# DETERMINATION OF DEGRADATION POTENTIAL OF INDIGENOUS MICROORGANISMS FOR TOLUENE

by

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It is certified that contents and form of thesis entitled

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**DEDICATED !!!**

*To my ever loving parents, sister, brothers, cousins, late grandmother & last but not the least my best friend whose countless prayers and faith in my abilities made me able to stand where I am*

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***MARIAM MANSOOR***

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| BTEX | Benzene, toluene, ethylbenzene, xylene |
| MSM | Mineral salt medium |
| EDTA | Ethylene diamine tetra acetic acid |
| PAHs | Polycyclic aromatic hydrocarbons |
| rpm | Revolutions per minute |
| $lb/cm^2$ | Pounds per centimeter square |
| OD | Optical density |
| 16S rRNA | 16S ribosomal ribonucleic acid |
| BLAST | Basic Local Alignment Search Tool |
| NCBI | National Center for Biotechnology Information |

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# ABSTRACT

Toluene is a cyclic monoaromatic hydrocarbon which is extensively used as an industrial solvent. It is among the major environmental pollutants in petroleum contaminated sites. Environmental and public health effects of toluene due to soil and water contamination requires remedial measures. It has been categorized as hazardous chemical on EPA list of priority pollutants. Biodegradation offers the most viable solution for remediation of petroleum contaminated sites. Present study was carried out to isolate the bacterial strains capable of degrading toluene efficiently. A total of thirteen toluene degrading bacteria were isolated from petroleum contaminated soil by enrichment culture technique. Mineral salt medium (MSM) having toluene as a sole carbon source was used to investigate the growth of isolated strains. Acclimatization study was carried out to screen out potential toluene degraders on the basis of optical density measured at 600 nm. Out of thirteen isolates, only four isolates showed efficient growth at different concentrations (15, 30, 45 and 60 mg/L) of toluene with maximum optical density of 0.85. Identification of potential strains was done by 16S rRNA gene sequencing process which identified strains as *Ochrobactrum anthropi*, *Ochrobactrum intermedium*, *Enterobacter cloacae* and *Pseudomonas aeruginosa.* Bench scale bioreactor system was established for biodegradation of toluene and its percentage removal was determined by using UV-visible Spectrophotometer. At 60 mg/L of toluene concentration, degradation efficiency of individual strains and consortium was found to be 62.9, 58.7, 72.1, 81.1 and 88.4 % respectively within 72 hours which indicated that efficiency enhanced when consortium of four isolates was used. A phylogenetic tree was developed by using MEGA 4 program to explore the lineage of potential toluene degraders. This study may play an important role in onsite bioremediation of petrochemical industrial waste.

*Chapter 1*

# INTRODUCTION

## 1.1 BACKGROUND

Monoaromatic hydrocarbons like benzene, toluene, ethylebenzene and xylene also known as BTEX represent a major class of environmental pollutants due to their increased toxic effects to a large number of organisms (Jo *et al*., 2008). They are extensively used chemicals in a number of industrial synthesis (Lin *et al*., 2010). The solubility of BTEX in water poses serious threat of groundwater pollution (Annesere *et al*., 2008). The higher motility of these compounds in soil and water is associated with low octanol-water partition coefficient, which ultimately leads to slow absorption and at the end, a preferential water transportation which favors the contamination of water resources, once they move around at a faster rate in these kinds of mediums (Nakhla, 2003).

BTEX are an important class of organic pollutants which are components of gasoline and fuels (Yadav and Reddy, 1993). BTEX compounds as major environmental contaminants are often present together at contaminated sites as a result of leakage of solvents, fuels and inappropriate waste management practices (Xu *et al*., 2003). The U.S. Environmental Protection Agency has categorized them as environmental priority pollutants indicating their immediate removal from contaminated sites (Dean, 1985).

The majority of aromatic hydrocarbons found in the environment are from the release of petroleum chemicals, processing and burning of fossil fuels. These also travel to marine and inland water systems by means of flowing surface water and wastewater. Monoaromatic compounds such as BTEX have water solubility that usually decreases as the number of benzene rings increases (Homg *et al*., 2009). The amount of individual aromatic hydrocarbons depend on their sources,

environmental factors and degradation kinetics. Currently the major solution towards the elimination of these compounds is microbial degradation (Wilson and Jones, 1993).

Severe exposure to gasoline and its other components such as benzene, toluene and xylene is directly related to nose, eye and throat infection, headache, vomiting and malfunctioning of liver, kidney and brain (EPA, 2004).

Improvement in quality of life demands efforts to combat such aromatic hydrocarbons waste by identifying cost effective and environment friendly remediation technologies. Recent developments have made microbial system and bioremediation programs the more nature friendly choice over the traditional methods of waste handling like incineration, filling, excavation etc. for the remediation of polluted soils and water systems (Labana *et al*., 2005).

Biodegradation of organic matter and toxic chemical compounds in soil and water is the most economical, efficient and environment friendly approach for elimination of such compounds from natural surroundings. (Furukawa *et al*., 1998). The working principle of this process is to increase the rate of natural degradation of contaminants by providing the microorganisms with specific conditions and nutrients which help them to degrade the desired contaminants.

The magnitude of groundwater pollution with BTEX hydrocarbons is increasing day by day, thus requiring more effective treatment methods to eliminate the risks caused by these compounds. The physical methods of treatment, besides very high operational and maintenance cost, account for elimination of the pollutants from environment without any transformation which results in accumulation of poisonous residues in the environmental systems.

Biological treatment such as “Bioremediation or Biodegradation” is considered to be an efficient clean technology, due to its simplicity, cost effectiveness as compared to other treatment methods.

According to the European Environment Agency (EEA), contaminated sites in Europe are nearly 250,000. If the present trend continues with the same rate, the number of sites requiring remediation will be increased by 50 % by 2050.

## 1.2 TOLUENE

Among all BTEX components, toluene also called methyl benzene is distributed in water, soil and industrial wastewater. It is a common aromatic hydrocarbon that is extensively used in manufacturing processes and as a solvent for resins, coatings, oil and paints. Toluene is carcinogenic and hardly soluble in water. Humans can be exposed to toluene either by inhalation or by means of ingestion.

Toluene causes damage to human kidney and liver even at very minute concentration. Every year large quantities of toluene are being released into the environment thus impairing the quality of air and causing health risk to humans. Maximum Contaminant Level for toluene is 1 ppm as mentioned by EPA. Long term exposure of toluene to industrial workers results in an increased risk of leukemia (Pratheesh and Jayachandran, 2012).

## 1.3 PRESENT STUDY

The process of biodegradation uses bacteria to degrade environmental pollutants and transform them into less detrimental products. It involves complete mineralization of organic compounds into water and carbon dioxide. The present study was conducted to investigate biodegradation of one of the most widely used monoaromatic hydrocarbons like toluene by using indigenous soil microorganisms isolated from petroleum contaminated soil.

## 1.4 OBJECTIVES OF STUDY

The objectives of the present study were:

1. Isolation, identification and characterization of toluene degrading bacteria
2. Determination of toluene degradation potential

Overall this research work is an initiative in developing remediation methodologies for cleaning up of toluene contaminated sites thus improving the overall quality of life and environment.

*Chapter 2*

# LITERATURE REVIEW

Petroleum compounds of crude oil and fossil fuel are the major pollutants in the environment. Petroleum hydrocarbons enter into the environment through accidental spillage and cause serious damage to the biotic and abiotic components of the environment (Prince and Roger, 1993). Although n-alkanes can be degraded very easily as compared to PAHs, long chain n-alkanes and branched -chain hydrocarbons which are hard to degrade.

