill mainly depend on the composition of the polluting petroleum product. Alkanes and aromatic hydrocarbons exhibit relatively low solubility in water with benzene being the most soluble at 1780 mg/L. The most biologically active substances, in terms of toxicity and bio-accumulation, have log octanol/water partition coefficient (K_{OW}) values between 2 and 6, and are referred to as lipophilic compounds (Trapp *et al*., 2001) Many

hydrocarbons fall into this group e. g. log K_{OW} for *n*-hexane and *n*-decane are 2.91 and 5.58, respectively. As petroleum products occur as complex mixtures of individual substances, concentration of individual components really matters for the ecological impact. For instance, n-alkanes are less toxic and persistent than aromatic compounds. In particular, the PAHs (e.g. benzo(a)pyrene), are carcinogenic and have been implicated in many a wide range of human health problems and also disease problems with aquatic organisms (Grimmer, 1991).

1.3.4.1 Toxicity to plants

PHC pollution leads to decrease in plant growth as well as in crop productivity. Many authors have reported a lower rate of seed germination in soil contaminated with petroleum or its derivatives. For example, Adam and Duncan (2002) screened different plant species including grasses, legumes, herbs and commercial crops for their ability to germinate in 25 and 50 g/kg diesel fuel contaminated soil. The authors observed delayed and decreased seed germination and suggested that this may be due to a volatile component (light hydrocarbons) which may be phytotoxic and capable of entering easily through the plant cell walls. Also, PHCs may form a film on the seed, preventing the entry of oxygen and water. Sharifi *et al*. (2007) observed the effect of 25, 50, 75, 100 g/kg of spent oil on seed germination, shoot height and biomass of six herbaceous plant species including one species of Fabaceae (*Medicago truncatular*), four species of Gramineae (*Bromous mermis, Secal seral, Triticum sativa* and *Agropyron deserterum*) and one species of Linaceae (*Linum ussitasimum*). The authors reported dose-dependent responses to the contaminated soils by all species. Only 16.2, 15 and 2.7% germination was seen in *A. deserterum*, *B. mermis* and *L. ussitasimum*, respectively, while 63.5% germination was observed in *M. truncatular*. Reduction in seedling height and biomass was also observed in all plant species. Similarly Ogbo (2009) studied effects of 1, 2, 3, 4 and 5% of diesel contamination on four crop plants *Arachis hypogaea, Vigna unguiculata, Sorghum bicolor* and *Zea mays*. They observed that all the test plants tolerated diesel fuel contamination at 1-3% levels of contamination as seed germination was between 89-33%. In presence of 1% diesel, 89% of seed germination was reported in *Z. mays* and *S. bicolor* but only 77 and 68% seeds germinated in *A. hypogaea* and *V. unguiculata* respectively. The authors also observed reduction in seed germination with increase in levels of

contamination in the four test plants. There was total inhibition of seed germination in *S. bicolor* and *V. unguiculata* in 4% diesel of contamination. Houshmandfar and Asli (2011) evaluated the effect of 2 ml/kg (v/w) of mixed gasoline and diesel fuel on seed germination and seedling growth characteristics of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), alfalfa (*Medicago sativa* L.) and clover (*Trifolium resupinatum* L.). The authors reported delayed onset of germination due to gasoline and diesel fuel mixture stress. They observed inhibition of germination with a decrease of 70.92%, 53.19%, 25.01% and 8.46% in wheat, 61.58%, 31.78%, 8.48% and 4.18% in barley, 85.97%, 35.84%, 7.68% and 2.15% in clover, and 91.76%, 56.68%, 37.55% and 26.26% in alfalfa plant after 5, 8, 11 and 14 days respectively. Siddiqui *et al.* (2001) also studied the effect of 0, 0.64, 1.6, 4.0 and 13.6% (w/w) diesel on the germination of perennial ryegrass. Germination of perennial ryegrass was linked to the decomposition of *n*-C_{10} and *n*-C_{11} and took 11 to 30 days depending on the levels of diesel added to the soil. Inhibition of seed germination until 24 weeks was observed with 13.6% (w/w) diesel.

Achuba (2006) studied the effect of various sublethal concentrations of crude oil on the growth and metabolism of cowpea (*Vigna unguiculata*) seedlings. The authors reported that 0.25%, 0.5%, 1.0% and 2.0% crude oil, induced stress in the seedlings which was evident from the dose dependent increase in free sugar, total protein and amino acids and decrease in chlorophyll contents of the leaves of seedlings. They also observed inhibition of amylase and starch phosphorylase, which affect starch assimilation.

Diesel oil can cause chronic or acute effects on the plants. Several studies have demonstrated the effect of oil pollution on plant growth in terms of reduced germination rate, root length, shoot length, plant weight etc. For instance, Njoku *et al.* (2009b) showed the effect of 0.2, 0.4, 0.6, 0.8 and 1% (v/w) of gasoline fuel/diesel mixture on the germination of seeds, survival of the seedlings and growth of *Vigna unguiculata* over a period of 10-38 days. A drastic decrease was seen in survival of seedlings with increasing concentrations of petroleum products. They also observed decreased % seed germination, dry weight, leaf area, shoot and root lengths of plants at all concentrations of gasoline fuel/diesel. Similarly, Saadoun and Al-Ghazawi (2010) studied toxicity of diesel towards plant seeds of *Atriplex halimus*, *Cochorus olitorius, Hordeum spontaneum* and *Triticum aestivum.* The authors reported 57.7 and

76.9% decline in seed germination of *C. olitorius* at 100 and 5000 mg/kg of diesel respectively after 10 days. While, seeds of *H. spontaneum*, *T. aestivum* and *A. halimus* showed a decline of less than 30 % at 100 mg/kg and 11.1, 20 and 29.4% decline at 5000 mg/kg of diesel, respectively. The authors also observed severe decline (> 50%) of *C. olitorius* sprouts' length and weight at 5000 mg/kg diesel as compared to the most resistant *T. aestivum* sprouts that showed < 15 % decline of both sprouts' length and fresh weight.

Akujobi *et al*. (2011) also studied the effect of 2, 4, 6, 8 and 10% diesel pollution and nutrient amendments on plant growth parameters of eggplant (*Solanum melongena*). The authors observed concentration dependent adverse effects of diesel pollution on plant growth parameters. Nutrient amendments such as poultry waste, pig waste, cow dung and inorganic fertilizer were able to remediate the effect of diesel oil pollution. Ehiagbonare *et al.* (2011) investigated the effect of 1, 2, 3, 4 and 5% of spent and unspent diesel on *Zea mays* and *Arachis hypogaea*. They reported 40% seed germination for *Z. mays* and 22% for *A. hypogaea* in spent diesel while only 10% for *Z. mays* and 0.5% for *A. hypogaea* in unspent diesel at 5% level of contamination. The authors also observed 75% and 80-83.4% reduction in radicle and plumule growth in *Z. mays* and *A. hypogaea* respectively in presence of 5% diesel.

1.3.4.2 Toxicity to animals/birds/humans

PHC constitutes mainly the aromatic compounds which are toxic, mutagenic or carcinogenic (Balba *et al.*, 1998). Birds and animals if ingest the oil from the environment may face problems such as congestion, pneumonia, emphysema and even death by breathing in droplets of oil, or oil fumes or gas. Ingestion may also lead to decreased absorption of nutrients and finally result in death of these birds and animals due to severe liver damage and anemia. Symptoms of crude oil toxicity include liver necrosis, blocking of the liver, fat disintegration and dissociation of hepatocytes (Sathishkumar *et al.*, 2008). Eventually food chain is also affected. Burrowing soil organisms may be affected by the reduced or total absence of aeration in hydrocarbon-polluted soils. Few studies in humans (reviewed by Aguilera *et al.*, 2010) have linked PHC pollution to lower respiratory problems, long term mental health effects, genotoxic damage, hormonal imbalance, reproductive and developmental toxic effects, skin and lung cancer etc.

1.3.4.3 Toxicity to microorganisms

Gill and Ratledge (1972) reported that *n*-alkanes are toxic to microorganisms. Walker *et al.* (1975) demonstrated the toxicity of crude and refined oil to natural bacterial populations from pristine sediments with refined oil being more toxic. The aromatics in crude oils such as α-pinene, limonene, camphene, and isobornyl acetate were found to be toxic to the microorganisms (Andrews *et al.*, 1980). Even for bacteria surviving in presence of dissolved aromatic hydrocarbons like naphthalene, increase in lag phase and decreased growth rate were observed (Calder and Lader, 1976). The toxic effects of cyclohexane in *Saccharomyces cerevisiae* reported by Uribe *et al.* (1990) included inhibition of oxygen uptake in intact cells and isolated mitochondria, impaired ATP synthesis and potassium uptake along with dissipation of the mitochondrial membrane potential. Sikkema *et al.* (1995) reviewed the mechanism of membrane toxicity of cyclic hydrocarbons. The accumulation of these compounds in the membrane of microorganisms has considerable effects on the structural and functional properties. Hydrocarbon insertion alters membrane structure by changing fluidity and protein conformations and results in disruption of the barrier and energy transduction functions while affecting membrane-bound and embedded enzyme activity (Van Hamme *et al.*, 2003). This toxicity to microorganisms exerts selective pressure changing the microbial diversity of the polluted soil as described in detail in section 1.9.

1.4 Treatment of contaminated soil

The toxic effects of PHC on different biotic components as described in section 1.3.4, demand for the remediation of the contaminated soils. Various physical, chemical and biological methods are available for the treatment of PHC contaminated soils. These methods can be employed to restore close to similar microbial diversity.

1.4.1 Physical methods

Most commonly used methods include: landfill, cap and contain and incineration. A landfill is a carefully engineered pit that is dug in the ground where the contaminated soil from actual site is excavated and put, then covered with soil and spread evenly in layers. This method aims to isolate and contain the hazardous waste and avoid pollution of ground water and surface water. In cap and contain method the

contaminated soil is treated on the site. The contaminated site is capped, as in landfill, and is monitored periodically for reduction in the contamination. Incineration is a disposal method that involves combustion of hazardous material. Incinerators convert this material into ash, gas, heat and steam.

1.4.2 Chemical methods

Ozonation and surfactant washing are the most commonly used chemical methods for PHC removal. For instance, Iturbe *et al*. (2004b) demonstrated *in situ* flushing with alternate periods of water and water/surfactant for the remediation of soil contaminated with 55,156 g/kg TPH. The authors reported 98% removal efficiency of TPH at the end of six weeks. Molecular ozone or its decomposition products (e.g., hydroxyl radicals) react with organic compounds to convert them into oxidized products, which are more water-soluble, less toxic and/or more bioavailable than parental compounds. For example, Jung *et al*. (2005) investigated the effects of chemical oxidation on the survival of indigenous microbes from field soil (gas station in Gwangju, Korea) contaminated with diesel fuel. The authors reported 50% TPH removal after 900 minutes of ozonation. They also observed that the indigenous microbes treated were very sensitive to ozone and thus a decrease in microbial population was observed with ozonation time. The costs for the clean-up of the contaminated sites with conventional physical and chemical techniques are enormous (Liu *et al.*, 2010). Also due to lack of public acceptance and technological complexities, these methods have not been successfully applied (Vidali, 2001; Liu *et al.*, 2010). Moreover, for these approaches the possible environmental hazards and their effect on soil diversity are of a major concern. Therefore, alternative methods such as biological methods to restore polluted sites in a safe, environmental friendly, less expensive and labor intensive way is need of the hour.

1.4.3 Biological methods

1.4.3.1 Phytoremediation/Rhizoremediation

Phytoremediation is the use of plants for *in situ* remediation of contaminated soil, sludge, sediment and groundwater through any of the mechanism: extraction, filtration, stabilization, degradation and evapotranspiration. A number of plants which have extensive fibrous roots such as common grasses, corn, wheat, soyabean, peas

and beans were studied for their rhizoremediation potential (Glick, 2003). Several trees of family *Salicaceae* (poplar and willow) which can grow fast and possess a deep rooting ability were planted to bioremediate soil polluted with 20,000 mg/kg gasoline and diesel compounds to a depth of 3 m (Trapp *et al.,* 2001). However, the authors observed that at high concentrations e.g. >5000 mg/kg hydrocarbons, willows could not be used for phytoremediation due to toxic effects. Thus, use of willows for bioremediation, *per se*, was restricted and localized and for low hydrocarbon contamination.

Pradhan *et al.* (1998) used phytoremediation technology for the treatment of soil contaminated with PAHs. Three plant species, alfalfa (*Medicago sativa),* switch grass *(Panicum virgatum)* and little bluestem grass *(Schizachyrium scoparium)* were found to remediate 72% of total PAH within 6 months. White *et al.* (2006) studied phytoremediation of two to four ring alkylated PAHs in crude oil-contaminated soil using treatment systems involving combination of fescue (*Lolium arundinaceum*), ryegrass (*Lolium multiflorum* L.) or bermudagrass (*Cynodon dactylon* L.). The authors reported a degradation pattern of 2-ring > 3-ring > 4-ring which decreased with increased alkylation of larger ringed structures. They also observed an increase in degradation with addition of plants and fertilizer. Tanee and Akonye (2009) observed that *Vigna unguiculata* could remediate 5% (v/w) crude oil polluted soil by reducing the total hydrocarbon contents by 54% within two months along with an improved growth and yield of the cassava crop in the phytoremediated soil.

After phytoremediation plants can then be subsequently harvested, processed and disposed. The plants usually influence rhizosphere microbial community which can play role in rhizoremediation (Lee *et al.,* 2008). However, numbers of plants are also reported to produce enzymes such as cytochrome P450 and peroxidase involved in the metabolism of *n*-alkanes. Vega-Jarquin *et al.* (2001) showed that the cell cultures of *Cinchona robusta* and *Dioscorea composita* could take up and metabolize *n*-hexadecane with the formation of hexadecanol and hexadecanoic acid. They further reported that the levels and activities of cytochrome P450 and peroxidase were increased suggesting their role in biotransformation of *n*-hexadecane. Diab (2008) evaluated potential of broad bean (*Vicia faba*), Maize (*Zea mays*) and wheat (*Triticum aestivum*) plants for their abilities to stimulate the microbial degradation of soil pollutants in desert soil contaminated with 2.2-2.3% crude petroleum oil. *V. faba*

exhibited effective degradation of PHCs as its rhizosphere soil showed 30% reduction of TPH as compared to 16.8 % and 13.7 % reduction in rhizosphere soils of *Z. mays* and *T. aestivum* respectively. The author also reported that *V. faba* rhizosphere was able to reduce 5.3 % of the hardly degradable resins fraction along with 47.0 % of the saturated and 26.2 % of the aromatics fractions as compared to 37.4 % and 8.2 % for *Z. mays* and 33.2 % and 3.9 % for *T. aestivum* rhizospheres respectively.

Phytoremediation is limited to the surface area and depth occupied by the roots. Secondly, the time required is also more as the plants involved grow slowly with low biomass. The leaching of contaminants into the groundwater cannot be avoided in plant-based systems of remediation (Kuiper *et al*., 2004).

1.4.3.2 Bioremediation using microorganisms

Microbial bioremediation refers to treatment processes that use microorganisms such as bacteria, fungi, yeast or their enzymes to break down hazardous substances into less toxic or nontoxic substances thereby restoring the contaminated site (Bhatnagar and Kumari, 2013). Land farming, composting, biopiling, slurry bioreactors, natural attenuation, bioventing, biosparging, biostimulation and bioaugmentation are different techniques which employ microorganisms for bioremediation (Kumar *et al*., 2011). Different aspects of microbial remediation of PHC contaminated soils are described in following sections.

1.5 Different microbial bioremediation techniques

Excavation involves removal of contaminated soils from sites with subsequent treatment either by land farming, composting, biopiling or slurry bioreactors. Land farming involves the spreading of excavated contaminated soils in a thin layer on the ground surface of a treatment site and stimulating aerobic microbial activity within the soils through aeration and/or the addition of nutrients, minerals, and water/moisture. Paudyn *et al*. (2008) reported remediation of diesel contaminated soils in Canadian Arctic by landfarming. The authors demonstrated enhanced bioremediation (90%) of 2800 mg/kg TPH when fertilizer was added and also a significant 80% loss of hydrocarbon due to aeration by rototilling during a 3-year period with rototilling every 4 days. Katsivela *et al*. (2005) in their study on bioremediation of petroleum waste sludge in landfarming sites reported an enhanced

degradation of PHC, particularly *n*-alkanes with 75-100% removal efficiency after 14 months. Kuo *et al*. (2011) used enhanced landfarming system to remediate diesel oil contaminated soils. The authors reported 92.4% remediation of 5,900 mg/kg TPH within 25 days using kitchen waste compost as an additive. They also observed TPH removal of 86.9, 83.1, 79.7 and 54% in presence of sludge, PH degrading bacteria, rice husks, and control reactors respectively.

Composting is a controlled biological process that treats organic contaminants using microorganisms under thermophilic conditions (40-50°C). Atagana (2008) demonstrated bioremediation of petroleum hydrocarbon contaminated soil containing 380000 mg/kg of TPH by composting over a period of 19 months. They observed reduction in TPH by 82% and selected hydrocarbon components like phenol, cresol, naphthalene, anthracene, phenanthrene, fluorene, pyrrole, pyrene, fluoranthene, chrysene and benzo(a)pyrene by 100%.

Biopiles stimulate aerobic microbial activity within the soils through aeration and/or adding together of minerals, nutrients, and moisture. Many organic pollutants especially petroleum hydrocarbons are successfully bioremediated using biopiles, at pilot and field level. Jorgensen and co-workers (2000) demonstrated remediation of lubricating oil and diesel contaminated soil by piling with bark chips and nutrients (N, P, K) with sufficient aeration using two commercially available mixed microbial inocula. The authors reported 71% decrease of 2400 mg/kg lubricating oil and 70% of 700 mg/kg diesel due to microbial activity within 5 months. The authors observed no particular effect of added inocula but enhanced activity of natural microbial community initially present in contaminated soils. Mohn *et al.* (2001) investigated biopiles for on-site bioremediation of diesel contaminated Arctic tundra soil. They observed 90-95% reduction in TPH content after 1 year in treatments with optimal conditions such as addition of ammonium chloride and sodium phosphates, inoculation with cold adapted, mixed microbial cultures and increased temperatures by covering biopiles with clear plastic covers. Iturbe *et al.* (2004a) used a combination of a physical and biological method to treat gasoline and diesel contaminated soils at field level. The authors reported that soil washing using nonionic surfactant and biopile removed 83 and 85% of 4500 mg/kg TPH respectively within 66 days using the autochthonous microflora.

Slurry bioreactors are utilized for treatment of contaminated soil, sediments, sludge and other solid or semi-solid waste in a controlled bioreactor. For example, Baptista *et al.* (2005) evaluated bioremediation of petroleum hydrocarbons in clay soil, supplemented with nutrients (N and P) to stimulate indigenous microorganisms using aerobic fixed bed reactors and observed 45% removal of 9700 mg/kg of TPH after 45 days. Chikere *et al.* (2012) used stirred-tank bioreactors stimulated with various nutrients (N, P, K, urea or poultry droppings) to treat hydrocarbon polluted marine sediment by natural microbial communities. The authors reported 75 to 95.5% degradation of 106 -116 mg/kg TPH and 93 to 98.9% of 95-104 mg/kg of PAHs over a period of 56 days.

Natural attenuation, bioventing, biosparging, biostimulation and bioaugmentation techniques involve treating contaminated sites in place whereby avoiding excavation and transport. These methods are more preferred due to lower operational cost and fewer disturbances to the contaminated sites.

Bioremediation which occurs without human intervention other than monitoring is often called natural attenuation. Rarely, oil contamination occurs suddenly (e.g. by tanker accidents or explosions), but more often it is a creeping contamination for long periods due to leakage of a pipeline, or a storage tank. This can be effectively bioremediated by the indigenous microflora which was adapted over the long period. The indigenous microflora uses the pollutants as a carbon and energy source (Watanabe, 2001). Although indigenous microflora in the soil degraded a wide range of target constituents of the oil, their population and efficiency were affected when toxic contaminant was present in relatively high concentrations (Mishra *et al*., 2001). Serrano *et al.* (2008) provided evidence for restoration of diesel contaminated soil by natural attenuation. They simulated a diesel fuel spill at a concentration of 1 L/ m^2 soil on a plot of agricultural land, and monitored natural attenuation of aliphatic hydrocarbons over a period of 400 days. According to the data on soil quality parameters *viz.* soil microbial mass and dehydrogenase activity, original levels were regained after 200 days. These observations were attributed to the presence of majority of aliphatic hydrocarbons at the surface (up to 10 cm deep) which were volatilized initially after spill and subsequently remaining were degraded by natural microbial flora.

Bioventing uses microorganisms to degrade organic constituents adsorbed on soils. The activity of native bacteria is enhanced by aeration. Lee and Swindoll (1993) showed feasibility of bioventing for *in situ* bioremediation of light hydrocarbons such as gasoline or diesel, heavier hydrocarbons such as fuel oils as well as other volatile and semi-volatile compounds such as dichloromethane, tetrachloroethene, chloroform and carbon tetrachloride. Moller *et al.* (1996) combined bioventing with nutrient addition (N and P) and inoculation with an oil degrading bacterium and showed 80% oil degradation in soil. Similarly, Mao and Yue (2010) used bioventing and microbe composting methods to simulate the remediation of the diesel contaminated soil. They achieved effective remediation after 45 days wherein over 64% diesel was removed from the soil having the original oil content of 50000 mg/kg. Biosparging is similar to bioventing which involves supply of air under pressure in the saturated zone so as to vaporize the volatile contaminants. The vaporized contaminants get carried to the unsaturated zone where they are degraded by microorganisms. Gasoline components such as benzene, toluene, ethylbenzene, and xylenes (BTEX) have been successfully degraded using biosparging with natural micro flora mainly *Candidauts magnetobacterium*, *Flavobacteriales bacterium*, and *Bacteroidetes bacterium* (Kao *et al.*, 2008).

Biostimulation utilizes indigenous microbial populations to remediate contaminated soils with and without addition of nutrients. In many studies on PHC bioremediation, natural attenuation by microorganisms was improved significantly by biostimulation and optimizing conditions such as temperature and addition of nutrients, agroindustry sludge, fertilizers, manure and crop residue addition. For instance, Agarry *et al*. (2010) used a combination of poultry manure, piggery manure, goat manure, and NPK fertilizer to remediate soil containing mixture of 10% (w/w) kerosene, diesel and gasoline. The authors observed 73%, 63%, 50%, and 39% TPH degradation in poultry manure, piggery manure, goat manure, and NPK fertilizer added soils after 4 weeks. Similarly, Coulon *et al*. (2005) studied the effects of nutrients and temperature on degradation of PHC in artificially contaminated sub-Antarctic soil with diesel or crude oil. The authors reported that increasing the temperature from 4°C to 20°C and addition of oleophilic fertilizer containing N and P increased the hydrocarbon degrading microbial abundance and TPH degradation. After 180 days the total alkane degradation was 77-95% and total PAHs was 80%.

Ka *et al*. (2001) monitored the size and metabolic activity of the bacterial community during biostimulation of fuel-contaminated soil using competitive PCR and RT-PCR. The authors reported that addition of N and P sources to microcosms of Arctic soil resulted in stimulation of biodegradation of hydrocarbon and the level in soil reduced by 42.35% in 29 days. Using molecular techniques the authors reported that there was overall increase in the metabolic activity of the microbial community of the microcosms. In the study by Liu *et al.* (2010) *in situ* biostimulation through adding manure was used to reclaim the oil sludge-contaminated soil in Shengli oilfield in China. They observed a reduction of 42.6% of 2410-2510 mg/kg of TPH after 360 days.

Molina-Barahona *et al*. (2004) studied the effects of moisture content, nutrients supplementation (C:N) and addition of common crop residues (corn or sugar cane) on PHC degradation by indigenous microflora in microcosms with 40000 mg/kg diesel. The authors observed 67% diesel removal in microcosms containing 100:10::C:N ratio, 30% moisture and 3% corn residue after 109 days. Mukherji *et al.* (2004) also studied the effect of nutrients (N:P), salinity and trace elemental formulations on the diesel degradation by ES1 cultures isolated from sea sediment obtained from the vicinity of an oil field. 39% loss of diesel was obtained over 8 days when the N:P was in the range of 2:1–5:1 and 0.5% salinity. The trace elemental formulations were seen to have a negative effect on the growth of the marine cultures. Spinelli *et al.* (2005) reported enhanced bioremediation of 50,000 L of diesel and gasoline /ha of soil using sludge from an agroindustry along with fertilizers (N:P:K). The authors observed that no detectable traces of the measured hydrocarbons at 170 days of treatment.

Bioaugmentation involves addition of specific microorganisms, native or exogenous to the contaminated sites for effective bioremediation. Bioaugmentation can be carried out by inoculating whole cells or encapsulating the cells in a carrier material. For instance, Catia *et al*. (2010) compared biodegradation of phenol by free and encapsulated cells of *Aspergillus* sp. strain isolated from a crude oil contaminated soil. The authors observed no significant difference in degradation potential between the two types but encapsulated cells adapted faster in batch cultures as compared to free cells. Gene bioaugmentation is also practiced wherein the inoculated culture transfers remediation genes to indigenous microorganisms. The observation that

introduced organisms have low survival rates following bioaugmentation led to investigations of use of naturally occurring horizontal gene transfer processes for introduction of remediation genes into a contaminated site. Horizontal gene transfer may occur via transformation, transduction, conjugation or exchange of genetic material such as plasmids (Gentry *et al.*, 2004).

Mishra *et al.* (2001) reported use of indigenous bacterial consortium grown in a laboratory as an inoculum to stimulate *in situ* bioremediation of oily-sludge contaminated soil. Bento *et al.* (2005) used a consortium of *Bacillus* species such as *B. cereus, B. sphaericus, B. fusiformis, B. pumilus, Acinetobacter junii* and *Pseudomonas* species for the degradation of hydrocarbons in soil. The microbial flora degraded light fraction (C_{12}- C_{23}) more efficiently than heavy (C_{23}–C_{40}) fractions of TPH. Furthermore, they compared the effectiveness of other methods namely, natural attenuation, biostimulation with bioaugmentation and found the latter treatment most effective. Ouyang *et al.* (2005) compared bio-augmentation and composting for remediation of oily sludge. They observed that bioaugmentation with the microbe preparation consisting of two *Rhodococcus* strains was more efficient with 45-53% decrease in oil contamination of oil sludge as compared to 31% by composting of the same sludge. Plaza *et al.* (2008) investigated petroleum waste remediation and toxicity reduction by five bacterial strains: *Ralstonia picketti* SRS (BP-20), *Alcaligenes piechaudii* SRS (CZOR L-1B), *Bacillus subtilis* (I'-1a), *Bacillus* sp. (T-1), and *Bacillus* sp. (T'-1), isolated from petroleum-contaminated soils. They observed that 91% PHCs was degraded in 30 days by the mixed bacterial cultures. Chang *et al.* (2011) demonstrated bioremediation of 4200 mg/kg Tapis crude oil contaminated soil by bioaugmentation of *Acinetobacter baumannii* T30C. The authors reported 42% degradation in bioaugmented and nutrient amended soils and 43% in non bioaugmented soil microcosms after 35 days. They observed only 15% degradation in bioaugmented microcosms. The study showed the requirement of nutrient amendment for stimulating growth of *A. baumannii* and indigenous microorganisms for TPH degradation.

1.6 Microorganisms involved in PHC degradation

Many microorganisms such as bacteria, fungi and microalgae have the ability to utilize petroleum hydrocarbons as the sole source of energy in their metabolism. According to van Beilen and Funhoff (2007) work of Söhngen N.L. published in 1913

on bacteria responsible for the disappearance of oil slicks on water had opened up the new area of tackling oil-pollution. Interestingly the work was initiated to find out the pathogenic nature of bacteria, if any. Eventually the focus shifted to the use of these organisms for bioremediation.

Numerous genera of bacteria are known as good hydrocarbon degraders. Most of them belong to *Aeromonas, Alcaligenes, Acinetobacter, Arthobacter, Bacillus*, *Brevibacterium, Flavobacterium, Geobacillus, Micrococcus, Mycobacterium, Ochrobactrum, Pseudomonas, Rhodococcus, Sphingomonas, Thermus* and *Xanthomonas* species (Atlas, 1981; Leahy and Colwell, 1990; Plaza *et al.*, 2008; van Beilen and Funhoff, 2007).

Rahman *et al.* (2002) used *Micrococcus* sp. GS2-22, *Corynebacterium* sp. GS5-66, *Flavobacterium* sp. DS6-86 and *Pseudomaonas* sp. DS10-129 individually as well as their consortium for degradation studies. They observed that the bacterial consortium degraded 78% of 1% crude oil which was higher than the % degradation by individual cultures. Schippers *et al.* (2005) reported two novel crude oil degrading bacteria, *Microbacterium oleivorans* sp. nov. and *Microbacterium hydrocarbonoxydans* sp. nov. Verma *et al.* (2006) tested the ability of three bacterial strains, *Bacillus* sp. SV9, *Acinetobacter* sp. SV4 and *Pseudomonas* sp., SV17 from contaminated soil in Ankleshwar (21°60' N 73°00' E), India for their ability to degrade the complex mixture of petroleum hydrocarbons (such as alkanes, aromatics, resins and asphaltenes), sediments, heavy metals and oily sludge. Wook *et al.* (2006) isolated a psychrotrophic *Rhodococcus* sp. from an oil-contaminated ground water which could degrade various petroleum hydrocarbons such as crude oil, diesel oil and gasoline by almost 90% within 20d and was able to grow in 7% NaCl at 4°C.

Auffret *et al*. (2009) reported two strains, *R. wratislaviensis* and *R. aetherivorans* that could degrade more than 15 petroleum compounds from a mixture of benzene, toluene, ethylbenzene, *m*-xylene, *p*-xylene, *o*-xylene, octane, hexadecane, 2,2,4-trimethylpentane [isooctane], cyclohexane, cyclohexanol, naphthalene, methyl *tert*-butyl ether [MTBE], ethyl *tert*-butyl ether [ETBE], *tert*-butyl alcohol [TBA], and 2-ethylhexyl nitrate [2-EHN]. The co-culture degraded 13 compounds completely. Interestingly, these strains had broad degradation capacities toward the more recalcitrant compounds like MTBE, ETBE, isooctane, cyclohexane, and 2-EHN. Olajide and Ogbeifun (2010) studied the hydrocarbon degrading potential

of *Proteus vulgaris* strain isolated from fish samples. John and Okpokwasili (2012) studied the utilization of kerosene, diesel oil, jet fuel and engine oil as carbon sources by autotrophic nitrifying bacteria, *Nitrosomonas* and *Nitrobacter* species and reported that mixed culture of the isolates degraded 52% of crude oil followed by 40% by *Nitrosomonas* sp. and 20%by *Nitrobacter* sp.

White-rot fungi like *Pleurotus tuberregium*, *Pleurotus ostreatus, Bjerkandera adusta, Irpex lacteus and Lentinus tigrinus* have been reported for degradation of polyaromatic hydrocarbons (Schutzendubel *et al.*, 1999; Isikhuemhen *et al.*, 2003). Further Isikhuemhen *et al.* (2003) examined the ability of *P. tuberregium* to bioremediate crude oil polluted soils and used it for the seed germination and seedling growth of *Vigna unguiculata*. The effect of various concentrations of crude oil on fungal populations of soil was investigated by Obire and Anyanwu (2009). These included *Alternaria*, *Aspergillus*, *Candida*, *Cephalosporium*, *Cladosporium*, *Fusarium*, *Geotrichum*, *Mucor*, *Penicillium*, *Rhizopus*, *Rhodotolura*, *Saccharomyces*, *Torulopsis* and *Trichoderma*. The authors reported the decreasing order of occurrence of a variety of fungal genera (fungal diversity) of both total fungi and petroleum-utilizers with increase in crude oil concentrations. They showed that higher concentrations of crude oil has adverse effect on fungal diversity while enhancing the population of a fewer fungi like *Aspergillus* sp., *Cladosporium* sp., *Mucor* sp., *Penicillium* sp. and *Trichoderma* sp. and *Candida* sp.

The yeast species described in literature as being able to use hydrocarbons as carbon source belong especially to the genera *Candida, Clavispora, Debaryomyces, Leucosporidium, Lodderomyces, Metschnikowia, Pichia, Rhodosporidium, Rhodotorula, Sporidiobolus, Sporobolomyces, Stephanoascus, Trichosporon* and *Yarrowia* (Csutak *et al.,* 2010). Sood and Lal (2009) isolated *Candida digboiensis* from soil samples contaminated with acidic oily sludge (pH 1–3) which could degrade 73% of the total petroleum hydrocarbons present in the medium at pH 3 in a week. Miranda *et al.* (2007) investigated the potential of the diesel degrading yeasts, *Rhodotorula aurantiaca* and *Candida ernobii.* They reported complete degradation of tetradecane, 5 methyl-octane and octadecane by *C. ernobii* and 93% for decane, 38.4% for nonane and 22.9% for dodecane with *R. aurantiaca* from 12% diesel after 20 days.

1.7 Factors affecting biodegradation

Various factors affect the process of bioremediation and have to be controlled and optimized. Major factors include presence of microbial population capable of degrading contaminant; availability of the contaminant to the microorganisms; environmental factors such as soil type, temperature, pH, moisture, nutrients and oxygen (Chikere *et al.*, 2011).

However, in case of oil pollution, the composition and inherent biodegradability of the petroleum hydrocarbon pollutant is the first and foremost important parameter. In general the susceptibility decreases in the order - n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes. Biodegradation rates have been shown to be highest for saturates, followed by the light aromatics, with high-molecular-weight aromatics and polar compounds exhibiting extremely low rates of degradation (Leahy and Colwell, 1990). The properties of petroleum hydrocarbon products which affected their biodegradation were chemical structure, degree of substitution, solubility, viscosity and concentration of its various components (Atlas, 1981; Jain *et al.,* 2011; Seo *et al.,* 2009). For example, Fedorak and Westlake (1981) reported more rapid degradation of simple aromatic (naphthalene and 2-methylnaphthalene) hydrocarbons as compared to *n*-alkanes.

Among physical factors, temperature plays an important role by affecting solubility of PHC and physiology of the microbial flora. For instance, Atlas (1975) observed that at low temperatures, the viscosity of the oil increased, while the volatility of the toxic low molecular weight hydrocarbons reduced, thereby delaying the onset of biodegradation. Nutrients such as nitrogen, phosphorus, and in some cases iron are important ingredients for successful biodegradation of hydrocarbon pollutants. Therefore, in many instances additions of nutrients becomes necessary to enhance the biodegradation of oil pollutant. However, excessive nutrient concentrations can also inhibit the biodegradation activity. Initial steps in the catabolism of PHC by bacteria and fungi involve the oxidation of the substrate by oxygenases, for which molecular oxygen is required. Hence, aerobic conditions are essential for PHC degradation.

Production and release of biosurfactants by the microbial community of the site is another essential step towards higher PHC degradation as it helps in emulsion formation, increasing the bioavailability/uptake by the microorganism. Therefore,

most of the bacteria which effectively degrade crude oil also exhibit strong emulsifying activity. Moisture content of the soils directly affects growth and metabolism of organisms and optimal rates of PHC biodegradation could be observed with 30 to 90% water saturation (Leahy and Colwell, 1990).

1.8 Mechanisms of PHC degradation

The metabolic pathways that hydrocarbon-degrading heterotrophs use can be either aerobic or anaerobic. The aerobic degradation usually proceeds more rapidly and is considered to be more effective than the anaerobic one.

1.8.1 Aerobic degradation

The degradation pathways of aliphatic hydrocarbons by microorganisms are extensively studied and reviewed (Watkinson and Morgan, 1990; Wentzel *et al.*, 2007; Rojo, 2009). Complete degradation of aliphatic hydrocarbons results in formation of carbon dioxide and water. Alkanes can be biodegraded by two pathways. The initial step of aerobic *n*-alkane degradation involves the enzymes that have a strict requirement of molecular oxygen, i.e., monooxygenases and dioxygenases. Table 1.1 shows some genera of microorganisms which metabolize various chain length *n*-alkanes along with the enzymes involved.

