DEGRADATION OF BENZENE USING RHIZOSPHERIC BACTERIA

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A thesis submitted in partial fulfilment of the requirement for the degree of Master of Science

IN

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CERTIFICATE

This dissertation submitted by **Mr. Muhammad Saad Bin Zahid** is accepted in its present form, by the Institute of Environmental Sciences and Engineering (IESE), School of Civil and Environmental Engineering (SCEE), National University of Sciences and Technology (NUST), Islamabad as satisfying the partial requirement for the degree of Master of Science in Environmental Science.

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DEDICATED….!!!

To my Nana Abu, Abu and Ammi

For their encouragement and support

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Muhammad Saad Bin Zahid

Table of Contents

LIST OF ABBREVIATIONS

mg/L	Milligram per Liter
Cu	Copper
Fe	Iron
IARC	International Agency for Research on Cancer
PGPR	Plant Growth Promoting Bacteria
BTEX	Benzene Toluene Ethylbenzene Xylene
CAA	Clean Air Act
EPA	Environmental Protection Agency
NESHAP	National Emissions Standards for Hazardous Air Pollutants
PAHs	Polycyclic Aromatic Hydrocarbons
API20E	Analytical Profile Index 20 *Enterobacteria*
EC	Electrical Conductivity
rRNA	Ribosomal Ribonucleic Acid
EMB	Eosin Methylene Blue
NCBI	National Center for Biotechnology Information
OD	Optical Density
BLAST	Basic Local Alignment Search Tool

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Rhizoremediation is considered as an environmental friendly technique to deal with the contamination of petroleum hydrocarbons. The current study aimed to check the potential of indigenous soil micro-organisms to degrade benzene. Soil samples were collected from petroleum-contaminated sites near Attock Oil Refinery, Rawalpindi, Pakistan, by brushing off the soil from roots of *Cannabis sativa*. The soil was processed for isolation of indigenous microbial strains. Sixteen bacterial strains isolated from the soil were screened for their potential to grow using benzene as sole carbon source. The strains were subjected to degrade 1000 mg/L benzene using M9 media. 16S rRNA gene sequencing was carried out for the potential performing strains. *Pseudomonas aeruginosa* (SAR-1) was the promising degrader with 85% degradation in 72 hours followed by *Bacillus cereus* (SAR-2) and *Acinetobacter junii* (SAR-3) with 83 and 70% degradation efficiency, respectively. A phylogenetic tree was developed by using TREEVIEW program to explore the lineage of better performing strains. These efficient degraders may be used as consortium for degradation of different compounds.

Chapter 1

INTRODUCTION

1.1 Background

Industrialization has resulted in the contamination of soil, air and water resources. Diesel fuel contaminates soil and water and it is categorized as hydrocarbon pollutant (Gallego *et al*., 2001). Petroleum is an important need of modern industrialized society, but it is source of environmental pollutants. Presence of petroleum hydrocarbons has adverse effects on human health and also on plants (Meudec *et al.,* 2007).

Useful products like kerosene, diesel and gasoline are produced after the processing of crude oil in an oil refinery (Gary and Handwerk, 1984). The crude oil has to go through separation or conversion, one or both of these processes to separate fractions (Hengstebeck, 1959). Unfortunately, these processes produce several aqueous waste products. Sour water is the wastewater that comes from the oil refineries and it contains hydrogen sulfide (Beychok, 1967).

Petroleum and its products such as diesel, gasoline, asphalts and natural gas are increasingly used as a result of modern human activities, and several marine and terrestrial sites have been contaminated. Petroleum hydrocarbons are composed of various proportions of short, medium, and long aliphatics (i.e. alkanes, alkenes), aromatics (e.g. benzene, toluene, ethyl benzene and xylene) as well as polycyclic aromatic hydrocarbons (known as PAHs; such as naphthalene, phenantherene, and pyrene) (Frick *et al.*, 1999). If hydrocarbons enter the food-chain, they can be

dangerous since few of them are persistent (Perelo, 2010). 20-50% of crude oil is made up of alkanes. Moreover, living organisms produce alkanes in the biosphere as waste products, structural elements, as chemo attractants or alkanes are also a part of defense mechanisms (Van Beilen *et al.,* 2007).

1.2 Benzene

Monoaromatic hydrocarbons such as benzene, toluene, ethylene, xylene and ethylbenzene are being known as representatives of environmental pollutants due to their known toxicity. Benzene is used on the large scale and it is produced from both natural processes and anthropogenic activities (Lin *et al.*, 2010; Adachi *et al.*, 2008). It is produced naturally from volcanos, forest fires, coal tar, petroleum and cigarette smoke. It is also used to produce rubbers, dyes, lubricants, drugs, pesticides, detergents and other synthetic fibers (ATSDR, 2011).

Benzene is considered as highly toxic chemical and it can cause serious health effects. It can be released from automobile stations, tobacco smoke, industrial emissions and vapors from products that comprise benzene like furniture polish, glues and paints. Spilling of benzene from landfills and hazardous waste sites can contaminate ground water (Daifullah and Girgis, 2003).

1.3 Harmful Effects of Benzene

If humans are exposed to benzene for more than 10 min at a very high level in air (10,000 to 20,000 mg/L) it can cause death. However, it causes dizziness, increased heartbeat, headache, drowsiness, confusion and unconsciousness at lower level (700 to 3,000 mg/L) (Daifullah *et al.*, 2003; ATSDR, 2011).

Level of benzene in rural atmosphere is less than in urban areas. At the same time, proximity to gas stations, petroleum refining processes, hazardous waste sites or petrochemical manufacturing sites results in higher levels of benzene exposure. Other than inhalation, people can be exposed to benzene through food, beverages, or drinking water. Food preservatives like sodium benzoate decarboxylate to benzene in the presence of ascorbic acid which itself is formed in the presence of metal catalysts like Cu (II) or Fe (III) (Lachenmeier *et al.*, 2008).

Benzene exposure causes chronic effects through blood. It decreases red blood cells by attacking bone marrow and ultimately leading towards anemia. It depresses the immune system and chances of infection are increased. Reproductive organs are also affected by benzene. Women who are exposed to higher levels of benzene for many months can experience decrease in size of ovaries and irregular menstrual periods (ATSDR, 2011). Benzene causes serious problems to human health which include cancer (Jiang *et al.*, 2011).

Due to the moderate water solubility, and relatively high vapor pressure, the major environmental sinks for benzene are atmosphere and the surface waters (Health Canada, 2009). According to International Agency for Research on Cancer (IARC), Benzene is a human carcinogen.

Major sources of benzene into water are landfill leachate, seepage and runoff from contaminated soils, discharge of untreated industrial wastewater and the leakage of gasoline from underground storage tanks (Daifullah and Girgis, 2003). Benzene gets released into soil through industrial discharges, leaks of gasoline from underground storage tanks and land disposal of benzene wastes. Benzene from the air can get deposited on the ground through rain or snow. Anyhow, benzene breaks down slowly in soil and water.

1.4 Benzene Removal

At present, soil remediation methods include physical, chemical and biological remediation. Bioremediation involves bio-augmentation which means the addition of bacteria into the soil for the degradation of pollutants. However, traditional methods of physical and chemical remediation are costly and not environmental friendly (Zhou and Song, 2004). Microorganisms are being used in the biological processes for the breakdown of environmental contaminants into less harmful products. The common remediation technology is bioaugmentation (Gentry *et al.,* 2004; Pandey *et al.,* 2009).

1.5 Biodegradation

Biodegradation involves different established microbial processes that happen in the natural ecosystems, for example detoxification, activation, mineralization or co-metabolism. However, we can also define biodegradation as natural degradation of organic compounds by the action of microorganisms, such as fungi, bacteria and actinomycetes (Hutchinson *et al.,* 2004).

Plant growth promoting rhizobacteria which are linked with roots of various plants are also called free-living beneficial soil bacteria. Increased level of nutrients for example, organic acids, amino acids and sugars result in the increased concentration of bacteria in the rhizosphere, and they can be used to support metabolism and growth of bacteria (Glick *et al.*, 1999).

Plant growth promoting bacteria which are free as wel s symbiotic increase the growth of plant by fixing nitrogen that can be used by plants, providing bio-available phosphorus that can be uptaken by plant, releasing plant hormones like gibberellins, auxins and cytokinens, and decreasing the levels of ethylene in plants (Glick *et al.*, 1999). The addition of PGPR can increase plant growth as well as decontamination of affected soils, hence its use in phytoremediation technique is now being considered to play a critical role (Mayak *et al.*, 2004).

For this study Benzene was selected because it is carcinogenic and mutagenic to humans and other animals and has more solubility as compared to other BTEX compounds. Exposure to even 1 mg/l of benzene has been reported to reduce blood cell count leading to hematotoxicity in factory workers (Jiang *et al.*, 2011).