Volatile organic compounds like aromatic hydrocarbons are the most widespread environmental contaminants. Hydrocarbon pollution in the seawater is designated as serious international environmental problem (Bao *et al*., 2012).

Hydrocarbon contamination in the environment arises from utilization of petroleum hydrocarbon resources, noxious air emissions from manufacturing processes, and the shipping or usage of petroleum products etc. These non-halogenated hydrocarbons are an important area of concern as they are very toxic and carcinogenic in nature, even in very minute quantities. Higher water solubility of BTEX, compared to other hydrocarbons leads to their increased mobility which enable them to become a part of subsurface water ultimately causing contamination of drinking water systems (Margesin *et al*., 2003). Regardless of many governmental and administrative interventions in different countries of the world, BTEX emissions rate in the environmental surroundings is still very high (Milna *et al*., 2007).

Both off shore and on shore spills of petroleum and petroleum products lead to serious environmental pollution and deterioration of quality of life. Large scale spill of hydrocarbons in oceanic regions, either accidental or human induced, poses serious risk to the marine environment.

In case of on shore oil spill, the petroleum hydrocarbons move towards the water table before becoming immobilized in the soil ecosystem. These components of hydrocarbons spread in a horizontal position on the groundwater system and ultimately reach pore places of soil along the transport route, resulting in soil and groundwater pollution at a large scale (Adebusoye *et al*., 2007).

Volatile organic compounds consist of significant fractions of toxic waste being handled and treated worldwide. They are considered to be harmful to public health, ecosystem and atmospheric environment (Adam *et al*., 2001). They are listed amongst major environmental pollutants due to toxic aspects of these chemicals. They volatize to air at a faster rate and distribute over remote regions due to high vapor pressure. Their presence in the air initiates various environmental issues like greenhouse gas effect, climate change, and ozone depletion (Durmusoglua *et al*., 2010).

In the U.S. volatile organic compounds emissions are strictly regulated by air quality assessment agencies at state and federal level (Fatehifar *et al*., 2008). This group of VOCs is regarded as **"regulated hazardous air pollutants"** according to United States Clear Air Act amendments projected in 1990 (Clean Air Act, 1990). Aromatic Hydrocarbons like benzene are classified as hazardous air pollutants and are restricted to 25 tons/y total amount of aromatic compounds and 10 tons/y of any other individual aromatic compound (Morrow and Lunsford, 1997).

BTEX are naturally present in different petroleum products like gasoline, crude oil and diesel oil. Benzene is extensively used in manufacturing of synthetic materials like paints, thinners, pesticides and plastics. Toluene and ethylbenzene are being used as industrial solvents for surface coatings, gums, paints and different types of oils. Ethylbenzene is used as fuel additive in gasoline

and aviation fuel (Buswell, 2001). Xylene is used as a solvent in rubber and leather manufacturing industries (U.S. EPA, 2006).

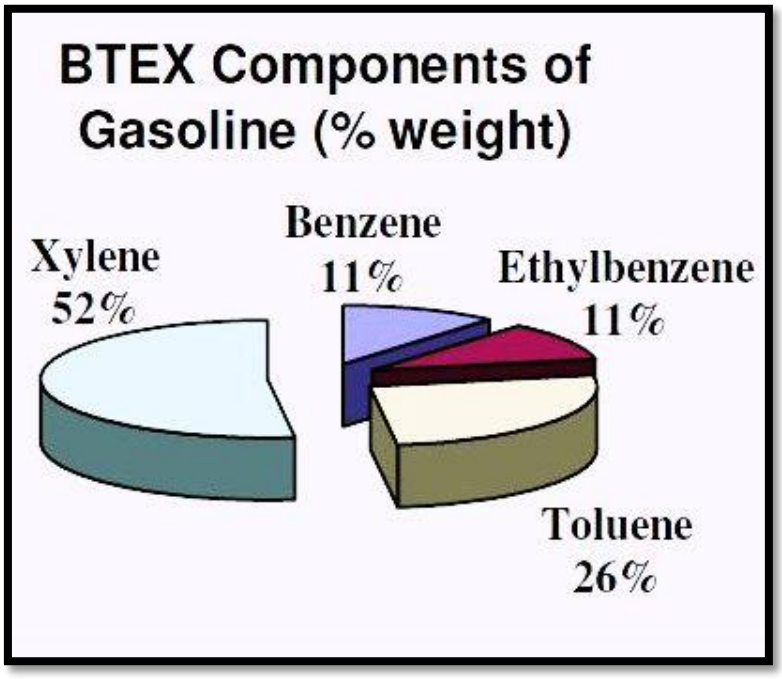


**Figure 2.1: BTEX Components of Gasoline**

Many restoration methods have been tested for the ultimate removal of toxic hydrocarbons from environenmnt since last decade. Biodegradation is an important approach to make use of biological processes to the maximum extent for rapid eradication of environmental contaminants (Hassanshahian *et al*., 2012).

## 2.1 Aromatic Hydrocarbons Contamination

The main source of aromatic hydrocarbons like BTEX pollution is the gasoline spill from improperly maintained underground gasoline storage tanks. Aromatic hydrocarbons enter into the environenmnt from leakage in pipelines, surface and underground spills and release from large gasoline facilities. Due to harmful effects of these chemicals on human health, the U.S. EPA has

modified maximum allowable levels of these pollutants in water for human use (U.S. EPA, 2006). Once these compounds become a part of the environment, they readily volatize, get attached to soil particles by getting into the soil pore places or biodegrade.

BTEX compounds stand as high as 80 % of the overall amount of volatile organic compounds in petrochemical related units and account for up to 59 % w/w of total gasoline contaminants (Kim and Lee, 2011).

Evaporation happens when these hydrocarbons volatize, which allows them to get into the atmosphere causing air pollution. Evaporation of the aromatic compounds takes place when gasoline is being pumped in the car causing characteristic smell. The same process occurs within the air spaces in soil. BTEX get dissolved into water channels causing groundwater contamination. If sufficient amount of oxygen is present then BTEX can also be degraded biologically though gradually (U.S. EPA, 2006).

## 2.2 Sources of Aromatic Hydrocarbons in Environment

BTEX as a mixture are naturally occurring components of crude oil and petroleum. For example benzene concentration in crude petroleum is up to 4 g/L. It can also be found in seawater system (0.8 ppb) in the locality of petrol and gasoline deposits (Atlas and Bragg, 2009). Gaseous emissions from volcanoes and forest fires constitute other natural sources of BTEX. Volatile organic compounds get into the environment through vehicular and air craft emissions, spills from different sources of petrol and losses during gasoline and other hydrocarbons marketing (Wang *et al*., 2010).

BTEX components are being produced and consumed during the processing of coal, crude oil, and refined petroleum products and during the manufacturing of daily consumer products like adhesives, inks, cosmetic and pharmaceutical items. BTEX fall under the category of most

abundantly produced chemical items including benzene, toluene, ethylbenzene and xylene with global annual production of nearly 8-10, 5-10, 4-9 and 10-15 million tons respectively. They can be introduced into the water through industrial wastewater and atmospheric emissions but discharge of BTEX into water is mostly due to petrol spills (Nie *et al*., 2013).

## 2.3 Health Effects of Aromatic Hydrocarbons

Volatile organic compounds can easily move into the atmosphere, so they can affect public health directly or indirectly. Short term exposure to BTEX compounds show potential toxicity to marine life in water systems (especially in confined regions) as well as toxic breathing hazards. Chronic exposure of these chemicals causes alterations in liver functioning and life threatening effects on central nervous system, lungs and heart. When humans are exposed to such toxic compounds in the form of a mixture, it leads to genetic, neurological, excretory and respiratory system malfunctioning (Dastgheib *et al*., 2011).