Table 1.1 Oxygenases involved in *n*-alkane oxidation

Enzymes	**Alkane (chain length)**	**Genera**	**Reference**
Methane monooxygenases	C_1-C_8	*Methylosinus, Methylococcus, Methylobacter,*	Fox *et al.*, 1989; Smith *et al.*, 1997; Hakemian and Rosenzweig, 2007
AlkB family of alkane monooxygenases	C_5-C_{16}	*Pseudomonas, Rhodococcus, Acenitobacter, Mycobacterium, Geobacillus*	Whyte *et al.*, 1997; Binazadeh *et al.*, 2009; Geißdo¨rfer *et al.*, 1999 Kelley *et al.*, 1990; Feng *et al.*, 2007
Bacterial P450 (CYP153, Class I)	C_5-C_{16}	*Mycobacterium,*	van Beilen *et al.*, 2005
Eukaryotic P450 (CYP52, Class II)	C_{10}-C_{16}	*Yarrowia, Candida, Lodderomyces*	Iida *et al.*, 2000; Mauersberger *et al.*, 1984
Dioxygenases	C_{10}-C_{30}	*Acenitobacter*	Maeng *et al.*, 1996

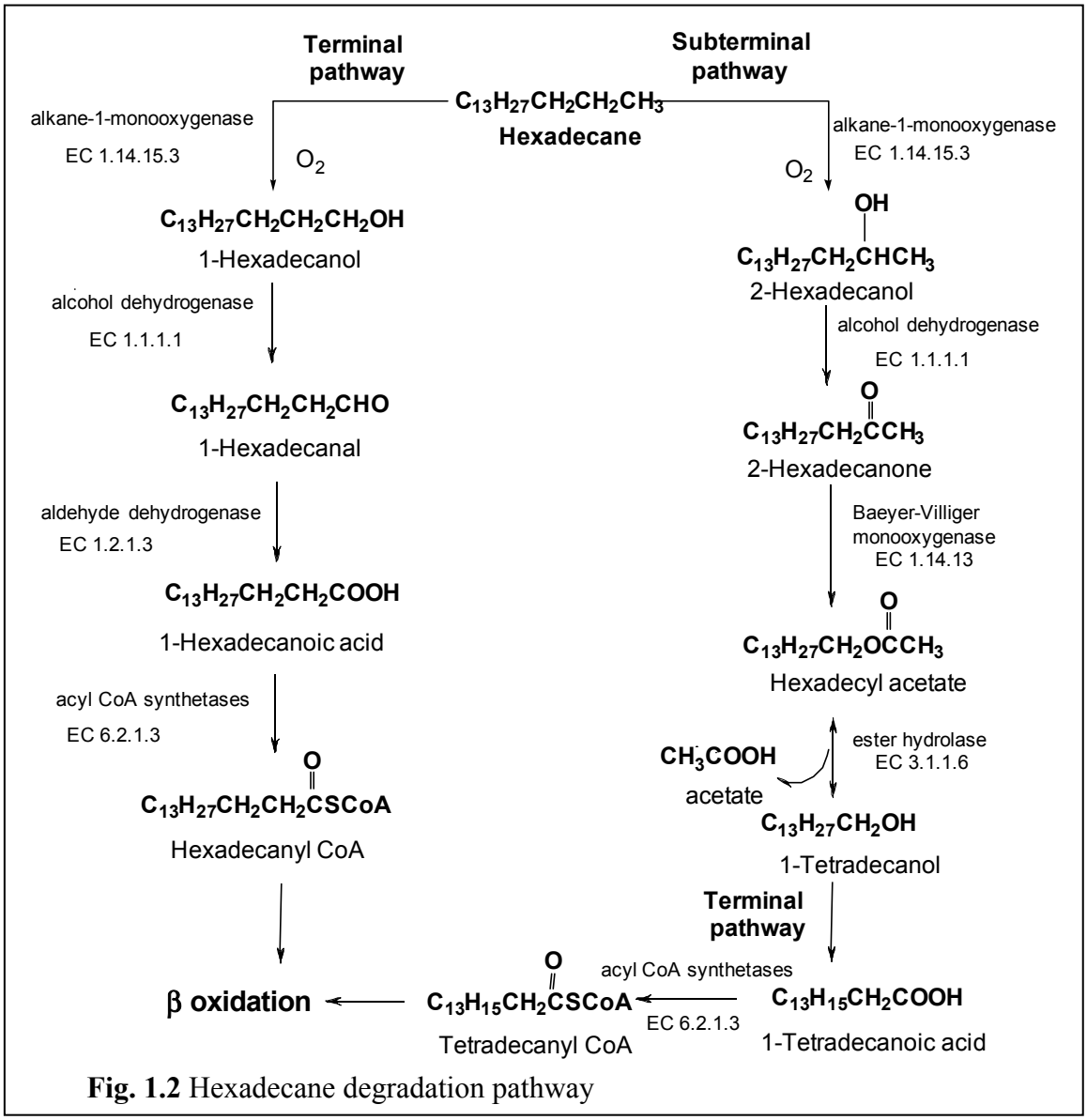
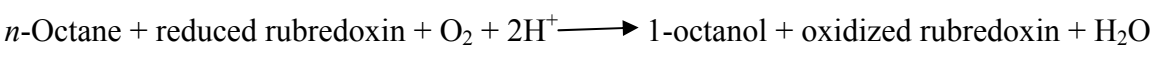


Fig. 1.2 Hexadecane degradation pathway

The first pathway involves the action of monooxygenases on *n*-alkanes at the first step. Oxidation of medium chain or long chain alkanes by aerobic microorganisms occurs *via* monoterminal (CH_3-CH_2-$(CH_2)_n$-CH_2-CH_3 to CH_3-CH_2-$(CH_2)_n$-CH_2-CH_2OH), biterminal (CH_3-CH_2-$(CH_2)_n$-CH_2-COOH to $HOCH_2$-CH_2-$(CH_2)_n$-CH_2-COOH) or subterminal (CH_3-CH_2-$(CH_2)_n$-CH_2-CH_3 to CH_3-CH_2-$(CH_2)_n$-CH_2-CHOH-CH_3) pathways as suggested by Berthe-Corti and Fetzner (2002). Hexadecane degradation pathway is depicted in Fig. 1.2.

The enzymes involved in the aerobic degradation of aliphatic hydrocarbons are: alkane 1 monooxygenase (EC 1.14.15.3), alcohol dehydrogenase (EC 1.1.1.1(NAD^+) or EC 1.1.1.2 ($NADP^+$) and aldehyde dehydrogenase (EC 1.2.1.3) and

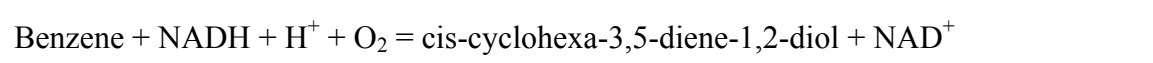
acyl CoA synthetase (EC 6.2.1.3). The monooxygenases initiate the alkane degradation.



n-Octane + reduced rubredoxin + O_2 + $2H^+$ ⟶ 1-octanol + oxidized rubredoxin + H_2O

The second pathway uses a dioxygenase at the first step which adds two oxygen atoms to the terminal methyl group of the alkane. This results in the formation of peroxide that is converted to fatty acid (Chikere *et al.*, 2011). The general degradation pathway for an *n*-alkane involves sequential formation of an aldehyde, an alcohol and a fatty acid. The fatty acid is cleaved by beta-oxidation, releasing carbon dioxide and forming a new fatty acid that is two carbon units shorter than the parent molecule (Watkinson and Morgan, 1990).

The general pathway for aromatic hydrocarbons involves cis-hydroxylation of the ring structure forming a diol (e.g. benzene) using dioxygenase. Benzene 1,2 – dioxygenase (EC 1.14.12.3) for instance catalyzes following reaction:



Benzene + NADH + H^+ + O_2 = cis-cyclohexa-3,5-diene-1,2-diol + NAD^+

The ring is oxidatively cleaved by dioxygenases, forming a dicarboxylic acid. Oxidation of substituted aromatics generally proceeds by initial beta-oxidation of the side chain, followed by cleavage of the ring structure (Diaz, 2004; Van Hamme *et al.*, 2003; Husain, 2008).

Naphthalene is one of the most common contaminants found in hydrocarbon polluted soils. Davies and Evans (1964) were the first to study the enzymes involved in naphthalene degradation by soil Pseudomonads. The degradation proceeds with the addition of two atoms of oxygen to the naphthalene ring to form cis-1,2-dihydroxy-1,2-dihydronaphthalene by 1,2-naphthalene dioxygenases (EC 1.14.12.12). The second step is catalyzed by naphthalene(+)-cis-dihydrodiol dehydrogenase (EC 1.3.1.29) which forms 1,2-dihydroxynaphthalene using NAD + as an electron acceptor. The next steps involve series of reactions to form salicylic acid which is further oxidized to catechol by salicylate hydroxylase (EC 1.14.13.1). In *Pseudomonas putida*, the genes specifying naphthalene catabolism are organized into two operons, *nah* and *sal*, and are carried on an 80kb plasmid, NAH7 (You *et al.*, 1988). Grimm and Harwood, 1997) reported that the catechol thus formed may undergo ring cleavage by a *meta* pathway to form pyruvate and acetaldehyde, or an

ortho - pathway to form succinyl-CoA and acetyl-CoA as end products. *Sphingomonas* sp. VKM B-2434 was reported to degrade naphthalene using a similar pathway (Baboshin *et al.,* 2008).

Aerobic degradation in soil is associated with a variety of microorganisms, including bacteria and fungi. *Pseudomonas* appears to be the most ubiquitous bacterium found in soil, and also in oil contaminated soil. Pseudomonads have high degradative potential e.g. *P. putida* and *P. fluorescens*. These have ability to adapt to many different hydrocarbons not solely with catabolic enzymes but also on metabolic regulation. Certain species of *Pseudomonas* have the capacity to degrade particular aliphatic hydrocarbons. For instance, one strain of *P. aeruginosa* was found to degrade C_6, C_8 and C_{10} compounds but failed to degrade longer aliphatic compounds while another strain showed degradation of longer chain compounds. These differences in the degradative capabilities prompted researchers to engineer novel strains genetically (Chapelle, 1999). *Acinetobacter* and *Rhodococcus* are two bacterial strains often associated with petroleum contaminated habitats. *Acinetobacter* utilized an alkane monooxygenase (terminal oxidation) to convert the hydrocarbon to a primary alcohol to allow the subsequent breakdown and utilization of the hydrocarbon (Fig. 1.2) (Geißdörfer *et al.,* 1999). *Rhodococcus* possessed an alkane monooxygenase as *Acinetobacter,* but exhibited subterminal oxidation leading to the production of a secondary alcohol and the subsequent ketone was further metabolized to a primary alcohol for further breakdown (Fig. 1.2) (Binazadeh *et al.*, 2009).

Filamentous fungi were also reported to degrade long chain alkanes. Hadibarata and Tachibana (2009) studied the degradation of n-eicosane (C_{20}) by *Trichoderma* sp. They observed that the fungus converted the alkane to the fatty acid nonadecanoic acid. While Zinjarde *et al.* (1998) reported hexadecane (C_{16}) degradation by a marine isolate *Yarrowia lipolytica*. Alkane monooxygenase/ hydroxylase is one of the key enzymes in the process of alkane degradation (Rojo, 2009). It has been detected in α-, β-, and γ-proteobacteria as well as the Actinobacteria (Vomberg and Klinner, 2000). Although many alkane monooxygenases have been reported to be involved in alkane degradation, Alk B is most important and prevalent in aerobic alkane degradation (Wang *et al.*, 2010).

The involvement of plasmids in *Pseudomonas* sp. in hydrocarbon degradation has been widely studied. Many plasmids have been characterized which encode

pathways for petroleum hydrocarbon degradation. Plasmids NAH, OCT and TOL are reported to involved in naphthalene, octane and toluene degradation (Chakrabarty *et al.*, 1973; Chakrabarty *et al.*, 1978; Yen and Gunsalus, 1982). Some of the plasmids related to naphthalene degradation in the genus *Pseudomonas* are listed in Table 1.2.

Table 1.2 Some of the plasmids related to naphthalene degradation in the genus *Pseudomonas*

Plasmid	Microorganism	Reference
pWW60	*Pseudomonas sp.* NCIB 9816	Cane and Williams, 1982
NAH7	*Pseudomonas putida*	Yen and Gunsalus, 1982
NPL1, pBS2	*Pseudomonas putida*	Boronin *et al*. 1993
pLIB119	*Pseudomonas stutzeri* 19SMN4	Rossello-Mora *et al*. 1994
pDTG1	*P. putida* strain NCIB 9816-4	Dennis and Zylstra, 2004
pND15	*Pseudomonas* sp. strain NGK 1	Subba *et al*. 2010

1.8.2 Anaerobic degradation

In contrast to the fact that aerobic microbial hydrocarbon metabolism was extensively investigated, the same was not true for anaerobic hydrocarbon degradation (Townsend *et al.,* 2004; Foght 2008). Under number of conditions such as in deep sediments, eutrophic lagoons, flooded soils, in oil reservoirs and stagnant fresh and ocean waters oxygen is not available. Ferric iron- reducing, sulfate-reducing and denitrifying bacteria have been reported to anaerobically utilize several alkanes, alkenes and alkylbenzenes from such sites (Heider *et al.*, 1999).

It was not until the late 1980s that photosynthetic *Blastochloris sulfoviridis,* denitrifying bacterium *Thauera aromatica,* plant associated *Azoarcus tolulyticus*, halotolerant *Halomonas,* ferric iron-reducing *Geobacter metallireducens*, to name a few were found to degrade *n*-alkanes, branched alkanes, and cycloalkanes under anoxic conditions (Aitken *et al.*, 2004; Heider *et al.*, 1999; Widdel and Rabus, 2001). In case of anaerobic toluene degradation, for instance, benzylsuccinate synthase (EC 4.1.99.11) catalyzed the first step of addition of toluene to fumarate to form benzylsuccinate (Heider *et al.*, 1999).

Anaerobic naphthalene degradation proceeds via carboxylation to form 2-naphtoate in sulfate-reducing bacteria. The identification of other metabolites in a sulfate-reducing enrichment culture indicated further metabolism of 2-naphthoate via

subsequent reduction of the two rings to yield decahydro-2-naphthoic acid (Zhang and Young, 1997; Meckenstock *et al.*, 2000). While Safinowski and Meckenstock (2006) reported that sulfate reducing culture(s) initiated the reaction in the naphthalene degradation pathway by methylation to 2-methylnaphthalene which then underwent oxidation to the 2-naphthoic acid. Anaerobic hydrocarbon degradation can technically be used in the bioremediation of some oil polluted sites which have limited aeration such as contaminated groundwater aquifers (Heider *et al.*, 1999).

1.9 Diversity of PHC contaminated soils

Soil is a complex and heterogeneous ecosystem which acts as a habitat to maintain biological activity, diversity, and productivity. It plays a role in filtering, buffering, storage and transformation of essential and non-essential chemicals along with biomass production and the cycling of nutrients and other elements. Soil biodiversity is generally high because soil contains all major groups of micro-organisms and fungi, green and blue-green algae and a great number of animal phyla (Lee, 1994; Nannipieri *et al.*, 2003). The soil microbial diversity is critical in maintenance of good soil health and fertility as these microorganisms have key roles in the many functional processes that support such systems (Kibblewhite *et al.*, 2008). These functional processes include nutrient recycling, regulation of organic matter dynamics, modification of soil physical structure, assistance to plant nutrient acquisition, mineralization, fixation and mobilization of nutrients, enhancement of plant health and biotic and abiotic stress tolerance.

PHC polluted soils are different from pristine soils due to changes in their biological as well as physicochemical properties (Robertson *et al.*, 2007). When PHC contamination occurs in soils, an initial reduction in soil microorganisms is observed (especially in soils that have not been previously polluted). However, the reduction is followed by a rapid increase in the number of microorganisms that are capable of degrading the contaminants (Seghers *et al.,* 2003). Though the number of soil microorganisms increase in PHC-polluted soils, species richness often decreases over time (Hofman *et al.,* 2004). In soils with high humus content, PHC toxicity to soil organisms have been found to be less severe (Salminen and Haimi, 1997). Nicolotti and Egli (1998) reported that some fungi may show resistance to PHC, even may benefit from the presence of the pollutants.

Microorganisms that can withstand and utilize hydrocarbons could only survive and grow in polluted soils affecting whole community structure (Evans *et al.*, 2004; Bordenave *et al.*, 2007; Popp *et al.*, 2006; Baek *et al.*, 2007). For example, *γ-proteobacteria* mainly *Pseudomonas* species were found to be selectively enriched after the contamination by hydrocarbons such as alkanes, and PAHs followed by *α-proteobacteria* and *β-proteobacteria* (Popp *et al.*, 2006). Yamane *et al.* (2008) characterized diversity of bacteria obtained directly from crude oil. They found presence of *Acinetobacter, Propionibacterium, Sphingobium* and *Bacillus*. Baek *et al.* (2007) studied crude oil contaminated sandy loam soil (pH 7.1) and found presence of *Mycobacterium, Nocardia, Thiocalovibrio, Pseudomonas citronellosis* and sulfur oxidizing bacteria. The change in microbial diversity of agricultural soil may have adverse effects on fertility and thus, on the crop yield. This necessitates the need for restoration of useful, similar or same diversity of agricultural soil, which can possibly be achieved by various bioremediation techniques.

There are many approaches towards studying the diversity of oil contaminated soil. Liquid enrichment cultures are routinely used in laboratory (Greene *et al.,* 2000). Vinas *et al.* (2002) reported that the enrichment with a specific hydrocarbon increased only those organisms, from a consortium, that could utilize that hydrocarbon as a sole source of carbon. In other words, the community structure obtained from the same soil after enrichment using two different hydrocarbons could be different (Baek and Kim, 2009). Use of molecular biology techniques like Denaturing Gradient Gel Electrophoresis (DGGE) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) were proved to be beneficial for judging the community structure of contaminated soil, which were independent of the ability of bacteria to grow in the culture media (Jung *et al.,* 2005; Kaplan and Kitts, 2004; Macnaughton *et al.*, 1999).

Ogino *et al.* (2001) studied community structure of contaminated soil by comparing it with untreated control soil. Hydrocarbon contamination increased the diversity of oil degraders especially *Pseudomonas* species, initially and subsequently it became similar to that of uncontaminated soil. Similarly, Kaplan and Kitts (2004) reported increase in the population of *Flavobacterium* and *Pseudomonas* at the end of first 3 weeks of oil contamination thereafter their abundance decreased. Macnaughton *et al.* (1999) reported similar observations for the coastal soils with experimental crude oil spill. Using phospholipid fatty acid (PLFA) analysis and 16S rDNA PCR-

DGGE to monitor *in situ* microbial community structures they reported that contaminated plot was dominated by *α-proteobacteria* which were not detected in uncontaminated plot.

Microbial mat is made up of complex communities which show diverse genetic and metabolic potential. Hence change in such communities after contamination is more pronounced. Bordenave *et al.* (2007) conducted a study on microbial mat contaminated with heavy (C_{23}–C_{40}) fuel oil. Immediately after contamination *γ-proteobacteria* mainly *Pseudomonas* species were abundant, while after 90 days bacilli and staphylococci increased in abundance to more than 50%. Abed *et al.* (2002) performed a study on microbial mat contaminated with diesel and obtained enrichment of *Phormidium*- and *Oscillatoria*-like cynobacteria, *γ*- and *β-proteobacteria*.

Different indices have been used to quantify diversity from soil. Diversity, *per se*, after contamination usually changed and subsequently gradually it decreased as easily biodegradable substrate were diminished (Popp *et al.*, 2006). Bundy *et al.* (2002) found that the community structure following diesel contamination on 3 different soil types was not similar. Hence each soil type bears a unique indigenous microbial community which needs to be studied before implementation of remedial measures.

1.10 Future prospects

The profile of oil contamination with respect to depth and the distance from the source of contamination, i.e. from heavy contamination to uncontaminated soil can be correlated with the microbial diversity. The distances of qualitative and quantitative changes in the diversity from the uncontaminated end can prompt to design the treatment for bioremediation. In case of accidental spillage, usually a large area is covered by the oil. On the other hand, contamination due to seepage may cover smaller area but to a greater depth. The depth of penetration is affected by factors like soil characteristics such as texture, structure, composition, and water saturation capacity (Massaud *et al.,* 2000). Usually TPH quantity was found to be decreased as depth of penetration increased (Al-Sarawi *et al*., 1997). Raymond *et al*. (1976) reported the oil contamination up to 30 m deep. However, the bioremediation using biostimulation method was achieved up to 15 m. The localized contamination of

petroleum hydrocarbons deep in the soil up to 3 m can be reduced using tree species which can withstand low level of diesel/gasoline contamination (< 1000 mg/kg soil) (Trapp *et al*. 2001). In the case of biopiling, the soil layers up to 0.5 m can be removed and further processed. While rhizo-remediation with grasses and the use of microorganisms having constitutive or induced capabilities to degrade hydrocarbons were found to be effective to remove the contaminants in the upper layers of soil up to 0.2 m.

However, the restoration of original microbial diversity and also the soil fertility is a main concern. Moreover, the studies are necessary to pin point the effect of oil contamination on unculturable diversity and the possibility to restore it too. It is indeed possible to restore useful and/ or similar diversity using bioremeditation techniques singly or in combination. But the translation of pilot level observations in to a technology which can be used in fields is a major challenge.

1.11 Objectives of present investigation

Based on aforementioned background, the specific objectives defined for present study were as follows –

- Isolation, identification and characterization of the microbial community of petroleum hydrocarbon contaminated sites.
- Identification of the pollutants present in the contaminated soils.
- Screening of the hydrocarbon degraders for their diesel degrading capability.
- Elucidating the effect of pollutants on soil communities and using bioremediation to restore the soil microbial community.

Chapter 2

Materials and Methods

CHAPTER 2

2.1 Materials

The materials and chemicals used in the present study were purchased from suppliers listed in the Table 2.1.

Table 2.1 Sources of chemicals and materials

Chemicals/Materials	Suppliers
Standard *n*-alkanes (C_7-C_{40}), 2-bromodecane, 1-hexadecanol, 2-hexadecanol, bovine serum albumin, Nicotinamide adenine dinucleotide (reduced) - NADH	Sigma-Aldrich, USA
n-Hexadecane	Hi Media Laboratories, India.
Dichloromethane, dimethyl sulfoxide, hexane, Folin–Ciocalteu reagent	Merck Specialties Pvt. Ltd., India.
Peptone, yeast extract	Hi Media Laboratories, India.
Taq DNA polymerase	BioResource Biotech Pvt. Ltd., India
DNA ladder (300-10,000 bp)	Bio-Rad Laboratories, USA
Polymerase chain reaction (PCR) primers: For amplification 16S_8F (5'- AGAGTTTGATC CTGGCTCAG-3') and 16S_1391R (5'- GACGGGCGGTGTGTRCA -3'); For sequencing 704F (5'- GTAGCGGTGAAATGCGTAGA-3') and 907R (5'-CCGTCAATTCMTTTGAG TTT-3')	BioResource Biotech Pvt. Ltd., India

All other chemicals, media, buffers etc. used were of analytical grade, procured from S.D.Fine-Chemicals Ltd.; Sisco Research Laboratoies Ltd.; Hi-Media Laboratories; Loba Chemie. The diesel used in this study was obtained from a local

service station in Pune, India. It was stored in airtight containers and sterilized by filtering through a Millipore 0.45μM pore size membrane filter.

2.2 Collection of PHC contaminated soil samples

PHC contaminated soil samples were collected from service stations and motor garages located as mentioned in Table 2.2. The samples were collected from the surface and subsurface layers (5-30 cm in depth) and passed through a 2 mm sieve, after removal of surface litter and stored at 4ºC in pre-sterilized bags. The uncontaminated soil was collected from an agriculture field close to Pune Site 1.

Table 2.2 Sites for collection of PHC contaminated soils

Code	Soil sample	Site
A	Pune Site 1 (MH)	
B	Pune Site 2 (MH)	18°31' N 73°51' E
C	Pune Site 3 (MH)	
D	Zahirabad Site 1(AP)	17°41' N 77°37' E
E	Jaigaon Site 1(WB)	26°34′ N 89°43′ E
F	Mangalabadi Site 1 (WB)	25°37′ N 88°07′ E
G	Mumbai Site 1(MH)	19°12' N 73°6' E

MH – Maharashtra, AP – Andhra Pradesh, WB – West Bengal

2.3 Physicochemical analysis of PHC contaminated soil samples

The physiochemical analysis of PHC contaminated soil samples and agriculture soil used for pot experiments was done at District Soil Survey and Soil Testing Laboratory, Pune, Maharashtra, India. The physical analysis of soil samples included the study of soil type, its pH, electric conductivity, moisture content, water holding capacity, and pore space. The chemical analysis included estimation of organic carbon, nitrogen and available phosphorous (Table 2.3).

Table 2.3 Physicochemical properties of PHC contaminated soil samples and normal soil

Soil sample / Property	A	B	C	D	E	F	G	Normal soil
pH	7.25	7.5	7.1	7.22	7.12	7.45	7.03	7.81
Electric conductivity (µs/cm)	0.4	0.42	0.43	0.47	1.11	0.53	0.45	0.32
Organic carbon (%)	3.36	7.33	4.34	2.32	2.25	5.42	3.3	0.87
Nitrogen content (%)	0.28	0.41	0.31	0.08	0.05	0.38	0.26	0.72
Available phosphorus (kg/hect)	10.0	9.34	9.52	13.57	12.79	8.67	10.89	14.01
Soil type	Sandy	Clay	Sandy	Sandy	Sandy Loam	Sandy Loam	Sandy	Clay
Moisture content (%)	1.6	0.6	1.71	1.84	1.82	2.25	1.27	9.85
Water holding capacity(%)	28.92	40.27	31.12	22.65	50.1	31.36	41.16	60.22
Pore space (%)	ND	ND	ND	24.7	53.05	ND	ND	63.8

ND: not determined

Total petroleum hydrocarbons (TPH) and *n*-alkanes present in the PHC contaminated soil samples were analyzed by gravimetry and gas chromatography (GC) as described by Schwab *et al.* (1999), Lin *et al.* (2005) and Lin and Mendelssohn (2009). Soil samples (10 g) were extracted with 30 ml of dichloromethane (DCM) thrice with continuous agitation for 30 min. The solvent fractions were pooled, passed through anhydrous sodium sulfate and evaporated under vacuum using a rotary evaporator to dryness. The extract was re-dissolved in 1 ml of DCM and used for GC (Varian CP-3800, Varian Inc., CA) for quantification of TPH and individual *n*-alkanes.

2.4 Composition of nutritional media

Compositions of different media used in the present study are given in Table 2.4.

Table 2.4 Media composition

Name of medium	Constituents (g/L)	Purpose
Luria Bertani broth - LB	Tryptone 10; NaCl 10; yeast extract 5; pH 7.0	Cultivation of bacterial cultures
Nutrient broth/ agar - NA	Peptone 10; NaCl 5; yeast extract 3; pH 7.0 Agar 20	Maintenance of bacterial cultures
Yeast extract peptone glucose broth/ agar (2%) - YPG	Glucose 10; yeast extract 3; peptone 5; pH 6.5 Agar 20	Maintenance of yeast cultures
Bushnell and Haas medium - BH	KH_2PO_4,1.0; K_2HPO_4, 1.0; NH_4NO_3, 1.0; $MgSO_4.7H_2O$, 0.2; $FeCl_3$, 0.05; $CaCl_2.2H_2O$, 0.02; pH 7.0 Agar 20	Preparation of inoculum, screening and isolation of diesel degraders

2.5 Enumeration of total heterotrophic bacteria in the soil samples

Heterotrophic bacteria present in the soil samples were enumerated by serial dilution and spread plating on NA medium. The plates were incubated for 48 h at 28°C. Colony Forming Units per gram of dry soil (CFU/g of dry soil) were counted.

2.6 Enrichment and isolation of diesel degraders

For the enrichment of diesel degrading bacteria from soil samples, 1 g of each sample was added to BH medium (Bushnell and Haas, 1940) containing 1% (w/v) diesel as the sole carbon source separately and incubated at 28°C under shaking (180 rpm) for 7 d. After incubation, serially diluted broth was spread plated on BH agar with 1% diesel as well as on NA. The plates were incubated for 48h at 28°C. The diesel degrading organisms from BH agar plates were isolated in pure form by subculturing.

The bacterial isolates obtained were numbered as I1 to I70 and the yeast isolates as Y1 to Y3. All bacterial isolates were maintained on NA slants, while yeast cultures were maintained on YPG slants at 4°C until further use and sub-cultured every 30 d. For long term preservation glycerol stocks were prepared and kept at -80°C.

2.7 Identification of diesel degrading organisms

Colony characteristics, gram nature, motility of isolates were studied. Identification of the bacterial isolates was done by carrying out different biochemical tests according to Bergey's Manual of Systematic Bacteriology, 2nd edition (Krieg and Holt, 1984) and 16S rRNA sequencing. Identification of yeasts was done based on the morphology and biochemical characteristics (Lodder, 1970).

The genomic DNA was isolated as described by Sambrook *et al.* (2001).Cells grown overnight in LB medium were harvested by centrifugation at 6000 rpm for 10 min at 4°C. The pellet was re-suspended in 5 ml GTE buffer (50 mM glucose, 25 mM Tris HCl, 10 mM EDTA, pH 8) and centrifuged at 7000 rpm for 15 min at 4°C. The pellet was re-suspended in 5 ml GTE buffer containing 1 mg/ml of lysozyme and kept at RT for 15 min. SDS (1% final concentration) was then added and kept at RT for 15 min. Equal volume of phenol equilibrated with 100 mM Tris, pH 8.0, was added and mixed thoroughly for 5 min. The contents were then centrifuged at 10000 rpm for 10 min at RT. The supernatant was transferred to another tube and equal volume of phenol-chloroform (1:1) mixture was added and centrifuged at 10000 rpm for 10 min at RT. The phenol-chloroform extraction was repeated until no interphase was observed. Final extraction was done with chloroform. The aqueous phase was collected in a fresh centrifuge tube, the salt concentration was adjusted to 0.15 M NaCl and two volumes of chilled ethanol were added. The spooled out DNA was transferred to another tube and centrifuged at 10000 rpm for 10 min at 4°C. DNA pellet was air dried completely and re-suspended in 100 µl of TE (10 mM Tris, 1 mM EDTA, pH 8) buffer and kept overnight at 4°C for dissolving high molecular weight DNA. Presence of DNA was confirmed by electrophoresis on 0.8% agarose gel.

The PCR was performed as described by Jadhav *et al.* (2013) using Applied Biosystems, model 9800 with 50 ng of DNA template in a total volume of 25 µl. The PCR master mixture contained 2.5 µl of 10X PCR reaction buffer (with 15 mM

$MgCl_2$), 2.5 µl of 2 mM dNTPs, 1.25 µl of 10 pmol/µl of each oligonucleotide primer - 16S_8F (5'- AGAGTTTGATC CTGGCTCAG-3') and 16S_1391R (5'-GACGGGCGGTGTGTRCA -3'), 0.2 µl of 5U/µl Taq DNA polymerase and 15.76 µl of glass-distilled water. Initial denaturation was done at 94°C for 3 min. Thirty-two cycles of amplification consisted of -denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 90 sec. A final extension at 72°C for 10 min was performed. The PCR product was purified by PEG-NaCl. The sample was mixed with 0.6 vol of PEG-NaCl [20% PEG (MW 6000) and 2.5 M NaCl] and incubated at 37°C for 20 min. The precipitate was collected by centrifugation at 3,800 rpm for 20 min. The pellet was washed with 70% ethanol, air dried and dissolved in 12 µl sterile distilled water.

The samples were sequenced using a 96-well Applied Biosystems sequencing plate as per the manufacturer's instructions. The thermocycling for the sequencing reactions was started with an initial denaturation at 94°C for 2 min, followed by 35 cycles of PCR consisting of denaturation at 94°C for 10 sec, annealing at 50°C for 10 sec, and extension at 60°C for 4 min using primers 704F (5'-GTAGCGGTGAAATGCGTAGA-3') and 907R (5'-CCGTCAATTCMTTTGAG TTT-3'). The samples were purified using standard protocols described by Applied Biosystems, Foster City, USA. To the purified product, 10 µl of Hi-Di formamide was added and vortexed briefly.

The DNA was denatured by incubating at 95°C for 3 min, kept on ice for 5-10 min, and was sequenced in a 3730 DNA analyzer (Applied Biosystems) following the manufacturer's instructions. The obtained sequences were analysed using Sequence Scanner (Applied Biosystems) software. The rDNA sequence contigs were generated using Chromas Pro and then analysed using online databases viz. NCBI-BLAST to find the closest match of the contiguous sequence.

2.8 Primary screening of isolates for PHC degradation

The isolates were first screened based on their capability to utilize 1% (w/v) diesel as a sole carbon source added in BH medium. The flasks were incubated at 28°C on an orbital shaker at 180 rpm for 48 h. Cell growth measured as optical density at 600 nm (OD_{600}) was used as a parameter for diesel degradation capability.

2.9 Alkane monooxygenase (AMO) assay

AMO assay was performed for isolates selected based on OD_{600} measurements. The cultures were grown in BH medium with 1% *n*-hexadecane, 0.5% *n*-hexadecane+ 0.5% glucose and 1% glucose at 28°C on an orbital shaker (180 rpm) for 72 h. After incubation, cells were harvested by centrifugation and suspended in 0.1 M phosphate buffer, pH 8.0. Cells were disrupted using Braun's homogenizer with 10 cycles of 30 s each. The samples were centrifuged at 12000 g for 15 min to obtain cell extract.

The activity of AMO was measured as described by Glieder *et al.* (2002). The oxidation of *n*-hexadecane was measured by monitoring the decrease in the A_{340} of NADH. The reaction mixture (1ml) contained 50-100 µl of cell extract, 0.01 M *n*-hexadecane in 1% DMSO and phosphate buffer (0.1 M, pH 8.0). The reaction was initiated by the addition of 125 µM NADH to the sample cuvette. A control in which *n*-hexadecane was omitted from the reaction mixture was run for each assay.

One unit of alkane monooxygenase activity was defined as the amount of enzyme required to oxidize 1 µmole of NADH per min per mg of protein under standard assay conditions.

Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

2.10 Screening of selected isolates for *n*-alkanes degradation

The isolates were inoculated (1 x 10^7 CFU/ml) in 50 ml BH medium supplemented with 1% (w/v) diesel. Uninoculated controls were used to estimate abiotic loss during the experiments. The cultures and controls were incubated at 28°C on an orbital shaker at 180 rpm for 7 d. Growth of the organisms was checked by spread plating and reported in terms of increase in CFU/ml. After incubation residual diesel was extracted and degradation of individual *n*-alkanes in diesel was quantified using GC as described in section 2.11.

2.11 Extraction and analysis of diesel range *n*-alkanes by GC

The diesel in the sample was extracted thrice with equal volumes of DCM, passed through anhydrous sodium sulfate and evaporated under vacuum using a rotary evaporator (Lin *et al.*, 2005). The extract was dissolved in 1 ml of DCM and analyzed by gas chromatography. The chromatographic analysis was performed on a Varian

CP-Sil 8B column with a flame ionization detector (FID) detector. Nitrogen was used as a carrier gas (1 ml/min) and injection volume was 1 µl. The initial column oven temperature was kept 50°C with a hold time of 1 min; further ramped at 5°C/min to 150°C and at 10°C/min to 300°C with a hold time 9 min. The injector temperature was set at 270°C while the FID temperature was set at 280°C (Mohanty and Mukherji, 2008).

2-bromodecane (5ng/µl) was used as an internal standard. *n*-Alkane identification and quantitation was done based on retention time and peak area of the C_7-C_{40} standards. The percentage degradation of the *n*-alkanes was calculated by comparison with the peak areas of the corresponding peaks in the un-inoculated controls.

2.12 Analysis of total petroleum hydrocarbons (TPH) by gravimetry

Analysis of TPH from soil was carried out by gravimetry. Soil sample (10 g) was extracted with DCM (Schwab *et al.*, 1999) and transferred to round bottom (RB) flasks for evaporation under vacuum and the un-evaporated diesel remaining in the RB flasks was weighed to the nearest 0.001 g. The TPH concentration was calculated and expressed as mg TPH/g of soil (Lin and Mendelssohn, 2009). The extract was re-dissolved in 1 ml of DCM and used for gas chromatography analysis.

2.13 Detection of alkane monooxygenase gene from selected isolates

Isolates I26, I38, I41 and I58 were grown on NA and single colony of each was used for colony PCR. Three PCR primer sets were used to detect genes encoding alkane monooxygenases present in the isolates as described by Kohno *et al.* (2002). The primers ALK-1F/ ALK1R, ALK-2F/ ALK2R and ALK-3F/ ALK3R (Table 2.5) were expected to generate PCR products of about 185 bp, 271 bp and 330 bp, respectively.