1.6 Objectives

Objectives of the study under discussion were

- Isolation and characterization of bacteria from the rhizosphere of *Cannabis sativa* irrigated with wastewater of petroleum refinery
- Assessment of benzene degradation potential of the bacteria under controlled conditions

1.7 Scope of the study

One of the major advantages of this study will be the removal of benzene from the wastewater of petroleum refinery and that will ultimately help towards improvement of human health as well as the environment. The bacterial strains which will help in the degradation of benzene will also be useful in removal of other volatile organic compounds such as toluene and xylene from the wastewater of oil, rubber and paint industries.

Chapter 2

LITERATURE REVIEW

2.1 Crude Oil

Crude oil is a mixture of many different chemical compounds. It contains very simple compounds such as methane as well as some other very complex hydrocarbons. Unlike water, crude oil will not evaporate, when heated to boiling and held there for quite a time (Leffler, 1979).

According to U.S. Energy Information Association (2009), crude oil is being used in many ways, petroleum products are being used by the world to move from one place to another and to merchandise by using gasoline and diesel in engines, as well as crude oil can be used to make crayons, plastics, CD's, bubble gum and ink (Gary and Handwerk, 1984).

2.2 Oil Refinery

An oil refinery converts crude oil into useful products like petroleum, diesel and kerosene oil etc. Different parts of the crude oil are used to make a wide range of products that are being used by consumers, by using the process of separation at an oil refinery. Separation and conversion are two main processes in almost all petroleum refineries.

During the process of separation, petroleummoves through a hot furnace in modern separation (Waddams, 1980). Liquids and vapors are then discharged into distillation towers, gasoline and liquid petroleum gas which are the lightest components, they rise

above and condensed back to liquids in the distillation tower, medium weight compounds stay in the middle and at the bottom, there are heavy liquids (Gary and Handwerk, 1984).

Cracking uses pressure and heat to break complex hydrocarbons into simpler ones, and hence it is the most commonly used conversion technique. It has been used in the refinery process since very long. (Hengstebeck, 1959).

2.3 Refinery Wastewater

Separation of crude oil into gasoline and other useful products uses water (Dold, 1989). Large quantities of wastewater are produced, which are contaminated with hydrocarbons or heavy metals. The amount of wastewater production is specific for every refinery and depends upon different parameters like treatment and disposal methods etc (UNEP, 1987). Water containing hydrogen sulfide is called sour water and it is the main wastewater from petroleum refinery (Dold, 1989).

2.4 Guidelines for Petroleum Refineries

The Clean Air Act (CAA) gives strength to the U.S.A. Environmental Protection Agency (EPA) for the development and enforcement of regulations in order to protect the general public from exposure to hazardous airborne contaminants. For protecting the public; Section 112 of the CAA, EPA established National Emissions Standards for Hazardous Air Pollutants (NESHAP).

If a refinery shows compliance with the benzene waste NESHAP 40 CFR 61 (subpart FF) and 40 CFR 63 (subpart R) it is considered to be in compliance with the refinery Maximum Achievable Control Technology (MACT) standard.

NESHAP is applicable to the following subsequent wastewater streams at petroleum refineries:

- Total benzene loading 10 Mg per year
- Flow rate 0.02 per minute.
- Benzene concentration 10 mg/L (mg/L by weight)
- Not exempt from controls under the benzene waste NESHAP

Table 2.1: World Bank Group Pollution Prevention and Abatement Handbook

Pollution	Approximate Quantities
Cooling systems	3.5-5 m^3 of wastewater generated per ton of crude
Polluted wastewater	BOD 150-250 mg/L
	COD 300-600 mg/L
	Phenol 20-200 mg/L
	Oil 100-300 mg/L (de-salter water)
	Oil 5000 mg/L in tank bottom
	Benzene 1-100 mg/L
	Heavy metals 0.1-100 mg/L
Solid waste & Sludge	3 to 5 kg /ton of crude (80 % considered as hazardous waste)
VOC emissions	0.5 to 6 kg/ton of crude
Others emissions	BTX 0.75 to 6 g/ton of crude Sulfur oxides 0.2-0.6 kg/ton of crude Nitrogen oxides 0.006-0.5 kg/ton of crude

Source: World Bank Group Pollution Prevention and Abatement Handbook

2.5 Hydrocarbons

Hydrocarbons are present in environment as gasses or tiny particles and they are composed of hydrogen and carbon. Crude oil contains hydrocarbons. They are released into the environment through diesel, petrol, crude oil and the oil products which are used in vehicles. Aromatic hydrocarbons are of main concern because they

contaminate environment by polluting air, water and soil. Three major classes of aromatic hydrocarbons are (i) polycyclic aromatic hydrocarbons (PAHs), (ii) heterocyclic aromatic hydrocarbons, and (iii) alkyl PAHs (McGuinness and Dowling, 2009). A large number of petroleum hydrocarbons can be strongly adsorbed on soil organic matter, trapped in small pores of soil (Hutchinson *et al.,* 2004) and long chain alkanes can be a factor in the formation of slicks and oil films resulting in the blockage of water, soluble nutrients and gas exchange (Leahy and Colwell, 1990).

2.6 Benzene

BTEX are very important materials for the chemical industry especially benzene which is used as a solvent in a number of processes. It is classified as a flammable, toxic and carcinogenic material (Das *et al.,* 2004; WHO, 2008; Jiang *et al.,* 2011) so its presence in the environment, usually in air and wastewater, even at low concentrations, is a major problem (Xie *et al.,* 2011). Benzene is an organic compound with a chemical formula C_6H_6. It is a colorless volatile liquid that is flammable in nature. Benzene enters into the environment naturally as well as anthropogenicaly. It is an essential part of crude oil and in released in the wastewater coming from printing, rubber, paint and petrochemical industries and it is also released during the preparation of phenol (Adachi *et al.,* 2008). Stringent regulations, therefore, have been imposed on the concentration levels of these compounds in wastewater discharge.

2.6.1 Physical and Chemical Properties of Benzene

Benzene (C_6H_6) is a colorless liquid with a characteristic sweet odor (ATSDR, 2011; Health Canada. 2009) and is both volatile and flammable. It is miscible with

polar solvents such as chloroform, acetone, alcohol, and carbon tetrachloride. It is relatively soluble in water. Even though benzene is a highly stable hydrocarbon, it reacts with other chemicals primarily by hydrogen atom substitution (Health Canada, 2009).

2.6.2 Industrial Applications of Benzene

Benzene has been in use as a solvent since years (Hindarso *et al.*, 2001) and as a synthetic intermediate for several chemicals such as carburetor cleaners, paint strippers, rubber cement, denatured alcohol, glue, liquid soap and polish for furniture (Adachi *et al.*, 2008). Because of the adverse effects on health due to benzene, it has been replaced by other chemicals in the recent years. Fig. 2.1 illustrates the major commodity and chemicals that are generated from benzene (Adachi *et al.*, 2008).

2.6.3 Benzene Metabolism

Metabolism is proved to be playing a crucial role in benzene toxicity. For example, benzene toxicity decreases due to competitive inhibition of metabolism by toluene. A decrease in toxicity was also observed due to decreased metabolism in Rodents when given a partial hepatectomy or mice deficient of the CYP2E1 gene. There is no indication of formed metabolites being affected by the administration route (Kim *et al.*, 2004). The process of benzene metabolism is very complex shown in Fig. 2.1 (PHG, 2001)

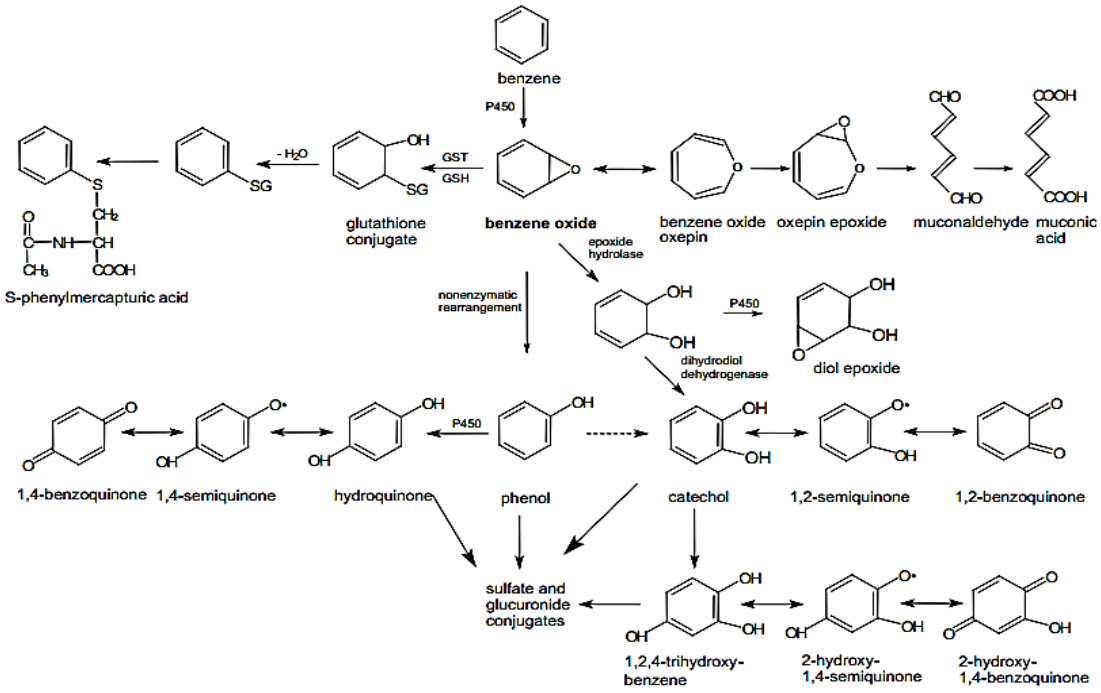

Fig 2.1 Metabolism of Benzene

2.6.4 Regulations for benzene

Benzene is at 4th number in the priority pollutants list by United States Environmental Protection Agency (Hazdat, 2006). Stringent regulations have, therefore, been imposed on the concentration levels of the compound in drinking water and wastewater discharge. Benzene is designated as the priority chemical that needs to be reduced to a very low level. According to United States Environmental Protection Agency, the maximum contaminant goal of benzene in drinking water is 0 mg/L, however the acceptable limit of benzene in drinking water is 0.005 mg/L, while that for treated industrial wastewater is 0.14 mg/L.