These chemicals are intense eye, skin and mucous membrane irritants and ultimately reduce bone marrow function and cause cancer. These chemicals persist in the environenmnt due to their wide spread applications in industrial processes (Tapilatu *et al*., 2010).

## 2.4 Biodegradation Techniques

The conventional approaches for management of oil leakage and spills are being confined to physical methods. On the other hand, biological methods for treatment can have benefit over the physicochemical processes in removing spills as they present bioremediation of oil fraction by bacteria (Chaillan *et al*., 2004). Biological decontamination of oil and its derivatives in polluted

surroundings is known to represent an efficient, economic and versatile alternate to other methods of waste treatment (Emtiazi *et al*., 2009).

The extent of bioremediation mostly depends on oil concentration, length of alkanes, biosurfactants and also on the type of microbes used for degradation (Cappello and Richard, 2012). It has been noticed that saturated components of crude oil are predominantly the alkanes of chain length ($C_{10}$-$C_{20}$) which can be easily degraded (Subarna and Raj, 2002). The degree of uptake and mineralization of many organic complexes depend on the quantity of compound.

High amount of hydrocarbons inhibit the process of degradation. It is done by either nutrient or oxygen shortage or via poisonous impacts applied by volatile organic compounds (Luis and Paul, 2000).

Das and Mukherjee (2007) reported biodegradation of petroleum hydrocarbons by *Acinetobacter* species isolated from petroleum polluted soil from North East India. Bacteria was capable of using n-alkanes as carbon source (Throne *et al*., 2007).

Bacterial species like *Gordonia, Brevibacterium, Aeromicrobium, Dietzia, 3Burkholderia* and *Mycobacterium* isolated from soil contaminated with petrol hydrocarbon are said to be the potential degraders of hydrocarbons (Jain *et al*., 2010). Daugulis and McCracken (2003) reported biodegradation of polycyclic aromatic hydrocarbons by *Sphingomonas* species.

Biodegradation of oil by synthetic microalgae-bacteria consortium was studied. Consortium that was made by *Scenedesmus obliquus* termed GH2 and other four crude oil degrading microorganisms with well-known degradation abilities, like *Sphingomonas* GY2B, *Burkholderia cepacia* GS3C, *Pandoraea pnomenusa* and *Pseudomonas* GP3A were said to be capable of eliminating alkanes totally, alkyl benzene and alkyl cycloalkanes within period of seven and ten

days correspondingly. Consortium also favorably degraded polycyclic aromatic hydrocarbons like phenanthrene and methylphenanthrene (Tang *et al*., 2012).

## 2.5 Aromatic Hydrocarbons Degradation

The bioremediation of gasoline and petrol hydrocarbons in the environment is a complicated phenomenon, its numerical and qualitative alterations mostly depend on the nature and quantity of crude oil present, the ambient and periodic environmental settings such as dissolved oxygen, optimal temperature (20-35 °C) and physical or chemical distribution of oil (Ubalua and Ezeronye, 2005).

Biodegradation of oil has been known to occur by attack of bacteria on light weight aromatics. High molecular weight aromatics like resins and asphaltenes are said to exhibit very low rates of degradation, though some studies conducted on bioremediation have stated their high removal rate under optimal conditions (Chang *et al*., 2013).

The rate of degradation usually increases with increase in temperature such as ecological system exposed to low temperature can degrade aromatic compounds at a much slower rate. The degradation of petroleum hydrocarbons in aquatic ecosystem is restricted mainly by micro and macro nutrients like nitrogen and phosphorus. Salinity and pressure factors are essential in deep sea parts. Likewise dissolved oxygen, nutrient concentration, moisture and pH are major elements in the determination of degradation.

Petrol mixtures bind to soil particles and they are hard to be removed (Barathi and Vasudevan, 2001). Petroleum hydrocarbons pollution in soil generally results in disproportion in carbon-nitrogen fraction which ultimately causes nitrogen shortage in oil water logged soil, therefore hindering the growth of microorganisms and consumption of carbon sources (Bajpai *et al*., 2011).

Moreover high concentration of degradable compounds in the upper layer of soil diminishes oxygen in soil and slows down the degree of oxygen circulation. Many native bacteria in water and soil have ability to remove petroleum toxins.

Petrol hydrocarbons can be biodegraded by varied groups of bacteria, which are habitual of consuming aromatic compounds as nutrient for growth (Das and Chandran, 2011). The biodegradation of complex compounds like crude oil and metals need bacterial consortium with extensive enzymatic capabilities (Bartha and Bossert, 1984).

Chang and Lee (2001) studied the degree of degradation of non-volatile petrol compounds and the supplementary bacterial activity in major cumulative sizes throughout a pilot-scale bio pile trial carried out at temperature 15 °C with soil having clayey texture, from a crude oil contaminated site in North of Canada. At the $65^{th}$ day of the experiment, 42 % of the $C_{16}$-$C_{34}$ hydrocarbons were found to be removed in the nutrient modified macro masses, parallel to 12 % in the meso aggregates (Brooijmans *et al*., 2009).

## 2.6 Toluene

The major sources of toluene discharge are production, transportation and consumption of petrol hydrocarbons, which have approximately 5-8 % of toluene. Its concentration in the air is found to be fairly low in remote areas, but concentration between 1.3-6.6 ppb is common in urban and suburban areas, with levels as much high as 351 ppb in areas of high traffic mass. Concentration at petrol stations can be as high as 2350 ppb. It is interesting to note that refilling a car adds considerably to daily toluene uptake (Erdogmus *et al*., 2013).

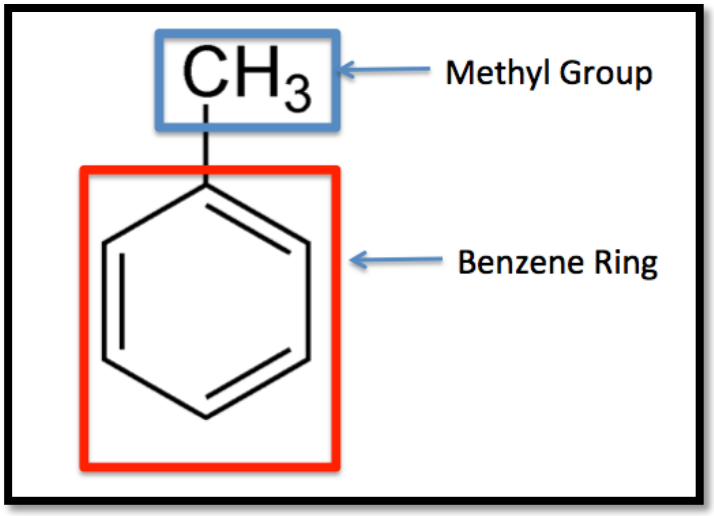


**Figure 2.2: Toluene Structure**

Toluene also known as methylbenzene is a well-known indoor air pollutant that is being used in daily household items such as nail polish, paints and also from cigarette smoke. Toluene is being detected in drinking water and its occurrence levels are usually less than 4 ppb but they can reach to 3500 ppb in groundwater systems from industrially contaminated sites.