The PCR mix of 20 µl contained the following: 5 µl template, 2 µl buffer (pH 8.0), 9.5 µl sterile de-ionized water, 1 µl dNTP mix (0.5 mM), 1 µl of each primer (0.5 µM) and 0.5 µl of Taq DNA polymerase (2.5 U). After initial denaturation at 98°C for 3 min PCR was carried out for 40 cycles, with denaturation at 98°C for 30 s, annealing at 42.8°C for 1 min, extension at 72°C for 1 min. The final extension was at 72°C for 7 min. PCR products (10 µl) were analyzed by electrophoresis on 1.0% agarose gel and staining with 0.5 µg/ml of ethidium bromide.

Table 2.5 Sequences of alkane monooxygenase (ALK) primers (Kohno *et al.*, 2002)

Primer	Sequence	Length (bp)	GC content (%)
ALK-1F	5′-CATAATAAAGGGCATCACCGT-3′	21	43
ALK1R	5′-GATTTCATTCTCGAAACTCCAAAC- 3′	24	38
ALK-2F	5′-GAGACAAATCGTCTAAAACGTAA-3′	23	35
ALK2R	5′-TTGTTATTATTCCAACTATGCTC-3′	23	30
ALK-3F	5′-TCGAGCACATCCGCGGCCACCA-3′	22	68
ALK-3R	5′-CCGTAGTGCTCGACGTAGTT-3′	20	55

2.14 Plasmid DNA detection

To check whether the diesel degrading ability was genomic or plasmid borne, plasmid isolation was done for few isolates (I26, I38, I41 and I58) as described by Sambrook *et al.* (2001) for low molecular weight / mini plasmids and by Kado and Liu (1981) for high molecular weight / mega plasmids.

E. coli DH5α was used as positive control. For mini-plasmid, culture grown overnight in BH medium with 0.5% diesel was pelleted by centrifugation at 12,000 g for 10 min at 4°C. The pellet was dried and re-suspended in 200 µl of ice-cold solution I (50 mM glucose, 25 mM Tris HCl, 10 mM EDTA, pH 8) and kept in ice for 10 min. 400 µl of freshly prepared solution II (1% SDS in 0.2 N NaOH) was added and kept in ice for 10 min. 300 µl of ice-cold solution III (5 M potassium acetate, 60 ml; glacial acetic acid, 11.5 ml; distilled water, 28.5 ml; pH 5.6) was added and mixed by inverting the tubes gently. The samples were centrifuged at 12,000 g for 10 min at 4°C. The supernatant was transferred to another tube and equal volume of chilled isopropanol was added. The contents were mixed properly, kept at -80°C for 30 min and then centrifuged at 12,000 g for 15 min at 4°C. Isopropanol was decanted and the pellet was re-suspended in 70% ethanol. The contents were mixed properly and centrifuged at 12,000 g for 10 min at 4°C. Plasmid DNA pellet was air dried completely and re-suspended in 40 µl of TE (10 mM Tris, 1 mM EDTA, pH 8) buffer containing 10 mg/ml RNase (Sigma, USA). Presence of plasmid was confirmed by 0.8% agarose gel electrophoresis and staining with 0.5 µg/ml of ethidium bromide.

For mega-plasmid, cells grown overnight in BH medium with 0.5% diesel were harvested by centrifugation. The pellet was re-suspended in 100 µl E buffer (20

mM Tris-acetate and 2 mM sodium salt of EDTA, pH 7.9) followed by addition of 300 µl lysing buffer (3% SDS and 50 mM Tris, pH 12.6). The tubes were incubated at 65°C in water bath for 90 min. After centrifugation at 12,000 g for 10 min at room temperature, supernatant were transferred to new tubes and equal volume of chilled isopropanol was added. The contents were mixed properly and kept at room temperature for 1 h. Plasmid pellet was obtained by centrifugation at 12,000 g for 15 min at 4°C. The pellet was dissolved in 40 µl TE buffer (pH 8) containing 10 mg/ml RNase. Presence of plasmid was confirmed by 0.8% agarose gel electrophoresis.

2.15 GC analysis of *n*-hexadecane degradation products

The isolates (I26, I38, I41, I58 and Y2) were grown on 1% (w/v) *n*-hexadecane in BH medium at 28°C on an orbital shaker (180 rpm). The cells were harvested after 3 d and re-suspended in 0.1 M phosphate buffer (pH 8.0). *n*-Hexadecane was added in 10 ml of the suspension to a final concentration of 2.0 mM and the tubes were kept at 30°C and 300 rpm for 30 min. Ethyl acetate (10 ml) was then added and the contents were centrifuged at 15,000 g and 4°C for 10 min. The organic phase was collected and analyzed by GC (Varian CP-3800) with a FID detector as described by Binazadeh *et al.* (2009). Nitrogen was used as a carrier gas (1 ml/min) and injection volume was 1 µl. The initial column oven temperature was kept at 100°C, further ramped at 15°C/min to 210°C with a hold time 2.67 min. The injector temperature was set at 280°C, while the FID temperature was 300°C. 1-hexadecanol and 2-hexadecanol were identified by matching retention times with authentic standards containing 2 mM 1-hexadecanol and 2-hexadecanol.

2.16 Biosurfactant production

Biosurfactant production by selected isolates was checked by inoculating (1×10^7 CFU/ml) the cultures in 50 ml BH medium supplemented with 1% (w/v) diesel. The cultures were incubated at 28°C on an orbital shaker at 180 rpm for 7 d. Emulsification index (E24%) was measured according to the method described by Cooper and Goldenberg (1987). Cell-free supernatant (2 ml) obtained after centrifugation of the cultures was added to 5 ml of diesel and vortexed for 2 min. The tubes were kept at room temperature for 24 h. Emulsification index was defined as the height of the emulsion layer divided by the total height and expressed as percentage.

Emulsification activity of the surfactant was determined by the method of Johnson *et al.* (1992). To 10 ml of cell free supernatant, 1 ml benzene was added and the mixture was vortexed for 10 min and left undisturbed. After 1 h, the optical density of the oil in water emulsion phase was recorded at 610 nm.

2.17 Microbial adhesion to hydrocarbon (MATH) assay

The hydrophobicity of the cells was measured by MATH assay (Rosenberg *et al.,* 1984). Cells of the selected isolates grown in BH medium supplemented with 1% (w/v) diesel were washed twice with phosphate buffer (0.1 M, pH 8.0). The cells were re-suspended in phosphate buffer to give an optical density of ~ 0.5 at 600 nm.

In two sets of cell suspension (2 ml), 500 µl of *n*-hexadecane and 500 µl of diesel were added separately. The tubes were vortexed for 3 min and oil and aqueous phase were allowed to separate for 1 h. Absorbance of the aqueous phase was measured at 600 nm. Hydrophobicity was expressed as the percentage of cell adherence to *n*-hexadecane or diesel and calculated as follows:

$100 \times [1 - (\text{OD of aqueous phase/OD of the initial cell suspension})]$.

2.18 Optimization of parameters for diesel degradation by *A. baumannii* using Taguchi approach

According to Design of Experiments techniques, Orthogonal Arrays (OAs) are employed in Taguchi's approach to systematically vary and test the different levels of each of the control factors (Phadke, 1989). The columns in the OA indicate the factor and its corresponding levels, and each row in the OA constitutes an experimental run, which is performed at the given factor settings. Eight different control factors were identified to evaluate their role in the objective function, i.e. optimization of medium for maximum diesel degradation. The factors selected were agitation, diesel concentration, nitrogen concentration, phosphorous concentration, pH, temperature, inoculum size and incubation time. The agitation factor has been assigned with only two levels namely shaking and no shaking, whereas, three levels were selected for the remaining seven factors.

Based on the number of factors and levels, L18 ($2^1X\ 3^7$) orthogonal array was used for the design. The L18 orthogonal array (OA) has 18 trials. Each column consists of multiple conditions depending on the levels assigned to each factor. In the

present study, all 8 columns were assigned with different factors. The agitation factor had nine level 1 and nine level 2 conditions. Other seven factors had six level 1, six level 2, and six level 3 conditions. Table 2.6 shows the layout of the L18 ($2^1X\ 3^7$) orthogonal array used in the present study.

Table 2.6 Design of experiment by L18 orthogonal array for diesel degradation

	Control factors assigned to columns							
Expt. No.	**Agitation**	**Diesel conc. (%) (w/v)**	**NH_4NO_3 conc. (%)**	**KH_2PO_4+ K_2HPO_4 conc. (%)**	**pH**	**Temp. (ºC)**	**Inoculum size (CFU/ml)**	**Incubation time (d)**
1	Shaking	1	0.1	0.01	6	28	10^6	5
2	Shaking	1	0.25	0.025	7	32	10^7	7
3	Shaking	1	0.5	0.05	8	37	10^8	9
4	Shaking	2.5	0.1	0.01	7	32	10^8	9
5	Shaking	2.5	0.25	0.025	8	37	10^6	5
6	Shaking	2.5	0.5	0.05	6	28	10^7	7
7	Shaking	5	0.1	0.025	6	37	10^7	9
8	Shaking	5	0.25	0.05	7	28	10^8	5
9	Shaking	5	0.5	0.01	8	32	10^6	7
10	No shaking	1	0.1	0.05	8	32	10^7	5
11	No shaking	1	0.25	0.01	6	37	10^8	7
12	No shaking	1	0.5	0.025	7	28	10^6	9
13	No shaking	2.5	0.1	0.025	8	28	10^8	7
14	No shaking	2.5	0.25	0.05	6	32	10^6	9
15	No shaking	2.5	0.5	0.01	7	37	10^7	5
16	No shaking	5	0.1	0.05	7	37	10^6	7
17	No shaking	5	0.25	0.01	8	28	10^7	9
18	No shaking	5	0.5	0.025	6	32	10^8	5

The 18 experiments were performed using *A. baumannii* as inoculum in 250 ml Erlenmeyer flasks containing 50 ml basal medium with 0.02% $MgSO_4$, 0.005% $FeCl_3$ and 0.002% $CaCl_2.2H_2O$. Other ingredients were added as mentioned in rows

of Table 2.6 for respective experiment. Late log phase culture (OD600~1), adapted to diesel (1%) was inoculated so as to achieve initial cell density of 10^6, 10^7 and 10^8 CFU/ml and the flasks were incubated at conditions mentioned in the Table 2.6. Uninoculated controls (18, corresponding to 18 experiments) were maintained under similar conditions. After indicated incubation time, the residual diesel from each flask was extracted thrice with equal volumes of DCM, passed through anhydrous sodium sulfate and evaporated under vacuum using a rotary evaporator. The residual extract was dissolved in 1 ml of DCM and analyzed by GC. All the experiments were performed in triplicates. Average results obtained were expressed in terms of % diesel degraded in comparison to controls.

2.19 Degradation of different concentrations of diesel by *A. baumannii* in soil microcosms

Agricultural soil (180 g) was sterilized by autoclaving thrice at 121°C for 15 min. The soil was filled in a series of pots, diesel at 0, 0.1%, 0.5% 1%, 2%, and 10% (w/w) concentrations was added separately. *A. baumannii* ($1x10^7$ CFU/gm) was added to each pot and mixed thouroughly. 20 ml of water was added to the pots every 24 h. After one week, the soil (10 g) from each pot was analyzed for residual *n*-alkanes and TPH as described in section 2.11 and 2.12.

2.20 Bioaugmentation of diesel contaminated soil microcosms with *A. baumanii*: Effect on soil microflora, diesel degradation and growth of *Arachis hypogaea*

Diesel [0, 0.1%, 1%, 2%, and 5% (w/w)] was added in soil (1 kg) and filled in pots to 10 cm depth. In one set of pots, $1x10^7$ CFU/g of *A. baumannii* was added and the other set was kept as un-inoculated control. Water (100 ml) was added to the pots every 24 h. At 0, 7, 14, 21, 28 d time points, soil samples were removed and analyzed for total heterotrophic count and % TPH degradation (Section 2.12).

A set of pots with similar treatment i.e. with and without *A. baumanii* was used to check the growth of *A. hypogaea* (groundnut). Seeds of *A. hypogaea* were obtained as a single batch from a local market in Pune, Maharashtra, India. The viability of the seeds was determined by floatation test using water. The seeds which remained at the bottom of the water were considered as potentially viable and those that floated on water were discarded. After 14 d of starting the experiment, ten seeds

of *A. hypogaea* were planted per pot at a depth of 2 cm and kept under partial shade. At the end of 28 d, % seed germination, total plant height, shoot and root length and biomass were determined. The % seed germination was calculated using the formula (Njoku *et al.*, 2009a).

% seed germination = (Number of seedlings that emerged from the soil / Number of seeds sown) × 100

Seeds which failed to sprout were considered as non germinatable (Achuba, 2006). Plant samples were collected from each pot by carefully uprooting the plants. The weights of the plants were measured. Shoot length was determined by measuring the plant from the base of each plant (above soil level) to the tip (Njoku *et al.*, 2009a) and root length was measured from the part of the stem buried in the soil to the longest rootlet (Ogbo *et al.*, 2010). The experiment was performed in triplicate.

2.21 Role of *A. baumannii* in restoration of soil bacterial flora

Agricultural soil (180 g) was sterilized by autoclaving thrice at 121°C for 15 min. The soil was spiked with 1% (w/w) diesel and filled in three (A, B and C) sets of pots. 1×10^7 CFU/g of *B. subtilis* (B2) was inoculated in all three sets and mixed thouroughly. Set A was inoculated with *A. baumannii* ($1x10^7$ CFU/gm) on 0 d and in Set B on 7 d. Set C was the uninoculated control. At 0, 7 and 14 d time points soil samples (1 g) were analyzed for count of B2.

Chapter 3
Results and Discussion

CHAPTER 3

Isolation and screening of efficient pollutant degraders is the first step in the development of a bioremediation process. Screening may be defined as the use of highly selective procedures to allow the isolation of only those microorganisms of interest from among a large microbial population. Thus, to be effective, screening must in one or few steps allow the discarding of many valueless microorganisms, while at the same time allowing the easy selection of the small percentage of useful microorganisms. Polluted soil is the obvious choice to look for such cultures. Therefore, in order to isolate PHC degraders, soil samples were collected from petroleum contaminated sites.

3.1 Physicochemical properties of PHC contaminated soil samples and normal soil

Total seven PHC contaminated soil samples were collected from service stations and motor garages located in different cities in Maharashtra, Andhra Pradesh and West Bengal, India. To check the effect of PHC pollution on different properties of soil, physico-chemical analysis of the collected soil samples and one uncontaminated agricultural soil was carried out. The results are given in Table 2.3.

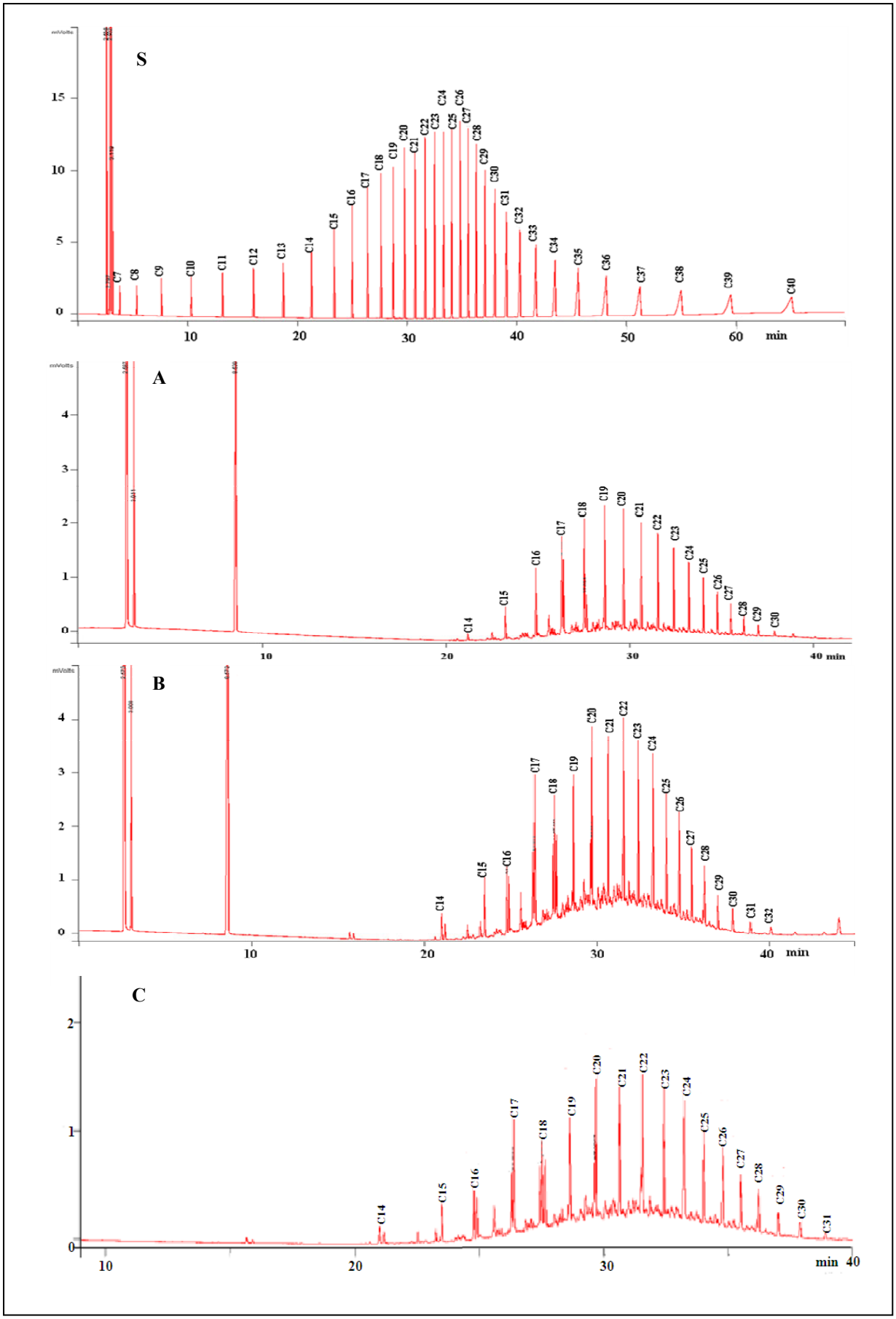
PHC pollution exerts adverse effects on soil conditions, microorganisms and plants (Uche *et al.*, 2011), leads to deterioration of soil structure, loss of organic matter contents, loss of soil mineral nutrients such as potassium, sodium, calcium, magnesium, nitrogen and sulphate, phosphate and nitrate (Akubugwo *et al.*, 2009). There was no significant change in the pH of the soils, however the electric conductivity was higher (0.4 to 1.11 µs/cm) in contaminated soil samples, which may be due to presence of metal or other ions (Pathak *et al.*, 2011). As expected due to hydrocarbons from the petroleum, the organic carbon content in all the contaminated soil samples was significantly higher than normal soil and ranged from 2.25 to 7.33%. The nitrogen and available phosphorous content of the soil samples was less than that of normal soil. Lower concentration of nitrogen, phosphorous and other mineral nutrients have been reported as limiting factors for the growth of microorganisms in PHC polluted environments (Rahman *et al.*, 2002).

The porosity and water holding capacity which determines the extent of water retention and aeration in the soil was also less in PHC contaminated soils as that of normal soil. These two properties are important for the growth of biotic components in the soil. Presence of PHC in the soil increases the soil hydrophobicity, reducing the water holding capacity of the soil (Osuji and Nwoye, 2007). Accordingly, the moisture content of the contaminated soils was observed to be far less (1.6-2.25%) than normal soil (9.85%). Bundy *et al.* (2002) have also reported that nutrient balance (C and N), pH and moisture content of soil were usually affected as a result of contamination by hydrocarbons. The altered physico-chemical properties of PHC contaminated soil makes it unfit for the growth of agricultural crops as well as the normal soil flora. Pathak *et al*. (2011) carried out the physico-chemical analysis of two PHC contaminated soils and reported high electric conductivity of 177.3 and 188 µs/cm for each soil sample. The authors also observed high carbon content of 4.96% and 4.33% for each soil sample as compared to 0.56 and 0.65% for uncontaminated soils. A decrease (25 to 90%) in the total N and P content were also seen in the PHC polluted soils.

3.2 *n*-alkanes and TPH levels in PHC contaminated soil samples

The TPH contents in the contaminated soils were found to be in the range of 1247±114 to 9666±543 mg/kg of soil (Table 3.1). TPH levels in the soil samples were higher than the global average permissible limit of TPH for soil (1000 mg/kg; Lotfinasabasl *et al.*, 2013), indicating high PHC pollution. Pathak *et al*. (2011) observed high concentrations of 11149 mg/kg and 14244 mg/kg TPH in soils contaminated with PHC and engine oil respectively as compared to uncontaminated soils (614 and 700 mg/kg). They suggested the probability of reduced microbial population in these polluted soil samples. Uche *et al*. (2011) also reported high TPH concentration (>200 mg/kg) in surface and sub surface soil samples collected from crude oil polluted sites which far exceeded the 50 mg/kg compliance baseline limit set for petroleum industries in Nigeria.

The *n*-alkanes present in the polluted soils were analyzed by GC and found to consist of C_{11}-C_{32} *n*-alkanes (Fig. 3.1). Crude oil usually consists of *n*-alkanes with n ranging from 8 to 32. It was observed that normal soil constituted negligible amount of TPH as compared to the PHC contaminated soil samples.


S
A
B
C
min

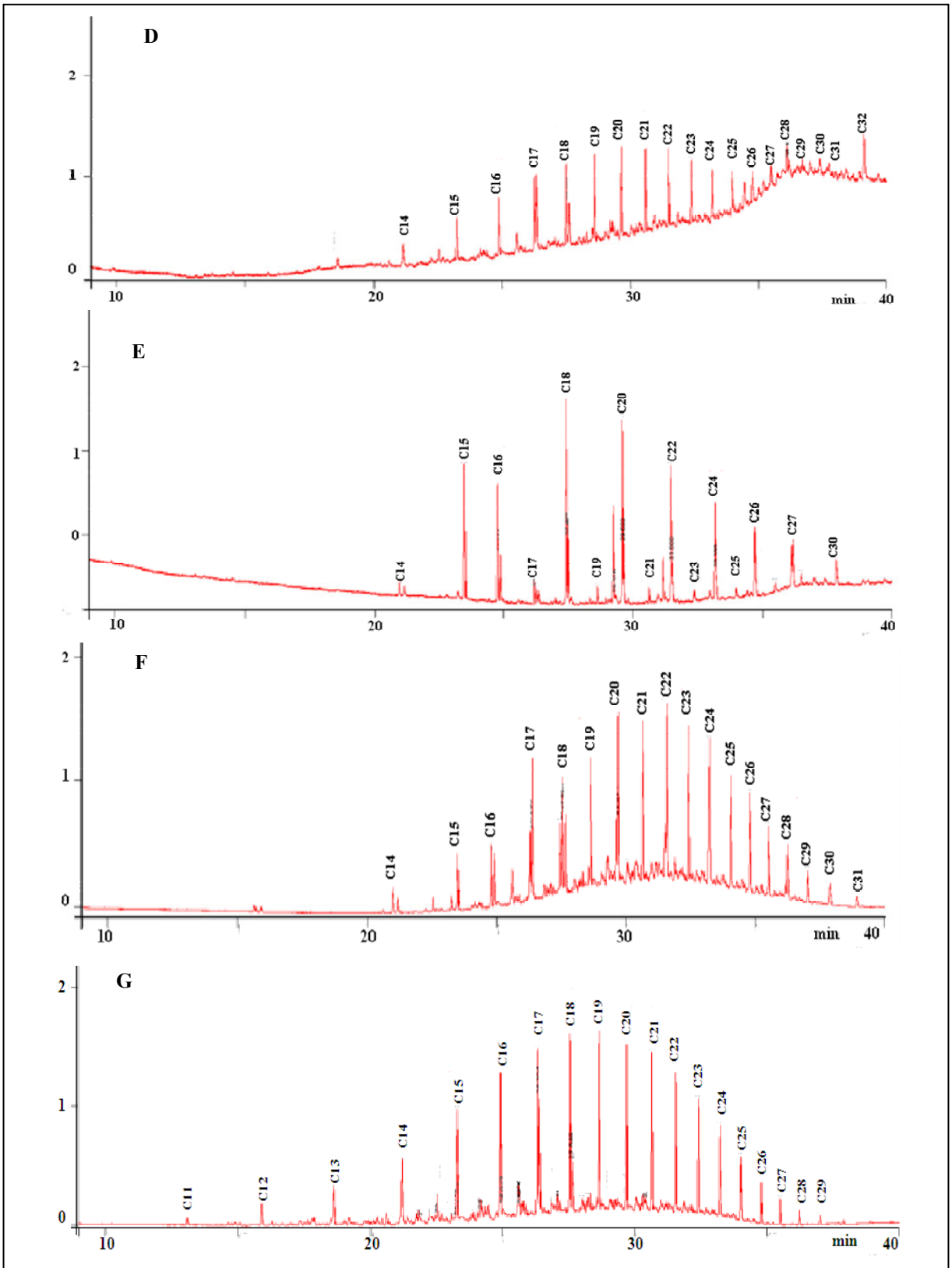


Fig. 3.1 The *n*-alkanes present in the PHC contaminated soil samples analyzed by Gas Chromatography. S) C_7-C_{40} alkane standard (1mg/ml) A) Pune Site 1 B) Pune Site 2 C) Pune Site 3 (Pune, Maharashtra) D) Zahirabad Site 1 E) Jaigaon Site 1 F) Mangalabadi Site 1 G) Mumbai Site 1.

The % *n*-alkanes of TPH for each soil sample is given in Table 3.1. Soil sample A, B and E contained C_{14}-C_{30}; soil D contained C_{14}-C_{32} while soil sample G contained C_{11}-C_{29} range *n*-alkanes respectively. C_{14}-C_{31} range *n*-alkanes were present in C and F soil samples. The *n*-alkanes were identified by comparison with the retention times of standard alkane mixture, C_7-C_{40} (Sigma-Aldrich, USA).

Kovats' retention index for all the alkane components was calculated using the formula –

$$KI = 100z + 100\left(\frac{\log t_s - \log t_z}{\log t_{z+1} - \log t_z}\right)$$

where: ts, tz and tz+1 - net to retention time of the compound of interest, and standards with z and (z+1) carbon numbers of *n*-alkanes.

Table 3.1 TPH and *n*-alkanes content of PHC contaminated soil samples and normal soil

Soil sample	TPH (mg/kg)	*n*-Alkanes (mg/kg)
A	2630±150	1277±55.5
B	9666±543	2608±342
C	4539±225	1263±105
D	1605±122	176±18.2
E	1247±114	149±9.7
F	4958±278	1251±63.1
G	4675±174	1247±54.4
Normal soil	72.4±29	-

The proportion of total *n*-alkanes was maximum in soil sample A (48.5% of TPH), whereas it was ~25-28% for samples B, C, F and G. *n*-alkanes were not found in normal soil and very less (~10-12% of TPH) in samples D and E. In all the samples, C_{17}-C_{26} range *n*-alkanes were more in quantity than other *n*-alkanes. For instance, quantities of individual *n*-alkanes C_{17}-C_{21} were in the range of 8-11% of TPH for all the samples, e.g. C_{18} and C_{20} in sample A were 10.4±0.9% and 10.88±1.1% of TPH, respectively.

3.3 Enrichment and isolation of diesel degrading microorganisms

The aerobic heterotrophic microorganisms were isolated using serial dilution of the soil samples and spread plating on NA. The total heterotrophic microbial count of PHC contaminated soil samples is given in Table 3.2. The count ranged from 2.3×10^7 to 2.71×10^9 CFU/g.

Table 3.2 Heterotrophic count and isolates obtained from PHC contaminated soil samples

Soil sample	Total heterotrophic count (CFU/g)	Isolates	Code
A	2.71×10^9	23	I1-I23
		03	Y1-Y3
B	1.2×10^9	04	I24-I27
C	1.33×10^9	08	I28-I35
D	5.15×10^8	06	I36-I41
E	2.9×10^8	07	I42-I48
F	2.2×10^8	09	I49-I57
G	2.3×10^7	13	I58-I70
Uncontaminated	9×10^9	-	-

Enrichment of diesel degrading microorganisms was done in BH medium containing 1% (w/v) diesel as the sole carbon source. After adding the soil, flasks were incubated at 28ºC at 180 rpm for 7 d. After incubation, spread plating on BH agar with 1% diesel led to isolation of 70 bacterial and 3 yeast isolates. The bacterial and yeast isolates were designated as I1-I70 and Y1-Y3, respectively. Maximum diesel utilizing isolates (23 bacteria and 3 yeasts) were obtained from Pune site 1. Only 4 isolates were obtained from Pune site 2, which may be due to high amount of TPH (9666±543 mg/kg) present in that soil.

3.4 Identification and phylogenetic analysis of the isolates

Colony characteristics, gram nature, motility of the bacterial isolates are given in Appendix I. Identification of the bacterial isolates was done by carrying out different biochemical tests (results are given in Appendix II) according to Bergey's Manual of Systematic Bacteriology, 2nd edition (Krieg and Holt, 1984) and 16S rRNA

sequencing (Table 3.3). Partial sequences of all the bacterial isolates have been deposited in the GenBank database under accession numbers KF318791 to KF318860. The bacterial isolates belonged to various genera such as *Acinetobacter*, *Achromobacter*, *Bacillus*, *Brevibacillus*, *Cellulomonas*, *Enterobacter*, *Exiguobacterium*, *Janibacter, Kocuria*, *Lysinibacillus*, *Microbacterium, Micrococcus, Novosphingobium*, *Ochrobactrum*, *Pseudomonas, Skermanella* and *Staphylococcus.* Based on the morphology and biochemical characteristics, the three yeast isolates were identified as belonging to the genera *Pichia*, *Rodotorula* and *Yarrowia* (Table 3.4 and Table 3.5).

All the above genera have been reported as hydrocarbon degraders in previous PHC biodegradation studies. Ahamed *et al*. (2010) isolated PHC degrading microbes from soil and water samples of ship-breaking yards at Vatiary and Kumira coast in Chittagong. The authors after enriching the samples in medium containing kerosene, diesel and engine oil as carbon source obtained isolates - *Listeria monocytogenes*, *Staphylococcus aureus, Pseudomonas alcaligenes, Listeria grayi, Bacillus pasteurii, Bacillus badius, Bacillus cirroflagellosus, Bacillus circulans, Bacillus brevis* and *Citrobacter freundii*. Ahmed *et al*. (2010) enriched samples collected from crude oil contaminated beach and isolated strains capable of growing on naphthalene, phenanthrene and fluoranthene. The authors identified the isolates as *Kocuria flava* which grew on naphthalene and phenanthrene while the second as *Kocuria rosea* which grew on all the three PAH. Chaudhary and Borah (2011) isolated two PHC degrading bacteria *Bacillus spp.* and *Corynebacterium spp*. from tannery effluent. Sohn *et al*. (2004) isolated *Novosphingobium pentaromativorans* sp. nov., a halophilic bacteria from muddy sediment of Ulsan Bay, Republic of Korea, which degraded high molecular mass polycyclic aromatic hydrocarbons of two to five rings.

Microorganisms that degrade petroleum hydrocarbons are widely distributed in nature and are not restricted to a few genera. The 70 isolates were from 5 different groups: Actinobacteria, α-Proteobacteria, β- Proteobacteria, γ-Proteobacteria and Firmicutes. Phylogenetic analysis of 16S rRNA sequences for bacterial isolates revealed that 67 % isolates belonged to Firmicutes, 10% to Actinobacteria and remaining 23% to α (6%), β (4%) and γ (13%) Proteobacteria (Fig.3.2). Similar pattern was observed for diversity of individual soil samples (Fig.3.3). The dominant species belonged to *Bacillus* followed by *Pseudomonas* and *Staphylococcus* species.

Table 3.3 Identification of the isolates

Isolates	GenBank Accession No.	Identified as	Isolates	GenBank Accession No.	Identified as
Soil A			I17	KF318841	*Microbacterium testaceum*
I1	KF318833	*Bacillus megaterium*	I18	KF318842	*Ochrobactrum anthropi*
12	KF318834	*Exiguobacterium* sp.	I19	KF318843	*Achromobacter xylosoxidans*
I3	KF318835	*Achromobacter xylosoxidans*	I20	KF318852	*Bacillus anthracis*
I4	KF318836	*Bacillus subtilis*	I21	KF318853	*Bacillus pumilis*
I5	KF318837	*Bacillus subtilis* subsp. *subtilis*	I22	KF318854	*Bacillus cereus*
I6	KF318838	*Lysinibacillus sphaericus*	I23	KF318855	*Staphylococcus* sp.
I7	KF318839	*Bacillus amyloliquefaciens*	Y1		*Pichia* sp.
I8	KF318844	*Bacillus subtilis* subsp. *subtilis*	Y2		*Yarrowia* sp.
I9	KF318845	*Bacillus subtilis* subsp. *spizizenii*	Y3		*Rhodotorula* sp.
I10	KF318846	*Bacillus pseudomycoides*	**Soil** B		
I11	KF318847	*Bacillus subtilis*	I24	KF318819	*Bacillus amyloliquefaciens*
I12	KF318848	*Bacillus subtilis* subsp. *subtilis*	I25	KF318820	*Bacillus subtilis* subsp. *subtilis*
I13	KF318849	*Bacillus anthracis*	I26	KF318821	*Bacillus subtilis*
I14	KF318850	*Bacillus subtilis* subsp. *subtilis*	I27	KF318822	*Bacillus subtilis*
I15	KF318851	*Bacillus amyloliquefaciens*	**Soil** C		
I16	KF318840	*Bacillus subtilis* subsp. *subtilis*	I28	KF318791	*Bacillus subtilis* subsp. *subtilis*

I29	KF318792	*Kocuria rhizophila*	I47	KF318808	*Bacillus subtilis* subsp. *subtilis*
I30	KF318793	*Enterobacter cloacae*	I48	KF318809	*Enterobacter cloacae*
I31	KF318794	*Cellulomonas flavigena*	**Soil** F		
I32	KF318795	*Pseudomonas mendocina*	I49	KF318815	*Pseudomonas aeruginosa*
I33	KF318796	*Bacillus subtilis* subsp. *subtilis*	I50	KF318816	*Kocuria rhizophila*
I34	KF318856	*Bacillus* sp.	I51	KF318817	*Bacillus subtilis* subsp. *subtilis*
I35	KF318859	*Bacillus subtilis* subsp. *subtilis*	I52	KF318818	*Staphylococcus haemolyticus*
Soil D			I53	KF318810	*Bacillus megaterium*
I36	KF318797	*Bacillus megaterium*	I54	KF318811	*Skermanella* sp.
I37	KF318798	*Bacillus subtilis subsp. subtilis*	I55	KF318812	*Bacillus subtilis*
I38	KF318799	*Microbacterium testaceum*	I56	KF318813	*Bacillus megaterium*
I39	KF318800	*Bacillus subtilis subsp. subtilis*	I57	KF318814	*Staphylococcus haemolyticus*
I40	KF318801	*Microbacterium testaceum*	**Soil** G		
I41	KF318802	*Pseudomonas aeruginosa*	I58	KF318823	*Acinetobacter baumannii*
Soil E			I59	KF318824	*Janibacter limosus*
I42	KF318803	*Pseudomonas fluorescens*	I60	KF318857	*Staphylococcus* sp.
I43	KF318804	*Exiguobacterium antarticum*	I61	KF318825	*Novosphingobium sp.*
I44	KF318805	*Bacillus subtilis* subsp. *subtilis*	I62	KF318826	*Achromobacter xylosoxidans*
I45	KF318806	*Bacillus subtilis*	I63	KF318860	*Bacillus pumilis*
I46	KF318807	*Brevibacillus brevis*	I64	KF318827	*Acinetobacter baumannii*

I65	KF318858	*Bacillus* sp.	I68	KF318830	*Bacillus subtilis*
I66	KF318828	*Bacillus subtilis* subsp. *subtilis*	I69	KF318831	*Novosphingobium sp.*
I67	KF318829	*Bacillus amyloliquefaciens*	I70	KF318832	*Pseudomonas stutzeri*

Table 3.4 Colony characteristics of yeast isolates

	Size	**Shape**	**Colour**	**Margin**	**Consistency**	**Opacity**	**Elevation**	**Appearance**
Y1	8 mm	Circular	Ivory white	Entire	Dry	Opaque	Raised	Dry
Y2	4 mm	Circular	Cream	Entire	Butyrous	Opaque	Raised at centre	Wrinkled
Y3	4 mm	Circular	Orange	Entire	Creamy	Opaque	Raised	Smooth and glossy

Table 3.5 Biochemical characterization of yeast isolates

Isolate	**Fermentation**										**Assimilation**								**Identified as***
	D-Glu		**D-Gal**		**Suc**		**Mal**		**Raff**		**D-Glu**	**D-Gal**	**L-Rha**	**Mal**	**Suc**	**Cello**	**Lact**	**Sali**	
	a	g	a	g	a	g	a	g	a	g									
Y1	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	*Pichia* sp.
Y2																			*Yarrowia* sp.
Y3	+	-	-	-	+	-	-	-	-	-	+	+	-	+	+	+	-	-	*Rhodotorula* sp.