2.6.5 Sources of Benzene in the Environment

According to EPA national priority list, benzene is present in almost 1000 of the 1684 hazardous sites (Hazdat, 2006). It can be present in the environment both from natural and industrial sources. Natural sources only account for a small amount of benzene released into the air, water, and soil. Natural sources of benzene in air include forest fires, gas emissions from volcanoes, crude oil leaks, plant volatiles, etc. Anthropogenic benzene emission can result from burning coal and oil, storage and waste operation, gasoline vapors, auto exhaust, chemical production and user facilities and tobacco smoke. Benzene is also released from hazardous waste sites which have been contaminated by benzene.

In general, the atmospheric residence time of benzene is only a few days due to chemical degradation reaction with hydroxyl radicals. Benzene in air can also be deposited on the ground by snow or rain. However, benzene in soil and water breaks down more slowly. In general, benzene in water and air is subject to volatilization, photo-oxidation and biodegradation. For water-associated benzene, biodegradation under aerobic conditions is an important environmental process.

2.6.6 Health Effects of Benzene

Benzene is highly toxic, carcinogenic and mutagenic to humans and other animals (Jiang *et al.*, 2011). Exposure to even 1 mg/L of benzene can reduce blood cell count leading to hematotoxicity in factory workers. The main sources of benzene exposure are industrial emissions, automobile stations, tobacco smoke, wastewater and vapors from products that comprise benzene like glues, paints and furniture polish. People can be exposed to benzene through drinking water, food or beverages. The major chronic effects of benzene exposure occur through the blood. It is found to depress the immune

system and cause excessive bleeding, as a result of which chances of infection are increased (Daifullah and Girgis, 2003).

2.7 Bacteria

Endophytic and rhizophytic bacteria are plant-associated bacteria. Bacteria present in the plant roots are called endophytic bacteria and they are beneficial to plant by promoting plant growth and they enhance biodegradation of soil pollutants (Weyens *et al.,* 2009). Rhizophytic bacteria are the bacteria which are present in the rhizosphere of plant, they are plant growth promoting bacteria (PGPR). They protect plant against pathogens and degrade contaminants (Glick, 2010).

2.7.1 Genetically Engineered Bacteria

In early 1980's genetically engineered micro-organisms started getting regulated by United States Environmental Protection Agency. There was a bloom in research about genetically engineered microbes during 1980's and 1990's. It gave birth to many companies who aimed to work on genetically engineered microbes, but they went out of business because of cost and some regulatory problems (Jiang *et al.,* 2005). Ananda Chakraborty created first genetically engineered microbe in 1971 and supreme court of United States approved it in 1980. It was capable to breakdown crude oil into its constituent parts, but unfortunately due to public concerns and regulatory issues, this microbe still sits on shelf and is unused (Obidimma *et al.,* 2010).

2.8 Rhizosphere

The soil present around the roots of plant is called rhizosphere. Bacteria present in such region are known as Rhizosphere bacteria and they promote plant growth and increase water uptake. Micro-organisms present in rhizosphere are capable for the remediation of contaminants. Microbial community in the rhizosphere have successfully degraded hydrocarbons (Hutchinson *et al.*, 2004).

As a result of environmental contaminations, several techniques have been proposed for soil remediation. One of them is introduction of external bacteria in the rhizosphere region. But this wasn't fully successful, there was competition among indigenous and exotic bacteria, and external bacteria failed to compete and couldn't survive (Afzal *et al.*, 2012).

2.9 Soil Remediation

Physical remediation, chemical remediation and bioaugmentation are the common remediation methods now a days. These conventional physical and chemical methods are costly and the end products are also considered as contamination, which need to be removed from the biosphere (Zhou and Song, 2004).

2.9.1 Bioremediation

It is the use of microorganisms for the remediation of contaminated soils or ground water. Researchers have already reported the ability of aerobic as well as anaerobic biodegradation of the contaminants and transformation process that ultimately describes the fate of the pollutant. Biodegradation of contaminants in the root zone has already been reported many times (Karthikeyan and Kulakow, 2003).

Bioremediation is one of the "Top 10 Biotechnologies to Improve Global Health". Bacteria transform toxic compounds into less harmful substances in a process known as microbial bioremediation. Some examples are benzene, toluene, etc. Bioremediation in which micro-organisms for example, fungi or bacteria are used to reduce hazardous compounds into less toxic substances is called microbial bioremediation (Pieper *et al.*, 2000).

2.10 Bacterial Identification

Identification of unknown bacterial cultures is mostly done through biochemical characterization. API20E (Analytical Profile Index 20 *Enterobacteria*) kits are used for this purpose. This kit contains 20 microtubes which in turn have dehydrated reagents and change in color is the determination of positive or negative tests.

For this purpose, Oxidase and Catalase tests are performed.

2.10.1 Phylogenetic Analysis

Phylogenetics can be defined as the study of evolutionary relationships while phylogenetic analysis can be defined as the estimation of these relationships. The history of evolution resulted from a phylogenetic tree is usually represented in the form of tree like drawings that show projected lineage of genetic relationships among organisms (Felsenstein, 1985).

2.10.2 Types of Phylogenetic Tree

Mainly there are two types of phylogenetic tree. Rooted and unrooted phylogenetic tree.

Rooted Tree

It is the type of the tree that has all the objects on it and shares a common ancestor. It has a particular root node

Unrooted Tree

This is the type of phylogenetic tree which has all the information about descendants but it doesn't have enough information to specify the common ancestor

2.10.3 Four Steps of a Phylogenetic Data Analysis

A straightforward phylogenetic analysis consists of four steps:

1. Alignment (both building the data model and extracting a phylogenetic dataset)
2. Determining the substitution model
3. Tree building
4. Tree evaluation

Alignment

Multiple sequence alignment is done in phylogenetic sequence. The current methods that include use of softwares like **CLUSTALW** align according to an explicitly phylogenetic criterion (Hughey *et al.,* 1996).

Determining the Substitute Model

Substitution model is of same importance as alignment and tree building.

Tree Building

Trees are built by the software (Saitou, 1996; Swofford *et al.,* 1996; Li, 1997)

Tree Evaluation

Evaluation of phylogenetic signal in the data and the robustness of trees can be done by different procedures (Swofford *et al.*, 1996; Li, 1997).

It can be concluded from the review of literature that benzene is a toxic compound and has serious health effects. There are certain processes available for its de-contamination but still there is need to find out an effective, fast and environmental friendly method. Exploiting the natural diversity of contaminated sites is a popular tool to find out effective degraders that has been explored in this study.

Chapter 3

MATERIALS AND METHODS

3.1 Study Zone

3.1.1 Attock Oil Refinery, Rawalpindi, Pakistan

Attock oil Refinery (Morgah), Rawalpindi is situated in Punjab and is the innovator crude oil purifying diligence in Pakistan, exact location is pointed out in Fig 3.1