**Table 2.1: Physical and Chemical Properties of Toluene**

| Name of Compound | Toluene |
|---|---|
| Physical State | Liquid |
| Molecular Weight | 92.14 g/mol |
| Color | Colorless |
| Odor | Sweet, Pungent, Benzene like |
| Boiling Point | 110.6 ˚C |
| Melting Point | -95 ˚C |

## 2.7 Toluene Degradation

Bioremediation of toluene happens in both groundwater and soil. On the other hand, it is observed to be slow particularly at higher concentration, which is lethal to bacteria. The use of acclimatized microbial community can allow fast degradation. Toluene is considered to be completely degraded in groundwater within a time span of 8 days which includes a lag phase of 3-4 days whereas bacterial community adapts to the specific growth conditions. In other case, it is also found that merely 1-2 % toluene is degraded in subsurface systems and the left over amount is being removed in period of one month in soil cores at different depths (Pufahl and Hiatt, 2012).

When toluene enters into the water, its removal rate can be faster or take some weeks which is dependent on different environmental conditions like temperature availability of nutrients, pH and rate of acclimatization. Evaporation of toluene occurs rapidly from water with an experimentally determined half-life of 2-8 hours.

Adenipekun and Clementina (2008) reported that *Pleurotus tuberregium* have the capacity to intensify nutrients in soils polluted with 1-30 % engine oil. Therefore fungi can also have potential to clean environment contaminated with oil and petrol components. A number of reports have been in knowledge about biodegradation of non-oxygenated compounds, there are only few studies present on degradation of BTEX compounds.

Nicholson and Fathepur (2005) investigated the biodegradation of BTEX in microcosm developed with soil from a non-contaminated site and from an oilfield in Oklahoma.

Hassan and coworkers (2012) studied the ability of *Alcanivorax* species HA03 to degrade BTEX as a sole source of carbon. This species was isolated from soda lakes located in Wadi E1Naturn. This statement that *Alcanivorax* can remove aromatic hydrocarbons magnifies the degradation

ability of this specific type of organisms because this species is primarily well-known for its ability to biodegrade PAHs.

Three microbial species identified as *Pseudomonas aeruginosa* (UKMP- 8T), *Rhodococcus* sp. M15-2 (UKMP-5T) and *Rhodococcus* sp. ZH8 (UKMP-7T) were isolated from groundwater contaminated with crude oil refinery waste. Four bacterial consortia were made from these three isolates by mixing isolated bacterial culture in subsequent proportions as follow: (*Pseudomonas aeruginosa*: *Rhodococcus* sp. M15-2, 1:1), (*Pseudomonas aeruginosa*: *Rhodococcus* sp. ZH8, 1:1), (*Rhodococcus* sp. M15-2i ZH8, 1:1) and (*Pseudomonas aeruginosa*: *Rhodococcus* sp. ZH8: *Rhodococcus* sp. M15-2, 1:1:1) correspondingly.

This synthetic consortium demonstrated different inclinations for nitrogen and when stimulated with favorable conditions like nitrogen concentration and growth in mineral salt medium, within a period of one week, all three isolates and four prepared consortia removed approximately 97.5-99.9 % of Tapis Massa oil (Hamzah *et al*., 2013).

Malik and Ahmed (2012) studied the biodegradation of petrol hydrocarbons by bacterial consortium. They stated that microbial removal of alkanes was maximum like 91 % for tridecane followed by pentadecane 78 %, octadecane 74 % whereas other alkanes showed 56-68 % after 24 days of incubation. Among the micro aromatic hydrocarbons like toluene, benzene and xylene, quickly evaporated at the $4^{th}$ day of incubation process, whereas the potential of polycyclic aromatic hydrocarbons like phenanthrene, anthracene and pyrene was calculated to be 46.2 and 55.4 % respectively at $24^{th}$ day of incubation.

## 2.8 Role of Consortium in Degradation of Toluene

A study on biodegradation of petrol hydrocarbons in a contaminated lake in Lagos, Nigeria was carried out and nine microbial cultures viz, *P. putida*, *P. aeruginosa*, *B. subtilis*, *Alcaligenes* sp., *Acinetobacter* sp., *Corynebacterium* sp., *Bacillus* sp., *Flavobacterium* sp. and *Micrococcus* sp., were purified from contaminated lake which are responsible for biodegradation of crude oil (Adebusoye *et al*., 2007).

*Halomonas shengliensis* (Wang *et al*., 2007), *Halomonas* strain C2SS100 (Mnif *et al*., 2009) and *Marinobacter aquaeolei* (Huu *et al*., 1999) were reported to be isolated from oil field, wastewater and other kind of saline environenmnt that have ability to biodegrade crude oil.

## 2.9 Toluene Degradation Pathway

A number of metabolic pathways have been explained for degradation of aromatic hydrocarbons such as, toluene can be degraded by bacteria in a variety of pathways. In the pathway coded by TOL plasmid, degradation of toluene results in the formation of benzyl alcohol, benzaldehyde and subsequently benzoate which is further converted to the TCA byproducts (Harayama *et al*., 1999).

The prime step involved in the degradation of toluene by *P. putida* F1 is the introduction of two OH groups to toluene which results in the formation of *cis*-toluene dihydrodiol. This compound ultimately gets converted into 3-methylcatechol (Panke *et al*., 1998).

Toluene gets oxidized from toluene 3- monooxygenase to *m*-cerol, which results in the formation of 3-methycatechol by action of another monooxygenase. This pathway is encoded by *Pseudomonas pickettii* PKO1 (Suyama *et al*., 1996).