D-Glu, D-glucose; D-Gal, D-galactose; Suc, sucrose; Mal, maltose; Raff, raffinose; L-Rha, rhamnose; Cello, cellobiose; Lact, lactose; Sali, salicin; a, acid production; g, gas production; +, positive; –, negative. *Identification based on Lodder (1970)

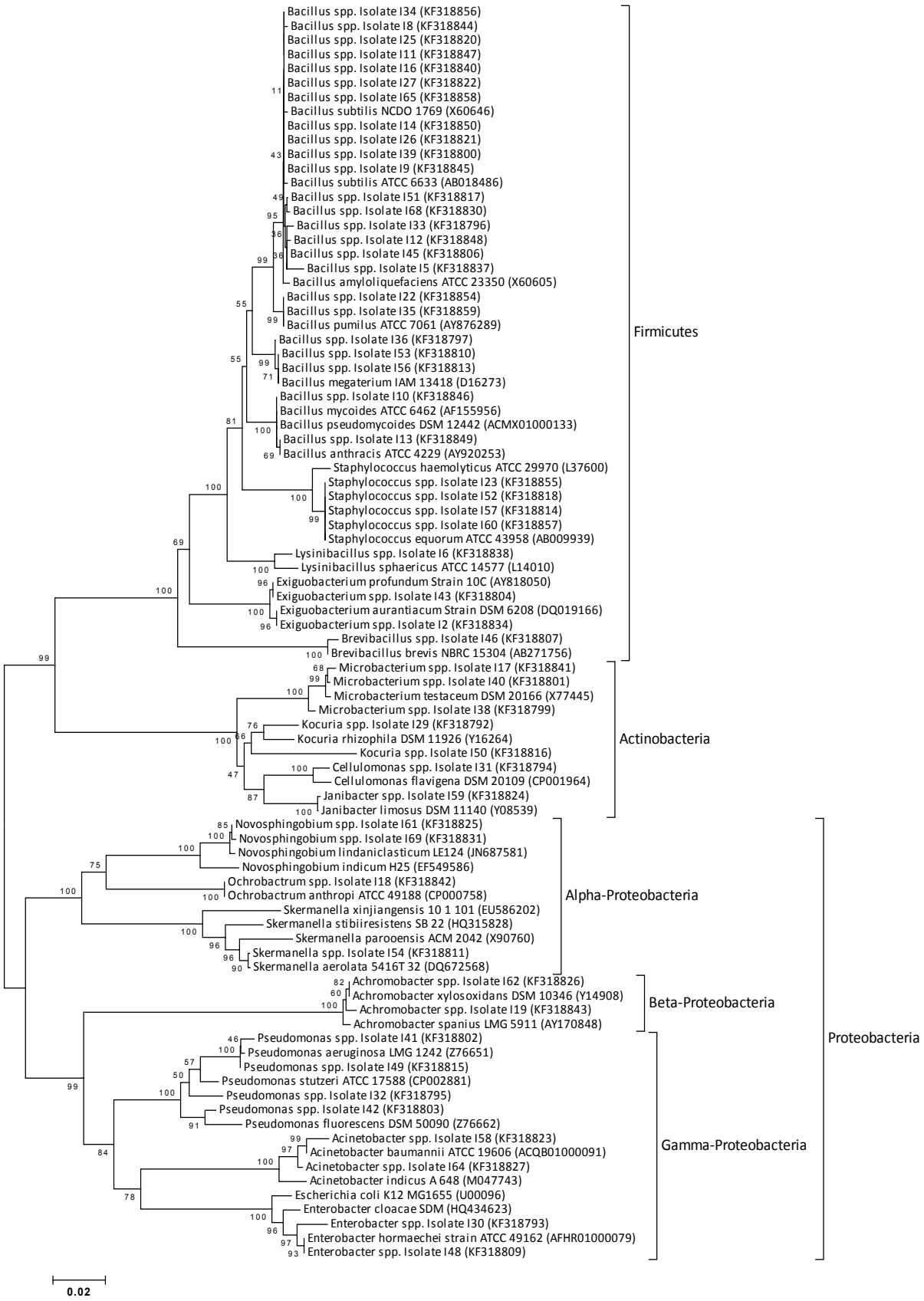


Fig. 3.2 Phylogenetic analysis of diesel degrading bacteria

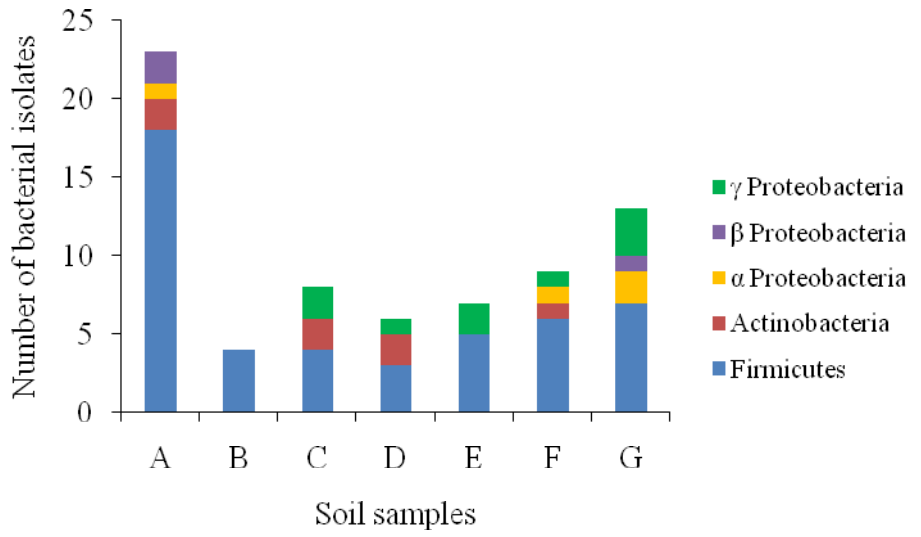


Fig. 3.3 Bacterial diversity of diesel degraders in individual soil samples

3.5 Primary screening for diesel degraders

Absorbance (OD_{600nm}) of the cells grown in a medium with petroleum as a sole carbon source has been used as an index of PHC biodegradation potential (Binazadeh *et al.*, 2009; Borah *et al.*, 2011; Celik *et al.*, 2008; Ciric *et al.*, 2010; Husain *et al.*, 2011; Sathishkumar *et al.*, 2008).

For primary screening, the isolates were grown in BH medium containing 1% (w/v) diesel as sole carbon source. Growth of the isolates was measured after 48 h in terms of OD_{600nm}.

Ciric *et al.* (2010) have differentiated growth of the alkane degraders based on OD_{600nm} using following criteria: No growth, OD_{600nm} 0.00–0.019; +, OD_{600nm} 0.02–0.099; ++, OD_{600nm} 0.1–0.2; +++, OD_{600nm} >0.2. In present study, the OD_{600nm} of the isolates ranged between 0.12±0.02 to 0.46±0.02 (Table 3.6). Forty isolates exhibited good (OD_{600nm} >0.2) growth on diesel. Based on the OD_{600} measurements, nine best isolates (OD_{600nm} >0.35) viz. *Bacillus amyloliquefaciens* (I7), *Bacillus* sp. (I34), *Bacillus subtilis* (I26), *Staphylococcus haemolyticus* (I57), *Microbacterium testaceum* (I38), *Pseudomonas aeruginosa* (I41), *Exiguobacterium antarticum* (I43), *Acinetobacter baumannii* (I58) and *Yarrowia* sp. (Y2) were selected for further studies.

Table 3.6 Preliminary screening of diesel degrading microorganisms - OD_{600}

Isolate	OD_{600}	Isolate	OD_{600}	Isolate	OD_{600}	Isolate	OD_{600}
I1	0.21±0.01	I20	0.15±0.02	I39	0.22±0.04	**I58**	**0.46±0.02**
12	0.34±0.04	I21	0.24±0.03	I40	0.15±0.01	I59	0.32±0.02
I3	0.14±0.05	I22	0.15±0.01	**I41**	**0.42±0.06**	I60	0.12±0.02
I4	0.33±0.04	I23	0.14±0.01	I42	0.23±0.04	I61	0.31±0.04
I5	0.14±0.01	I24	0.25±0.02	**I43**	**0.38±0.10**	I62	0.15±0.04
I6	0.18±0.02	I25	0.26±0.06	I44	0.22±0.04	I63	0.33±0.03
I7	**0.38±0.01**	**I26**	**0.42±0.01**	I45	0.20±0.04	I64	0.16±0.01
I8	0.17±0.02	I27	0.31±0.02	I46	0.21±0.04	I65	0.27±0.05
I9	0.29±0.03	I28	0.19±0.04	I47	0.23±0.04	I66	0.28±0.02
I10	0.15±0.01	I29	0.18±0.02	I48	0.22±0.04	I67	0.12±0.08
I11	0.19±0.02	I30	0.24±0.05	I49	0.15±0.05	I68	0.27±0.05
I12	0.20±0.05	I31	0.16±0.02	I50	0.15±0.03	I69	0.34±0.03
I13	0.30±0.06	I32	0.17±0.01	I51	0.20±0.04	I70	0.16±0.01
I14	0.15±0.01	I33	0.15±0.01	I52	0.22±0.04	Y1	0.21±0.03
I15	0.15±0.01	**I34**	**0.38±0.03**	I53	0.18±0.03	**Y2**	**0.42±0.02**
I16	0.16±0.01	I35	0.14±0.02	I54	0.19±0.04	Y3	0.24±0.02
I17	0.33±0.03	I36	0.20±0.04	I55	0.16±0.03		
I18	0.17±0.02	I37	0.21±0.04	I56	0.14±0.03		
I19	0.15±0.02	**I38**	**0.40±0.02**	**I57**	**0.39±0.03**		

3.6 Alkane monooxygenase assay

Alkane monooxygenase activity of the nine isolates was carried out by NADH depletion assay as described by Glieder *et al.* (2002). The enzyme, alkane 1 monooxygenase (EC 1.14.15.3) initiates the alkane degradation pathway (Watkinson and Morgan, 1990), (Fig. 3.4). The decline in the absorbance at 340 nm due to NADH oxidation allows monitoring of the rate of the first step of *n*-alkane oxidation reaction catalyzed by alkane monooxygenase. NADPH has also been reported as a cofactor for alkane monooxygenases in *Acinetobacter* sp. Strain ADP1 and *Pseudomonas oleovorans* GPo1 (Geißdo¨rfer *et al.*, 1999; Staijen *et al.*, 2000).

The alkane 1 monooxygenase activity for the 9 isolates was in the range 0.5±0.06 to 8.6±1.1 U/mg protein when grown in the presence of 1% hexadecane as

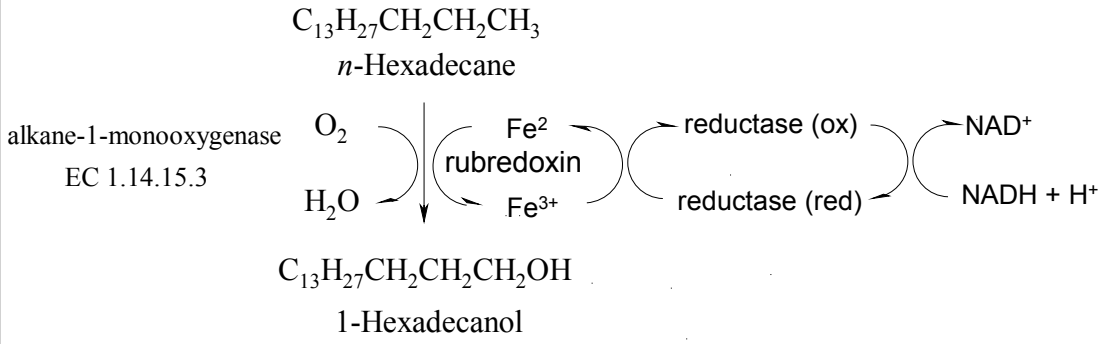


Fig. 3.4 Reaction catalyzed by alkane-1-monooxygenase

carbon source (Table 3.7). *A. baumannii* showed the highest activity of 8.6±1.1 units/mg protein followed by *Yarrowia* sp. (7.2±0.8 U/mg), *M. testaceum* (6.6±0.6 U/mg) and *P. aeruginosa* (5.7±0.8 U/mg). AMO activity was not detected when NADPH was used as a cofactor in the assay. Secondly, no activity was observed when the isolates were grown in medium containing glucose as the sole carbon source indicating that it is an inducible enzyme. Staijen *et al.* (2000) have also reported that alkane hydroxylase/monooxygenase in *Pseudomonas oleovorans* GPo1 is an inducible enzyme system. Similarly, Mishra and Singh (2012) observed that alkane hydroxylase activity was induced during *n*-hexadecane degradation in three bacteria, *P. aeruginosa*, *Rhodococcus* sp. NJ2 and *Ochrobactrum intermedium* P2. *Rhodococcus* sp. *Rhodococcus* sp. NJ2 exhibited maximum alkane hydroxylase induction (185 µmol/mg protein) after 2 days, while *P. aeruginosa* PSA5 and *O. intermedium* P2 showed maximum induction of this enzyme (89.83 µmol/mg protein for *P. aeruginosa* PSA5 and, 186.01 µmol/mg protein for *O. intermedium* P2) after 6 and 8 days, respectively. Li *et al*. (2013) reported NADH dependent linear alkane monoterminal oxygenase in cold tolerant *Pusillimonas* sp. strain T7-7 which utilized diesel (C_5 to C_{30} *n*-alkanes) as a sole carbon and energy source. The authors observed that purified large subunit of the monooxygenase was able to oxidize alkanes ranging from pentane (C_5) to tetracosane (C_{24}) using NADH as a cofactor, with greatest activity on the pentadecane (29 U/mg). AMO activity (2.61-22.91 U/mg) was seen on several alkane derivatives, including nitromethane and methane sulfonic acid. The authors found that the enzyme was specific for NADH. *Yarrowia lipolytica* var. indica. a marine yeast isolated in our laborated showed 8.82±0.12 U/mg of protein AMO activity in presence of NADH and 0.92±0.18 U/mg of protein when NAD(P)H

was the cofactor (unpublished data). Five isolates having high alkane monoxygenase activity were screened for diesel degradation.

Table 3.7 Alkane monooxygenase activity in the selected isolates

Code	Isolate	Carbon source	U/mg
I58	*A. baumannii*	1% H	8.6±1.1
		0.5% H + 0.5% G	5.8±1.4
		1% G	ND
I41	*P. aeruginosa*	1% H	5.7±0.8
		0.5% H + 0.5% G	3.6±0.3
		1% G	ND
I38	*M. testaceum*	1% H	6.6±0.6
		0.5% H + 0.5% G	3.5±0.3
		1% G	ND
I26	*B. subtilis*	1% H	3.6±0.1
		0.5% H + 0.5% G	1.5±0.1
		1% G	ND
I34	*S. haemolyticus*	1% H	2.3±0.1
		0.5% H + 0.5% G	2.4±0.2
		1% G	ND
I43	*E. antarticum*	1% H	5.1±0.1
		0.5% H + 0.5% G	2.8±0.2
		1% G	ND
I57	*S. haemolyticus*	1% H	1.7±0.1
		0.5% H + 0.5% G	0.7±0.1
		1% G	ND
I7	*B. amyloliquefaciens*	1% H	0.5±0.06
		0.5% H + 0.5% G	ND
		1% G	ND
Y2	*Yarrowia* sp.	1% H	7.2±0.8
		0.5% H + 0.5% G	4.3±0.3
		1% G	ND

1% H - 1% Hexadecane; 1% G - 1% Glucose, 0.5% H + 0.5% G - 0.5% Hexadecane + 0.5% Glucose, ND - not detected.

3.7 Screening of selected isolates for diesel range *n*-alkanes degradation

Degradation of diesel range *n*-alkanes by the selected isolates in BH + 1% (w/v) diesel is depicted in Fig. 3.5.

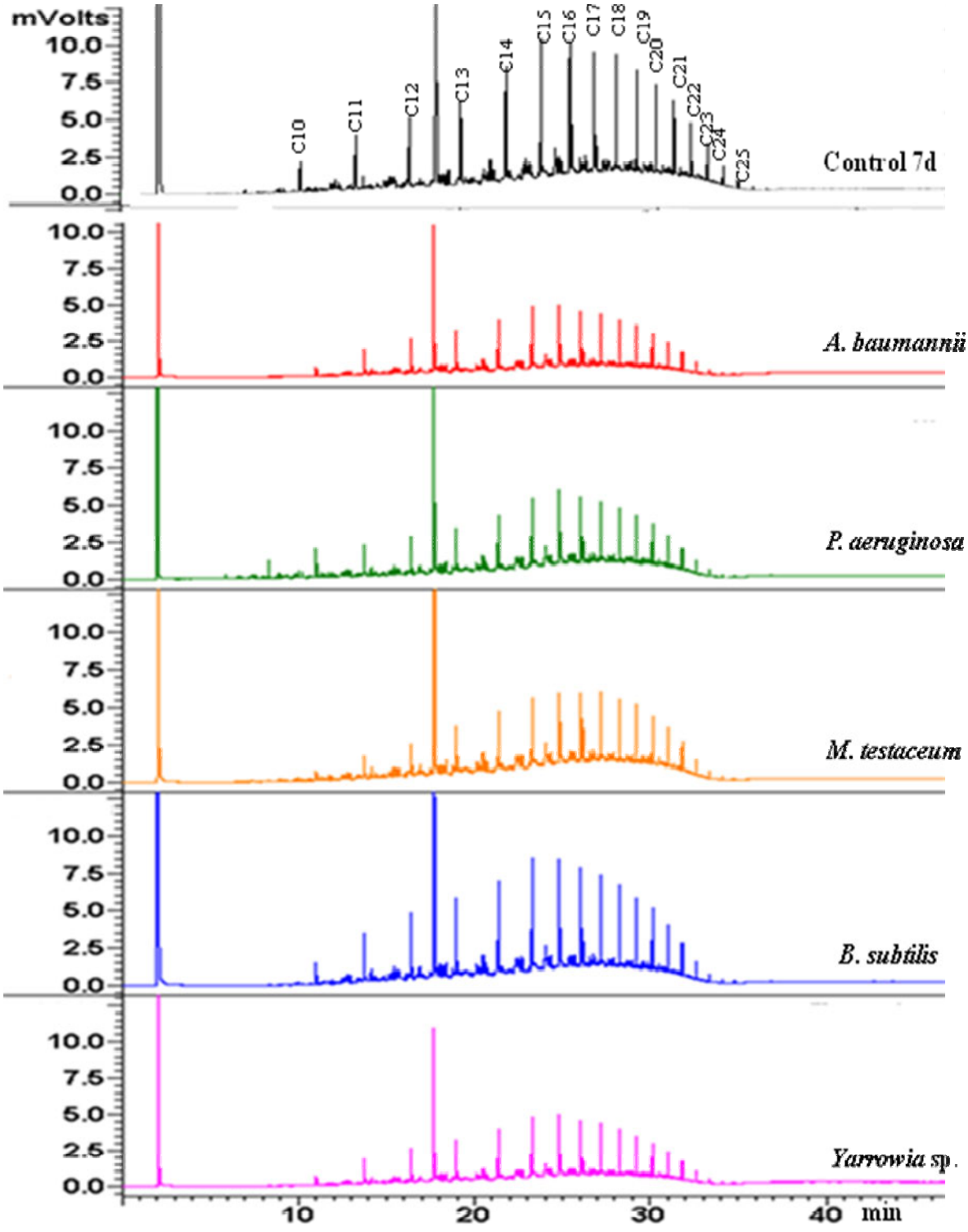


Fig.3.5 GC profile of selected isolates after 7d of incubation in BH containing 1% (w/v) diesel as sole carbon source

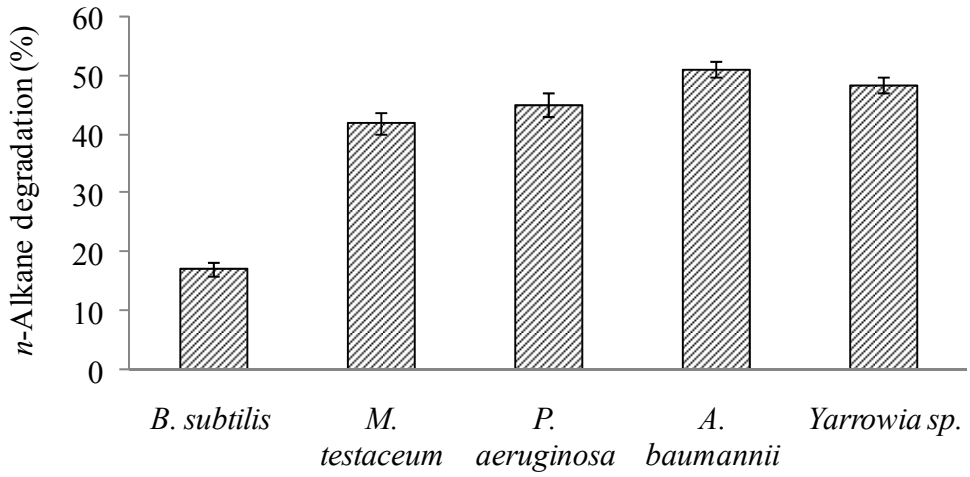


Fig. 3.6 Degradation of diesel range *n*-alkanes by selected isolates

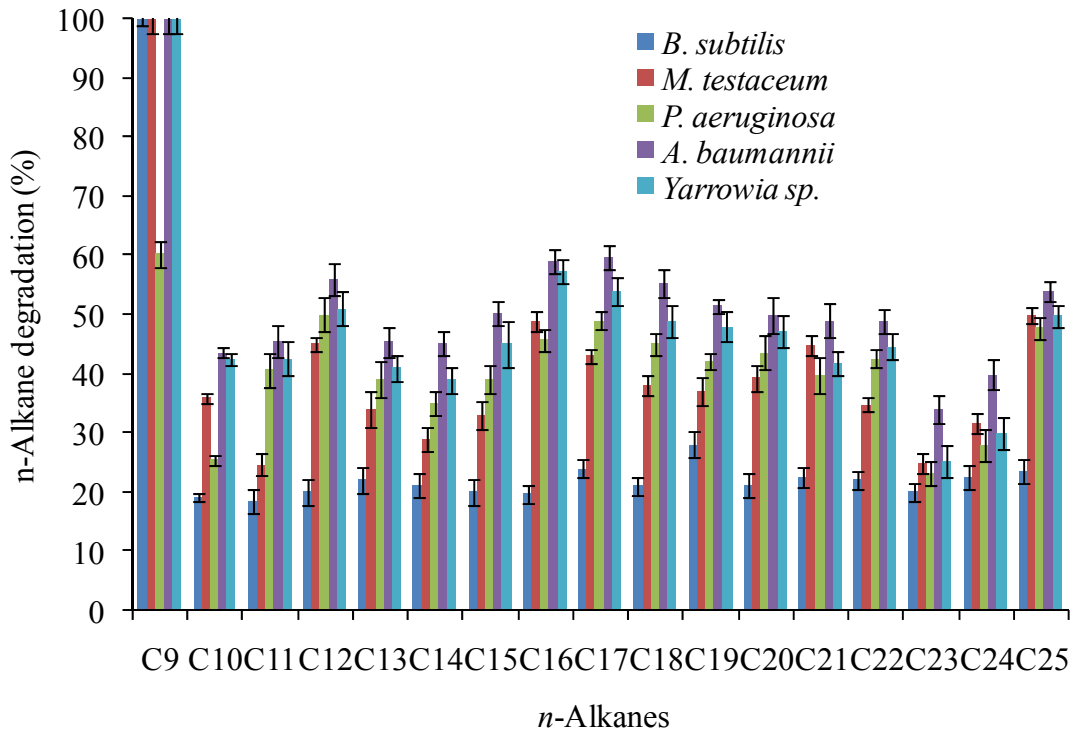


Fig. 3.7 Degradation of diesel range *n*-alkanes (C_9-C_{25}) by selected isolates

GC analysis indicated that these five isolates could utilize diesel range *n*-alkanes, however, the efficiency of utilization varied among them with *A. baumannii* being the best (51.1±1.3% degradation within 7 d) (Fig. 3.6). Except *B. subtilis,* which could degrade only 17.2±1.3%, other cultures showed degradation efficieny of >40%.

Yarrowia sp. degraded 48.5±1.4% *n*-alkanes followed by *P. aeruginosa* (45.1±2.1%) and *M. testaceum* (41.9±1.9%) respectively.

When the individual *n*-alkane degradation profile of the five isolates was compared a similar trend was observed with *A. baumannii* being the best. *B. subtilis* degraded least % of individual *n*-alkanes, while the degradation by *M. testaceum*, *P. aeruginosa*, and *Yarrowia* sp. was comparable. Hexadecane (C_{16}) is one of the major components of diesel (Chenier *et al.*, 2003) and was seen to be degraded in the range of 19.6±0.8 to 59.0±3.5% (Fig. 3.7). The absence of C_8 in control samples indicated its complete removal due to abiotic loss. *P. aeruginosa* was observed to degrade only 60.2±2.1% of C_9 while the remaining isolates showed 100% degradation. In case of *A. baumannii,* among individual *n*-alkanes, toxic short chain *n*-alkanes C_{10} and C_{11} were good substrates with degradation of 43.5±2.6 and 45.5±2.7% respectively. Whereas, long chain *n*-alkanes C_{16} and C_{20} - C_{25} were degraded in varying proportions which were 59.4±3.5, 49.8±2.5, 48.9±1.5, 49±2.1, 34.1±2.8, 39.8±3.1 and 53.9±3.9% respectively. *Yarrowia* sp. and *M. testaceum* were observed to degrade 42.4±1% and 35.8±0.9% of C_{10} respectively after 7d while, *Yarrowia* sp. and *P. aeruginosa* degraded 42.6±2.8% and 40.6±2.8% of C_{11} respectively. Long chain *n*-alkanes C_{20} - C_{25} were degraded in varying propotions (19.9±1.6% to 49.8±1.8%) by *B. subtilis, M. testaceum*, *P. aeruginosa*, and *Yarrowia* sp.

Abdel-Megeed *et al.* (2010) reported hexadecane degradation by *Pseudomonas putida, Rhodococcus erythroplotis and Bacillus thermoleovorans.* Martins *et al.* (2012) studied the ability of *Yarrowia lipolytica* to degrade *n*-alkane fraction of crude oil. Many microorganisms including bacteria, fungi and yeast have been reported to degrade alkanes as sole source of carbon and energy (Jagadevan and Mukherji, 2004; van Beilen *et al.*, 2003). *A. dieselolei* was reported to degrade a broader spectrum of *n*-alkanes with chain length C_5 to C_{36} (Liu and Shao, 2005). *Acinetobacter calcoaceticus* S30 and *Alcaligenes odorans* P20 grew very well on *n*-alkanes up to C33 (Lal and Khanna, 1996). *P. aeruginosa* K1 and *Rhodococcus equi* P1 were able to degrade alkanes of chain lengths from C_7 to C_{28} (Ko and Lebeault, 1999). Most of the oil-degrading *P. aeruginosa* strains preferentially degrade middle to moderately long chain alkanes (Norman *et al.*, 2002). Das and Mukherjee (2006) compared the crude petroleum-oil *n*-alkane (C_{14}-C_{30}) biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa*. They found that *P. aeruginosa* was

more efficient as it degraded 80% of *n*-alkanes after 120 d as compared to 53.4% degradation by *B. subtilis*.

Similiarly Verma *et al.* (2006) tested the ability of *Bacillus* sp. SV9, *Acinetobacter* sp. SV4 and *Pseudomonas* sp., SV17 to degrade *n*-alkanes fraction of oily sludge. They reported that *Bacillus* sp. SV9 was able to degrade 88.9±1.24% of C_{12}-C_{30} *n*-alkanes in 5d as compared to 77.8±2.62% and 65.6±3.28% by *Acinetobacter* sp. SV4 and *Pseudomonas* sp., SV17 respectively. Wongsa *et al.* (2004) reported that *P. aeruginosa s*train WatG had ability to degrade a broad range of hydrocarbons consisting of middle to moderately long carbon chains *n*-alkanes (up to C_{24}). Hasanuzzaman *et al.* (2007) examined strain WatG for its ability to degrade very long chain *n*-alkanes up to C_{40} in a minimal liquid medium.

3.8 Detection of alkane monooxygenase gene from selected isolates

Smits *et al*. (1999) were one of the earliest to report the use of PCR primers to detect alkane degrading bacteria. The authors developed highly degenerate oligonucleotides for PCR amplification of genes related to *Pseudomonas oleovorans* GPo1 and *Acinetobacter* sp. ADP1 alkane hydroxylases, based on a number of highly conserved sequence motifs. They observed PCR products of the expected size in isolates growing on medium (C_6-C_{11}) or long chain *n*-alkanes (C_{12}-C_{16}). Strains that were unable to grow on *n*-alkanes did not yield PCR products with homology to alkane hydroxylase genes.

Kohno *et al*. (2002) designed three combinations of PCR primer sets and gene probes based on homologous regions within a variety of alkane hydroxylase genes registered in GenBank for extensive detection of alkane-degrading bacteria. They classified alkane hydroxylase genes into 3 groups, Group I encoding alkB which catalyzed medium chain length (C_6-C_{12}) *n*-alkanes via a terminal oxidation pathway with monooxygenase systems. The genes classified into Group II encoded alkM and catalyzed long chain alkanes (>C_{12}) via terminal oxidation with hydroxylase (monooxygenase) systems or with dioxygenase systems. The genes classified into Group III encoded alkB or alkB1 and were unknown for substrate specificity, *n*-alkane oxidation pathways, and oxidation systems.

Using the approach of Kohno *et al*. (2002), AMO gene was detected in all the seventy bacterial isolates using the three primers, ALK-1F/ ALK1R, ALK-2F/

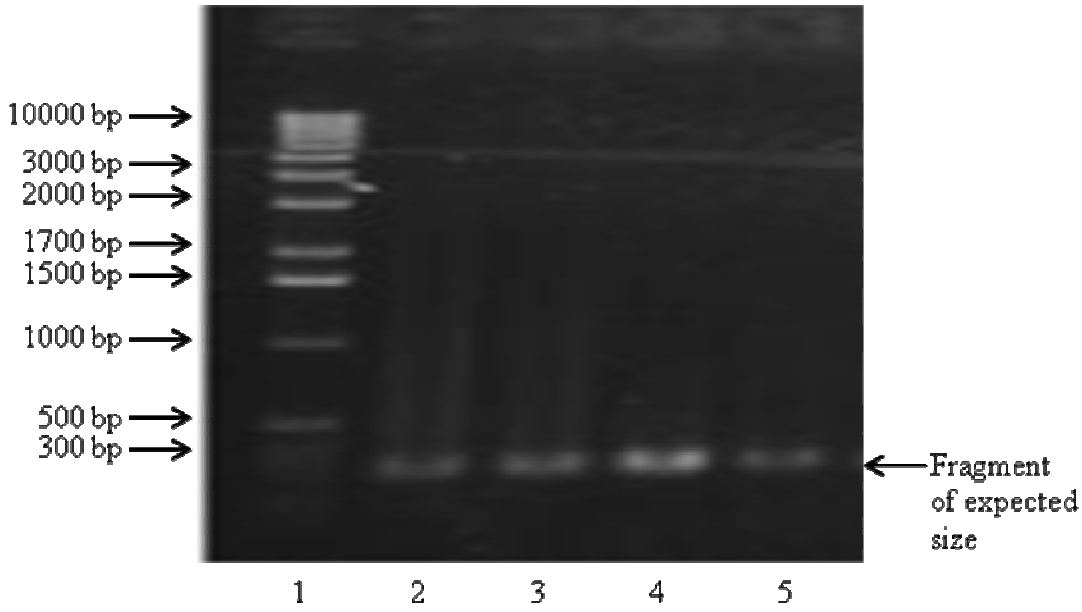


Fig. 3.8 PCR amplification of the alkane monooxygenase gene from selected isolates **Lane 1 –** Gene ruler DNA ladder mix (100bp-10kb), **Lane 2 -** *B. subtilis,* **Lane 3 -** *M. testaceum,* **Lane 4 –** *P. aeruginosa*, **Lane 5 -** *A. baumannii*

ALK2R and ALK-3F/ ALK3R. The fragments of expected size for each primer were amplified for all the seventy isolates (Appendix III).

Fragments of expected size (~271 bp) were amplified from isolates *B. subtilis* (I26), *M. testaceum* (I38), *P. aeruginosa* (I41) and *A. baumannii* (I58) using ALK 2F plus ALK 2R primer set (Fig. 3.8). After presence of alkane monooxygenase gene was confirmed, the selected isolates were subjected to plasmid isolation.

3.9 Plasmid DNA detection in selected isolates

Catabolic pathways, which encoded different hydrocarbon degradation routes, are frequently located on plasmids, although degradative genes can be located on either chromosome or plasmid (Coral and Karagoz, 2005). Mengoni *et al.* (2007) suggested that genes involved in alkane degradation in *A. venetianus* VE-C3 are carried by both the chromosome and the plasmids. While in *Acinetobacter* sp. strain ADP1 and M1 all the genes for alkane monoxygenase complex are located only on the chromosome (Ratajczak *et al.*, 1998 and Tani *et al.*, 2001). Mirdamadian *et al.* (2010) determined the plasmid profiles of five bacterial strains from genera *Pseudomonas*, *Rhodococcus*, *Micrococcus* and *Bacillus* isolated from petroleum-contaminated soils. They

demonstrated by curing experiments that except *Bacillus* sp. biodegradation ability proved to be plasmid related in all the remaining bacterial isolates. The plasmid content of 17 *Acinetobacter* strains was analyzed and it was revealed that 10 out of 17 strains harbored plasmids (Mara *et al.*, 2012).

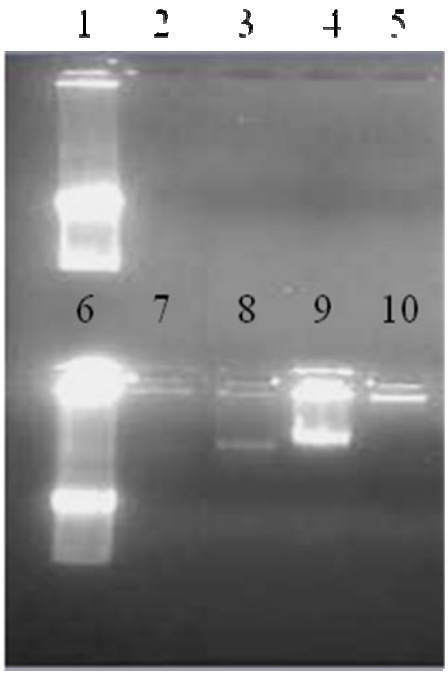


Fig. 3.9 – Detection of plasmid in selected isolates. Low molecular weight method - Lane 1 and 6 - Positive control *E. coli* DH5α. Lane 2 - *B. subtilis* (I 26), Lane 3 - *Microbacterium sp.* (I 38), Lane 4 - *P. aeruginosa* (I 41), Lane 5 - *A. baumannii* (I 58); High molecular weight method - Lane 7 - *B. subtilis* (I 26), Lane 8 - *Microbacterium sp.* (I 38), Lane 9 - *P. aeruginosa* (I 41), Lane 10 - *A. baumannii* (I 58)

Plasmid detection was done in the isolates *B. subtilis* (I26), *Microbacterium sp.* (I38), *P. aeruginosa* (I41) and *A. baumannii* (I58) to check whether their diesel degrading ability was genomic or plasmid borne. Sambrook *et al*. (2001) method based on alkaline lysis indicated that all the isolates were free of low molecular weight plasmid DNA. Whereas, Kado and Liu (1981) method based on alkaline lysis and thermal treatment showed the presence of high molecular weight plasmid DNA in *Microbacterium sp.* (BD38) and *P. aeruginosa* (I41) while *B. subtilis* (I 26) and *A. baumannii* (I58) were free of high molecular weight plasmid DNA (Fig. 3.9).

The observation that the hydrocarbon degrading genes are chromosomal in *B. subtilis* (I26) and *A. baumannii* (I58) is very important. As the degradation is chromosomal based the use of these cultures becomes more dependable in

bioremediation. The results were comparable with that of Decorosi *et al.* (2006), Pollisi *et al.* (1990), Ratajczak *et al.* (1998) and Tani *et al.* (2001). Andretta *et al.* (2004) and Tondo *et al.* (1998) attributed the presence of degradative genes on chromosome to the necessity of bacterial cells to detoxify its surrounding environment in order to survive. They reasoned that the toxic compounds were always present in nature exerting a selective pressure on microorganisms during evolution, justifying the presence of stable biodegrading routes in the bacterial metabolism.