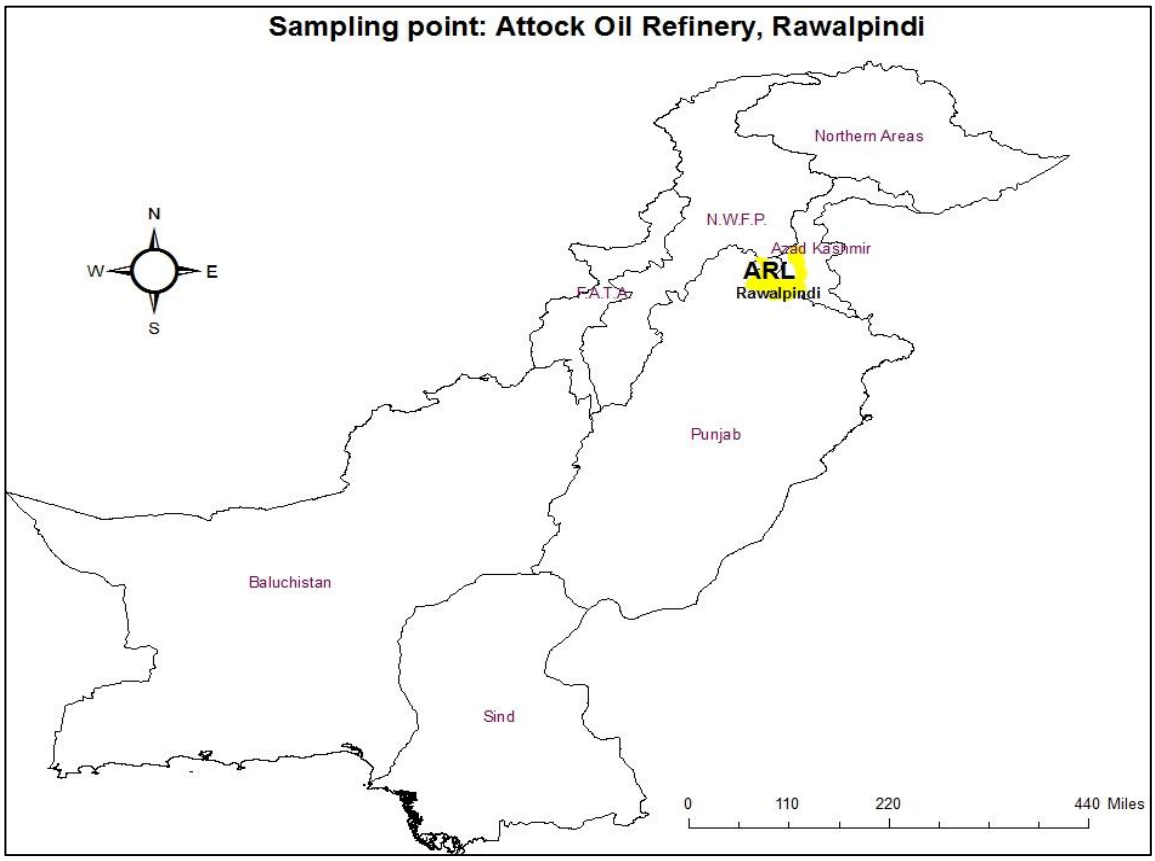

Fig 3.1: Plot of the study zone

3.1.2 Soil sampling

Benzene degrading indigenous microorganisms were isolated from the soil as described in standard methods.

The soil samples were collected by brushing off the soil from the roots of *Cannabis sativa*. The samples were carried to IESE, NUST in polyethylene bags. Soil samples were air dried for the period of 24 h, they were then sieved by using 2-mm sieve and finally they were crushed by using pestle and mortar. These soil samples were stored at 4 °C in bottles.

3.2 Physico-Chemical Properties

The physic-chemical properties of soil i.e. pH, electrical conductivity (EC), and soil texture were analyzed by using standard laboratory procedures.

3.2.1 pH and Electrical Conductivity

The pH and electrical conductivity of the soil samples were calculated by using portable pH/EC meter. Suspension of soil was made in distilled water with a ratio of 1:9 (10 g soil; 90 mL distilled water). The combination was moderately agitated for 1 min after 10 min time span for 30 min. The electrode was dipped in the soil suspension and readings were taken at room temperature (Ryan *et al.*, 1996).

3.2.2 Texture

Soil texture was determined with the help of Bouyocous Method (1962). The soil samples were sieved by using 2-mm sieve to remove large particles. The samples were then ionized with hot hydrogen peroxide (50 mL distilled water with 5 mL of 30% H_2O_2) to remove organic matter. The soil samples were placed in special dispersion cups and distilled water was added to cover the 2.5 inches above the rim of each cup. 5 mL (10%) sodium hexametaphosphate was added to each sample and this was followed by stirring for 5 min. The treated soil suspension was then transferred to Buoyocous cylinder (1 Litre capacity) and filled with distilled water to 1000 mL with the hydrometer in the suspension. Then the hydrometer was removed, suspension was shaken. After 20 sec hydrometer was inserted, and was read after another 20 sec and the 40 sec reading was recorded. Hydrometer was removed from the suspension and temperature was recorded. After removing the hydrometer and recording the reading the suspension was re- shaken and left for 2 h.

Since the soil settled in 40 sec, the weight of sand in the sample was obtained by subtracting the corrected hydrometer reading from total weight of sample. The percentage of sand was also recorded and calculated. Second reading was taken after 2 h interval, silt had settled out of suspension. Clay was left in the suspension. Hydrometer was inserted after 2 h. The corrected hydrometer reading after 2 h represented the grams of clay in suspension. The percentage of silt was calculated by subtracting percentage of sand and clay from 100 and recorded.

3.3 Washing and Sterilization of Glassware

All the glassware to be used in the experiments was washed with the help of the tap water, then dipped in distilled water and rinsed. For the purpose of sterilization, the glassware was autoclaved at 121 °C, 15 psi pressure for 15 min. The disinfected glassware was oven dried at 105 °C for 2 h.

3.4 Preparation of Media

Merck KGA nutrient agar was used for the experimental studies. The media was prepared according to the manufacturer's instructions. Conical flasks containing nutrient agar were used to make nutrient agar plates.

The flasks were autoclaved at 121 °C, 15 psi pressure for 15 min. Plates of nutrient agar were made in sterile conditions of laminar flow hood and incubated for 24 h at 37 °C. These plates were used for isolation, purification, inoculum preparations and degradation studies (Ramanathan and Lalithakumari, 1999)

3.5 Isolation of Benzene Degrading Bacteria

The bacterial cultures which were able to degrade benzene specifically were isolated from hydrocarbon contaminated soil and the process of isolation was carried out by standard serial dilution technique followed by the plating method. For this procedure, six disinfected test tubes were arranged in a test tube rack. These were marked as 10^{-1} to 10^{-6}. Nine milliliter sterilized distilled water was added to each test tube. One gram of soil was added to the first test tube in the series marked as 10^{-1}. A homogenized soil suspension was made by energetic shaking on vortex for 5 min. One milliliter soil suspension was taken from this tube and was added to the subsequent tube in the

series which was named as 10^{-2}, and the same procedure was repeated for the next tubes. 100 µl soil suspensions were taken from 10^{-3} to 10^{-6} dilution and was spread on nutrient agar plates and the plates were incubated at 37 °C for 24 hours. The bacterial colonies were counted on the colony forming unit. Colony forming unit was calculated with the help of the following formula:

CFU/g soil = number of colonies × dilution factor × (10) …. Equation (1)

3.6 Maintenance of Bacterial Culture

The isolated bacterial strains were streaked onto nutrient agar plates and the growth was observed after incubation at 37 °C for 24 hours and the cultures were maintained at 4 °C in order to preserve it for longer period. The bacterial culture was sub-cultured after every two weeks for the experimental studies.

3.7 Identification

Identification was done by performing gram staining, biochemical tests, 16S rRNA gene sequencing and phylogenetic tree.

3.7.1 Morphological Characteristics

Gram staining was used to observe cell morphology.

Gram staining

Bacterial colony was picked from and equally spread on the clean glass slide containing a small drop of water. Slide was air dried and heat fixed.

Then, crystal violet was applied on the snear for one min and washed with distilled water. Then, iodine solution was applied for a min and washed with distilled water. Then decolorizing agent was spread on the slide for 30 sec, then the glass slide was air dried and finally safranin was applied for one min. Gram positive cells appeared purple while gram negative cells appeared pink when observed under oil immersion at 100x resolution using microscope.

3.7.2 Biochemical Characterization

Biochemical characterization was done using different biochemical tests. The details are presented below

Oxidase Test

Oxidase test determines the presence of an intracellular oxidase enzyme by bacteria. Some bacteria may produce more than one type of oxidase enzyme. Oxidase Reagent is used to detect the presence of oxidase enzymes produced by a variety of bacteria. Oxidase test was performed by allowing oxidase reagent to reach room temperature before its use, then a small piece of filter paper was placed into a sterile petri dish and filter paper was moistened with 1 to 2 drops of oxidase reagent. The bacterial colony to be tested was touched with the help of loop and it was smeared onto filter paper. Appearance of purple color on the filter paper within 30 sec showed positive results.

Catalase Test

Catalase activity was determined by adding few drops of 3 percent hydrogen peroxide on 24-48 h grown cultures and tubes were examined for evolution of oxygen. Appearance of bubbles in the tubes was taken as a positive test for catalase presence.

MacConkey Agar Test

MacConkey Agar is a specialized bacterial growth medium that is selective for Gram-negative bacteria. It inhibits the growth of gram positive bacteria. For this purpose, MacConkey Agar was prepared according to the manufacturer's instructions. Bacterial cultures were streaked and the plates were incubated for 24 h at 37 °C. Bacterial cultures which turned pink after the period of 24 h were 'Lactose fermenters'. It is the bacteria which eats lactose and turns pink and not the media.

EMB Agar Test

Eosin Methylene Blue (EMB) Agar, is used for the isolation and differentiation of Gram-negative enteric bacilli. For this purpose, EMB agar was prepared according to the manufacturer's instructions and was incubated for 24 h at 37 °C. Bacterial cultures were streaked and the plates were incubated for 24 h at 37 °C. Bacterial cultures which turned blue after the period of 24 h were 'Lactose fermenting bacteria'.