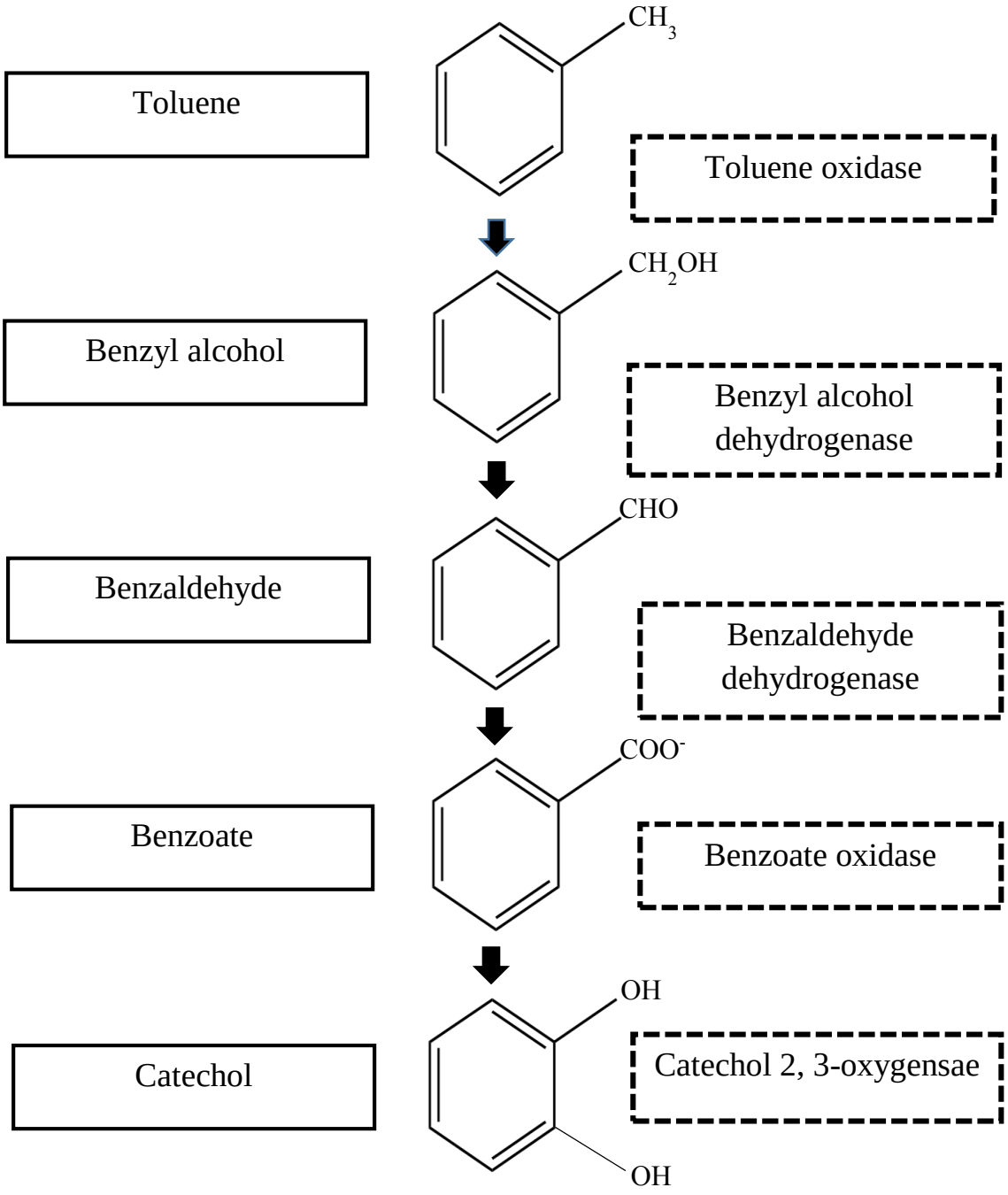


**Figure 2.3: Degradation Pathway of Toluene**

## 2.10 Regulations on Toluene

U.S. EPA has customized permissible levels for pollutants in drinking water being supplied by public water systems. They are called Maximum Contaminant Levels (MCLs). For the development of these levels, U.S. EPA uses traditional assumptions, thereby certifying proper safety of public health. In case of supposed or known pollutants, the calculation of MCLs is dependent on hypothesis that average adult person weighs (70 kg) and consumes almost 2 L of water/day over a life span of 70 years (U.S. EPA, 2012).

**Table 2.2: Max. Contaminant Levels of BTEX**

| Chemicals (mg/L = ppm) | Maximum Contaminant Levels (MCLs) |
|---|---|
| Benzene | 0.005 |
| Toluene | 1 |
| Ethylbenzene | 0.7 |
| Xylene | 10 |

## 2.11 Industrial Applications of Toluene

Toluene production rate has increased from 5.1 billion lbs. in 1984 to 6.3 billion lbs. in 1992. It was projected in 1985 that industries consumed toluene as mentioned: Benzene production 45 %, industrial solvent 9 % and gasoline blending 36 %. The main chemical use for toluene is the manufacturing of benzene, benzoic acid, dyes, explosives and many other organic compounds such as solvents for paints, gums, resins; antifreezes and in fuel blending process (U.S. EPA, 2012).

*Chapter 3*

# MATERIALS AND METHODS

The present study was conducted in Environmental Microbiology Teaching Laboratory, Institute of Environmental Sciences and Engineering at the National University of Sciences and Technology, Islamabad.

Biodegradation studies of toluene were carried out by using indigenous soil microbes in bench scale bioreactors. The increase in growth of microorganisms determined the utilization potential of monoaromatic hydrocarbon by microorganisms and lead to decrease in organic matter. The quantitative analysis was done by UV- visible spectrophotometer to correlate the reduction in monoaromatic hydrocarbon with the corresponding growth of microbes in order to determine the extent of degradation.

## 3.1 Chemicals and Standards

The monoaromatic hydrocarbon used in this study was purchased from Merck. The standard analyte of toluene and extraction solvent n-hexane were also purchased from Merck.

## 3.2 Concentration of Aromatic Hydrocarbons

For growth studies, different concentrations of toluene were used in mineral salt medium to determine the utilization potential of bacteria. 15, 30, 45, 60 and 100 mg/L concentrations were used for acclimatization studies and 60 mg/L was used for degradation studies in bench scale bioreactors in present research work.

## 3.3 Preparation of Culture Media

### 3.3.1 Washing and Sterilization of Glassware

For experimental work, all glassware was dipped in solution of chromic acid for 24 hours, then washed thoroughly with tap water and then finally rinsed with distilled water. For sterilization, glassware was wrapped in aluminum foil and autoclaved at 121 °C and 15 lb/cm² pressure for 15 minutes. The sterilized glassware was then oven dried at 105 °C for 1 hour before use. All glassware for spectrophotometric analysis was prepared prior to sampling through washing with phosphate free detergent then with chromic acid and then rinsing with distilled water followed by n-hexane.

### 3.3.2 Preparation of Media

#### 3.3.2.1 Mineral Salt Medium (MSM)

The composition of mineral salt medium is shown in Table 3.1.

**Table 3.1: Composition of Mineral Salt Medium**

| Sr. No. | Chemicals | Quantity (g/L) |
|---|---|---|
| 1 | $KH_2PO_4$ | 0.5 |
| 2 | $NH_4Cl$ | 1 |
| 3 | NaCl | 0.01 |
| 4 | $MnSO_4$ | 0.0003 |
| 5 | $ZnSO_4$ | 0.0004 |
| 6 | EDTA | 0.001 |

### 3.3.2.2 Other Media

For preparing fresh cultures and bacterial inoculum, nutrient agar plates were prepared according to manufacturer's instructions. For this purpose 28 g of nutrient agar (Merck KGA) was mixed in 1 L distilled water and autoclaved at 121 °C and 15 lb/cm² pressure for 15 minutes. The nutrient agar was poured in autoclaved oven dried petri plates. Sterility test was performed by placing petri plates in incubator at 37 °C for 24 hours (Kao *et al*., 2005).