3.10 GC analysis of *n*-hexadecane degradation products

Oxidation of medium chain or long chain *n*-alkanes by aerobic microorganisms occurs *via* terminal (CH_3-CH_2-$(CH_2)_n$-CH_2-CH_3 to CH_3-CH_2-$(CH_2)_n$-CH_2-CH_2OH) or subterminal (CH_3-CH_2-$(CH_2)_n$-CH_2-CH_3 to CH_3-CH_2-$(CH_2)_n$-CH_2-$CHOH$-CH_3) pathways as suggested by Berthe-Corti and Fetzner (2002). The enzyme involved in the first step of aerobic degradation of aliphatic hydrocarbons is alkane-1-monooxygenase (EC 1.14.15.3) which either produces a primary or a secondary alcohol. To investigate the pathway for biodegradation of long chain *n*-alkanes (*n*-hexadecane), the metabolites 1-hexadecanol and 2-hexadecanol were analyzed during the alkane degradation. If the organisms followed the terminal oxidation pathway, 1-hexadecanol was detected and the presence of 2-hexadecanol was seen during a subterminal oxidation pathway. As seen from Fig 3.10, presence of 2-hexadecanol indicated that isolates I26 (*B. subtilis*), I38 (*Microbacterium* sp.), I41 (*P. aeruginosa*), I58 (*A. baumannii*) and Y2 (*Yarrowia* sp.) followed the subterminal oxidation pathway for *n*-alkane degradation.

Ladd (1956) first suggested that subterminal oxidation may be an important oxidative route for microbes based on observation that an alkane-utilizing (C_8–C_{16}) *Corrynebacterium* oxidized undecan-2-one faster than n-decylaldehyde. Forney and Markovetz (1970) reported oxidation of tridecane to tridecan-2-ol and undecan-1-ol by *Pseudomonas aeruginosa*, suggesting presence of a subterminal alkane degradation pathway. Subterminal oxidation has been detected for longer alkanes in *Rhodococcus* (Whyte *et al*., 1998; Binazadeh *et al*., 2009) and *Acinetobacter* sp. (Minerdi *et al*., 2012). Whyte et al. (1998) investigated the alkane catabolic pathway of psychrotroph *Rhodococcus* sp. strain Q15. The authors detected hexadecane and dodecane metabolic intermediates (1-hexadecanol and 2-hexadecanol and 1-dodecanol and 2-dodecanone, respectively) by solid phase microextraction GC-MS

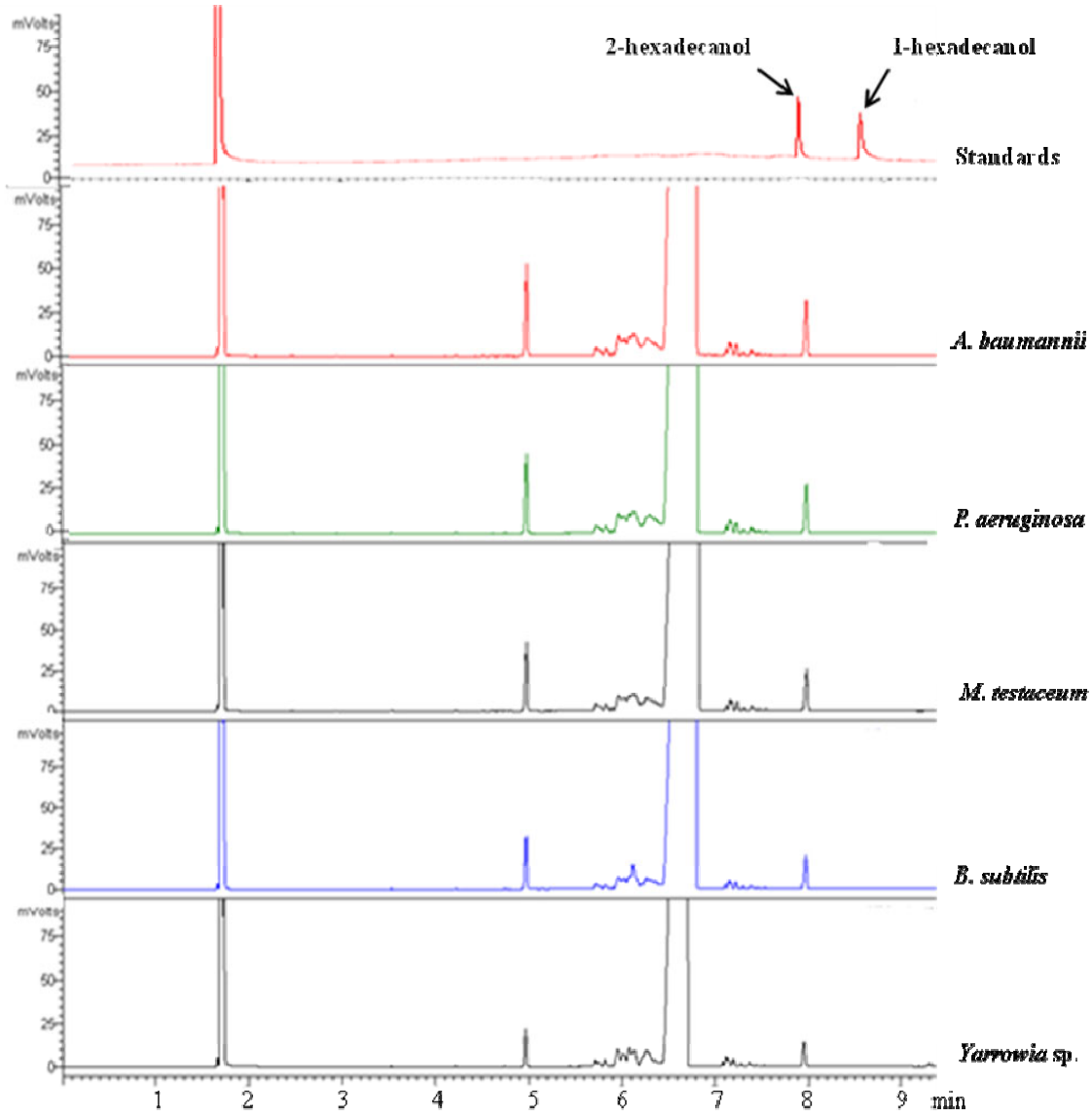


Fig. 3.10 GC chromatograms of the *n*-hexadecane degradation by the isolates showing presence of 2-hexadecanol

and suggested that Q15 oxidized alkanes by both the terminal and subterminal oxidation pathway. Binazadeh *et al*. (2009) studied *n*-dodecane degradation in *Rhodococcus* sp. Moj-3449 and detected presence of 2-dodecanol and 2-dodecanone, indicating presence of a subterminal oxidation pathway in this strain. Minerdi *et al*. (2012) demonstrated expression of genes coding for terminal alkane hydroxylase (*alk*B) and Baeyer-Villiger monooxygenase (*alm*A) involved in the initial and final steps of terminal and subterminal oxidation, respectively by *Acinetobacter radioresistens* strain S13. The two genes were differentially expressed according to the presence of medium (C_{14} and C_{16}) or long chain (C_{24} and C_{36}) *n*-alkanes.

3.11 Production of biosurfactant by the isolates

The Emulsification Index - E24(%) was determined for nine isolates, *B. amyloliquefaciens* (I7), *B. subtilis* (I26), *Bacillus* sp. (I34), *S. haemolyticus* (I57), *M. testaceum* (I38), *P. aeruginosa* (I41), *E. antarticum* (I43), *A. baumannii* (I58) and *Yarrowia* sp. (Y2). After 7d in BH medium with 1% (w/v) diesel, *A. baumannii* and *P. aeruginosa* showed highest E24% of 61.86±4.2 and 60.86±4.8% respectively (Fig. 3.11).

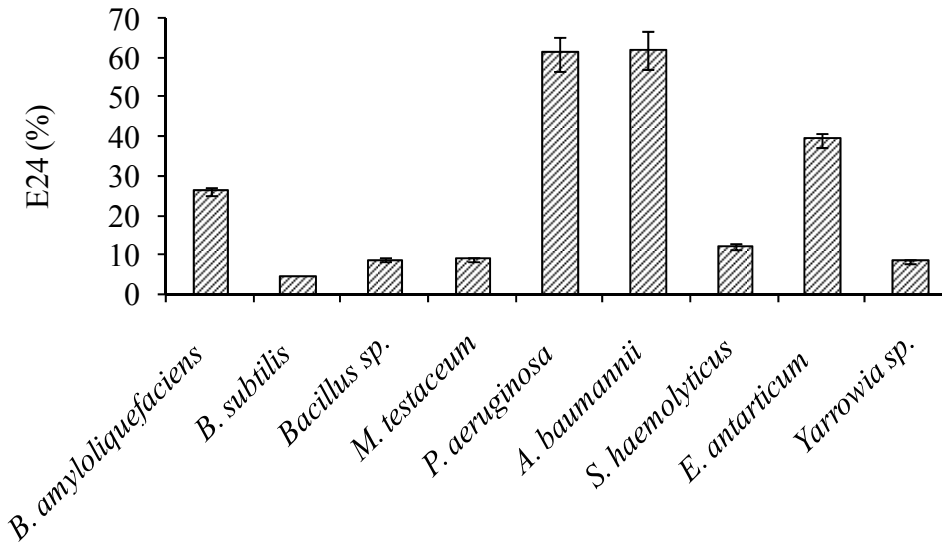


Fig. 3.11 Emulsification Index; E24 (%) of selected isolates in BH + 1% (w/v) diesel

E24 (%) is a measure of the emulsification ability of biosurfactant produced by the organisms. Production of biosurfactants by microorganisms increases the surface area of hydrophobic contaminants like PHC in soil or water and thus their aqueous solubility and concomitant microbial degradation (Karanth *et al.,* 1999). Thus, strong emulsifying activity exhibited by *A. baumannii* and *P. aeruginosa* will be useful in bioremediation of PHC in soils.

Jadhav *et al*. (2013) studied biosurfactant production by *Oceanobacillus* sp. BRI 10 isolated from Antarctic sea water. The authors observed maximum (E24 = 55%) in the medium containing glucose (3%) and ammonium chloride (0.48%) pH 8.0 at 30 °C after 48 h. The isolate was found to emulsify lubricant oil, crude oil, diesel and kerosene in following order: kerosene > lubricant oil > diesel > crude oil. The authors suggested that *Oceanobacillus* sp. BRI could be used to degrade diverse hydrocarbon pollution.

Different screening methods have been reported in literature for investigating biosurfactants and bioemulsifiers. MATH assay is one such screening method (Pruthi and Cameotra, 1997; Satpute *et al.*, 2010). Prabhu and Phale (2003) reported that growth associated extracellular biosurfactant production and modulation of cell surface hydrophobicity plays an important role in hydrocarbon uptake in *Pseudomonas* sp. Strain PP2. In MATH assay, cells are partitioned between aqueous and hydrophobic phase (eg. water and hexadecane) and cell surface hydrophobicity (%) is measured. MATH assay was performed for the selected isolates, however, no correlation between biodegradation of diesel by the isolates and cell surface hydrophobicity (Table. 3.8) was observed.

Table 3.8 MATH assay of selected isolates

Isolate	**Cell surface hydrophobicity (%)**	
	***n*-hexadecane**	**diesel**
B. amyloliquefaciens (I7)	18.9±1.4	26.1±1.1
B. subtilis (I26)	13.8±0.9	4.31±0.8
S. haemolyticus (I34)	14.1±1.2	9.2±1.3
M. testaceum (I38)	10.0±0.6	12.7±0.9
P. aeruginosa (I41)	19.58±1.1	2.61±0.1
E. antarticum (I43)	16.83±1.2	13.2±0.7
S. haemolyticus (I57)	14.46±1.1	11.6±0.6
A. baumannii (I58)	3.92±0.1	2.7±0.3
Yarrowia sp. (Y2)	22.8±1.8	20.0±1.8

Table 3.9 Emulsification activity (OD_{610}) of selected isolates

Isolate	**Emulsification activity (OD_{610})**
B. amyloliquefaciens	0.5±0.02
P. aeruginosa	1.65±0.1
E. antarticum	1.55±0.1
A. baumannii	1.98±0.09

Emulsification activity (OD_{610}) is another screening test. Emulsification activity using benzene as the hydrocarbon was determined for isolates showing high E24 index in BH medium with 1% (w/v) diesel by the method of Johnson *et al*. (1992). Highest emulsification activity was observed for *A. baumannii* followed by *P. aeruginosa*, *E. antarticum* and *B. amyloliquefaciens* (Table 3.9).

Gurjar *et al*. (1995) studied bioemulsifier production by *Bacillus stearothermophilus* VR-8 in in presence of 4% crude oil. The emulsification activity (OD_{610}) using benzene as the hydrocarbon was 1.11±0.04 and between 0.43±0.01 to 0.69±0.03 in presence of other hydrocarbons such as hexane, dodecane, octadecane, naphthalene and crude oil. Similarly, Jadhav *et al*. (2013) also used emulsification activity (OD_{610}) to compare emulsification of different hydrocarbons by biosurfactant isolated from *Oceanobacillus* sp. BRI 10. They observed 0.64±0.03, 0.57±0.01, 0.43±0.02, and 0.60±0.02 emulsification activity in presence of kerosene, diesel, crude oil and lubricant oil, respectively.

3.12 Biodegradation studies with *A. baumannii*

Based on the results of diesel degradation, individual alkane degradation profile, and characterization studies, *A. baumannii* was selected as best isolate and used in further biodegradation and bioaugmentation studies.

3.12.1 Effect of varying concentrations of diesel on its degradation by *A. baumannii*

The diesel range *n*-alkane degradation by *A. baumannii* in the presence of 1-10 % diesel as sole carbon source was investigated. With varying concentrations of diesel, *A. baumannii* could degrade 50.8±1.8% of *n*-alkanes when the concentration of diesel was 1 % within 7 d, while it could degrade 22.5±2.8 and 8.9±0.9% of *n*-alkanes when the concentration was 5 and 10 % respectively (Fig 3.12). This may be due to the toxic effects of diesel on microorganisms at higher concentrations and it has been observed that degradation is generally unfavorable at concentrations higher than 1 or 1.5% (Shukor *et al.*, 2009). It was evident from decrease in the number of bacteria with increase in diesel concentration. Degradation of 6% diesel has been reported but in presence of 0.2% w/v glucose and 0.1% w/v yeast extract (Kwapisz *et al.*, 2008).

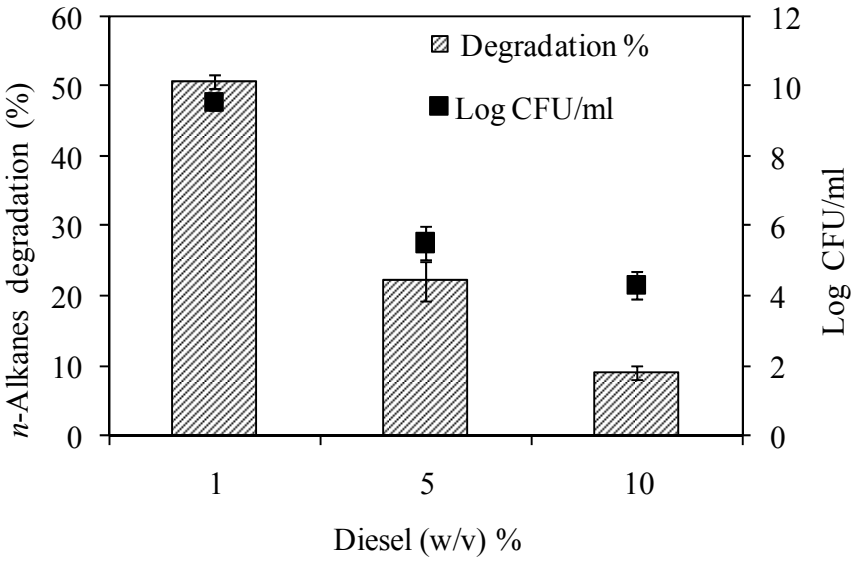


Fig. 3.12 Effect of initial diesel concentration on biodegradation of diesel by *A. baumannii*

3.12.2 Effect of incubation time on diesel degradation by *A. baumannii*

Time course of *n*-alkane degradation pattern by *A. baumannii* in presence of 1% (w/v) diesel showed that degradation increased from 11.7±2.4% at 24 h to 26.8±3.0% on 3rd day and reached 51.1±1.3% after 7d (Fig. 3.13a). *A. baumannii* utilized diesel as sole source of carbon and energy, which was evident from the increase in cell number from 2×10^7 cfu/ml to 8.7 x 10^9 CFU/ml after seven days.

The % degradation for individual *n*-alkanes was also increased with time and in the range of 43.5 - 53.9% on 7th day. The long chain *n*-alkanes, C_{12}, C_{16}, C_{17} and C_{18} were preferred substrates with more than 55% degradation. C_{10} and C_{11} which were reported to be toxic for seed germination (Siddiqui *et al.*, 2001) were degraded ~48 and 47% respectively (Fig. 3.13b). When the flasks were further incubated till 30 days, complete degradation of all diesel range *n*-alkanes except $C_{15,}$ C_{16} and C_{18} was observed (Fig. 3.14).

Utilization of *n*-alkanes in the range of C_{10} to C_{44} has been described in many strains of the genus *Acinetobacter* (Throne-Holost *et al.*, 2006). Sakai *et al.* (1994) have reported an *Acinetobacter* strain capable of degrading very long chain *n*-alkanes (up to C_{44}). However, it could grow on such hydrocarbons only in the presence of a chemical detergent. Lal and Khanna (1996) reported degradation of two types of Indian crude oil (Bombay High and Gujarat) by *Acinetobacter calcoaceticus* and

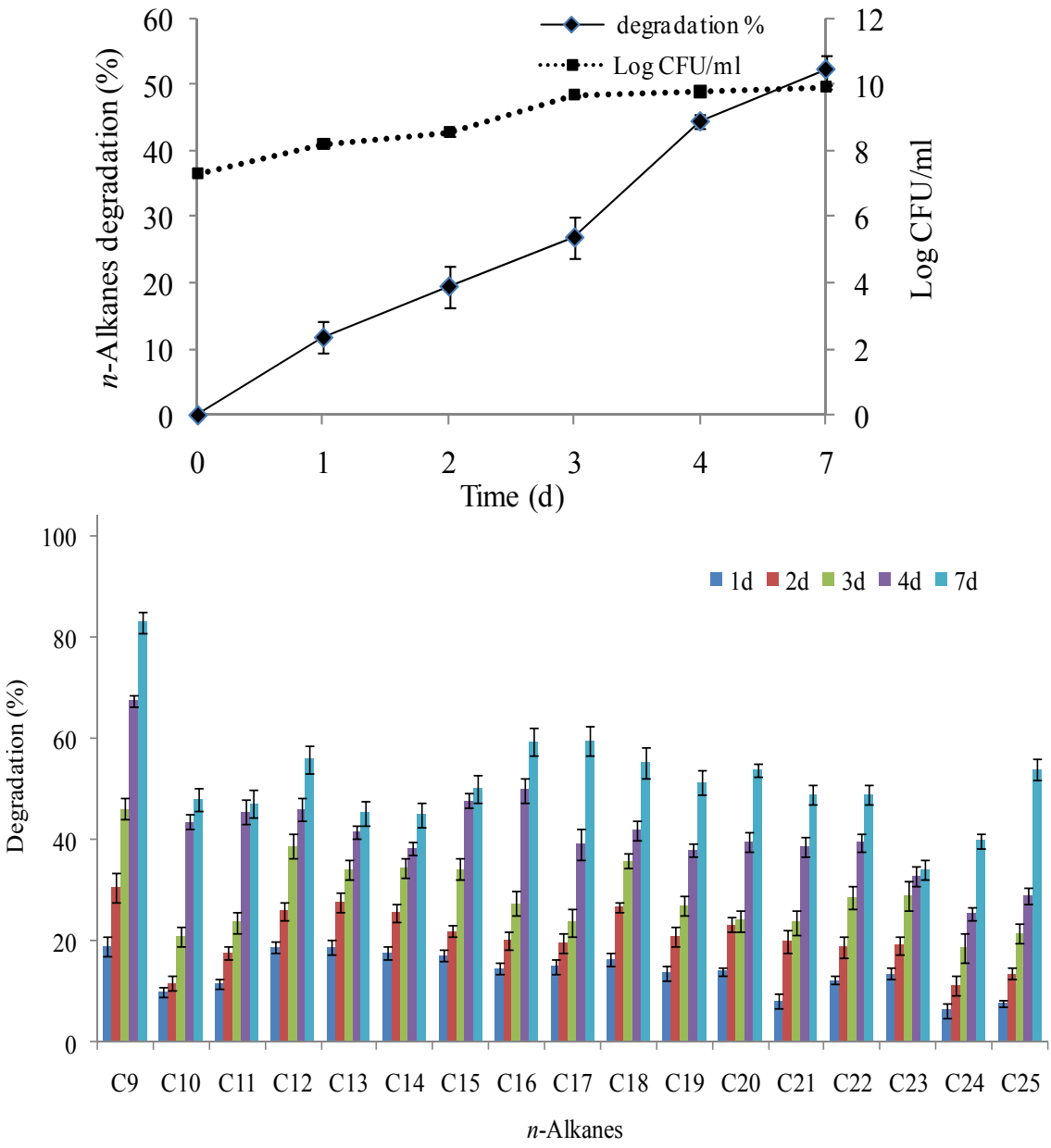


Fig. 3.13 a) Time course of diesel range *n*-alkanes degradation by *A. baumannii* b) degradation of individual diesel range *n*-alkanes (C_{10}-C_{25}) by *A. baumannii*

Alcaligenes odorans. The authors observed 50% and 45% degradation of 1% Bombay High crude oil and only 29% and 37% of 1% Gujarat crude oil (heavy crude oil) after 15 days by *A. calcoaceticus* and *A. odorans* respectively. *A. calcoaceticus* degraded more of the *n*-alkanes fraction (43% and 25%) than the aromatics fraction of both crude oils. GC fingerprinting of *n*-alkane fraction showed major degradation of C_{17}, C_{18}, C_{19}, C_{20}, C_{22}, C_{23} and C_{24} of crude oil. Basuki *et al*. (2011) reported that

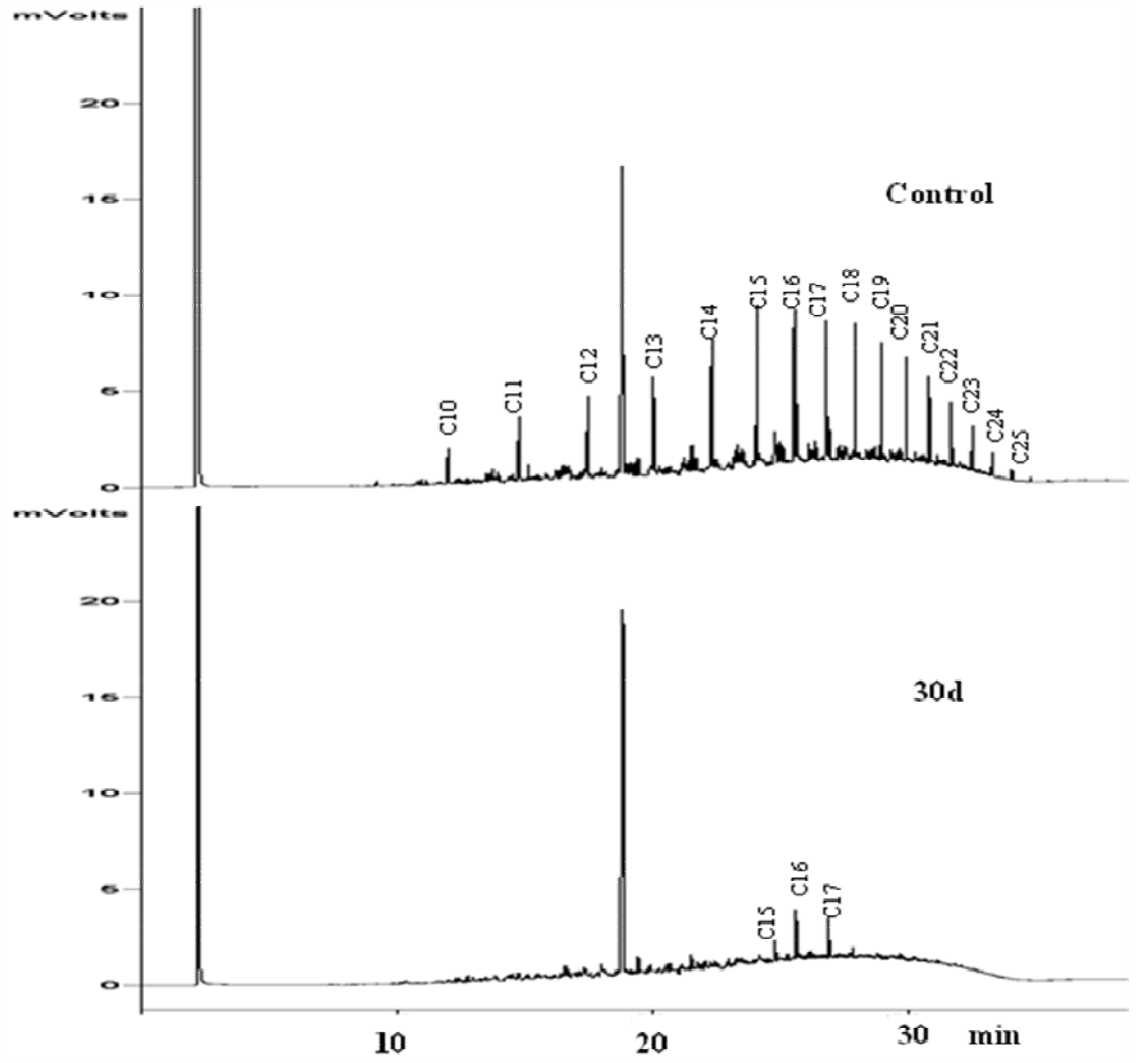


Fig. 3.14 Degradation of diesel range *n*-alkanes by *A. baumannii* after 30d in BH medium

Acinetobacter junii TBC 1.2V could degrade short and long chain hydrocarbon significantly from used engine oil.

3.12.3 Optimization of parameters for diesel range *n*-alkanes degradation by Taguchi Design of Experiments (DOE) approach

Petroleum hydrocarbon degradation by microorganisms was found to be influenced by nutritional and environmental conditions such as agitation, pH, temperature, inoculum size, incubation time, carbon source, nitrogen source, phosphorous source and their concentration (Aghamiri *et al*., 2011). For effective removal of petroleum hydrocarbons it becomes highly essential to optimize all the culture conditions and composition of media. Conventional process optimization procedures involve altering

of "one variable at a time" (OVAT) keeping all other variables or parameters constant and assumes that the process parameters do not interact and that the outcome is a direct function of the single variable (Rao *et al*., 2008). However, the observed behavior of a process results from the interactive influences of the various variables. Therefore, optimization of parameters by the conventional approach becomes erroneous (Tupe *et al*., 2007).

Table 3.10 Diesel range *n*-alkane degradation by *A*. *baumannii* and the SN ratios calculated using 'smaller the better' approach

Expt. No.	% degradation of diesel range *n*-alkanes	% residual diesel range *n*-alkanes (Mean)	Mean sum of squares	SN ratio
1	36.4±1.3	63.54	4037.33	-36.06
2	48.9±1.8	51.03	2604.06	-34.16
3	**58.7±2.0**	41.25	1701.56	-32.31
4	34.2±2.1	65.80	4329.64	-36.36
5	24.9±2.9	75.10	5640.01	-37.51
6	28.3±2.3	71.70	5140.89	-37.11
7	16.5±1.9	83.43	6960.56	-38.43
8	12.9±1.5	87.04	7575.96	-38.79
9	11.5±1.3	88.42	7818.10	-38.93
10	27.1±2.2	72.87	5310.04	-37.25
11	23.1±2.7	76.86	5907.46	-37.71
12	32.6±2.9	67.37	4538.72	-36.57
13	18.1±1.1	81.87	6702.70	-38.26
14	16.1±1.9	83.90	7039.21	-38.48
15	13.4±1.6	86.58	7496.10	-38.75
16	4.5±0.5	95.46	9112.61	-39.60
17	8.7±0.6	91.22	8321.09	-39.20
18	3.3±0.3	96.68	9347.02	-39.71

Statistical optimization methods such as response surface methodology (RSM) and Taguchi approach can take into account the interaction of variables in generating the process response. Huang *et al*. (2008) used RSM for optimization of nutrient components for diesel oil degradation by *Rhodococcus erythropolis*. Similarly, Mohajeri *et al*. (2010) used this statistical design approach for optimizing biodegradation of weathered crude oil in coastal sediments. Taguchi method of orthogonal array (OA) experimental design (DOE) involves the study of any given system by a set of independent variables (factors) over a specific region of interest (levels) (Periasamy and Palvannan, 2010).

As seen in Table 3.10, diesel range *n*-alkane degradation efficiency under the 18 different experiments varied significantly. These 18 experiments represent different combinations of the factors and levels, and can be considered as environmental and nutritional stress variability. Diesel range *n*-alkane degradation was maximal for 3rd experimental trial. The preferred parameter settings were determined through analysis of the "signal-to-noise" (SN) ratio where factor levels that maximize the appropriate SN ratio were optimal.

There are three standard types of SN ratios depending on the desired performance response: "smaller the better", "nominal the best" and "larger the better". These SN ratios are derived from the quadratic loss function and are expressed in a decibel scale. For diesel range n-alkane degradation, the average residual diesel range n-alkane concentrations in the 18 experiments were used as raw data and its square values were termed as mean sum of squares (*Yi2*). The S/N ratios were calculated by using "smaller the better" approach and following formula.

$$SNs = -10\log (1/n\sum Yi^2)$$

Where, Y is the observed value and n is the number of test results. The SN ratios for *A. baumannii* are given in Table 3.10. Once all of the SN ratios have been computed for each run of an experiment, a graphical approach was applied to analyze the data. In the graphical approach, the SN ratios and average response are plotted for each factor against each of its levels (Fig. 3.15). Similarly, the SN ratios were used to plot the graphs for interaction of different factors with each other at different levels. The graphs are then examined to "pick the winner", i.e., pick the factor level, which (1) best maximize SN and (2) bring the mean on economical level.

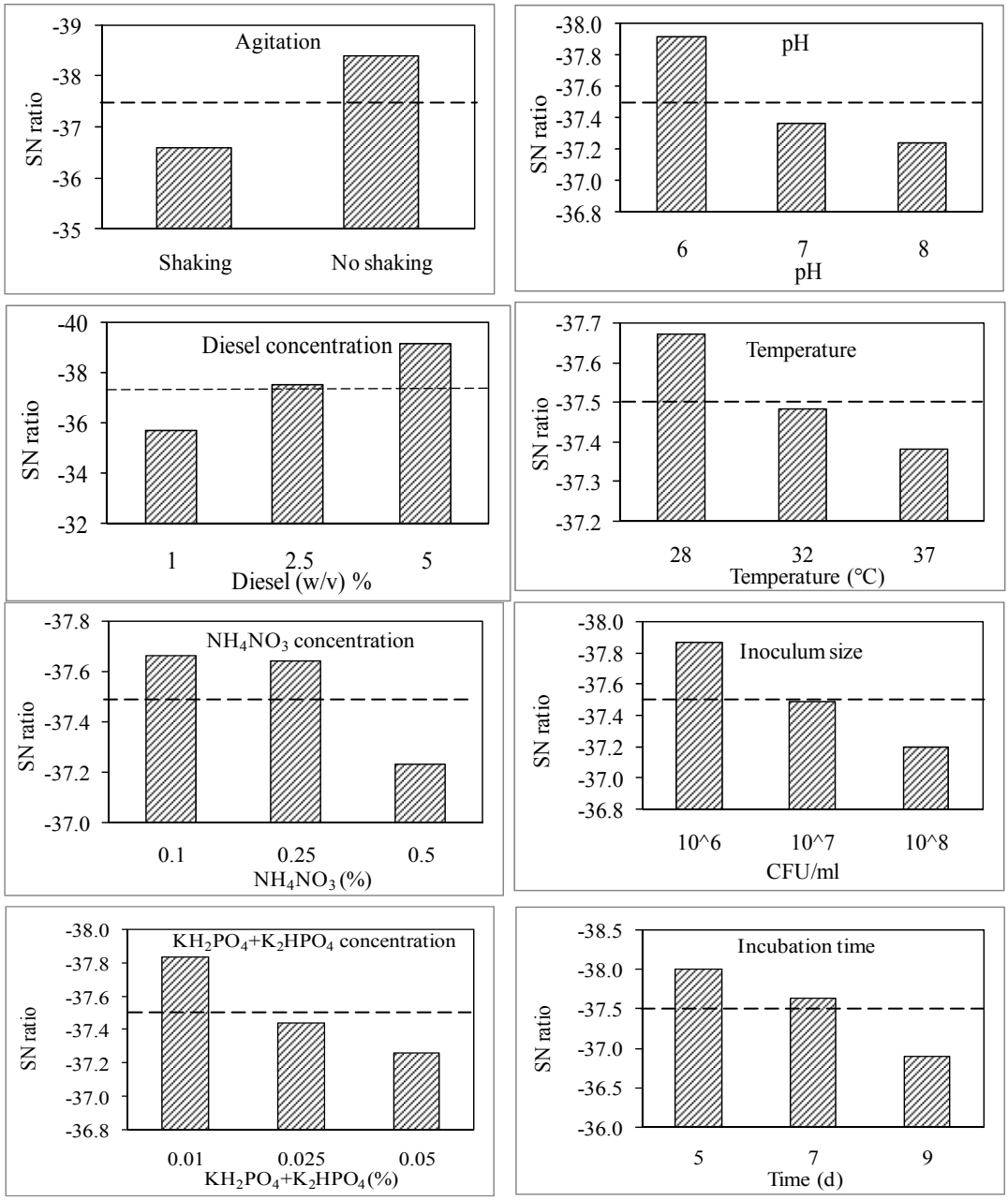


Fig. 3.15 The SN ratios and average response plots for different parameters. (Average SN ratio is presented as dotted line)

Analysis of variance (ANOVA) was used to analyze the results of the OA experiment and to determine the variation and percent contribution due to each factor. ANOVA with the percentage of contribution of each factor with interactions is given in Table 3.11. The percentage contribution was calculated for each individual factor by the ratio of pure sum to the total sum of the squares. Parameters were ranked based on contribution to the process performance enhancement. For predicting the performance under optimum conditions, factors with F =<1.5 were pooled.

Table 3.11 Analysis of variance (ANOVA)

CONTROL FACTORS \ LEVELS	Degrees of Freedom	Sum of Squares	Mean Square	Factor Effect (%)	F before pooling	empty or pooled F=<1.5	**F After pooling**
Agitation	1	14	14	22	22	no	**29**
Diesel conc. %	2	36	18	57	29	no	**37**
NH_4NO_3 conc. %	2	1	0	1	1	pooled	**-**
KH_2PO_4+ K_2HPO_4 conc. (%)	2	1	1	2	1	pooled	**-**
pH	2	2	1	2	1	pooled	**-**
Temperature ºC	2	0	0	0	0	pooled	**-**
Inoculum size CFU/ml	2	1	1	2	1	pooled	**1**
Incubation time d	2	4	2	6	3	no	**4**

Diesel concentration was the most significant factor followed by agitation for the overall performance of the process. The optimum parameters obtained for diesel range n-alkane degradation by *A. baumannii* were as follows: shaking, diesel concentration (w/v) 1%, NH_4NO_3 concentration 0.5 %, KH_2PO_4+K_2HPO_4 concentration 0.05 %, pH 8.0, temperature 37ºC, inoculum size 10^8 cfu/ml and incubation time 9 d. At this optimum condition, diesel range *n*-alkane degradation by *A. baumannii* was found to be 58.7±2.0%.

Taguchi design was successfully applied to test the relative importance of medium components and environmental factors on diesel range *n*-alkane degradation by *A. baumannii*. The results would further facilitate economic design of the large scale bioreactor system.

Aghamiri *et al*. (2011) used Taguchi method for optimization of crude oil bioremediation in soil using mixed cultures of three *Pseudomonas* strains and obtained 64-68% oil removal under optimum conditions. Aeration and bacterial inoculum size were found out to be important parameters affecting the process. Castorena-Cortés *et al*. (2009) used microcosm assays and Taguchi DOE to assess the biodegradation of an oil sludge produced by a gas processing unit. The authors

performed a Taguchi L_9 experimental design with 4 variables, moisture, nitrogen source, surfactant concentration and oxidant agent and 3 levels and showed that moisture and nitrogen source are the major variables that affect TPH degradation. TPH removal of 56,092 mg/kg/dry matter was observed in the best experimental treatment with 70% moisture, no oxidant agent, 0.5% of surfactant and NH_4Cl as nitrogen source.