Motility Test

Microscopy is the most accurate way to determine motility of fresh culture of bacteria. For this purpose, fresh culture of bacteria was placed in the middle of a glass

slide and a smear was made with the help of a drop of autoclaved distilled water. It was covered with a cover slip. It was then observed under the microscope by using a drop of oil immersion. Bacteria could be seen moving under the microscope.

3.7.2 16S rRNA Gene Sequencing

The bacterial strains with potential degradation capabilities were preserved in glycerol and stored at -20 °C. The isolates were sent to Overseas Genome Analysis Department Macrogen Inc. Korea for 16S rRNA sequencing.

3.7.3 Phylogenetic Tree

Sequences obtained were analyzed using BLAST search at National Center for Biotechnology Information (NCBI) databases. The sequences were aligned using CLUSTALW after complete deletion of the mismatch sequences. A phylogenetic tree constructed by using the TREEVIEW program illustrates the phylogenetic relatedness of identified strains to the selected strains obtained from GenBank (NCBI).

3.8 Biodegradation Studies

3.8.1 Benzene used for Studies

Benzene used in this study belongs to the class of aromatic hydrocarbons. Commercial grade materials were used in the experiments, because they are of same nature to which micro-organisms are likely to be exposed in the environment. Other chemicals used were of analytical grade.

3.8.2 Concentrations of Benzene

For growth and degradation studies different concentrations of benzene were used to determine the utilization and degradation potential of bacterial species. Concentrations of 250, 500, 750 and 1000 mg/L were used during the experiment.

3.8.3 Preparation of Inocula

The inocula for the experiment of benzene degradation studies were prepared by growing bacterial cultures on nutrient agar plates for 24 h at 37 °C. The culture plates were washed with 10 mL autoclaved distilled water having pH 7 and used as inocula for degradation experiments.

Acclimatization of bacterial strains was done in 250 mL flask containing distilled water, M9 media and different concentrations of benzene (250, 500, 750 and 1000 mg/L) as a single carbon source to study the growth pattern of bacterial strains in relation to benzene. All experiments were carried out in triplicate.

3.8.4 Benzene Degradation in Orbital Shaker

For a batch experiment (3 days) the degradation potential of different bacterial strains was determined using flask shaker. In 250 mL conical flask, 140 mL distilled water, 60 mL M9 media, 5 mL of different benzene concentrations were added and was inoculated with 5 mL of strains and incubated on orbital shaker at ambient temperature at 120-180 rpm (Ramanathan and Lailithakumari, 1999). Control samples with equal volumes of media having different concentrations of benzene but no bacterial inoculum were used in order to analyze initial total organic carbon content.

Table 3.1 Data from Orbital Shaker

Orbital Shaker	Specifications
Speed	120-180 rpm
Total volumetric load	250 mL
Temperature	Ambient
Sample collection	24 h
Size of inoculums	5 mL

3.8.5 Sampling Procedure

For the first day, 5 mL samples were extracted from experiment flask after every hour for 6 h and then at the period of 24, 48 and 72 h under sterilized conditions of laminar flow hood. These original samples were used to check benzene degradation on UV Spectrophotometer.

3.9 Analytical Procedure

3.9.1 Optical Density

The growth of bacterial strains was determined by measuring the optical density (OD) of different bacterial strains at 600 nm by using spectrophotometer (spectronic) before starting the batch experiment. For calibration of instrument, 10 mL distilled water was used.

3.9.2 UV Spectrophotometer

Benzene shows absorbance at a wavelength of 254 nm. So, the samples which were extracted from experiment flasks were then analyzed on UV Spectrophotometer at the wavelength of 254 nm in order to determine the residual concentration of benzene. The benzene degradation was further calculated.

Chapter 4

RESULTS AND DISCUSSION

4.1 Physico-chemical Properties of soil

Physico-chemical properties of soil samples were determined and soil pH was 6.9. Electrical conductivity was 1.50 dS/m and soil texture was sandy loam, which makes it suitable for the growth of plants.

4.2 Bacterial Strains

In order to observe the ability of soil microorganisms to degrade benzene, sixteen visually distinct bacterial strains were isolated from the soil. These bacterial species were thought to be able to use petroleum hydrocarbon as sole carbon source (Walker and Colwell, 1977). However, enzyme systems and nutritional capabilities are also important factors which make the petroleum degraders to survive in adverse environmental conditions. Most of rhizospheric bacteria are detrimental to plant parasites, act by metabolic-by products, enzyme and toxins, such as antibiotics, butyric acid, hydrogen sulphide or increase of chitinases, peroxidases and other products associated with induced systemic resistance (Siddiqui *et al.*, 1999).

4.3 Obtaining screened bacterial cultures

Continuous exposure to increasing concentrations of the targeted compounds to the microorganisms which can efficiently degrade those compounds is a commonly used

enrichment technique (Ling *et al.*, 2011, Masakorala *et al.*, 2013). In the present study, same technique has been used to isolate benzene degrading bacteria. The ability of bacterial strains to survive in benzene was measured by calculating their biomass in terms of optical density. All the sixteen bacterial strains were subjected to enrichment with M9 minimal medium, distilled water and benzene as sole carbon source. Mazeo *et al.* (2010) reported that biodegradation occurs as a result of consortium of different microorganisms which includes algea, bacteria and fungi etc. However, present study consisted of changes in microbial community only.

The results regarding optical density of sixteen isolated bacterial strains in response to exposure of 250 mg/L benzene are presented in Fig 4.1. Out of sixteen strains namely SAR-1 to SAR-16, four strains were unable to grow at this concentration. These were SAR-13 to SAR-16, all other strains had optical density greater than 0.5. These strains were then cultured on media containing 500 mg/L of benzene and results are shown in Fig 4.2. Out of these 12 strains, only seven had optical density greater than 0.7 and other five strains namely SAR-8 to SAR-12 were unable to grow using benzene as sole carbon source. These seven strains (SAR-1 to SAR-7) were acclimatized at 750 mg/L of benzene and results are shown in Fig 4.3. All the seven strains were able to grow and an increase in optical density was observed for all the seven bacterial strains. SAR-1 had maximum optical density i-e 2.1, while that for SAR-2 was 1.7 and SAR-3 had an optical density of 1.6. These seven acclimatized bacterial strains were further used for the biodegradation of benzene.

Our findings coincide with researches where microorganisms use hydrocarbons as their sole carbon source (Sharma *et al.*, 2014) reported the growth of bacteria on media that contained liquid hydrocarbon (benzene, toluene, octane & heptane) as sole carbon source.

Vasudevan and Rajaram (2001) reported changes in microbial population when they were subjected to targeted compounds, which could be due to interactions between microbial populations. Ting *et al.* (1999) reported the decrease after initial increase of microbial populations in nutrient systems.

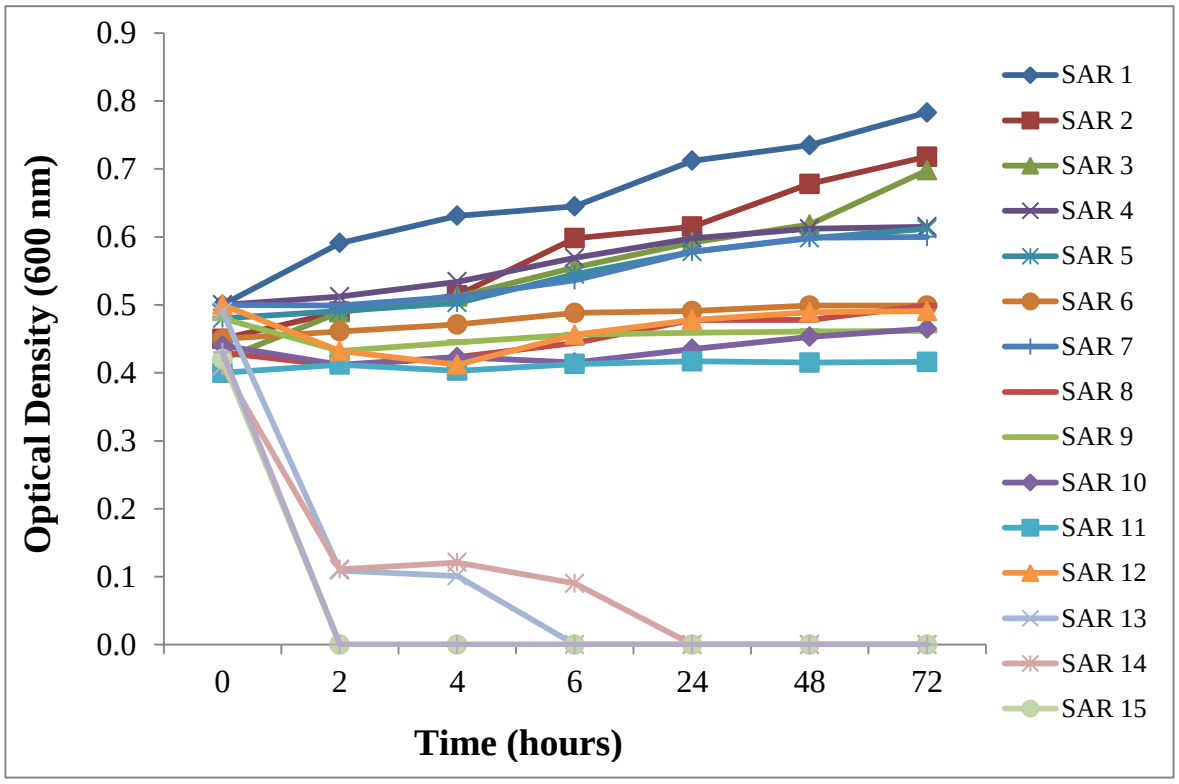

Fig 4.1 Growth of bacterial strains (Benzene = 250 mg/L)