# EXPERIMENTAL SETUP

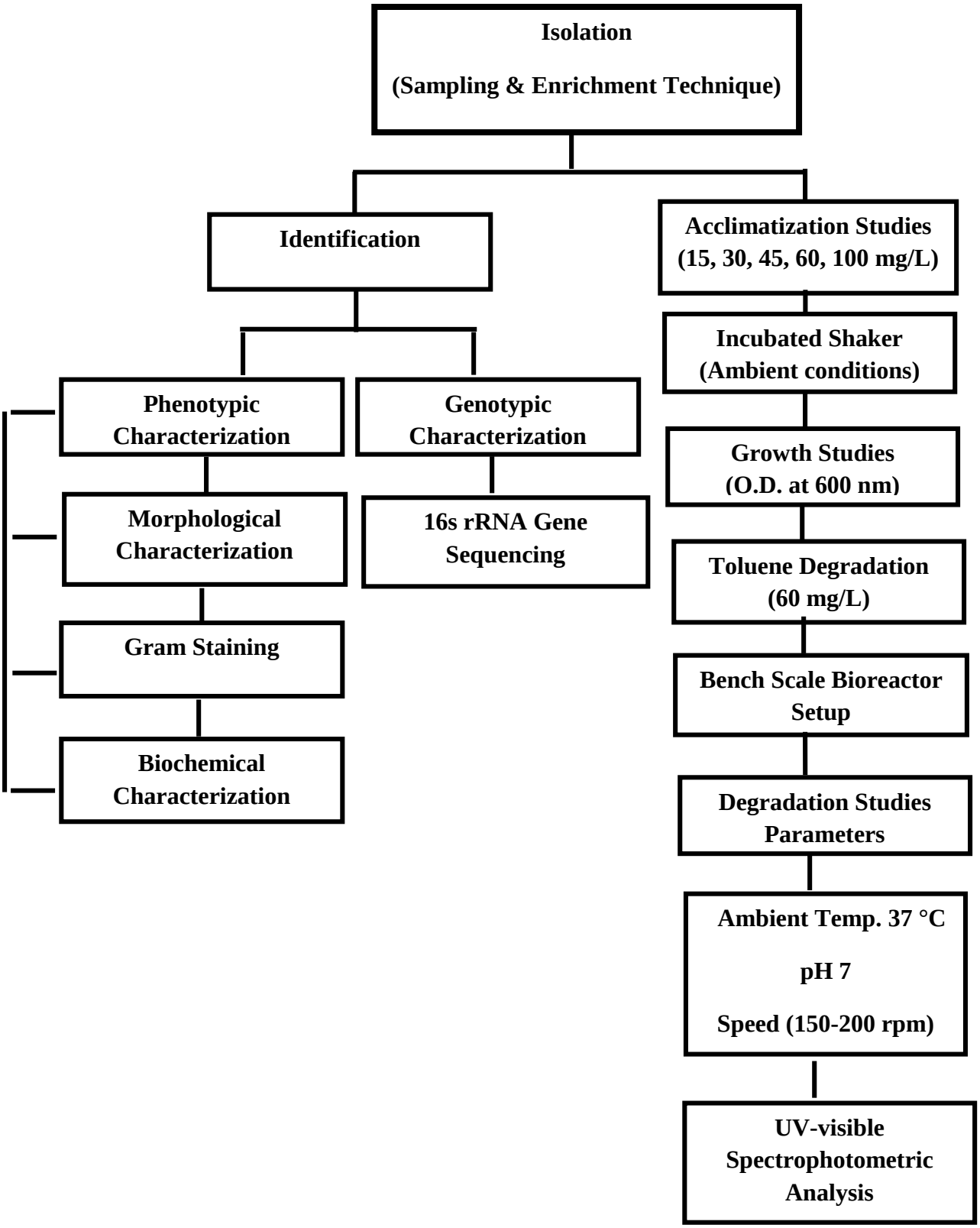


**Figure 3.1: Experimental Setup of Toluene Degradation**

## 3.4 Isolation

### 3.4.1 Sampling

Soil samples were collected from Attock oil Refinery Morgah, Rawalpindi situated in Punjab. Soil samples were taken from the top 0-15 cm of soil from three different sites. Composite sample was made by mixing soils from each site. Soil was kept at 4 $^{o}$C in sterile polythene bags before bacterial isolation. The pH of soil sample was measured using portable pH meter (Nannipieri, 1994). Soil samples were air dried for a period of 24 hours, then sieved by using 2-mm sieve and finally crushed by using pestle and mortar.

### 3.4.2 Enrichment Technique

Toluene degrading species were isolated using enrichment culture technique. 10 g of soil was inoculated into 250 ml volume Erlenmeyer flask containing 100 ml of sterilized mineral salt medium having pH 7 with 15 mg/L of toluene. The flask was incubated at 37 $^{o}$C with shaking at 150-200 rpm. After two weeks of incubation, 10 ml of sample from flask was transferred to another flask with freshly prepared 100 ml mineral salt medium containing different concentrations of toluene (30 and 45 mg/L) as a sole carbon source respectively. After one week of incubation, serial dilutions of the enriched culture were prepared. Six sterilized test tubes were arranged in a test tube rack. These were marked as $10^{-1}$ to $10^{-6}$. 9 ml autoclaved distilled water was added to each test tube. 1 ml of enriched culture was added to first test tube in the series marked as $10^{-1}$ and homogenized by vigorous shaking on vortex for 2 minutes. 1 ml of sample was taken from this tube and added to subsequent tube in the series named as $10^{-2}$.The same procedure was repeated for next tubes. 0.l ml of sample from $10^{-3}$ to $10^{-6}$ dilutions was poured and spread on toluene coated nutrient agar plates and the plates were incubated at 37 $^{o}$C for 24 hours. The bacterial

colonies were counted on colony counter. Colony forming unit was calculated with the help of following formula:

Cfu/g of soil = number of colonies x dilution factor x 10

### 3.4.3 Maintenance of Bacterial Culture

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

## 3.5 Identification

Identification of isolated strains was done with the help of colony morphology, gram staining, biochemical test (Collins and Lyne, 1985) and growth on selective media.

### 3.5.1 Morphological Characterization

#### 3.5.1.1 Colony Morphology

Observation of single colony (derived from a single cell) plays a key role in description and identification of unknown microorganisms. The single colony was picked and streaked on nutrient agar plate repeatedly until the pure culture was obtained in the form of single isolated strain. Colony was observed at each step of purification in terms of color (from naked eye under microscope), shape, margin, size, texture, elevation and pigmentation.

**Table 3.2: Morphological Characteristics**

| Morphological Characteristics | Description |
|---|---|
| Colony size | Puntiform, Small, Large |
| Margins | Entire, Undulate, Curled, Lobate |
| Forms | Circular, Irregular, Filamentous, Rhizoid |
| Texture | Creamy, Mucoid, Dry |
| Elevation | Raised, Convex, Umbonate |
| Color | Yellow, Orange, Pale Yellow, Off-White |

### 3.5.1.2 Cell Morphology

Cell morphology was observed through Gram staining method under oil immersion at 100X resolution using light microscope. Cells were identified as gram positive or gram negative cocci, bacilli or cocci-bacilli.

### 3.5.2 Biochemical Characterization

### 3.5.2.1 Catalase Test

Catalase is an enzyme produced by bacteria which decomposes hydrogen peroxide into oxygen and water. Usually catalase production is characteristic of aerobes and facultative anaerobes. The reaction taking place in bacterium by catalase production is reported as

$$2H_2O_2 + \text{Catalase} \longrightarrow 2H_2O + O_2$$

0.3 % hydrogen peroxide solution was prepared. A clean slide was taken and 18-24 hours fresh culture was placed onto it with the help of sterile inoculating loop.

Hydrogen peroxide solution was poured onto the growth. The immediate effervescence (bubble production) indicated positive catalase test whereas no bubble production indicated negative result (Reiner and Roger, 2012).

#### 3.5.2.2 Oxidase Test

In this test tetra-methyl-p-phenyenediamine dihydrochloride (TMPD) is used which is oxidized by cytochrome oxidase and transformed into a colored product i.e. dark blue or purple which indicates the presence of cytochrome oxidase, also known as indophenols oxidase. TMPD solution was prepared. The autoclaved filter paper strips were dipped in the solution and air-dried. Bacterial culture was picked with the help of inoculating loop and rubbed on dried filter paper strips. Change of bacterial culture color to blue is an indication of positive result while no change is a sign of negative result.

#### 3.5.2.3 Citrate Utilization Test

This test is performed for the ability of bacteria to convert citrate into oxaloacetate. In this media, citrate is the only carbon source available to the bacteria. If it cannot use citrate then it will not grow. These organisms also convert ammonium dihydrogen phosphate to ammonia and ammonium hydroxide which creates an alkaline environment in the medium. At pH 7.5 or above bromothymol blue turns royal blue. At neutral pH, bromothymol blue is green. Simmon's agar plates were prepared and inoculated with 24 hours fresh culture. After inoculation, plates were incubated at 37 $^{o}$C for 24 hours.