3.12.4 Diesel range *n*-alkane degradation profile of *A. baumannii* at different concentrations of diesel in soil microcosms

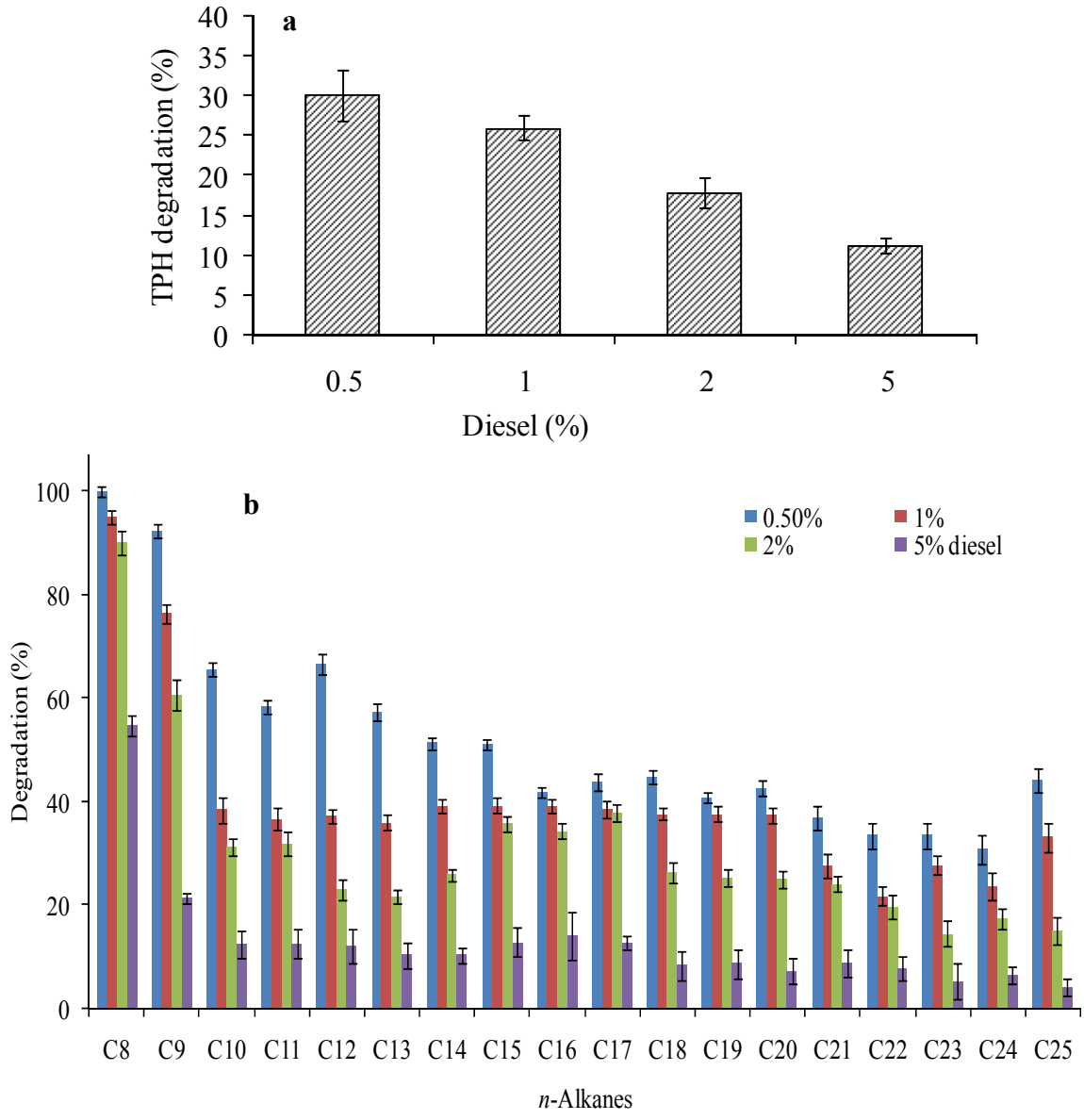


Fig. 3.16 a) Total Petroleum Hydrocarbons (TPH) degradation and b) individual diesel range *n*-alkanes degradation by *A. baumannii* in soil microcosms

In soil microcosms, *A. baumannii* could degrade 30.2±3.2, 26.0±1.5; 17.9±2.0 and 11.3±1.0 % of TPH from soils with 0.5, 1, 2, 5 % (w/w) of diesel respectively within 7d (Fig. 3.16a)., whereas, *n*-alkanes degradation was 50.2±5.9, 40.8±4.5 for 0.5 and 1 % (w/w) diesel, respectively at the end of 7d. It decreased to 31.0±2.8 and 11.9±2.4 % for 2 and 5 % (w/w) of diesel respectively. C_{10} and C_{11} *n*-alkanes which are reported to be toxic to seed germination (Siddiqui *et al.*, 2001) were degraded 65.3±1.2 and 58.3±1.3% degradation of 0.5% and 38.3±2.5 and 36.6±2.1 % of 1% diesel respectively (Fig. 3.16b).

3.13 Bioaugmentation of diesel contaminated soil microcosms with *A. baumannii*

Bacterial communities in contaminated soils tend to be dominated by the strains that can survive toxicity and are able to utilize the contaminant itself for growth. As a response to bioremediation treatment, these populations may begin to actively degrade the pollutants and detoxify the soil, allowing other quiescent/starving populations to increase in numbers. It may result in a quantitative increase in the number of bacterial cells and of their activity, implying a succession of leading bacterial groups and the change of the observable diversity (Øvrea˚s *et al.*, 1998; MacNaughton *et al.*, 1999; Iwamoto *et al.*, 2000; Ranjard *et al.*, 2000). The aim of the experiment was to investigate the response of a bacterial population to a bioremediation treatment of contaminated soil. In laboratory microcosms, we subjected the polluted soil to bioaugmentation with *A. baumannii* and followed the quantitative dynamics of the cultivable bacterial population.

Bioaugmentation studies using *A. baumannii* were performed to check its potential to degrade diesel in spiked soil microocosms and reclaimation of polluted soil in terms of restoration of the orignal bacterial flora and plant growth. For soil microcosms, 1.0 kg soil was spiked with 0.5%, 1%, 2% and 5% (w/w) diesel and 10^7 CFU/g of *A. baumannii* was added. Two controls (without *A. baumannii*) were run in parallel, wherein one microcosm was with diesel while the second was without diesel. After two weeks, *Arachis hypogaea* (groundnut) seeds were sown in the soils.

The effect of bioaugmentation with *A. baumannii* on total heterotrophic count and TPH degradation in soil microcosms having different diesel concentrations is presented in Fig. 3.17.

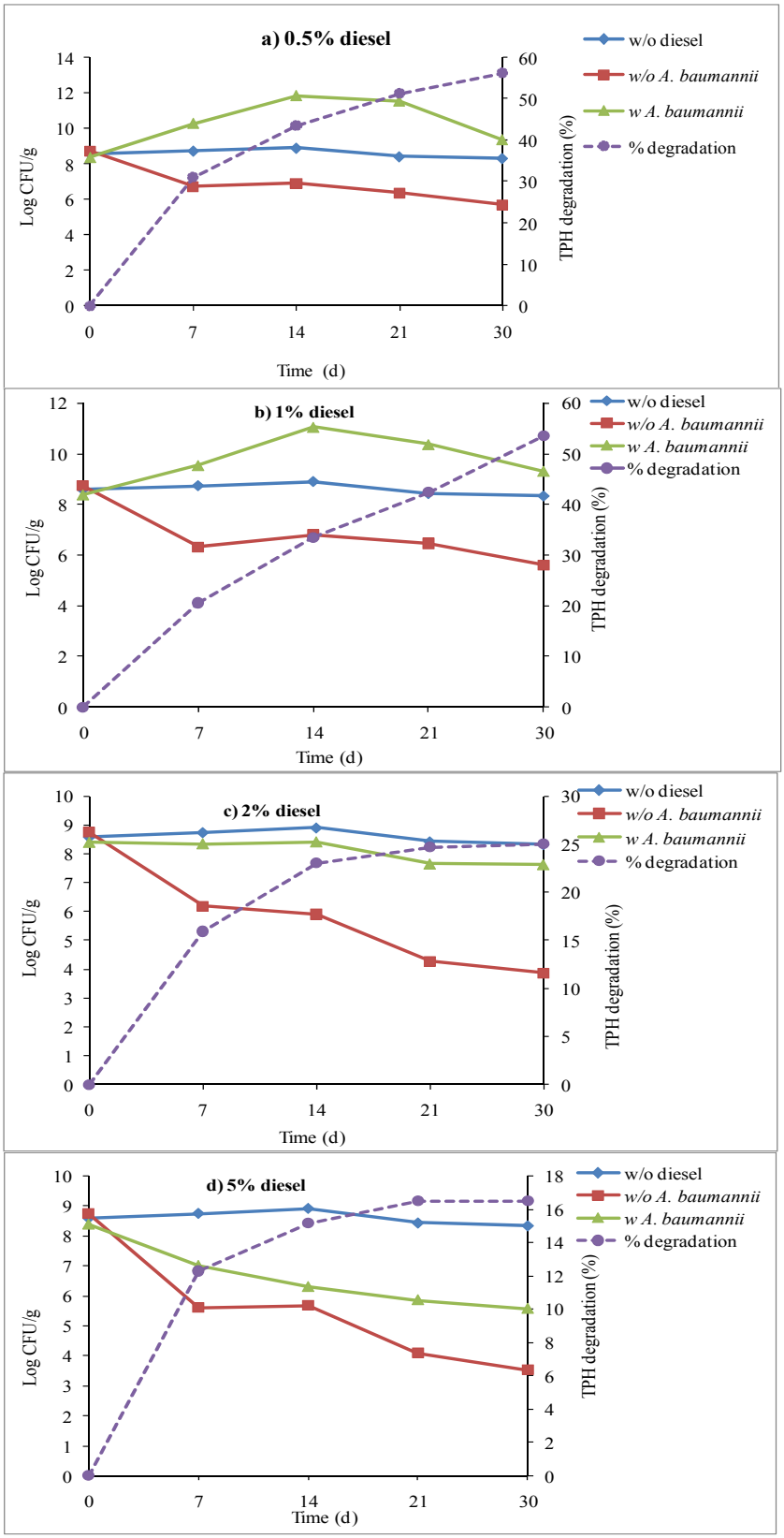


Fig. 3.17 Changes in total heterotrophic count and TPH degradation (%) in different soil microcosms with added diesel concentration a) 0.5 % b) 1 % c) 2 % d) 5 % (w/w).

The degradation of TPH is an important aspect for successful restoration of the soil bacterial flora. The TPH of of 0.5% diesel spiked soil were degraded up to 56.2±2.2% after 30d. Also within 30 d time, 53.5±1.9, 25.3±1.8 and 16.5±0.9% TPH degradation was observed for 1, 2 and 5% diesel respectively (Fig. 3.17).

The original count of bacteria in the control devoid of diesel was $4.6\pm0.5\times10^{8}$ CFU/g and it remained in the range of $2.1\text{-}8.2\pm0.3\text{-}0.8\times10^{8}$ CFU/g over the period of 30d. In the uninoculated control the bacterial count was drastically reduced from $5.5\pm0.2\times10^{8}$ CFU/g to $5.4\pm0.6\times10^{5}$ CFU/g at 0.5% diesel and $3.3\pm0.3\times10^{3}$ CFU/g at 5% diesel indicating a negative effect of diesel on the soil bacterial flora. In the soils with 0.5% disel and bioaugmented with *A. baumannii* the count of bacteria increased to $7.7\pm0.5\text{x}10^{11}$ after 14d (Fig. 3.17a). It was $3.8\pm0.2\times10^{11}$ CFU/g on 21 d which then reduced to $2.5\pm0.2\times10^{9}$ CFU/g on 30d. Similar trend was observed for 1% diesel concentration (Fig. 3.17b).The count after 30 d reduced to $5.4\pm0.2\times10^{7}$ and $4.3\pm0.2\times10^{5}$ CFU/g in bioaugmented soil microcosms containing 2 and 5 % diesel respectively indicating the toxic effect of diesel at such high concentration (Fig. 3.17c and d). Ueno *et al*. (2006) reported 51% TPH degradation in one week through *P. aeruginosa* strain WatG bioaugmentation in stimulated soil microcosms.

According to Adam and Duncan (2002), petroleum hydrocarbons may form a film on the seed, preventing the entry of oxygen and water.Thus, presence of petroleum hydrocarbons in the soils adversely affects the germination of seeds in soils (Ogbo *et al.*, 2010). In present study, decrease in % seed germination and all plant growth parameters were seen in uninoculated controls. The diesel at all concentrations delayed the germination of *A. hypogaea* by 2 d. Only 40±0.3, 35±0.4, and 25±0.2% seed germination was seen at 0.5, 1, and 2% (w/w) diesel respectively. Total inhibition of germination was observed at 5 % (w/w) diesel. The growth in presence of 0.5, 1 and 2% diesel was stunted. Similarly plant fresh weight was also seen to be decreased by 54.8 and 51.7% at 1 and 2% diesel. The reduction was also found to be dependent on diesel concentration (Fig. 3.18 and 3.19). Similar observations were reported by Agboidi and Enujeke, (2012) on *A. hypogaea*; Akujobi *et al.*, (2011) on *Solanum melongena*; Christo, (2010) on *Vigna radiata* and *A. hypogaea* and Ogbo *et al.*, (2010) on *Zea mays.*

A. hypogaea showed improved growth in bioaugmented soils as compared to uninoculated control soils (Fig. 3.19).

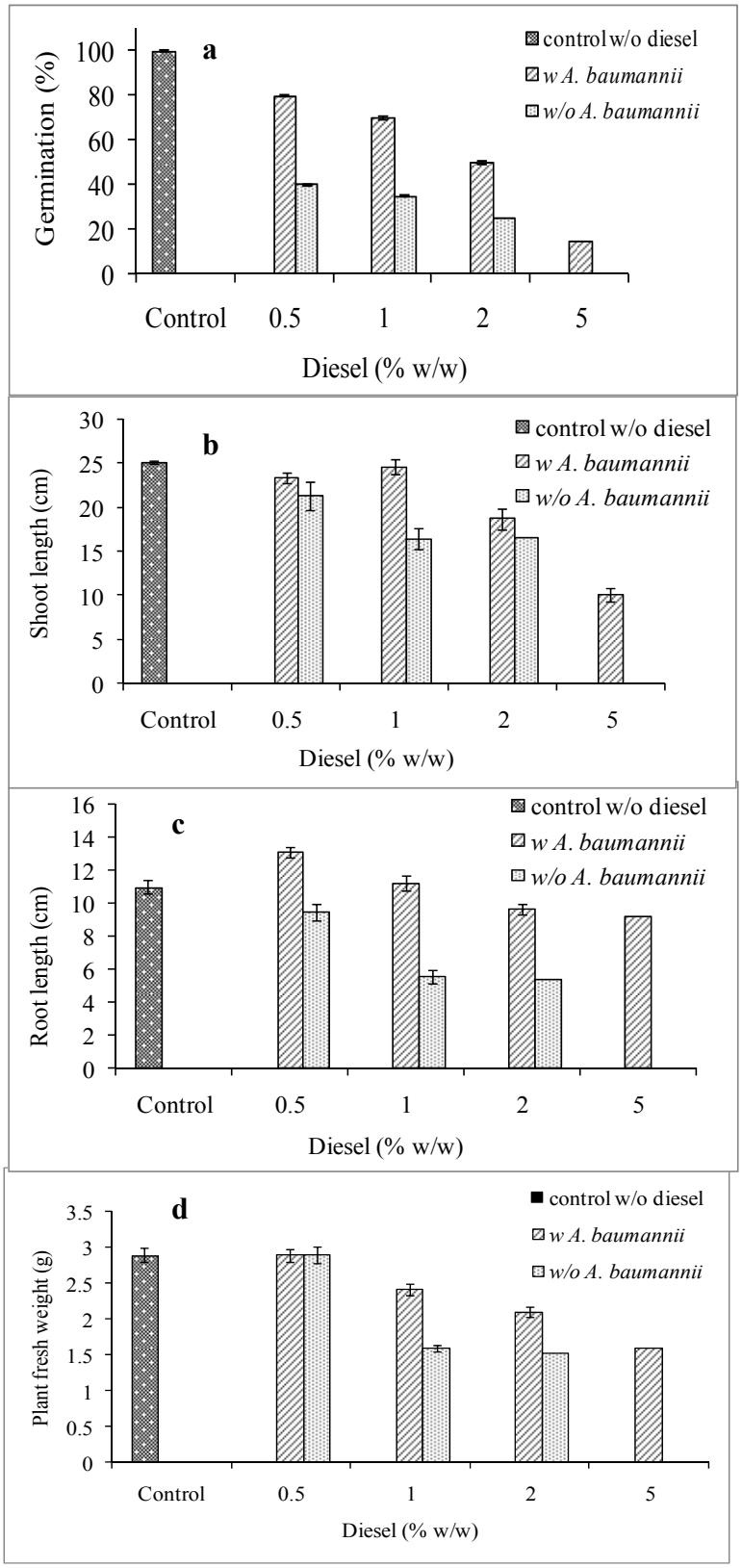


Fig. 3.18 Effect of bioaugmentation by *A. baumannii* on growth parameters of *A. hypogaea* - a) % seed germination, b) shoot length, c) root length and d) plant fresh weight of *A. hypogaea.* in presence of different concentrations of diesel

It was observed that seed germination doubled as compared to uninoculated controls in 0.5, 1 and 2% diesel containing soils. An increase in the growth parameters of the plants like total plant height, shoot and root length and plant fresh weight was seen in the presence of 0.5 to 2% diesel.

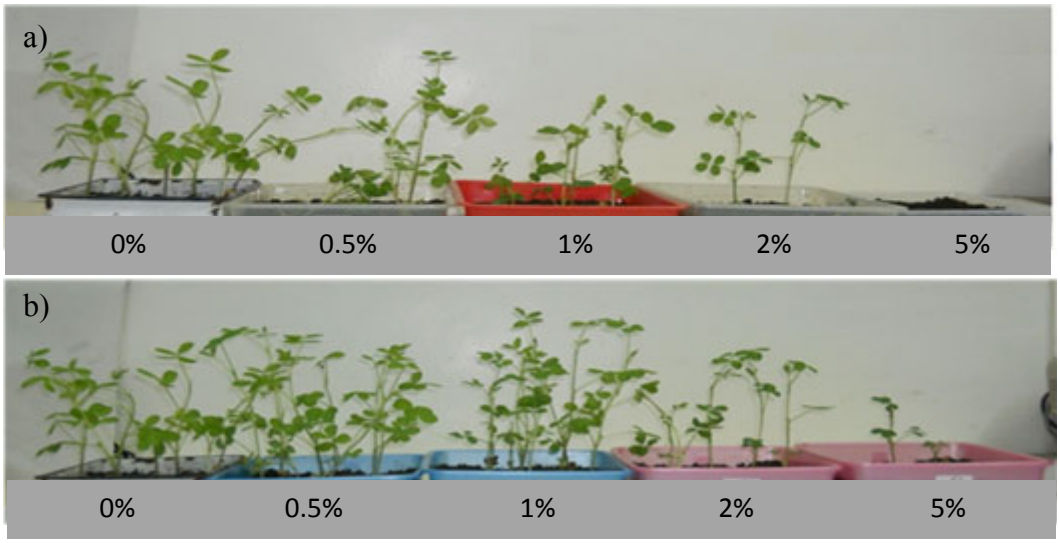


Fig. 3. 19 Growth of *A. hypogaea* in soils with different concentrations of diesel and a) without *A. baumannii*; b) bioaugmented with *A. baumannii*

All these results indicated that bioremediation of diesel spiked soils by *A. baumannii* could efficiently restore the conditions appropriate for the germination and growth of *A. hypogaea.*

Mukherjee and Bordoloi (2011) demonstrated bioremediation and reclamation of 20% (v/w) PHC contaminated soils using combination of *Bacillus subtilis* DM-04 and *Pseudomonas aeruginosa* M. The authors reported 76% TPH degradation in 180 days and observed that the reclaimed soil supported the germination and growth of crop plants *Cicer aretinum* and *Phaseolus mungo*.

3.14 Role of *A. baumannii* in restoration of soil bacterial flora

To confirm the role of *A. baumannii* in restoration of soil bacterial flora, *B. subtilis* (B2) isolated from agriculture soil was used as a model organism. Sterile soil was spiked with 1% (w/w) diesel and 1×10^7 CFU/g of B2 were inoculated in 3 (A, B, C) sets.

In set A, inoculated with 1×10^7 CFU/g *A. baumannii* on 1st d, the count of B2 increased to $5.1\pm0.6\times10^7$ CFU/g after 7 d, whereas in other sets the count was

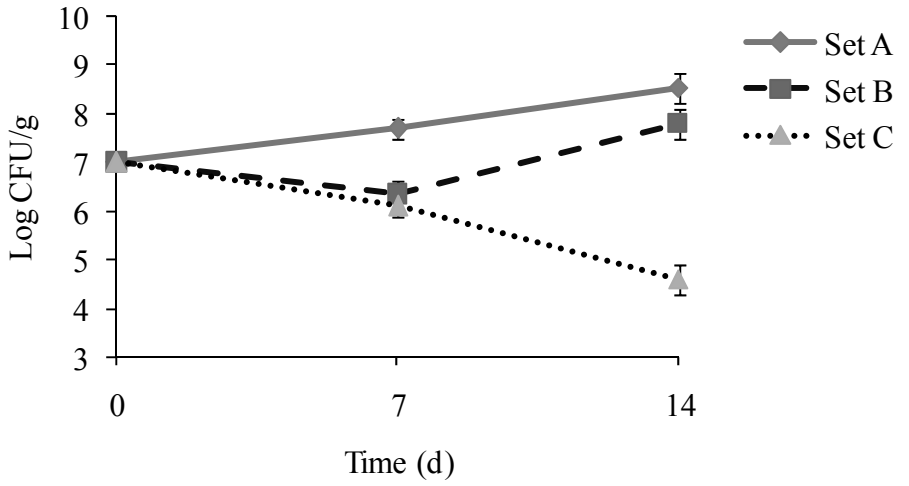


Fig. 3. 20 Restoration of model organism *B. subtilis* in diesel spiked soil using *A. baumannii.* Set A) inoculated with *A. baumannii* at 0 d, Set B) inoculated with *A. baumannii* after 7 d and Set C) without *A. baumannii*

reduced to $2.2\pm0.2\times10^{6}$. *A. baumannii* (1×10^{7} CFU/g) was inoculated in set B on 7 d (Fig. 3.20). The count after 14 d in set A and B increased, whereas it decreased for set C as compared to count on 7^{th} d confirming the role of *A. baumannii* in restoration of B2 in soil with 1% (w/w) diesel.

Chapter 4
Summary and Conclusions

CHAPTER IV

A detailed literature search indicated that soil pollution due to diesel and crude oil spills is becoming a widespread environmental problem of ever-increasing importance. Soil pollution due to diesel/PHC adversely reduces soil fertility and negatively influences plant growth and production and also leads to reduction in soil microflora diversity and population. Due to the toxic effects of PHC, it is necessary to remediate the contaminated soil. The cost for the clean-up of the contaminated sites with conventional physical and chemical techniques is enormous. Also due to lack of public acceptance and technological complexities, these methods are not successfully applied. Moreover, for these approaches the possible environmental hazards and their effect on soil diversity are of a major concern. Therefore, alternative methods such as bioremediation to restore polluted sites in a safe, environmental friendly and less expensive and labor intensive way are warranted.

Bioaugmentation involves the use of specific microorganisms, indigenous or exotic for effective bioremediation. It can be employed to restore useful, similar or same microbial diversity of the contaminated soils and get the desired level of soil fertility and crop yield. Therefore, the work presented in this thesis was initiated with an aim to isolate efficient diesel/PHC degrading microorganisms and use them in bioremediation of PHC polluted soils so as to restore soil microbial diversity and conditions appropriate for plant growth.

The findings of the present investigation can be briefly summarized as follows:

Analysis of seven PHC soil samples from different regions of India revealed that they were contaminated with PHC. The level of TPH in the soil samples were found to be in the range of 1247 to 9666 mg/kg of soil. Soil sample B (Pune site 2) had the highest (9666 mg/kg) TPH content. Only four bacterial isolates belonging to genus *Bacillus* were isolated from this soil. This may be attributed to the spore bearing capacity of these organisms due to which they could survive in high concentration of TPH. GC analysis showed that the *n*-alkanes present in the PHC contaminated soil samples were in the range of C_{12}-C_{32} indicating presence of PHC pollutants. Only soil sample G (Mumbai site 1) showed the presence of C_{11}, C_{12} and C_{13} *n*-alkanes. *A.*

baumannii, the best degrader in this study was isolated from this soil sample. *A. baumannii* was observed to degrade maximum % of diesel range *n*-alkanes as compared to the other isolates. This capability may be due to the adaptation of *A. baumannii* to the range of *n*-alkanes present in soil sample G.

The soil samples were also analyzed for their physico-chemical properties. The carbon content and extent of contamination was directly proportional. It was also found that the soil samples were deficient in nitrogen and phosphorous content. The electric conductivity of soil samples were high while moisture content, water holding capacity and pore size was reduced. The contaminated soils showed altered physico-chemical properties which make them unfit for the growth of agricultural crops as well at the normal soil flora. The total heterotrophic microbial count of PHC contaminated soil samples ranged from 2.3×10^7 to 2.71×10^9 CFU/g.

Total 70 bacterial (I1-I70) and 3 yeast isolates (Y1-Y3) were obtained by enrichment in selective minimal media (Bushnell-Hass) containing diesel as a sole source of carbon. Phylogenetic analysis of 16S rRNA sequences for bacterial isolates revealed that 67% isolates belonged to Firmicutes, 10% to Actinobacteria and remaining 23% to α, β and γ Proteobacteria. Similar pattern was observed for diversity of individual soil samples. The isolates were members of various genera such as *Acinetobacter*, *Achromobacter*, *Bacillus*, *Brevibacillus*, *Cellulomonas*, *Enterobacter*, *Exiguobacterium*, *Janibacter, Kocuria*, *Lysinibacillus*, *Microbacterium, Micrococcus, Novosphingobium*, *Ochrobactrum*, *Pseudomonas, Skermanella* and *Staphylococcus.* The dominant species belonged to *Bacillus* followed by *Pseudomonas* and *Staphylococcus* species. Based on the morphology and biochemical characteristics, the three yeast isolates were identified as belonging to the genera *Pichia*, *Rodotorula* and *Yarrowia.* The above bacterial and yeast genera have been reported among PHC degraders. Partial sequences of all the bacterial isolates have been deposited in the GenBank database under accession numbers KF318791 to KF318860.

OD_{600nm} was used as an index of PHC biodegradation potential. The isolates were tested for their growth in terms of OD_{600nm} in BH medium containing diesel as sole source of carbon. The OD_{600nm} of the isolates ranged between 0.12±0.02 to 0.46±0.02. Forty isolates exhibited good (OD_{600nm} >0.2) growth on diesel. Based on the OD_{600} measurements 9 isolates (with OD_{600nm} >0.35) - *Bacillus amyloliquefaciens* (I7), *Bacillus* sp. (I34), *Bacillus subtilis* (I26), *Staphylococcus haemolyticus* (I57), *Microbacterium testaceum* (I38), *Pseudomonas aeruginosa* (I41), *Exiguobacterium*

antarticum (I43), *Acinetobacter baumannii* (I58) and *Yarrowia* sp. (Y2) were selected for further studies.

The enzyme, alkane 1 monooxygenase (AMO) initiates the alkane degradation pathway. AMO activity for the 9 isolates was in the range 0.5±0.06 to 8.6±1.1 U/mg protein when grown in the presence of 1% hexadecane as carbon source. *A. baumannii* showed the highest activity of 8.6±1.1 U/mg protein followed by *Yarrowia* sp. 7.2±0.8. AMO activity was found to be inducible.

When five isolates with high AMO activity were checked for diesel utilization, it was observed that the efficiency of utilization of diesel range *n*-alkanes varied among them with *A. baumannii* being the best (51.1±1.3% degradation within 7 d). *M. testaceum*, *P. aeruginosa* and *Yarrowia* sp. could degrade 41.9±1.9, 45.1±2.1 and 48.5±1.4% respectively, whereas *B. subtilis* could degrade only 17.2±1.3%. A similar trend was observed for individual *n*-alkane degradation profile of the five isolates with *A. baumannii* being the best. *P. aeruginosa* was observed to degrade only 60.2±2.1% of C_9 while the remaining isolates showed 100% degradation. C_{10} and C_{11} which are reported to be toxic to seed germination were degraded by *A. baumannii* upto 48.0±1.2 and 47.1±1.5% respectively within 7d. *A. baumannii* showed complete degradation of C_{10} and C_{11} and long chain *n*-alkanes C_{20} -C_{25} after 1 month. *Yarrowia* sp. and *M. testaceum* were observed to degrade 42.4±1% and 35.8±0.9% of C_{10} respectively after 7d while, *Yarrowia* sp. and *P. aeruginosa* degraded 42.6±2.8% and 40.6±2.8% of C_{11} respectively. Absence of C_8 in control samples indicated its complete removal due to abiotic loss. A combination of the five isolates, *A. baumannii*, *B. subtilis*, *M. testaceum*, *P. aeruginosa*, and *Yarrowia* sp. with their individual *n*-alkane degrading capabilities can lead to improved PHC degradation. When the individual *n*-alkane degradation profile of the 5 isolates was compared, it was observed that *A. baumannii* showed maximum degradation.

Polymerase chain reaction using specific primer sets in the selected isolates confirmed the presence of catabolic gene for alkane monooxygenases. Fragment of expected size (271 bp) was amplified. High molecular weight plasmid was present in *Microbacterium sp.* and *P. aeruginosa*. Absence of plasmid in *A. baumannii* indicated that the required genes for hydrocarbon degradation activity are located on its genome. Presence of 2-hexadecanol in the medium indicated that *B. subtilis*, *Microbacterium* sp., *P. aeruginosa*, *A. baumannii* and *Yarrowia* sp. followed the subterminal oxidation pathway for *n*-alkane degradation.

A. baumannii and *P. aeruginosa* showed highest E24% of 61.86±4.2 and 60.86±4.8% respectively after growth in BH medium with diesel for 7 d. The emulsification activity of *A. baumannii* was 1.98±0.09 in BH medium with 1% diesel. High emulsifying activity exhibited by *A. baumannii* and *P. aeruginosa* will be useful in bioremediation of PHC in soils. No correlation was observed between biodegradation and cell surface hydrophobicity.

Based on the results of diesel degradation, individual alkane degradation profile, and characterization studies, *A. baumannii* was selected as best isolate and used in further biodegradation and bioaugmentation studies. *A. baumannii* was tested for its ability to degrade higher concentrations of diesel. It could degrade 50.8±1.8% of *n*-alkanes from 1 % added diesel, while it could degrade 22.5±2.8 and 8.9±0.9% of *n*-alkanes when the concentration was 5 and 10 % respectively.

Time course of *n*-alkane degradation pattern by *A. baumannii* in presence of 1% (w/v) diesel showed that degradation increased from 11.7±2.4% at 24 h to 26.8±3.0% on 3rd day and reached 51.1±1.3% after 7d. The % degradation for individual *n*-alkanes was also increased with time and in the range of 43.5 - 53.9% on 7th d. Initial inoculum of 1×10^7 CFU/ml reached to 8.6×10^9 after 7 d. Increase in inoculum size of *A. baumannii* in medium containing diesel as sole source of carbon indicated its diesel degrading capabilities.

Optimization of parameters for diesel range *n*-alkane degradation by *A. baumannii* in liquid medium was done by Taguchi design of experiments and optimum conditions obtained were - shaking, diesel 1%, NH_4NO_3 concentration 0.5 %, KH_2PO_4:K_2HPO_4 0.05:0.05%, pH 8.0, temperature 37ºC, inoculum size 10^8 CFU/ml and incubation for 9 d. At the optimum conditions, diesel range *n*-alkane degradation by *A. baumannii* was 58.75±2.3%. Taguchi design was applied to find the relative importance of environmental factors and medium components on diesel range *n*-alkanes degradation by *A. baumannii.*

The diesel degrading ability of *A. baumannii* in soil microcosms was also checked. *A. baumannii* could degrade 30.2±3.2, 26.0±1.5; 17.9±2.0 and 11.3±1.0% of TPH from 0.5, 1, 2, 5 % (w/w) of diesel respectively within 7 d, whereas, *n*-alkanes degradation was 50.2±5.9, 40.8±4.5 for 0.5 and 1 % (w/w) diesel, respectively at the end of 7 d. It decreased to 31.0±2.8 and 11.9±2.4 % for 2 and 5 % (w/w) of diesel respectively. C_{10} and C_{11} *n*-alkanes which are reported to be toxic to seed germination were degraded 65.3±1.2 and 58.3±1.3% degradation of 0.5% and 38.3±2.5 and

36.6±2.1 % of 1% diesel respectively. *A. baumannii* was observed to degrade TPH and *n*-alkanes in soil microcosms to a lesser extent than in liquid medium as PHC degrading process is known and reported to be highly aerobic.

Bioaugmentation studies using *A. baumannii* were performed to check its potential to degrade diesel in spiked soil microocosms and reclaimation of polluted soil in terms of restoration of the orignal bacterial flora and plant growth. For soil microcosms, soil was spiked with 0.5%, 1%, 2% and 5% (w/w) diesel and 10^7 CFU/g of *A. baumannii* was added. After two weeks, *Arachis hypogaea* (groundnut) seeds were sown in the soils.

The TPH of 0.5% diesel spiked soil were degraded up to 56.2±2.2% after 30d. Also 53.5±1.9, 25.3±1.8 and 16.5±0.9% TPH degradation was observed for 1, 2 and 5% diesel respectively. The degradation of TPH is an important aspect for successful restoration of the soil bacterial flora. The changes in bacterial flora in soil microcosms were also observed. The original count of bacteria in the control devoid of diesel was $4.6\pm0.52\times10^8$ CFU/g and it remained in the range of $2.1\text{-}8.2\pm0.33\text{-}0.81\times10^8$ CFU/g over the period of 30d. In the uninoculated control the bacterial count was drastically reduced from $5.5\pm0.2\times10^8$ CFU/g to $5.4\pm0.6\times10^5$ CFU/g at 0.5% diesel and $3.3\pm0.3\times10^3$ CFU/g at 5% diesel indicating a negative effect of diesel on the soil bacterial flora. In the soils with 0.5% diesel bioaugmented with *A. baumannii* the count of bacteria increased to $7.7\pm0.5\text{x}10^{11}$ after 14 d. It was $3.8\pm0.2\times10^{11}$ CFU/g on 21 d which then reduced to $2.5\pm0.2\times10^9$ CFU/g on 30 d. Similar trend was observed for 1% diesel concentration. In soil microcosms bioaugmented with *A. baumannii* containing 2 and 5 % diesel the bacterial count after 30 d was seen to reduce to $5.4\pm0.2\times10^7$ and $4.3\pm0.1\times10^5$ CFU/g respectively indicating the toxic effect of diesel at such high concentration.

A. hypogaea showed improved growth performance in bioaugmented soils as compared to uninoculated control soils. It was observed that seed germination doubled as compared to uninoculated control in 0.5, 1 and 2% diesel containing soils. An increase in the growth parameters of the plants like total plant height, shoot and root length and plant fresh weight was seen in the presence of 0.5 to 2% diesel. Shoot length was seen to be more by 2.0 and 8.2 cm and root length by 3.6 and 5.6 cm respectively at 0.5 and 1% diesel indicating successful bioaugmentation.

To confirm the role of *A. baumannii* in restoration of soil bacterial flora, *B. subtilis* (B2) isolated from agriculture soil was used as a model organism. Sterile soil

was spiked with 1% (w/w) diesel and 1×10^7 CFU/g of B2 were inoculated in 3 (A, B, C) sets. In set A, inoculated with 1×10^7 CFU/g *A. baumannii* on 1st d, the count of B2 increased to $5.1\pm0.6\times10^7$ CFU/g after 7 d, whereas in other sets the count was reduced to $2.2\pm0.2\times10^6$. *A. baumannii* (1×10^7 CFU/g) was inoculated in set B on 7 d. Increase in the count after 14 d in set A, B and further decrease in set C in comparison with count on 7th d confirmed the role of *A. baumannii* in restoration of B2 in soil with 1% (w/w) diesel. Therefore, biougmentation with *A. baumannii* resulted in reclaimation of soil contaminated with diesel, as evident from the germination and growth of *A. hypogaea* and restored bacterial diversity.