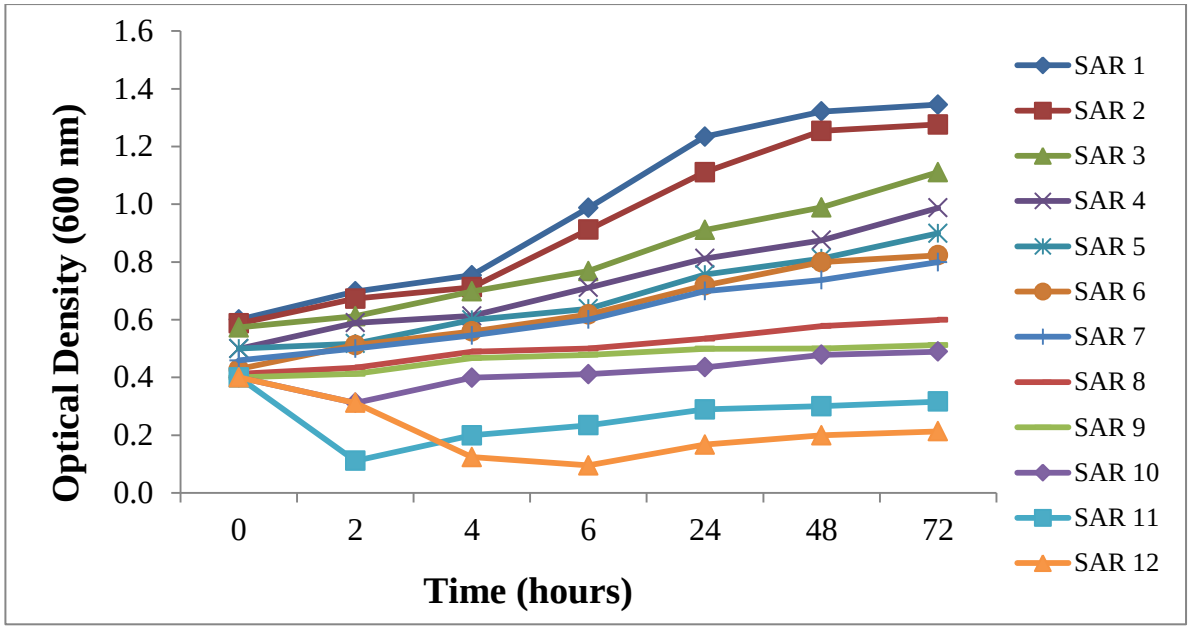

Fig 4.2 Growth of bacterial strains (Benzene = 500 mg/L)

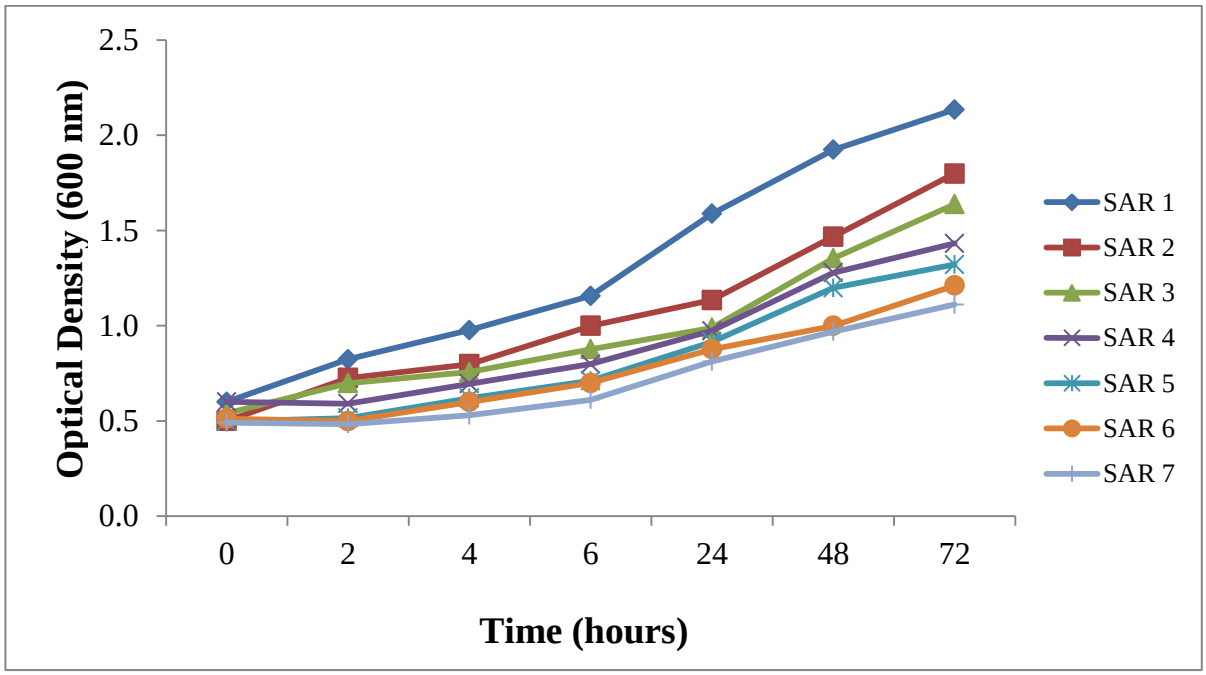

Fig 4.3 Growth of bacterial strains (Benzene = 750 mg/L)

4.4 Biodegradation of Benzene (1000 mg/L)

Residual concentration of benzene was determined with UV spectrophotometer in order to estimate the biodegradation of benzene. It was confirmed that decrease in the concentration of benzene is the result of biodegradation, because controlled culture showed no consistent reduction in the concentration of benzene. Comparison of profiles of different cultures to degrade benzene showed that the degradation occurred slowest in SAR-3.

For the biodegradation of 1000 mg/L of benzene, SAR-1 degraded 68% benzene within first 24 h, and 77% degradation was achieved at 48^{th} h, ultimately 85% degradation was achieved in 72 h while optical density had a maximum value of 2.5 during the same time frame (Fig 4.4). SAR-2 degraded 55 % benzene in first 24 h, 73% in 48 h and finally degrading 83% benzene in 72 h and optical density for SAR-2 was 2.3 (Fig 4.5). SAR-3 degraded 43% , 57% and 70% benzene in 24, 48 and 72 h respectively and optical density was 2.1 (Fig 4.6).

Other workers have already reported analogous results which indicated the hydrocarbon degradation in oil contaminated soil by microbial populations (Margesin *et al.,* 2003).

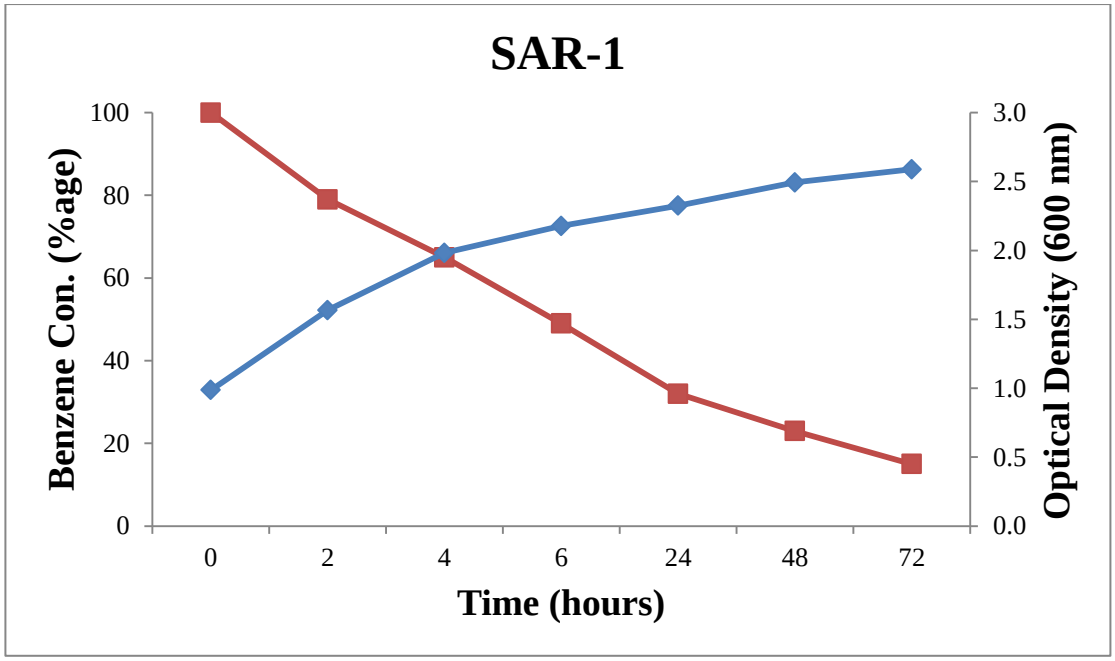

Fig 4.4 Comparison of optical density and decrease in benzene concentration by SAR-1