### 3.5.3 Molecular Characterization

#### 3.5.3.1 16S rRNA Gene Sequencing

Bacteria isolated from petroleum contaminated soil were further preserved for gene sequencing analysis. Bacterial isolates fully grown on nutrient agar plates were wiped gently with autoclaved distilled water with the help of glass rod. Prepared bacterial inoculum was transferred into eppendorf and centrifuged for 15 minutes at 2000 rpm to separate supernatant from bacterial culture and finally pellet of bacterial culture was settled down at the bottom of eppendorf. Supernatant was removed. For sample preservation, 100 µl of 50 % glycerol and 300 µl of 30 % nutrient broth were added to eppendorf containing bacterial culture. Prepared samples were preserved at -20 °C. For 16S rRNA gene sequencing process, preserved samples were sent to Genome Analysis Department Macrogen Inc. Korea.

#### 3.5.3.2 Phylogenetic Analysis

The term phylogeny or evolutionary tree symbolizes the connection amongst group of organisms that is called taxa. The tree describes the evolution of genetically related groups of organisms that belong to collective ancestors. The tips of phylogenetic tree exemplifies the set of descendent taxa which are often species. Phylogenetic tree is also called “Dendogram”. After 16S rRNA gene sequencing, sequences were processed through BLAST nucleotide search from databases of National Center for Biotechnology Information (NCBI). Then by using FASTA, low quality genomic sequences were removed. Resulted sequences were run in MEGA4 software for construction of phylogenetic tree. It exhibits the phylogenetic relation and link of identified strains with strains selected from Genbank. An extensive phylogenetic variety of microbes has ability to

degrade contaminants aerobically. *Pseudomonas* sp. and closely linked organisms are being studied because of their ability to degrade pollutants (Wacket, 2003).

## 3.6 Acclimatization Studies

### 3.6.1 Screening of Toluene Degrading Bacterial Strains

The bacterial colonies (Initial O.D. = 0.3) isolated through enrichment culture technique were acclimatized individually by inoculating into 100 ml of mineral salt medium (pH 7) and varying concentrations of toluene (15, 30, 45, 60 and 100 mg/L) as single carbon source. Flasks were placed at orbital shaker at 150 rpm for a period of four days at room temperature (Hamzah *et al*., 2011). All experiments were run in triplicates. Sample having toluene but no bacterial culture was set as control to estimate abiotic loss of toluene.

**Table 3.3: Technical Data for Orbital Shaker**

| **Orbital shaker** | **Labcon Spo-MP 8** |
|---|---|
| Speed | 150 rpm |
| Operating temperature | Ambient 37 °C |
| Volumetric load | 250 ml |
| Retention time | 72 hours |
| Size of inoculum | 10 Cfu/ml |

### 3.6.2 Sampling Procedure

At the first day, 5 ml of sample was taken from each flask after every hour for a period of six hours then at $24^{th}$, $48^{th}$ and $72^{nd}$ hours under sterilized conditions of laminar flow hood.

### 3.6.3 Analytical Procedure

The growth of bacterial strains was determined by measuring turbidity in terms of optical density (O.D.) at 600 nm by using T60 UV- visible spectrophotometer. For calibration of instrument, 5 ml of distilled water was used.

## 3.7 Selection of Potential Toluene Degraders

Out of 13 isolates, only four, able to show growth at different concentrations of toluene (15, 30, 45 and 60 mg/L) were selected for further degradation studies.

## 3.8 Biodegradation Studies

### 3.8.1 Preparation of Inoculum for Biodegradation Study

The inoculum for degradation experiment was prepared by growing selected isolates (SMM1, SMM2, SMM3, and SMM4) on nutrient agar plates and incubated for 24 hours at 37 °C. After 24 hours, culture plates were washed with autoclaved distilled water having pH 7 and inoculated in bioreactors containing 2 L of sterile mineral salt medium and 60 mg/L of toluene. Optical Density of bacterial inoculum was set to be 0.3 after inoculation (Zhao *et al*., 2009). Each experiment was run in triplicates to validate the accuracy of results.

### 3.8.2 Bench Scale Bioreactor Set up for Degradation of Toluene

For the present study, a bench scale bioreactor system was established. The experimental setup was run as a closed system without any air sparging to avoid substrate loss due to its volatile nature.

The system was operated aerobically with ambient temperature and pH remained at 7 (Reardon *et al*., 2002). The bioreactors were covered with aluminum foil to prevent photo-degradation and evaporation losses of toluene. Selected isolates were sustained in sterile mineral salt medium having 60 mg/L of toluene to determine degradation potential of isolated strains. Mineral salt medium having toluene but no bacterial culture was set as control to validate performance of selected strains. Samples were collected from each reactor at regular intervals (0, 2 4, 6, 24, 48 and 72) with the help of disposable pipettes and extraction of toluene residues for spectrophotometric analysis was done by using n-hexane as extraction solvent.

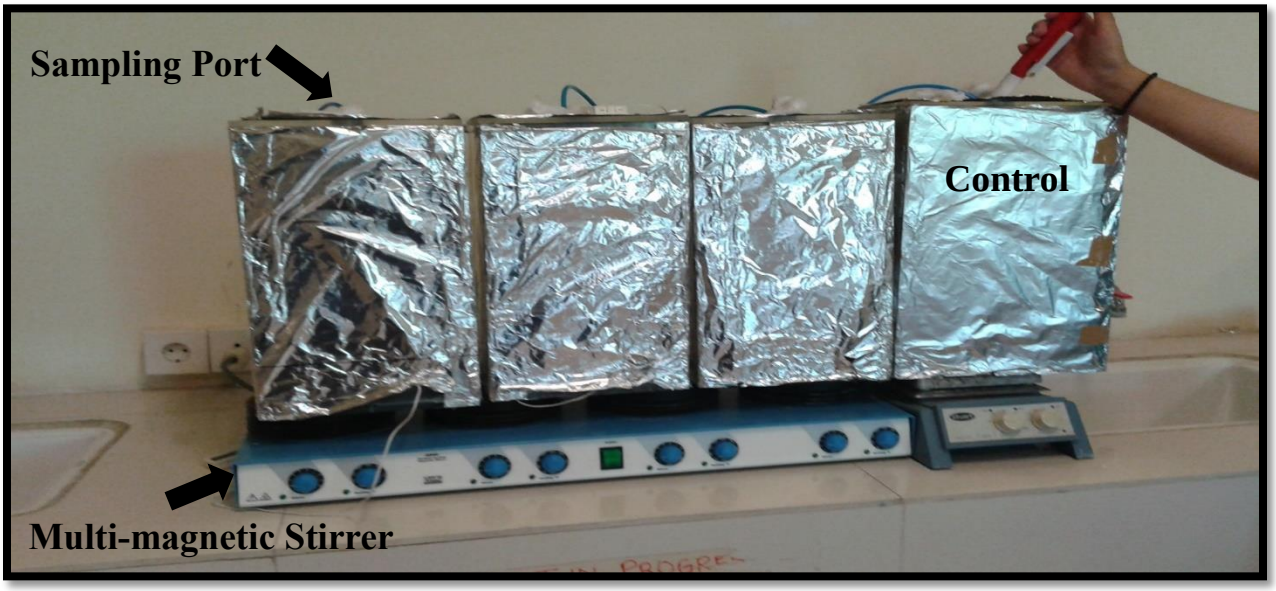


**Figure 3.2: Bench Sale Bioreactor Setup for Biodegradation**

### 3.8.3 Liquid-liquid Extraction

Samples were extracted from bioreactors at regular intervals (0-72 hours) in order to determine the rate of toluene biodegradation. 4 ml of sample was taken in test tube and 2 ml of n-hexane was added into it (Emtiazi *et al*., 2005). The mixture was gently shaken for few minutes and left for

some time until two distinct layers were apparent. The organic layer was extracted with the help of sterile glass pipette and extracted sample was further analyzed through T60 UV-visible spectrophotometer to determine the amount of residual toluene.