Thus, the salient findings of the thesis can be summarized as follows:

- 70 bacterial and 3 yeast isolates were isolated and identified from PHC polluted soil samples.
- Bioremediation of PHC contaminated soils using *A. baumannii* was successfully demonstrated.
- Consequently plant growth and soil bacterial flora could be restored in bioremediated soils.

Appendix

APPENDIX I

Colony characteristics of the bacterial isolates

Isolate	Size	Shape	Colour	Margin	Consistency	Opacity	Elevation	Gram Nature	Motility	Spores
I1	5 mm	circular	cream	entire	butyrous	opaque	raised at edges	positive rods	non motile	-
I2	3 mm	circular	pearly white	entire	butyrous	opaque	raised	positive rods	non motile	-
I3	1 mm	circular	yellow	entire	mucoid	opaque	raised	negative rod	motile	-
I4	4 mm	circular	cream	irregular	slimy	opaque	flat	positive rods	motile	central
I5	5 mm	circular	cream	lobate	slimy	opaque	flat	positive rods	motile	central
I6	pin point	circular	white	entire	butyrous	opaque	raised	positive rods	non motile	-
I7	1 mm	circular	pale white	irregular	dry	opaque	flat	positive rods	non motile	-
I8	3 mm	circular	cream	entire	mucoid	opaque	flat	positive rods	motile	central
I9	5 mm	circular	cream	entire	dry	opaque	flat (crumpled)	positive rods	motile	central
I10	pin point	circular	pale white	entire	butyrous	opaque	raised	positive rods	non motile	-
I11	5 mm	circular	cream	lobate	slimy	opaque	flat	positive rods	motile	central
I12	6 mm	circular	pale white	entire	slimy	opaque	umbonate	positive rods	motile	central

I13	4 mm	circular	off white	entire	mucoid	opaque	raised	positive long rods in chains	non motile	-
I14	5 mm	circular	cream	irregular	slimy	opaque	flat	positive rods	motile	central
I15	1 mm	circular	pale white	irregular	dry	opaque	flat	positive rods	non motile	-
I16	4 mm	circular	pale white	entire	slimy	opaque	flat	positive rods	motile	central
I17	2 mm	circular	shiny yellow	entire	butyrous	transluscent	raised	positive rods	non motile	-
I18	3 mm	circular	beige	entire	mucoid	transluscent	raised	negative rod	motile	-
I19	1 mm	circular	yellow	entire	mucoid	opaque	raised	negative rod	motile	-
I20	2 mm	circular	off white	entire	mucoid	opaque	raised	positive long rods in chains		
I21	3 mm	circular	cream	entire	mucoid	opaque	raised	positive rods	non motile	-
I22	6 mm	circular	white	irregular	butyrous	opaque	flat	positive rods	motile	central
I23	pin point	circular	pale white	entire	butyrous	transluscent	slightly raised	positive cocci	non motile	-
I24	1 mm	circular	yellow	irregular	butyrous	opaque	raised	positive rods	non motile	-
I25	5 mm	circular	cream	lobate	slimy	opaque	flat	positive rods	motile	central
I26	3 mm	circular	cream	entire	mucoid	opaque	flat	positive rods	motile	central
I27	1 mm	circular	cream	entire	mucoid	opaque	flat	positive rods	motile	-

I28	4 mm	circular	cream	entire	slimy	opaque	flat	positive long thick rods		
I29	1 mm	circular	orange	entire	mucoid	opaque	raised	positive coccoid	non motile	-
I30	pin point	circular	shiny white	entire	butyrous	opaque	convex	negative short rods	motile	-
I31	pin point	circular	white	entire	butyrous	opaque	raised	positive curved rods	motile	-
I32	2 mm	circular	brown	entire	slimy	opaque	raised	negative rods	motile	-
I33	5 mm	circular	cream	entire	butyrous	opaque	raised in centre	positive rods	motile	central
I34	2 mm	circular	cream	entire	slimy	opaque	flat	positive rods	non motile	-
I35	3mm	circular	cream	irregular	slimy	opaque	raised in centre	positive rods	motile	central
I36	5 mm	circular	cream	entire	butyrous	opaque	raised at edges	positive rods	non motile	-
I37	3 mm	circular	cream	irregular	slimy	opaque	flat	positive rods	motile	central
I38	3 mm	circular	shiny yellow	entire	butyrous	transluscent	raised	positive rods	non motile	-
I39	5 mm	circular	cream	irregular	mucoid	opaque	flat	positive rods	motile	central
I40	3 mm	circular	shiny orange	entire	butyrous	opaque	raised	positive rods	non motile	-

I41	3 mm	circular	brown	entire	slimy	transluscen	raised	negative rods	motile	-
I42	2 mm	irregular	fluorescent green	entire	mucoid	transluscent	flat	negative rods	motile	-
I43	3 mm	circular	pearly white	entire	butyrous	opaque	raised	positive rods	non motile	-
I44	5 mm	circular	pale white	irregular	slimy	opaque	raised in centre	positive rods	motile	central
I45	2 mm	circular	pearly white	entire	butyrous	opaque	raised	positive rods	motile	central
I46	3 mm	circular	pale yellow	irregular	butyrous	transparent	flat	positive short rods	motile	terminal
I47	5 mm	circular	cream	lobate	slimy	opaque	flat	positive rods	motile	central
I48	pin point	circular	shiny white	entire	butyrous	opaque	convex	negative short rods	motile	-
I49	2 mm	circular	brown	entire	slimy	opaque	raised	negative rods	motile	-
I50	1 mm	circular	orange	entire	mucoid	opaque	raised	positive coccoid	non motile	-
I51	3 mm	circular	cream	entire	slimy	opaque	raised at edges	positive rods	motile	central
I52	pin point	circular	pale white	entire	butyrous	transluscent	slightly raised	positive cocci	non motile	-
I53	3 mm	circular	white	irregular	butyrous	opaque	raised in centre	positive rods	non motile	-

I54	1 mm	circular	apricot	irregular	slimy	opaque	raised	negative thin rods	motile	-
I55	6 mm	circular	pale white	irregular	slimy	opaque	flat (concentric circles)	positive rods	motile	central
I56	3mm	circular	cream	irregular	butyrous	opaque	raised in centre	positive rods	non motile	-
I57	pin point	circular	pale white	entire	butyrous	transluscent	slightly raised	positive cocci	non motile	-
I58	2 mm	circular	pale yellow	irregular	butyrous	opaque	convex	negative rods	non motile	-
I59	2 mm	circular	pale yellow	entire	butyrous	opaque	raised	positive short rods	non motile	-
I60	pin point	circular	yellow	entire	butyrous	transluscent	slightly raised	positive cocci	non motile	-
I61	1 mm	circular	yellow	entire	butyrous	opaque	slightly raised	negative rods	non motile	-
I62	1 mm	circular	yellow	entire	mucoid	opaque	raised	negative rod	motile	-
I63	3 mm	circular	cream	entire	mucoid	opaque	raised	positive rods	non motile	-
I64	2 mm	circular	pale yellow	irregular	butyrous	opaque	convex	negative rods	non motile	-
I65	3 mm	circular	cream	entire	slimy	opaque	flat	positive rods	non motile	-
I66	5 mm	circular	cream	lobate	slimy	opaque	flat	positive rods	motile	central

I67	1 mm	circular	pale white	irregular	dry	opaque	flat	positive rods	non motile	-
I68	4 mm	circular	cream	irregular	slimy	opaque	flat	positive rods	motile	central
I69	1 mm	circular	pale yellow	entire	butyrous	opaque	slightly raised	negative rods	non motile	-
I70	2 mm	circular	brown	entire	slimy	opaque	raised	negative rods	motile	-

APPENDIX II

Biochemical characteristics of the bacterial isolates

Isolate	Sugar fermentation						Ind.	MR	VP	Cit.	Gel.	Cat.	Oxi.	Urease	Star.	Identified as Genus
	D-Glu a/g	D-Rib a/g	D-Xyl a/g	D-Lac a/g	Suc a/g	Mann a/g										
I1	+/-	-	-	+/-	+/-	-	-	-	-	+	-	+	-	-	+	*Bacillus*
I2	+/-	-	-	-	-	+/-	-	+	-	-	-	+	+	-	-	ND
I3	+/-	-	+/-	-	-	-	-	-	-	-	-	+	+	-	+	ND
I4	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I5	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I6	+/-	-	+/-	-	-	-	-	-	-	-	+	+	-	-	-	ND
I7	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I8	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I9	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I10	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I11	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I12	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I13	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*

I14	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I15	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I16	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I17	+/-	-	+/-	-	+/-	-	+	+	-	-	-	+	-	+	-	ND
I18	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	ND
I19	+/-	-	+/-	-	-	-	-	-	-	-	-	+	+	-	+	ND
I20	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I21	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	-	*Bacillus*
I22	+/-	-	-	+/+	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I23	-	-	-	-	-	+/-	-	-	-	-	-	+	+	-	-	*Staphylococcus*
I24	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I25	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I26	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I27	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I28	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I29	+/-	-	-	+/-	+/-	-	-	-	-	-	+	+	-	-	-	*Kocuria*
I30	+/-	-	-	+/+	+/-	-	-	+	-	+	+	+	-	+	-	*Enterobacter*
I31	+/-	-	-	-	+/-	-	-	-	-	-	-	+	-	-	+	ND
I32	-	-	-	-	-	-	-	+	-	+	+	+	+	-	-	*Pseudomonas*
I33	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*

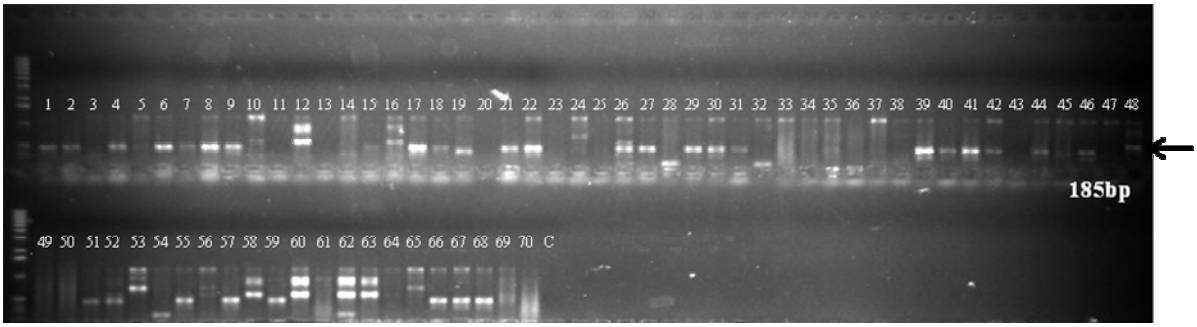
I34	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I35	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I36	+/-	-	-	+/-	+/-	-	-	-	-	+	-	+	-	-	+	*Bacillus*
I37	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I38	+/-	-	+/-	-	+/-	-	+	+	-	-	-	+	-	+	-	ND
I39	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I40	+/-	-	+/-	-	+/-	-	+	+	-	-	-	+	-	+	-	ND
I41	-	-	-	-	-	-	-	+	-	+	+	+	+	-	-	*Pseudomonas*
I42	-	-	-	-	-	-	-	+	-	+	+	+	+	-	-	*Pseudomonas*
I43	+/-	-	-	-	-	+/-	-	+	-	-	-	+	+	-	-	ND
I44	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I45	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I46	+/-	+/-	-	-	+/-	-	-	-	-	-	+	+=	-	-	+=	ND
I47	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I48	+/-	-	-	+/+	+/-	-	-	+	-	+	+	+	-	+	-	*Enterobacter*
I49	-	-	-	-	-	-	-	+	-	+	+	+	+	-	-	*Pseudomonas*
I50	+/-	-	-	+/-	+/-	-	-	-	-	-	+	+	-	-	-	*Kocuria*
I51	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I52	-	-	-	-	-	+/-	-	-	-	-	-	+	+	-	-	*Staphylococcus*
I53	+/-	-	-	+/-	+/-	-	-	-	-	+	-	+	-	-	+	*Bacillus*

I54	+/-	-	-	+/-	-	+/-	-	-	-	+	-	+	+	+	-	ND
I55	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I56	+/-	-	-	+/-	+/-	-	-	-	-	+	-	+	-	-	+	*Bacillus*
I57	-	-	-	-	-	+/-	-	-	-	-	-	+	+	-	-	*Staphylococcus*
I58	+/-	-	+/-	+/-	-	-	-	-	-	+	-	+	-	+	-	ND
I59	+/-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	ND
I60	-	-	-	-	-	+/-	-	-	-	-	-	+	+	-	-	*Staphylococcus*
I61	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	ND
I62	+/-	-	+/-	-	-	-	-	-	-	-	-	+	+	-	+	ND
I63	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	-	*Bacillus*
I64	+/-	-	+/-	+/-	-	-	-	-	-	+	-	+	-	+	-	ND
I65	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I66	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I67	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I68	+/-	-	-	+/-	+/-	-	-	-	+	+	-	+	-	-	+	*Bacillus*
I69	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	ND
I70	-	-	-	-	-	-	-	+	-	+	+	+	+	-	-	*Pseudomonas*

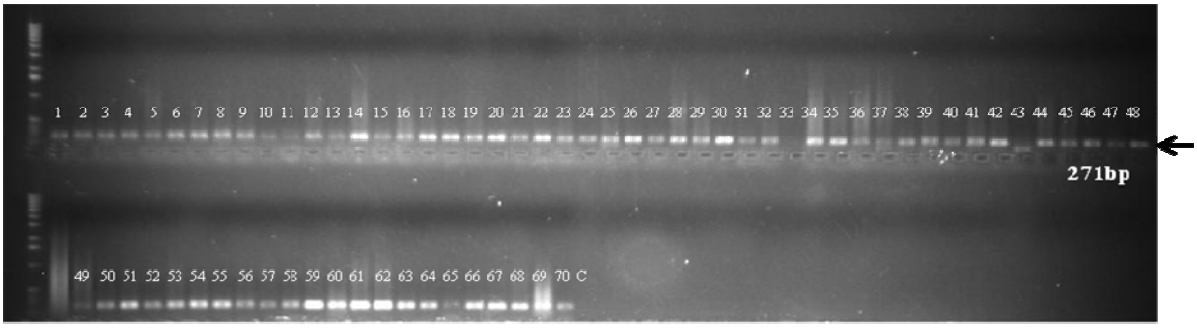
Fermentation: D-Glu, D-glucose; D-Rib, D-Ribose; D-Xyl, D-Xylose; Lact, lactose; Suc, Sucrose; Mann, Mannitol; Ind., Indole; MR, Methyl Red; VP, Voges-Prauskauer; Cit., Citrate utilization; Gel., Gelatin liquefaction; Cat., Catalase; Oxi., Oxidase; Star., Starch hydrolysis.
a, acid production; g, gas production; +, positive; –, negative.

APPENDIX III

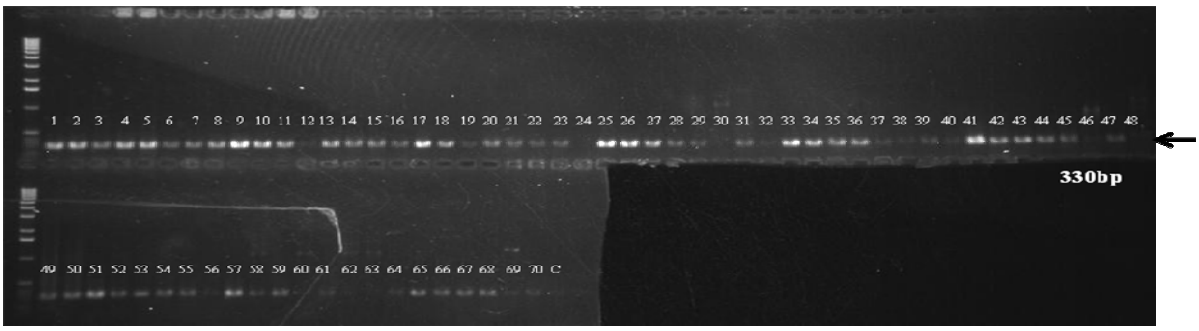
Detection of alkane monooxygenase (AMO) gene from bacterial isolates



a) PCR amplification of the AMO gene from the isolates I1- I70 with ALK 1F/1R primers. 41% of the 70 bacterial isolates showed amplification with ALK 1F/1R primers. C – *Escherichia coli* (negative control)



b) PCR amplification of the AMO gene from the isolates I1- I70 with ALK 2F/2R primers. 98% of the 70 bacterial isolates showed amplification with ALK 2F/2R primers. C – *E. coli* (negative control)



c) PCR amplification of the AMO gene from the isolates I1- I70 with ALK 3F/2R primers. 88% of the 70 bacterial isolates showed amplification with ALK 3F/2R primers. C – *E. coli* (negative control)

References

Abdel-Megeed, A., Al-Harbi1, N. and Al-Deyab, S. (2010) Hexadecane degradation by bacterial strains isolated from contaminated soils. Afr. J. Biotechnol. 9: 7487-7494.

Abed, R. M. M., Safi, N. M. D., Koster, J., de Beer, D., El-Nahhal, Y., Rullkotter, J. and Garcia-Pichel, F. (2002) Microbial diversity of a heavily polluted microbial mat and its community changes following degradation of petroleum compounds. Appl. Environ. Microbiol. 68: 1674-1683.

Achuba, F. I. (2006) The effect of sublethal concentrations of crude oil on the growth and metabolism of cowpea (*Vigna unguiculata*) seedlings. Environmentalist. 26: 17-20.

Adam, G. and Duncan, H. J. (2002) Influence of diesel fuel on seed germination. Environ. Pollut. 120: 363-370.

Agarry, S., Owabor, C. and Yusuf, R. (2010) Bioremediation of soil artificially contaminated with petroleum hydrocarbon oil mixtures: evaluation of the use of animal manure and chemical fertilizer. Biorem. J. 14:189-195.

Agboidi, O. M. and Enujeke, E. C. (2012) Effects of spent motor oil on soil physico-chemical properties and growth of *Arachis hypogaea*. Global J. Biosci. Biotechnol. 1: 72-74.

Aghamiri, S., Kabiri, K. and Emtiazi, G. (2011) A novel approach for optimization of crude oil bioremediation in soil by the Taguchi method. J. Pet. Environ. Biotechnol. 2: 2-6.

Aguilera, F., Méndez, J., Pásaroa, E. and Laffona, B. (2010) Review on the effects of exposure to spilled oils on human health. J. Appl. Toxicol. 30: 291-301.

Ahamed, F., Hasibullah, M., Ferdouse, J. and Anwar, M. N. (2010) Microbial degradation of petroleum hydrocarbon. Bangladesh J. Microbiol. 27: 10-13.

Ahmed, R. Z., Ahmed, N. and Gadd, G. M. (2010) Isolation of two *Kocuria* species capable of growing on various polycyclic aromatic hydrocarbons. Afr. J. Biotechnol. 9: 3611-3617.

Aitken, C., Jones, D. and Larter, S. (2004) Anaerobic hydrocarbon biodegradation in deep subsurface oil reservoirs. Nature. 431: 291-294.

Akubugwo, E. I., Ogbuji, G. C., Chinyere, C. G. and Ugbogu, E. A. (2009) Physicochemical properties and enzymes activity studies in a refined oil contaminated soil in Isiukwuato, Abia state, Nigeria. Biokemistri. 21: 79-84.

Akujobi, C. O., Onyeagba, R. A., Nwaugo, V. O. and Odu, N. N. (2011) Effect of nutrient amendments of diesel oil polluted soil on plant growth parameters. Curr. Res. J. Biol. Sci. 3: 421-429.

Al-Sarawi, M., Massaud, M. S. and Al-Abdali, F. (1997) Preliminary assessment of oil contamination levels in soils contaminated with oil lakes in the Greater Burgan oil fields, Kuwait. Water Air Soil Pollut. 106: 493-504.

Andretta, C. W., Rosa, R. M., Tondo, E. C., Gaylarde, C. C. and Henriques, J. A. (2004) Identification and molecular characterization of a *Bacillus subtilis* IS13 strain involved in the biodegradation of 4,5,6-trichloroguaiacol. Chemosphere. 55: 631-639.

Andrews, R., Parks, T. and Spence, K. (1980) Some effects of Douglas fir terpenes on certain microorganisms. Appl. Environ. Microbiol. 40: 301-304.

Atagana, H. (2008) Compost bioremediation of hydrocarbon-contaminated soil inoculated with organic manure. Afr. J. Biotechnol. 7:1516-1525.

Atlas, R. M. (1975) Effects of temperature and crude oil composition on petroleum biodegradation. J. Appl. Microbiol. 30: 396-403.

Atlas, R. M. (1981) Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol. Rev. 45: 180-209.

Auffret, M., Labbe, D., Thouand, G., Greer, C. W. and Fayolle-Guichard, F. (2009) Degradation of a mixture of hydrocarbons, gasoline, and diesel oil additives by *Rhodococcus aetherivorans* and *Rhodococcus wratislaviensis* Appl. Environ. Microbiol. 75: 7774-7782.

Baboshin, M., Akimov, V., Baskunov, B., Born, T., Khan, S. and Golovleva, L. (2008) Conversion of polycyclic aromatic hydrocarbons by *Sphingomonas* sp. VKM B-2434. Biodegradation. 19: 567-576.

Baek, K. H. and Kim, H. S. (2009) Microbial community structure in hexadecane and naphthalene enriched gas station soil. J. Microbiol. Biotechnol. 19: 651-657.

Baek, K. H., Yoon, B. D., Kim, B. H., Cho, D. H., Lee, I. S., Oh, H. M. and Kim, H. S. (2007) Monitoring of microbial diversity and activity during bioremediation of crude oil contaminated soil with different treatments. J. Microbiol. Biotechnol. 17: 67-73.

Balba, M. T., Al-Awadhi, N. and Al-Daher, R. (1998) Bioremediation of oil contaminated soil: Microbiological methods for feasibility assessment and field evaluation. J. Microbiol. Meth. 32: 155-164.

Baptista, J. S., Cammarota, C. M. and Carvalhofreire, D. D. (2005) Production of CO_2 in crude oil bioremediation in clay soil. Braz. Arch. Biol. Technol. 48: 249-255.

Basuki, W., Syahputra, K., Suryani, A. T. and Pradipta, I. (2011) Biodegradation of used engine oil by *Acinetobacter junii* TBC 1.2. Indonesian J. Biotechnol. 16: 132-138.

Bento, F. M., Camargo, F. A. O., Okeke, B. C. and Frankenberger, W. T. (2005) Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresour. Technol. 96: 1049-1055.

Berthe-Corti, L. and Fetzner, S. (2002) Bacterial metabolism of *n*-alkanes and ammonia under oxic, suboxic and anoxic conditions. Acta Biotechnol. 22: 299-336.

Bhatnagar, S. and Kumari, R. (2013) Bioremediation: A sustainable tool for environmental management - A review. Ann. Rev. Res. Biol. 3: 974-993.

Binazadeh, M., Karimi, I. A. and Li, Z. (2009) Fast biodegradation of long chain *n*-alkanes and crude oil at high concentrations with *Rhodococcus* sp. Moj-3449. Enzyme Microb. Technol. 45: 195-202.

Borah, D. (2011) Isolation and molecular characterization of hydrocarbon degrading microorganisms isolated from automobile engines. Dev. Microbiol. Mol. Biol. 2: 23-28.

Bordenave, S., Goni-Urriza, M. S., Caumette, P. and Duran, R. (2007) Effects of heavy fuel oil on the bacterial community structure of a pristine microbial mat. Appl. Environ. Microbiol. 73: 6089-6097.

Boronin, A. M., Filonov, A. E., Gayazov, R. R. Kulakova, A. N. and Mshensky, Y. N. (1993) Growth and plasmid encoded naphthalene catabolism of *Pseudomonas putida* in batch culture. FEMS Microbiol. Lett. 113: 303-308.

Bundy, J. G., Paton, G. I. and Campbell, C. D. (2002) Microbial communities in different soil types do not converge after diesel contamination. J. Appl. Microbiol. 92: 276-288.

Bushnell, L. D. and Haas, H. F. (1940) The utilization of certain hydrocarbons by microorganisms. J. Bacteriol. 41: 653-673.

Calder, J. and Lader, J. (1976) Effect of dissolved aromatic hydrocarbons on the growth of marine bacteria in batch cultures. Appl. Environ. Microbiol. 32: 95-101.

Cane, P. A. and Williams, P. A. (1982) The plasmid-coded metabolism of naphthalene and 2-methylnaphthalene in *Pseudomonas* strains: phenotypic changes correlated with structural modification of the plasmid pWW60-1. J. Gen. Microbiol. 128: 2281-2290.

Catia, T. D. P, Mariano, M., Janaína, F., Medeiros, B., Susana, J. K., Carlos, A. and Veiga, B. (2010) Biodegradation of phenol by free and encapsulated cells of a new *Aspergillus* sp. isolated from a contaminated site in southern Brazil. Afri. J. Biotechnol. 9: 6716-6720.

Celik, G. Y., Aslim, B. and Beyatli, Y. (2008) Enhanced crude oil biodegradation and rhamnolipid production by *Pseudomonas stutzeri* strain G11 in the presence of Tween-80 and Triton X-100. J. Environ. Biol. 29: 867-870.

Chakrabarty, A., Chou, G. and Gunsalus, I. (1973) Genetic regulation of octane dissimilation plasmid in *Pseudomonas* (incompatibility). Proc. Natl. Acad. Sci. U.S.A. 70: 1137-1140.

Chakrabarty, A., Friello, D. and Bopp, L. (1978) Transposition of plasmid DNA segments specifying hydrocarbon degradation and their expresssion in various microorganisms Proc. Natl. Acad. Sci. U.S.A. 75: 3109-3112.

Chang, L., Ibrahim, D. and Omar, I. (2011) A laboratory scale bioremediation of Tapis crude oil contaminated soil by bioaugmentation of *Acinetobacter baumannii* T30C. Afr. J. Microbiol. Res. 5: 2609-2615.

Chapelle, F. (1999) Bioremediation of petroleum hydrocarbon contaminated ground water: The perspectives of history and hydrology. Ground Water 37: 122-132.

Chaudhary, V. K. and Borah, D. (2011) Isolation and molecular characterization of hydrocarbon degrading bacteria from tannery effluent. Int. J. Plant Anim. Environ. Sci. 1: 110-114.

Chenier, M. R., Beaumier, D., Roy, R., Driscoll, B. T., Lawrence, J. R. and Greer, C. W. (2003) Impact of seasonal variations and nutrient inputs on nitrogen cycling and degradation of hexadecane by replicated river biofilms. Appl. Environ. Microbiol. 69: 5170-5177.

Chikere, C., Chikere, B. and Okpokwasili, G. (2012) Bioreactor based bioremediation of hydrocarbon polluted Niger Delta marine sediment, Nigeria. 3 Biotech. 2: 53-66.

Chikere, C., Okpokwasili, G. and Chikere, B. (2011) Monitoring of microbial hydrocarbon remediation in the soil. 3 Biotech. 1: 117-138.

Christo, I. E. (2010) Effects of crude oil polluted soil on the germination and yield of mungbean *(Vigna radiata(L) WIL C.)* and groundnut *(Arachis hypogaea)*. Int. Sci. Res. J. 2: 70-73.

Ciric, L., Philp, J. C. and Whiteley, A. S. (2010) Hydrocarbon utilization within a diesel degrading bacterial consortium. FEMS Microbiol. Lett. 303: 116-122.

Cooper, D. G. and Goldenberg, B. G. (1987) Surface active agents from two *Bacillus* species. Appl. Environ. Microbiol. 53: 224-229.

Coral, G. and Karagoz, S. (2005) Isolation and characterization of phenanthrene degrading bacteria from a petroleum refinery soil. Ann. Microbiol. 55: 255-259.

Castorena-Cortés, G., Roldán-Carrillo, T., Zapata-Peñasco, I., Reyes-Avila, J., Quej-Aké, L., Marín-Cruz, J. and Olguín-Lora, P. (2009) Microcosm assays and Taguchi experimental design for treatment of oil sludge containing high concentration of hydrocarbons. Bioresour. Technol. 100: 5671-5677.

Coulon, F., Pelletier, E., Gourhant, L. and Delille, D. (2005) Effects of nutrient and temperature on degradation of petroleum hydrocarbons in contaminated sub Antarctic soil. Chemosphere. 58: 1439-1448.

Csutak, O., Stoica, I., Ghindea, R., Tanase, V. and Vassu, T. (2010) Insights on yeast bioremediation processes. Rom. Biotechnol. Lett. 15: 5066-5071.

Das, K. and Mukherjee, A. K. (2006) Crude petroleum oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum oil contaminated soil from North-East India. Bioresour. Technol. 98: 1339-1345.

Davies, J. and Evans, W. (1964) Oxidative metabolism of naphthalene by soil Pseudomonads. Biochem. J. 91: 251-261.

Decorosi, F., Mengoni, A., Baldi, F. and Fani, R. (2006) Identification of alkane monoxygenase genes in *Acinetobacter venetianus* VE-C3 and analysis of mutants impaired in diesel fuel degradation. Ann. Microbiol. 56: 207-214.

Demirel, Y. (2012) Energy: Production, Conversion, Storage, Conservation, and Coupling. Green Energy and Technology. Springer-Verlag London Limited. pp. 27-70.

Dennis, J. J. and Zylstra, G. J. (2004) Complete sequence and genetic organization of pDTG1, the 83 kilobase naphthalene degradation plasmid from *Pseudomonas putida* strain NCIB 9816-4. J. Mol. Biol. 341:753-768.

Diab, E. (2008) Phytoremediation of oil contaminated desert soil using the rhizosphere effects of some plants. Res. J. Agric. Biol. Sci. 4: 604-610.

Diaz, E. (2004) Bacterial degradation of aromatic pollutants: a paradigm of metabolic versatility. Int. Microbiol. 7: 173-180.

Ehiagbonare, J. E., Obayuwana, S., Aborisade, W. T. and Asogwa, I. (2011) Effect of unspent and spent diesel fuel on two agricultural crop plants: *Arachis hypogea* and *Zea mays*. Sci. Res. Essays 6: 2296-2301.

Evans, F. F., Rosado, A. S., Sebastian, G. V., Casella, R., Pedro, Machado, L. O. A., Holmstrom, C., Kjelleberg, S., van Elsas, J. D. and Seldin, L. (2004) Impact of oil contamination and biostimulation on the diversity of indigenous bacterial communities in soil microcosms. FEMS Microbiol. Ecol. 49: 295-305.

Fedorak, P. M. and Westlake, D. W. (1981) Microbial degradation of aromatics and saturates in Prudhoe Bay crude oil as determined by glass capillary gas chromatography. Can. J. Microbiol. 27: 432-43.

Feng, L., Wang, W., Cheng, J., Ren, Y., Zhao, G., Gao, C., Tang, Y., Liu, X., Han, W., Peng, X., Liu, R. and Wang, L. (2007) Genome and proteome of long chain alkane degrading *Geobacillus thermodenitrificans* NG80-2 isolated from a deep-subsurface oil reservoir. Proc. Natl. Acad. Sci. U.S.A. 104: 5602-5607.

Foght, J. (2008) Anaerobic Biodegradation of aromatic hydrocarbons: pathways and prospects. J. Mol. Microbiol. Biotechnol. 15: 93-120.

Forney, F. W., and Markovetz, A. J. (1970) Subterminal oxidation of aliphatic hydrocarbons. J. Bacteriol. 102: 281-282.

Fox, B., Froland, W., Dege, J. and Lipscombs, J. (1989) Methane monooxygenase from *Methylosinus trichosporium* OB3b. J. Biol. Chem. 264: 10023-10033.

Gallego, J. L., Lpredo, R. J., Llamas, J. F., Vazquez, F. and Sanchez, J. (2001) Bioremediation of diesel-contaminated soils: Evaluation of potential *in situ* techniques by study of bacterial degradation. Biodegradation 12: 325-335.

Garg, P. (2012) Energy scenario and vision 2020 in India. J. Sustainable Energy Environ. 3: 7-17.

Geißdo¨rfer, W., Kok, R., Ratajczak, A., Hellingwerf, K. and Hillen, W. (1999) The Genes *rubA* and *rubB* for alkane degradation in *Acinetobacter* sp. strain ADP1 are in an operon with *estB*, encoding an esterase, and *oxyR*. J. Bacteriol. 181: 4292-4298.

Gentry, T., Rensing, C. and Pepper, I. (2004) New approaches for bioaugmentation as a remediation technology. Crit. Rev. Environ. Sci. Tech. 34: 447-494.

Gill, C. and Ratledge, C. (1972) Toxicity of *n*-alkanes, *n*-alk-l-enes, *n*-alkan-1-o1s and *n*-alkyl -l-bromide towards yeasts. J. Gen. Microbiol. 72: 165-172.

Glick, B. R. (2003) Phytoremediation: synergistic use of plants and bacteria to clean up the environment. Biotechnol. Adv. 21: 383-393.

Glieder, A., Farinas, E. T. and Arnold, F. H. (2002) Laboratory evolution of a soluble, self sufficient, highly active alkane hydroxylase. Nat. Biotechnol. 20: 1135-1139.

Greene, E. A., Kay, J. G., Jaber, K., Stehmeier, L. G. and Voordouw, G. (2000) Composition of soil microbial communities enriched on a mixture of aromatic hydrocarbons. Appl. Environ. Microbiol. 66: 5282-5289.

Grimm, A. and Harwood, C. (1997) NahY, a catabolic plasmid-encoded receptor required for chemotaxis of *Pseudomonas putida* to the aromatic hydrocarbon naphthalene. Appl. Environ. Microbiol. 83: 4111-4115.

Grimmer, G., Brune, H., Dettbarn, G., Jacob, J., Misfeld, J., Mohr, U., Naujack, K. W, Timm, J. and Wenzel-Hartung, R. (1991) Relevance of polycyclic aromatic hydrocarbons as environmental carcinogens. Fresenius J. Anal. Chem. 339: 792-795.

Gurjar, M., Khire, J. M. and Khan, M. I. (1995) Bioemulsifier production by *Bacillus stearothermophilus* VR-8 isolate. Lett. Appl. Microbiol. 21: 83-86.

Hadibarata, T. and Tachibana, S. (2009) Microbial degradation of *n*-eicosane by filamentous fungi. In: Interdisciplinary studies on environmental chemistry-environmental research in Asia (eds. Y. Obayashi, T. Isobe, A. Subramanian, S. Suzuki, S. Tanabe), Terrapub, pp. 322-329.

Hakemian, A. and Rosenzweig, A. (2007) The biochemistry of methane oxidation. Ann. Rev. Biochem. 76: 223–241.

Harayama, S., Kishira, H., Kasai, Y. and Shutsubo, K. (1999) Petroleum biodegradation in marine environments. J. Mol. Microbiol. Biotechnol. 1: 63-70.

Hasanuzzaman, M., Ueno, A., Ito, H., Ito, Y., Yamamoto, Y., Yumoto, I. and Okuyama, H. (2007) Degradation of long chain *n*-alkanes (C_{36} and C_{40}) by *Pseudomonas aeruginosa* strain WatG. Int. Biodeter. Biodegr. 59: 40-43.

Heider, J., Spormann, A., Beller, H. and Widdel, F. (1999) Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiol. Rev. 22: 459-473.

Hofman, J., Svihalek, J. and Holoubek, I. (2004) Evaluation of functional diversity of soil microbial communities - a case study. Plant Soil Environ. 50: 141-148.

Houshmandfar, A. and Asli, D. E. (2011) Seed germination and seedling growth of wheat, barley, alfalfa and clover as affected by gasoline and diesel fuel mixture. Adv. Environ. Biol. 5: 1250-1255.

Huang, L., Ma, T., Li, D., Liang, F., Liu, R. and Li, G. (2008) Optimization of nutrient component for diesel oil degradation by *Rhodococcus erythropolis*. Marine Pollut. Bull. 56: 1714-1718.

Husain, I. A. F., Alkhatib, M., Zahangir Alam M. D. and Muyibi, S. A. (2011) Kinetic study of a bacterial consortium isolated from soil contaminated with crude oil. Aust. J. Basic Appl. Sc. 5: 925-930.

Husain, S. (2008) Literature overview: Microbial metabolism of high molecular weight polycyclic aromatic hydrocarbons. Remed. J.18: 131-160.