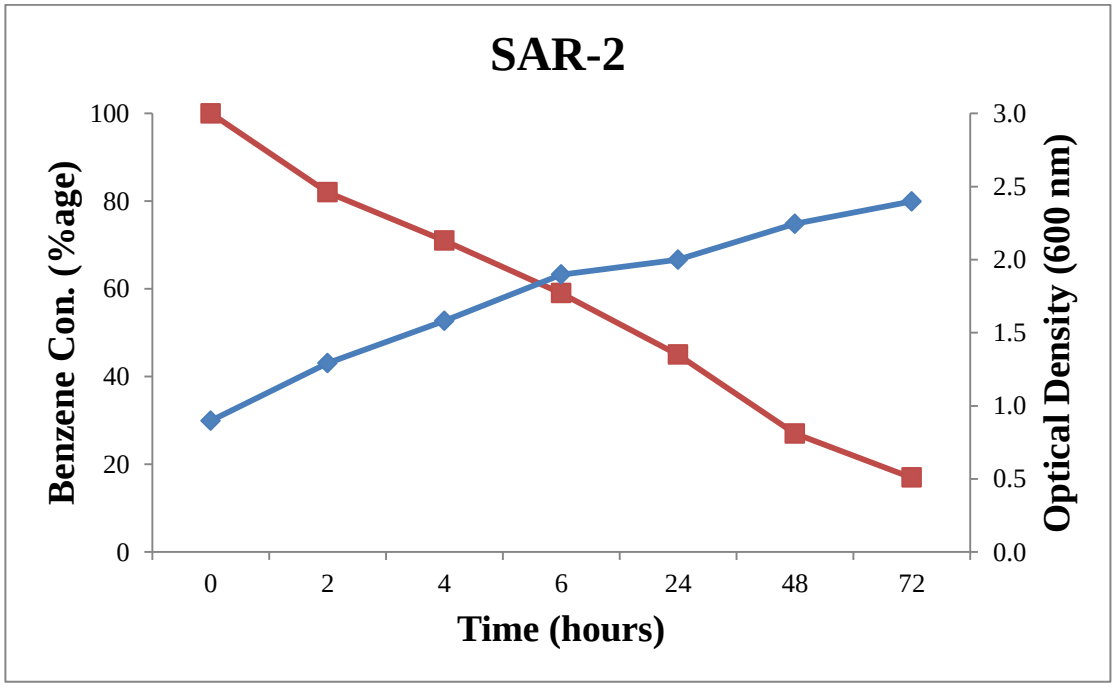

Fig 4.5 Comparison of optical density and decrease in benzene concentration by SAR-2

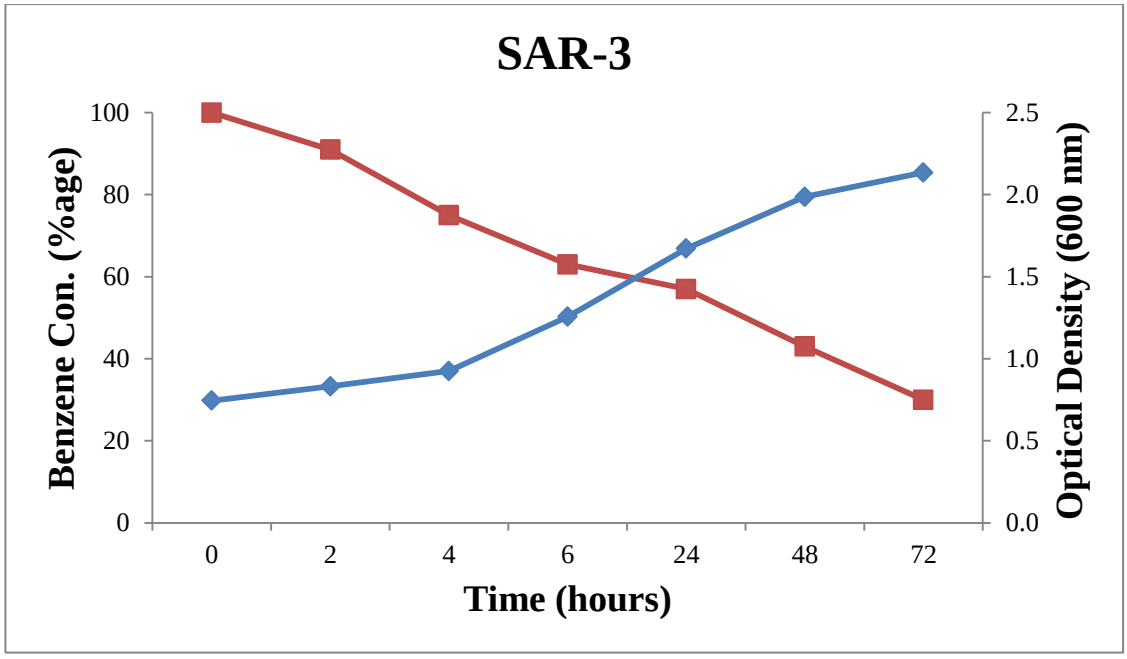

Fig 4.6 Comparison of optical density and decrease in benzene concentration by SAR-3

4.5 Morphological Characterization

Cell morphology was determined by performing gram staining, results are shown in Table 4.1.

Table 4.1: Cell morphology of the bacterial isolates obtained from hydrocarbon contaminated soil

Bacterial Isolates	Gram stain	Shape	Arrangement	Motility
SAR 1	Negative	Cocci	Chain	+
SAR 2	Positive	Bacilli	Single	+
SAR 3	Negative	Cocci	Group	+
SAR 4	Negative	Bacilli	Single	_
SAR 5	Negative	Bacilli	Single	_
SAR 6	Negative	Bacilli	Single	+
SAR 7	Negative	Bacilli	Pairs	+

All of the strains were gram negative except SAR-2, which was gram positive. Except two strains i.e. SAR-1 and SAR-3 which were cocci all of the bacterial isolates were bacilli in shape, these two strains were present in group and chain conformations. Strains SAR-2, SAR-4, SAR-5 and SAR-6 appeared to exist single. SAR-7 was the only bacterial isolate to have existed in pairs. SAR-4 and SAR-5 were non motile while all other bacterial isolates were motile when observed under microscope at 100x.

4.6 Biochemical Characterization

Biochemical characterization was done by performing different biochemical tests, results are shown in Table 4.2.

Table 4.2: Results of Biochemical tests of bacterial isolates

Bacterial Isolates	Oxidase	Catalase	MacConkey Agar	EMB Agar
SAR 1	Positive	Positive	Positive	Positive
SAR 2	Negative	Positive	Negative	Negative
SAR 3	Negative	Positive	Positive	Positive
SAR 4	Positive	Positive	Positive	Positive
SAR 5	Negative	Positive	Positive	Positive
SAR 6	Positive	Positive	Positive	Positive
SAR 7	Positive	Positive	Positive	Positive

All seven bacterial isolates were able to produce catalase. Strains SAR-2, SAR-3 and SAR-5 were not able to produce oxidase.

4.7 Identification of Potential Performing Benzene Degraders

Out of seven bacterial strains, top three benzene degraders were subjected to sequence analysis. This analysis was performed as described by (Jan-Roblero *et al.,* 2008). *Alcanivorax borkumensis* was the first ever hydrocarbonclastic bacteria to have its genome sequenced (Martins, 2008). Full length 16S rRNA gene sequencing of SAR1- SAR3 was carried out from overseas Genome Analysis Department Macrogen Inc. Korea. They were screened and noise was removed manually. They were identified by performing a BLAST search (Altschul *et al.,* 1997) of the National Center for Biotechnology Information (NCBI) databases revealing up to 97% similarity to different bacterial species. Schloss and Handlesman in 2004 considered the limit of 97% for species identification. Complete alignment was done by using

CLUSTALW. A phylogenetic tree, constructed by using the TREEVIEW program illustrates the phylogenetic relatedness of identified strains, shown in Figure 4.7.