Iida, T., Sumita, T., Ohta, A. and Takagi, M. (2000) The cytochrome P450ALK multigene family of an *n*-alkane-assimilating yeast, *Yarrowia lipolytica*: cloning and characterization of genes coding for new CYP52 family members. Yeast. 16: 1077-1087.

Isikhuemhen, O., Anoliefo, G. and Oghale, O. (2003) Bioremediation of crude oil polluted soil by the white rot fungus *Pleurotus tuber-regium* (Fr.) Sing. Environ. Sci. Pollut. Res. 10: 108-112.

Iturbe, R., Flores, C., Chavez, C., Bautista, G. and Torres, L. (2004a) Remediation of contaminated soil using soil washing and biopile methodologies at a field level. J. Soils Sediments 4: 115-122.

Iturbe, R., Flores, C., Chávez, C., Ramírez, A. and Torres, L. G. (2004b) *In Situ* flushing of contaminated soils from a refinery: Organic compounds and metal removals. Remed. J. 14: 141-152.

Iwamoto, T., Tani, K., Nakamura, K., Suzuki, Y., Kitagawa, M., Eguchi, M. and Nasu, M. (2000) Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE. FEMS Microbiol. Ecol. 32: 129-141.

Jadhav, V. V., Yadav, A., Shouche, Y. S., Aphale, S., Moghe, A., Pillai, S., Arora, A. and Bhadekar, R. K. (2013) Studies on biosurfactant from *Oceanobacillus* sp. BR1 10 isolated from Antarctic sea water. Desalination. 318: 64-71.

Jagadevan, S. and Mukherji, S. (2004) Successful *in situ* oil bioremediation programmes – key parameters. Ind. J. Biotechnol. 3: 495-501.

Jain, P., Gupta, V., Gaur, R., Lowry, M., Jaroli D. and Chauhan, U. (2011) Bioremediation of petroleum oil contaminated soil and water. Res. J. Environ. Toxicol. 5: 1-26.

John, R. C. and Okpokwasili, G. C. (2012) Crude oil-degradation and plasmid profile of nitrifying bacteria isolated from oil-impacted mangrove sediment in the Niger Delta of Nigeria. Bull. Environ. Contam. Toxicol. 88: 1020-1026.

Johnson, V., Singh, M. and Saini, V. S. (1992) Bioemulsifier production by an oleaginous yeast *Rhodotorula glutinis* IIP-30. Biotechnol. Lett. 14: 487-490.

Jorgensen, K., Puustinen, J. and Suortti, A. (2000) Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles. Environ. Pollut. 107: 245-254.

Jung, S. Y., Lee, J. H., Chai, Y. G. and Kim, S. J. (2005) Monitoring of microorganisms added into oil contaminated microenvironments by Terminal Restriction Fragment Length Polymorphism analysis. J. Microbiol. Biotechnol. 15: 1170-1177.

Ka, J., Yu, Z. and Mohn, W. (2001) Monitoring the size and metabolic activity of the bacterial community during biostimulation of fuel-contaminated soil using competitive PCR and RT-PCR. Microb. Ecol. 42: 267-273.

Kado, C. I. and Liu, S. T. (1981) Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145: 1365-73.

Kao, C., Chen, C., Chen, S., Chien, H. and Chen, Y. (2008) Application of *in situ* biosparging to remediate a petroleum-hydrocarbon spill site: Field and microbial evaluation. Chemosphere. 70: 1492-1499.

Kaplan, C. W. and Kitts, C. L. (2004) Bacterial succession in a petroleum land treatment unit. Appl. Environ. Microbiol. 70: 1777-1786.

Karanth, N. G. K., Deo, P. G. and Veenanadig, N. K. (1999) Microbial production of biosurfactant and their importance. Ferment. Sci. Technol. 77: 116-126.

Katsivela, E., Moore, E., Maroukli, D., Strömpl, C., Pieper, D. and Kalogerakis, N. (2005) Bacterial community dynamics during *in situ* bioremediation of petroleum waste sludge in landfarming sites. Biodegradation 16: 169-180.

Kelley, I., Freeman, J. and Cerniglia, C. (1990) Identification of metabolites from degradation of naphthalene by a *Mycobacterium* sp. Biodegradation 1: 283-290.

Kibblewhite, M. G., Ritz, K. and Swift, M. J. (2008) Soil health in agricultural systems. Phil. Trans. R. Soc. B 363: 685-701.

Ko, S. H. and Lebeault, J. M. (1999) Effect of a mixed culture on co-oxidation during the degradation of saturated hydrocarbon mixture. J. Appl. Microbiol. 87: 72-79.

Kohno, T., Sugimoto, Y., Sei, K. and Mori, K. (2002) Design of PCR primers and gene probes for general detection of alkane degrading bacteria. Microb. Environ. 17: 114-121.

Krieg, N. R. and Holt, J. G. (1984) Bergey's Manual of Systematic Bacteriology, Vol. 1, eds: Williams and Wilkins. Baltimore.

Kuiper, I., Lagendijk, E., Bloemberg, G. Lugtenberg, B. (2004) Rhizoremediation: A beneficial plant-microbe interaction. Mol. Plant-Microbe Interact. 17: 6-15.

Kumar, A., Bisht, B. S., Joshi., V. D. and Dhewa, T. (2011) Review on bioremediation of polluted environment: A management tool. Int. J. Environ. Sci. 1: 1079-1093.

Kuo, Y., Wang, S., Kao, C., Chen, C. and Sung, W. (2011) Using enhanced landfarming system to remediate diesel oil contaminated soils. Appl. Mech. Materials 121-126: 554-558.

Kwapisz, E., Wszelaka, J. Marchut, O. and Bielecki, S. (2008) The effect of nitrate and ammonium ions on kinetics of diesel oil degradation by *Gordonia alkanivorans* S7. Int. Biodeterior. Biodegrad. 61: 214-222.

Ladd, J. N. (1956) The oxidation of hydrocarbons by soil bacteria. Aust. J. Biol. Sci. 9: 92-97.

Lal, B. and Khanna, S. (1996) Degradation of crude oil by *Acinetobacter calcoaceticus* and *Alcaligenes odorans*. J. Appl. Bacteriol. 81: 355-362.

Leahy, J. G. and Colwell, R. R. (1990) Microbial degradation of hydrocarbons in the environment. Appl. Environ. Microbiol. 54: 305-315.

Lee, K. E. (1994) The biodiversity of soil organisms. Appl. Soil Ecol. 1: 251-254.

Lee, M. and Swindoll, C. (1993) Bioventing for *in situ* remediation. Hydrological Sci. 38: 4-8.

Lee, S. H., Lee, W. S., Lee, C. H. and Kim, J. G. (2008) Degradation of phenanthrene and pyrene in rhizosphere of grasses and legumes. J. Hazard. Mater. 153: 892-898.

Li, P., Wang, L. and Feng, L. (2013) Characterization of a novel Rieske type alkane monooxygenase system in *Pusillimonas* sp. strain T7-7. J. Bacteriol. 195:1892-1901.

Lin, Q. and Mendelssohn, I. (2009) Potential of restoration and phytoremediation with *Juncus roemerianus* for diesel contaminated coastal wetlands. Ecol. Engineering. 35: 85-91.

Lin, T. C., Young, C. C., Ho, M. J., Yeh, M. S., Chou, C. L., Wei, Y. H. and Chang, J. S. (2005) Characterization of floating activity of indigenous diesel assimilating bacterial isolates. J. Biosci. Bioeng. 99: 466-472.

Liu, C. and Shao, Z. (2005) *Alcanivorax dieselolei* sp. nov., a novel alkane-degrading bacterium isolated from sea water and deep-sea sediment. Int. J. Syst. Evol. Microbiol. 55: 1181-1186.

Liu, W., Luo, Y., Teng, Y., Li, Z., and Ma, L.Q. (2010) Bioremediation of oily sludge-contaminated soil by biostimulating indigenous microbes. Environ. Geochem. Health. 32: 23-29.

Lodder, J. (1970) In: Lodder, J., Kregger, V. R. (Eds.), The Yeast: A Taxonomic Study. North Holland Publishing Co., Holland, 36-104.

Lotfinasabasl, S., Gunale, V. R. and Rajurkar, N. S. (2013) Petroleum hydrocarbons pollution in soil and its bioaccumulation in mangrove species, *Avicennia marina* from Alibaug mangrove ecosystem, Maharashtra, India. Int. J. Advancements in Res. Technol. 2: 1-7.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Macnaughton, S. J., Stephen, J. R., Venosa, A. D., Davis, G. A., Chang, Y. J. and White, D. C. (1999) Microbial population changes during bioremediation of an experimental oil spill. Appl. Environ. Microbiol. 65: 3566-3574.

Maeng, J., Sakai, Y. Tani, Y. and Kato, N. (1996) Isolation and characterization of a novel oxygenase that catalyzes the first step of *n*-alkane oxidation in *Acinetobacter* sp. strain M-1. J. Bacteriol. 178: 3695-3700.

Mara, K., Decorosi, F., Viti, C., Giovannetti, L., Papaleo, M. C., Maida, I., Perrin, E., Fondi, M., Vaneechoutte, M., Nemec, A., van den Barselaar, M., Dijkshoorn, L. and Fani, R. (2012) Molecular and phenotypic characterization of *Acinetobacter* strains able to degrade diesel fuel. Res. Microbiol. 163:161-172.

Mau, L. and Yue, Q. (2010) Remediation of diesel contaminated soil by bioventing and composting technology. In: Proceedings of International Conference on Challenges in Environmental Science and Computer Engineering. 2: 3-6.

Martins, F. F., Ferreiraa, T. F., Azevedob, D. A. and Coelhoa, M. A. Z. (2012) Evaluation of crude oil degradation by *Yarrowia lipolytica*. Chem. Eng. Trans. 27: 223-228.

Massaud, M. S., Al-Sarawi, M. and Wahba, S. A. (2000) Variations in the chemical properties of soils contaminated with oil lakes in the greater Burgan oil fields, Kuwait. Water Air Soil Pollut. 118: 281-297.

Mauersberger, S. Schunck, W. and Muller, H. (1984) The induction of cytochrome P-450 in the alkane utilizing yeast *Lodderomyces elongisporus*: Alterations in the microsomal membrane fraction. Appl. Microbiol. Biotechnol. 19:29-35.

Meckenstock, R., Annweiler, E., Michaelis, W., Richnow, H. and Schink, B. (2000) Anaerobic naphthalene degradation by a sulfate reducing enrichment culture. Appl. Environ. Microbiol. 66: 2743-2747.

Mengoni, A., Ricci1, S. Brilli, M., Baldi, F. and Fani, R. (2007) Sequencing and analysis of plasmids pAV1 and pAV2 of *Acinetobacter venetianus* VE-C3 involved in diesel fuel degradation. Ann. Microbiol. 57: 521-526.

Minerdi, D., Zgrablic, I., Sadeghi, S. J. and Gilardi, G. (2012) Identification of a novel Baeyer-Villiger monooxygenase from *Acinetobacter radioresistens*: close relationship to the *Mycobacterium tuberculosis* prodrug activator EtaA. Microb. Biotechnol. 5: 700-716.

Miranda, R., Silva de Souza, C., Gomes, E., Lovaglio, R., Lopes, C. Vieira de Queiroz Sousa, M. (2007) Biodegradation of Diesel Oil by Yeasts Isolated from the vicinity of Suape Port in the State of Pernambuco –Brazil. Braz. Arch. Biol. Technol. 50: 147-152.

Mirdamadian, S. H., Emtiazi, G., Golabi, M. H. and Ghanavati, H. (2010) Biodegradation of petroleum and aromatic hydrocarbons by bacteria isolated from petroleum contaminated soil. J. Pet. Environ. Biotechnol. 1: 1-5.

Mishra, S., Jyot, J., Kuhad, R. and Lal, B. (2001) Evaluation of inoculum addition to stimulate *in situ* bioremediation of oily sludge contaminated soil. Appl. Environ. Microbiol. 67: 1675–1681.

Mishra, S. and Singh, S. N. (2012) Microbial degradation of *n*-hexadecane in mineral salt medium as mediated by degradative enzymes. Bioresour. Technol. 111: 148-154.

Mohajeri, L., Aziz, H., Isa, M. and Zahed, M. (2010) A statistical experiment design approach for optimizing biodegradation of weathered crude oil in coastal sediments. Bioresour. Technol. 101: 893-900.

Mohanty, G. and Mukherji, S. (2008) Biodegradation rate of diesel range *n*-alkanes by bacterial cultures *Exiguobacterium aurantiacum* and *Burkholderia cepacia* . Int. Biodeterior. Biodegrad. 61: 240-250.

Mohn, W., Radziminski, C., Fortin, M. and Reimer, K. (2001) On site bioremediation of hydrocarbon-contaminated Arctic tundra soils in inoculated biopiles. Appl. Microbiol. Biotechnol. 57: 242-247.

Molina-Barahona, L., Rodriguez-Vazquez, R., Hernandez-Velasco, M., Vega-Jarquin, C., Zapata-Perez, O., Mendoza-Cantu, A. and Albores, A. (2004) Diesel removal from contaminated soils by biostimulation and supplementation with crop residues. Appl. Soil Ecol. 27: 165-175.

Moller, J., Winther, P., Lund, B., Kirkebjerg, K. and Westermann, P. (1996) Bioventing of diesel oil contaminated soil: comparison of degradation rates in soil based on actual oil concentration and on respirometric data. J. Indust. Microbiol. 16: 110-116.

Mukherjee, A. K. and Bordoloi, N. K. (2011) Bioremediation and reclamation of soil contaminated with petroleum oil hydrocarbons by exogenously seeded bacterial consortium: a pilot-scale study. Environ. Sci. Pollut. Res. Int. 18: 471-478.

Mukherji, S., Jagadevan, S., Mohapatra, G., Vijay, A. (2004) Biodegradation of diesel oil by an Arabian Sea sediment culture isolated from the vicinity of an oil field. Bioresour. Technol. 95: 281-286.

Nannipieri, P., Ascher, J., Ceccherini, M. T., Landi, L., Pietramellara, G. and Renella, G. (2003) Microbial diversity and soil functions. Eur. J. Soil Sci. 54: 655-670.

Nicolotti, G. and Egli, S. (1998) Soil contamination by crude oil: impact on the mycorrhizosphere and on the vegetation potential of forest trees. Environ. Pollut. 99: 37-43.

Njoku, K. L., Akinola, M. O. and Ige, T. O. (2009a) Comparative effects of diesel fuel and spent lubricating oil on the growth of *Zea Mays* (Maize). Amer-Eurasian J. Sustain. Agric. 3: 428-434.

Njoku, K.L., Akinola, M. O. and Taiwo, B. G. (2009b) Effect of gasoline diesel fuel mixture on the germination and the growth of *Vigna unguiculata* (Cowpea). Afr. J. Environ. Sci. Technol. 3: 466-471.

Norman, R. S., Frontera-Suau, R. and Morris, P. J., (2002) Variability in *Pseudomonas aeruginosa* lipopolysaccharide expression during crude oil degradation. Appl. Environ. Microbiol. 68: 5096-5103.

Nwaoguikpe, R. N. (2011) The effect of crude oil spill on the ascorbic acid content of some selected vegetable species: *Spinacea oleraceae*, *Solanum melongena* and *Talinum triangulare* in an oil polluted soil. Pakistan J. Nutr. 10: 274-281.

Obire, O. and Anyanwu, E. (2009) Impact of various concentrations of crude oil on fungal populations of soil. Int. J. Environ. Sci. Tech. 6: 211-218.

Ogbo, E. M. (2009) Effects of diesel fuel contamination on seed germination of four crop plants – *Arachis hypogaea, Vigna unguiculata, Sorghum bicolor* and *Zea mays*. Afr. J. Biotechnol. 8: 250-253.

Ogbo, E. M., Tabuanu, A. and Ubebe, R. (2010) Phytotoxicity assay of diesel fuel-spiked substrates remediated with *Pleurotus tuberregium* using *Zea mays*. Int. J. Appl. Res. Nat. Prod. 3: 12-16.

Ogino, A., Koshikawa, H., Nakahara, T. and Uchiyama, H. (2001) Succession of microbial communities during a biostimulation process as evaluated by DGGE and clone library analyses. *J. Appl. Microbiol.* 91: 625-635.

Okoh, A. I. (2006) Biodegradation alternative in the cleanup of petroleum hydrocarbon pollutants. Biotechnol. Mol. Biol. Rev. 1: 38-50.

Olajide, P. O. and Ogbeifun, L. B. (2010) Hydrocarbon biodegrading potentials of a *Proteus vulgaris* strain isolated from fish samples. Am. J. Appl. Sci. 7: 922-928.

Osuji, L. and Nwoye, I. (2007) An appraisal of the impact of petroleum hydrocarbons on soil fertility: the Owaza experience. Afr. J. Agric. Res. 2: 318-324.

Ouyang, W., Liu, H., Murygina, V., Yu, Y., Ziu, Z. and Kalyguzhnyi, S. (2005) Comparison of bioaugmentation and composting of oily sludge: a field-scale study in China. Process Biochem. 40: 3761-3768.

Øvrea°s, L., Jensen, S., Daae, F. L. and Torsvik, V. (1998) Microbial community changes in a perturbed agricultural soil investigated by molecular and physiological approaches. Appl. Environ. Microbiol. 64: 2739-2742.

Pathak, H., Bhatnagar, K. and Jaroli, D. P. (2011) Physico-chemical properties of petroleum polluted soil collected from Transport Nagar (Jaipur). Ind. J. Fund. Appl. Life Sc. 1: 84-89.

Paudyn, K., Rutter, A., Kerry Rowe, R. and Poland, J. (2008) Remediation of hydrocarbon contaminated soils in the Canadian Arctic by landfarming. Cold Regions Sci. Technol. 53: 102-114.

Periasamy, R. and Palvannan, T. (2010) Optimization of laccase production by *Pleurotus ostreatus* IMI 395545 using the Taguchi DOE methodology. J. Basic Microbiol. 50: 548-556.

Phadke, M. S. (1989). Quality engineering using robust design. Prentice Hall, Englewood Cliffs, New Jersey. pp. 41-65.

Plaza, G., Jangid, K., Lukasik, K., Nalecz-Jawecki, G., Berry, C. and Brigmon, R. L. (2008) Reduction of petroleum hydrocarbons and toxicity in refinery wastewater by bioremediation. Bull. Environ. Contam. Toxicol. 81: 329-333.

Polissi, A., Bestetti, G., Bertoni, G., Galli, E. and Deho, G. (1990) Genetic analysis of chromosomal operons involved in degradation of aromatic hydrocarbons in *Pseudomonas putida* TMB. J. Bacteriol. 172: 6355-6362.

Popp, N., Schlomann, M. and Mau, M. (2006) Bacterial diversity in the active stage of a bioremediation system for mineral oil hydrocarbon contaminated soils. Microbiol. 152: 3291-3304.

Prabhu, Y. and Phale, P. S. (2003) Biodegradation of phenanthrene by *Pseudomonas* sp. strain PP2: novel metabolic pathway, role of biosurfactant and cell surface hydrophobicity in hydrocarbon assimilation. Appl. Microbiol. Biotechnol. 61: 342-351.

Pradhan, S., Conrad, J., Paterek, J. and Srivastava, V. (1998) Potential of phytoremediation for treatment of PAHs in soil at MGP sites. J. Soil Contam. 7: 467-480.

Pruthi, V. and Cameotra, S. (1997) Rapid identification of biosurfactant producing bacterial strains using a cell surface hydrophobicity technique. Biotechnol Tech. 11: 671-674.

Rahman, K. S., Banat, I. M., Thahira, J., Thayumanavan, T. and Lakshmanaperumalsamy, P. (2002) Bioremediation of gasoline contaminated soil by a bacterial consortium amended with poultry litter, coir pith and rhamnolipid biosurfactant. Bioresour. Technol. 81: 25-32.

Ranjard, L., Nazaret, S., Gourbie`re, F., Thioulouse, J., Linet, P. and Richaume, A. (2000) A soil microscale study to reveal the heterogeneity of Hg(II) impact on indigenous bacteria by quantification of adapted phenotypes and analysis of community DNA fingerprints. FEMS Microbiol. Ecol. 31: 107-115.

Rao, R., Kumar, C., Prakasham, S. and Hobbs, P. (2008) The Taguchi methodology as a statistical tool for biotechnological applications: A critical appraisal. Biotechnol. J. 3: 510-523.

Ratajczak, A., Geibdo¨rfer, W. and Hillen, W. (1998) Alkane hydroxylase from *Acinetobacter* sp. strain ADP1 is encoded by *alk*M and belongs to a new family or bacterial integral membrane hydrocarbon hydroxylases. Appl. Environ. Microbiol. 64: 1175-1179.

Raymond, R. L., Hudson, J. O. and Jamison, V. W. (1976) Oil degradation in soil. Appl. Environ. Microbiol. 31: 522-532.

Robertson, S. J., McGill, W. B., Massicotte, H. B. and Rutherford, P. M. (2007) Petroleum hydrocarbon contamination in boreal forest soils: A mycorrhizal ecosystems perspective. Biol. Rev. 82: 213-240.

Rojo, F. (2009) Degradation of alkanes by bacteria. Environ. Microbiol. 11: 2477-2490.

Rossello-Mora, R. A., Lalucat, J. and Garcia-Valdes, E. (1994) Comparative biochemical and genetic analysis of naphthalene degradation among *Pseudomonas stutzeri* strains. Appl. Environ. Microbiol. 60: 966-972.

Rosenberg, M. (1984) Bacterial adherence to hydrocarbons; a useful technique for studying cell hydrophobicity. FEMS Microbiol. Lett. 22: 289-295.

Saadoun, I and Al-Ghazawi, Z. (2010) Toxicity of diesel fuel towards plant seeds as reflected by seed germination outcomes, sprout length and fresh weight. Amer-Eurasian J. Agric. Environ. Sci. 8: 167-172.

Safinowski, M. and Meckenstock, R. (2006) Methylation is the initial reaction in anaerobic naphthalene degradation by a sulfate reducing enrichment culture. Environ. Microbiol. 8: 347-352.

Sakai, Y., Maeng, J. H., Tani, Y. and Kato, N., (1994) Use of long chain *n*-alkanes (C_{13}-C_{44}) by an isolate, *Acinetobacter* sp. M-1. Biosci. Biotechnol. Biochem. 58: 2128-2130.

Salminen, J. and Haimi, J. (1997) Effects of pentachlorophenol on soil organisms and decomposition in forest soil. J. Appl. Ecol. 34: 101-110.

Sambrook, J.; Maccallum, P. and Russell, D. (2001) Molecular Cloning: A Laboratory Manual. 3rd. Cold Spring Harbor Press, NY.

Sathishkumar, M., Binupriya, A., Baik S. and Yun, S. (2008) Biodegradation of crude oil by individual bacterial strains and a mixed bacterial consortium isolated from hydrocarbon contaminated areas. Clean. 36: 92-96.

Satpute, S. K., Dhakephalkar, P. K., Banpurkar, A. G., Banat, I. B. and Chopade, B. A. (2010) Methods for investigating biosurfactants and bioemulsifiers: A review. Crit. Rev. Biotechnol. 30: 127-144.

Schippers, A., Bosecker, K., Spröer, C. and Schumann, P. (2005) *Microbacterium oleivorans* sp. nov. and *Microbacterium hydrocarbonoxydans* sp. nov., novel crude oil degrading gram positive bacteria. Int. J. Syst. Evol. Microbiol. 55: 655-660.

Schutzendubel, A., Majcherczyk, A., Johannes, C. and Hüttermann, A. (1999) Degradation of fluorene, anthracene, phenanthrene, fluoranthene, and pyrene lacks connection to the production of extracellular enzymes by *Pleurotus ostreatus* and *Bjerkandera adusta*. Int. Biodeterior. Biodegrad. 43: 93-100.

Schwab, A. P., Su , J., Wetzel, S., Pekarek, S. and Banks, M. K. (1999) Extraction of petroleum hydrocarbons from soil by mechanical shaking. Environ. Sci. Technol. 33: 1940-1945.

Seghers, D., Bulcke, R., Reheul, D., Siciliano, S. D., Top, E. M. and Verstraete, W. (2003) Pollution induced community tolerance (PICT) and analysis of 16S rRNA genes to evaluate the long-term effects of herbicides on methanotrophic communities in soil. Eur. J. Soil Sci. 54: 679-684.

Seo, J. S., Keum, Y. S. and Li, Q. X. (2009) Bacterial degradation of aromatic compounds. Int. J. Environ. Res. Public Health. 6: 278-309.

Serrano, A., Gallego, M., González, J. and Tejada, M. (2008) Natural attenuation of diesel aliphatic hydrocarbons in contaminated agricultural soil. Environ. Pollut. 151: 494-502.

Sharifi, M., Sadeghi, Y. and Akharpour, M. (2007) Germination and growth of six plant species on contaminated soil with spent oil. Int. J. Environ. Sci. Technol. 4: 463-470.

Shukor, M., Hassan, N., Jusoh, A., Perumal, N., Shamaan, N., MacCormack, W. and Syed, M. (2009) Characterization of a diesel degrading strain isolated from a hydrocarbon contaminated site. J. Environ. Biol. 30: 1-6.

Siddiqui, S., Adams, W. and Schollion, J. (2001) The phytoxicity and degradation of diesel hydrocarbons in soil. J. Plant Nutr. Soil Sci. 164: 631-635.

Sikkema, J., Bont, J. and Poolman, B. (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. 59: 201-222.

Smith, K., Costello, A. and Lidstrom, M. (1997) Methane and trichloroethylene oxidation by an estuarine methanotroph, M*ethylobacter* sp. strain bb5.1. Appl. Environ. Microbiol. 63: 4617-4620.

Smits, T. H, Röthlisberger, M, Witholt, B. and van Beilen, J. B. (1999) Molecular screening for alkane hydroxylase genes in Gram negative and Gram positive strains. Environ. Microbiol. 1: 307-317.

Snape, I., Riddle, M. J., Stark, J. S., Cole, C. M., King, C. K., Duquesne, S. and Gore, D. B. (2001) Management and remediation of contaminated sites at Casey Station, Antarctica. Polar Rec. 37: 199-214.

Sohn, J. H., Kwon, K. K., Kang, J. H., Jung, H. B. and Kim, S. J. (2004) *Novosphingobium pentaromativorans* sp. nov., a high molecular mass polycyclic aromatic hydrocarbon degrading bacterium isolated from estuarine sediment. Int. J. Syst. Evol. Microbiol. 54: 1483-1487.

Sood, N. and Lal, B. (2009) Isolation of a novel yeast strain *Candida digboiensis* TERI ASN6 capable of degrading petroleum hydrocarbons in acidic conditions. J. Environ. Manage. 90: 1728-1736.

Spinelli, L., Schnaid, F., Selbach, P., Bento, F. and Oliveira, J. (2005) Enhancing bioremediation of diesel oil and gasoline in soil amended with an agroindustry sludge. J. Air Waste Manage. Assoc. 55: 421-429.

Staijen, I. E., van Beilen, J. B. and Witholt, B. (2000) Expression, stability and performance of the three component alkane monooxygenase of *Pseudomonas oleovorans* in *Escherichia coli*. Eur. J. Biochem. 267: 1957-1965.

Subba, R. M., Kishore, G. and Rambabu, C. (2010) Plasmid mediated Naphthalene Degradation in *Pseudomonas* sp. strain NGK 1. Curr. Trends Biotechnol. Pharm. 4: 702-707.

Tanee, F. and Akonye, L. (2009) Effectiveness of *Vigna unguiculata* as a phytoremediation plant in the remediation of crude oil polluted soil for Cassava (*Manihot esculenta*; Crantz) cultivation. J. Appl. Sci. Environ. Manage. 13: 43-47.

Tani, A., Ishige, T., Sakai, Y. and Kato, N. (2001) Gene structures and regulation of the alkane hydroxylase complex in *Acinetobacter* sp. strain M-1. J. Bacteriol. 183: 1819-1823.

Throne-Holst, M., Markussen, S., Winnberg, A., Ellingsen, T. E., Kotlar, H. K. and Zotchev. S. B. (2006) Utilization of *n*-alkanes by a newly isolated strain of *Acinetobacter venetianus*: the role of two AlkB-type alkane hydroxylases. Appl. Microbiol. Biotechnol. 72: 353–360.

Tondo, E. C., Andretta, C. W. S., Souza, C. F. V., Monteiro, A. L., Henriques, J. A. P. and Ayub, M. A. Z. (1998) High biodegradation levels of 4,5,6-trichloroguaiacol by *Bacillus* sp. isolated from cellulose pulp mill effluent. Rev. Microbiol. 29: 265-271.

Townsend, G., Prince, R. and Suflita, J. (2004) Anaerobic biodegradation of alicyclic constituents of gasoline and natural gas condensate by bacteria from an anoxic aquifer. FEMS Microbiol. Ecol. 49: 129-135.

Trapp, S., Köhler, A., Larsen, L., Zambrano, K. and Karlson, U. (2001) Phytotoxicity of fresh and weathered diesel and gasoline to willow and poplar trees. J. Soils Sediments 1: 71-76.

Trofimov, S. Y. and Rozanova, M. S. (2003) Transformation of soil properties under the impact of oil pollution. Eurasian Soil Sci. 36: 82-87.

Tupe, S., Rajwade, J. and Paknikar, K. (2007) Taguchi approach significantly increases bioremediation process efficiency: a case study with Hg (II) removal by *Pseudomonas aeruginosa.* Lett. Appl. Microbiol. 45: 36-41.

Uche, O. M., Owhondah, W. M. and Augustine, U. A. (2011) The Omoku old pipeline oil spill: Total hydrocarbon content of affected soils and the impact on the nutritive value of food crops. Archiv. Appl. Sc. Res. 3: 514-521.

Ueno, A., Hasanuzzaman, M., Yumoto, I. and Okuyama, H. (2006) Verification of degradation of *n*-alkanes in diesel oil by *Pseudomonas aeruginosa* strain WatG in soil microcosms. Curr. Microbiol. 52: 182-185.

Uribe, S., Rangel, P., Espinola, G. and Aguirre, G. (1990) Effects of cyclohexane, an industrial solvent, on the yeast *Saccharomyces cerevisiae* and on isolated yeast mitochondria. Appl. Environ. Microbiol. 56: 2114-2119.

van Beilen, J. and Funhoff, E. (2007) Alkane hydroxylases involved in microbial alkane degradation. Appl. Microbiol. Biotechnol. 74: 13-21.

van Beilen, J. B., Li1, Z., Duetz, W.A., Smits, T. H. M. and Witholt, B. (2003) Diversity of alkane hydroxylase systems in the environment. Oil Gas Sci. Technol. 58: 427-440.

van Beilen, J., Holtackers, R., Luscher, D., Bauer, U., Witholt, B. and Duetz, W. (2005) Biocatalytic production of perillyl alcohol from limonene by using a novel *Mycobacterium* sp. cytochrome P450 alkane hydroxylase expressed in *Pseudomonas putida.* Appl. Environ. Microbiol. 71: 1737-1744.

Van Hamme, J., Singh, A. and Ward, O. (2003) Recent advances in petroleum microbiology. Microbiol. Mol. Biol. Rev. 67: 503-549.

Vega-Jarquin, C., Dendooven, L., Magana-Plaza, I., Thalasso, F. and Ramos-Valdivia, A. (2001) Biotransformation of *n*-hexadecane by cell suspension cultures of *Cinchona robusta and Dioscorea composita*. Environ. Toxicol. Chem. 20: 2670-2675.

Verma, S., Bhargava, R. and Pruthi, V. (2006) Oily sludge degradation by bacteria from Ankleshwar, India. Int. Biodeterior. Biodegrad. 57: 207-213.

Vidali, M. (2001) Bioremediation. An overview. Pure Appl. Chem. 73: 1163-1172.

Vinas, M., Grifoll, M., Sabate, J. and Solanas, A. M. (2002) Biodegradation of a crude oil by three microbial consortia of different origins and metabolic capabilities. J. Ind. Microbiol. Biotechnol. 28: 252-260.

Vomberg, A. and Klinner, U. (2000) Distribution of alkB genes within *n*-alkane degrading bacteria. J. Appl. Microbiol. 89: 339-348.

Walker, J. D., Seesman, P. A. and Colwell, R. R. (1975) Effect of South Louisiana crude oil and No. 2 fuel oil on growth of heterotrophic microorganisms, including proteolytic, lipolytic, chitinolytic and cellulolytic bacteria. Environ. Pollut. 9: 13-33.

Wang, W., Wang, L. and Shao, Z. (2010) Diversity and abundance of oil degrading bacteria and alkane hydroxylase (alkB) genes in the subtropical seawater of Xiamen Island. Microb. Ecol. 60: 429-439.

Watanabe, K. (2001) Microorganisms relevant to bioremediation. Curr. Opin. Biotechnol. 12: 237-241.

Watkinson, R. and Morgan, P. (1990) Physiology of aliphatic hydrocarbon degrading microorganisms. Biodegradation 1: 79-92.

Wentzel, A., Ellingsen, T., Kotlar, H., Zotchev, S. and Throne-Holst, M. (2007) Bacterial metabolism of long chain *n*-alkanes. Appl. Microbiol. Biotechnol. 76: 1209-1221.

White, P., Wolf, D., Thoma, G. and Reynolds, C. (2006) Phytoremediation of alkylated polycyclic aromatic hydrocarbons in a crude oil-contaminated soil. Water Air Soil Pollut. 169: 207-220.

Whyte, L. G., Hawari, J., Zhou, E., Bourbonnière, L., Inniss, W. E., and Greer, C. W. (1998) Biodegradation of variable chain length alkanes at low temperatures by a psychrotrophic *Rhodococcus* sp. Appl. Environ. Microbiol. 64: 2578-2584.

Whyte, L., Bourbonniere, L. and Greer, C. (1997) Biodegradation of petroleum hydrocarbons by psychrotrophic *Pseudomonas* strains possessing both alkane (*alk*) and naphthalene (*nah*) catabolic pathways. Appl. Environ. Microbiol. 63: 3719-3723.

Widdel, F. and Rabus, R. (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr. Opin. Biotechnol. 12: 259-276.

Wongsa, P., Tanaka, M., Ueno, A., Hasanuzzaman, M., Yumoto, I. and Okuyama, H. (2004) Isolation and characterization of novel strains of *Pseudomonas aeruginosa* and *Serratia marcescens* possessing high efficiency to degrade gasoline, kerosene, diesel oil, and lubricating oil. Curr. Microbiol. 49: 415-422.

Wook, R. H., Joo, Y. H., Youn-Joo, A. and Kyung-Suk, K. (2006) Isolation and characterization of psychrotrophic and halotolerant *Rhodococcus* sp. YHLT-2. J. Microbiol. Biotechnol. 16: 605-612.

Yamane, K., Maki, H., Nakayama, T., Nakajima, T., Nomura, N., Uchiyama, H. and Kitaoka, M. (2008) Diversity and similarity of microbial communities in petroleum crude oils produced in Asia. Biosci. Biotechnol. Biochem. 72: 2831-2839.

Yen, K. and Gunsalus, I. (1982) Plasmid gene organization: Naphthalene/salicylate oxidation. J. Bacteriol. 162: 1008-1013.

You, I. S., Ghosal, D. and Gunsalus, I. C. (1988) Nucleotide sequence of plasmid NAH7 gene nahR and DNA binding of the nahR product. J. Bacteriol. 170: 5409-5415.

Zhang, X. and Young, L. (1997) Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. Appl. Environ. Microbiol. 63: 4759-4764.

Zinjarde, S., Pant, A. and Deshpande, M. V. (1998) Dimorphic transition in *Yarrowia lipolytica* isolated from oil polluted sea water. Mycol. Res. 102: 553-558.

LIST OF PUBLICATIONS/PRESENTATIONS

Book chapter

- **S. V. Kulkarni**, A. S. Palande and M. V. Deshpande. (2012). Bioremediation of Petroleum Hydrocarbons in Soils. *Microorganisms in Environmental Management: Microbes and Environment*, Eds. T. Satyanarayana, B. N. Johri and A. Prakash. Springer. 589-606.

Presentations

- **S. Kulkarni**, A. Kasav, A. Sen, G. Kulkarni, Y. Shouche, José Ruiz-Herrera and M. V. Deshpande. Biodegradation of diesel using potential hydrocarbon degraders isolated from petroleum hydrocarbon polluted sites. Presented in poster session at AMI 2009; The 50th Annual Association of Microbiologists of India (AMI) Conference held on December 15-18, 2009 at National Chemical Laboratory, Pune.

Research paper based on additional work

- Palande, A. S., **Kulkarni S. V.**, Leon-Remirez, C., Campose-Gongora, E., Ruiz-Herrera, J. and Deshpande, M. V. (2014) Dimorphism and hydrocarbon metabolism in *Yarrowia lipolytica* var. indica. Arch. Microbiol.
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