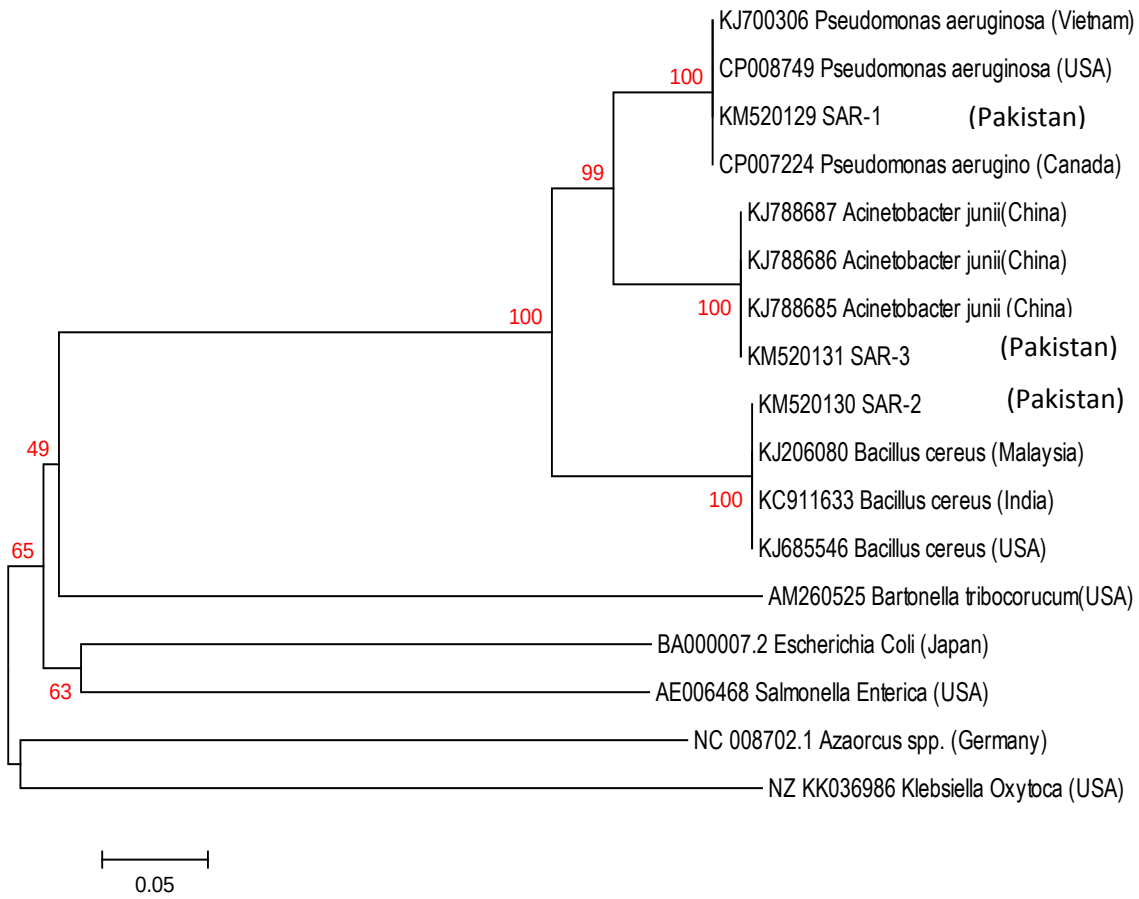

Fig 4.7 Phylogenetic tree showing the relatedness of bacterial strains. All 16s rRNA sequences were received from Genome Analysis Department Macrogen Inc. Korea (GeneBank accession numbers KM520129- KM520131). Alignments were made using CLUSTALW. Tree was constructed using TREEVIEW program. Genus names were identified as *Pseudomonas, Bacillus* and *Acinetobacter.*

SAR 1, SAR 2 and SAR 3 were identified as *Pseudomonas aeruginosa, Bacillus cereus* and *Acinetobacter junii* respectively. 16S rRNA sequences of organisms

related to benzene degradation were submitted to GenBank under accession numbers KM520129, KM520130 and KM520131. The data is now available online at http://www.ncbi.nlm.nih.gov/.

Cunnigham *et al.* (1996) reported that microorganisms present in rhizosphere of different plants might be responsible for the degradation of petroleum hydrocarbons. Liu *et al.* (2014) mentioned that *alkB* genes are involved in the degradation of aromatic hydrocarbons. (Kim et al., 2004; Bagneris et al., 2005) reported that dioxygenases and monooxygensases enzymes initiate by oxidizing aromatic ring of BTEX compounds. Ridgway *et al.* (1990) reported *Pseudomonas aeruginosa* and Dou *et al.* (2008) reported *Bacilus cereus* are helpful in BTEX biodegradation. Ijah and Antai (2003) reported that bacillus species are helpful in clearing oil spills due to their resistant endospores. Lin *et al.* (2014) demonstrated that 75% phenanthrene was degraded by *Pseudomonas sp*. BZ-3 of petroleum hydrocarbon at initial concentration of 50 mg/L in 7 days. Three strains were isolated by Zhang *et al.* (2012) which were able to degrade n-alkanes up to n-C40. Removal efficiency of each strain increased up to 10% within 7 days, under aerobic conditions. The three strains were identified as *Pseudomonas aeruginosa.* Saleh *et al.* (2014) reported degradation of aromatic hydrocarbons including benzene and phenanthrene by *Cupriavidus gilardii, Pseudomonas sp., Bacillus cereus* and *Paenibacillus ehimensis.* In our case, *Pseudomonas aeruginosa* degraded 85% of 1000 mg/L benzene in 72 h. When the concentration of benzene was 1.62 µg/L, they achieved 22.2 % degradation. Sharma *et al.* (2014) reported 66% degradation of diesel hydrocarbons including benzene by *Pseudomonas aeruginosa* in 30 days.

Dou *et al.* (2010) reported complete degradation of 150 mg/L benzene within 25 days with the help of *Bacillus cereus*. In the present study, *Bacillus cereus* was able to degrade 83% of 1000 mg/L benzene in 72 hours.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

Based upon the experimental results, statistical analysis and bioinformatics data, following conclusions can be drawn from the present study.

1. Total 16 bacterial strains were isolated from the soil based upon morphological and biochemical tests. During the screening process for the degradation capability for increasing concentrations of benzene, only 7 were able to grow on 1000 mg/L of benzene while others were eliminated upon to exposure to 250, 500 or 750 mg/L of benzene.
2. In degradation studies with the selected seven strains at 1000 mg/L of benzene, three strains out-competed others. Prominent benzene degraders were identified as *Pseudomonas aeruginosa, Bacillus cereus* and *Acinetobacter junii* which degraded 85, 83 and 70% benzene, respectively in 72 h period.

5.2 RECOMMENDATIONS

It has been a ground breaking work in terms isolation, identification and degradation studies for benzene at the department. Following are some of the suggestions for carrying forward the work reported here:

1. These bacterial strains may be studied in combination for the remediation of contaminated soils and water with benzene and other pollutants of same nature. These could have synergistic effect in consortium and could be more effective for degradation of target compound(s).
2. Complete understanding of mechanisms can help to develop bench and pilot scale reactors for the degradation of benzene and other petrochemicals. Relationship with various physical factors can help to improve efficiency and effectiveness of these strains.
3. Identification of genes responsible for benzene degradation in these bacterial strains could help to exploit their maximum potential for degradation through modification of relevant gene expression.

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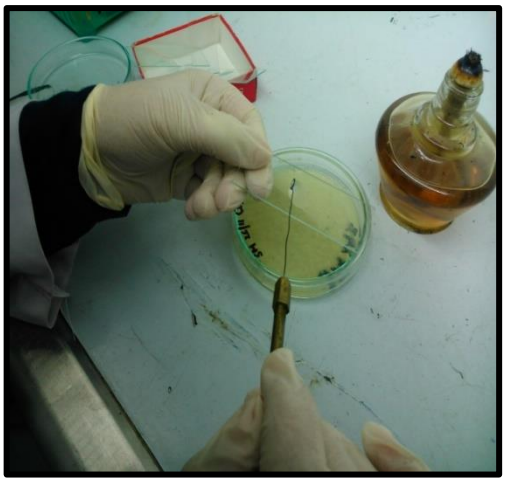
ANNEXURES

Rhizospheric Soil

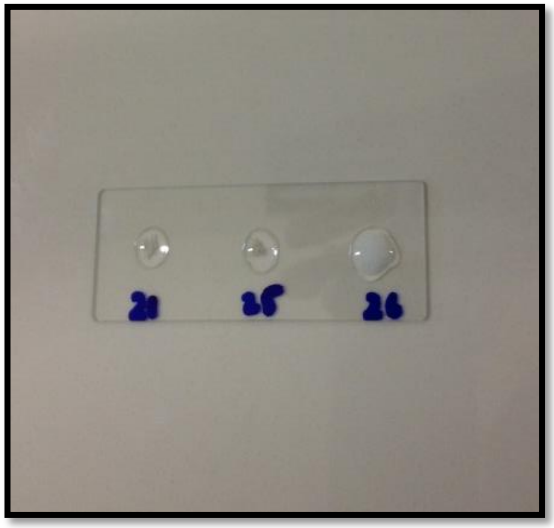


Gram Staining

Eosin Methylene Blue Agar

Macconky Agar

Catalase Test



Oxidase Test

Experimental Setup