## 3.9 UV-visible Spectrophotometric Analysis

A spectrophotometer is an analytical tool to measure the amount of photons (intensity of light) being absorbed after it passes through sample. With the help of spectrophotometer, the quantity of a known chemical substance can also be analyzed by measuring intensity of light to be detected. Spectrophotometry is a technique which measures to what extent a chemical material absorbs light by determining the amount of light as a ray of light passes through sample.

The key principle of spectrophotometer is that every substance transmits or absorbs beam of light over a wide range of wavelength. Spectrophotometry is widely used technique for quantitative analysis in different fields. UV-visible spectrophotometer uses light over a wide ultraviolet and visible range (185-400 nm), (400-700 nm) of electromagnetic radiation spectrum respectively.

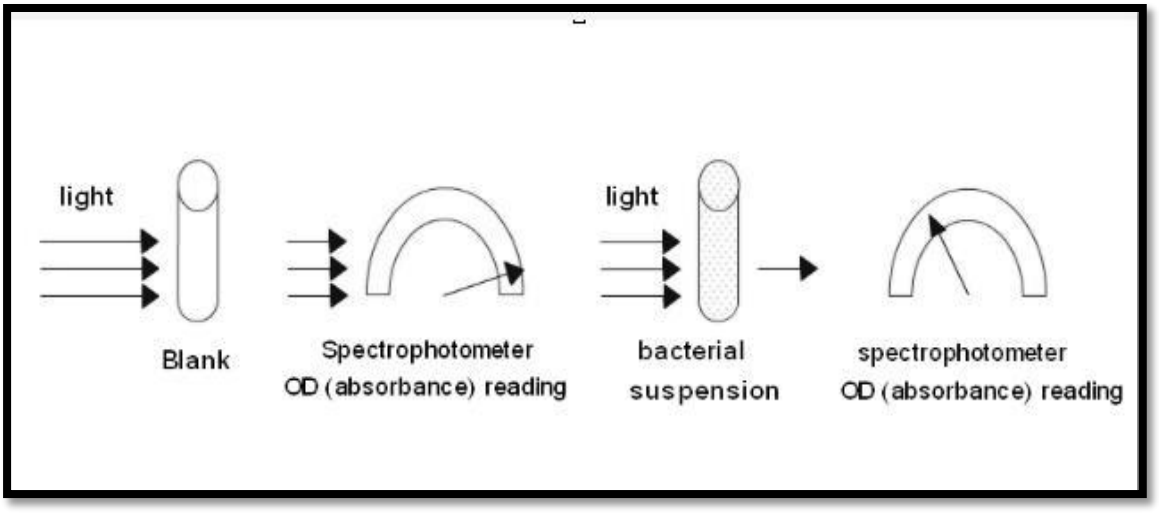


**Figure 3.3: Simplified Mechanism of UV-visible Spectrophotometer**

## 3.10 Toluene Removal Assay

Toluene shows absorbance at a wavelength of 265 nm. Samples extracted at regular intervals were then analyzed on UV-visible spectrophotometer at wavelength of 265 nm in order to determine residual concentration of toluene. The biomass of the bacterial strains were also measured in terms of optical density at 600 nm wavelength. Graphs were plotted between percentage degradation and optical density to determine the degradation potential of each isolate (Abari *et al*., 2012).

*Chapter 4*

# RESULTS AND DISCUSSION

## 4.1 Isolation of Toluene Degrading Bacteria

Bacteria that can degrade petrol hydrocarbons are being isolated from different environments, predominantly form petrol contaminated soil (Whyte *et al*., 1997). Assessments of indigenous bacterial strains are required so that microbial community configuration can be linked with capacity to biodegrade target contaminants (Alquati *et al*., 2005). An environmental pollutant acts on the indigenous biotic system, thus removing or selecting microbes according to their sensitivity in the presence of poisonous agent. Temperature and pH of soil were determined onsite and they were found to be 21 ºC and 7.13 respectively. A total of thirteen strains were isolated form petrol contaminated soil (Table 4.1).

**Table 4.1: Bacteria Isolated from Petroleum Contaminated Soil**

| Soil Sample | Isolated Strains (Designated Names) |
|---|---|
| Petroleum contaminated soil | SMM1, SMM2, SMM3, SMM4, SMM5, SMM6, SMM7, SMM8, SMM9, SMM10, SMM11, SMM12, SMM13 |

## 4.2 Morphological Characterization

The isolated strains were assigned codes as SMM1 - SMM13 as mentioned in Table 4.1. Isolated bacterial strains were studied for their form, color, size, elevation, margin and texture. Colony and

cell morphology of isolates are reported in Table 4.2 and 4.3. Majority of isolates (6/13) were observed to appear off white followed by white, green and pale yellow in appearance. The margins of strains were entire and undulate, whereas the elevation of majority of isolates was flat followed by raised, convex and umbonate. In terms of texture, 53 % isolates were creamy and 46 % isolates were dry. In terms of form, 77 % isolates were circular whereas only 23 % were irregular. 23 % of isolates were small followed by large and puntiform colony size.

**Table 4.2: Colony Morphology of Isolated Bacteria**

| Bacterial Strains | Colony size | Margins | Forms | Texture | Elevation | Color |
|---|---|---|---|---|---|---|
| **SMM1** | Large | Undulate | Circular | Creamy | Umbonate | Off white |
| **SMM2** | Large | Undulate | Circular | Creamy | Convex | Off white |
| **SMM3** | Puntiform | Entire | Circular | Dry | Flat | Off white |
| **SMM4** | Puntiform | Entire | Circular | Dry | Flat | Green |
| **SMM5** | Puntiform | Entire | Circular | Dry | Flat | Pale yellow |
| **SMM6** | Large | Undulate | Irregular | Creamy | Umbonate | White |
| **SMM7** | Small | Entire | Circular | Dry | Flat | Off white |
| **SMM8** | Large | Undulate | Irregular | Creamy | Umbonate | White |
| **SMM9** | Puntiform | Entire | Circular | Dry | Flat | Light yellow |
| **SMM10** | Puntiform | Entire | Circular | Dry | Flat | Light yellow |
| **SMM11** | Small | Undulate | Irregular | Creamy | Umbonate | Off white |
| **SMM12** | Small | Entire | Circular | Creamy | Raised | Off white |
| **SMM13** | Large | Undulate | Circular | Creamy | Convex | White |

**Table 4.3: Cell Morphology of Isolated Bacteria**

| Bacterial Strains | Gram Reaction | Shape | Arrangement |
|---|---|---|---|
| **SMM1** | Negative | Cocci | Single |
| **SMM2** | Negative | Cocci | Group |
| **SMM3** | Negative | Cocci | Group |
| **SMM4** | Negative | Bacilli | Single |
| **SMM5** | Positive | Cocci | Pair |
| **SMM6** | Positive | Bacilli | Single |
| **SMM7** | Positive | Bacilli | Group |
| **SMM8** | Positive | Bacilli | Single |
| **SMM9** | Positive | Cocci | Pair |
| **SMM10** | Positive | Bacilli | Single |
| **SMM11** | Negative | Cocci | Group |
| **SMM12** | Positive | Bacilli | Group |
| **SMM13** | Negative | Bacilli | Single |

Out of thirteen isolates, six were gram negative and seven were gram positive when observed under microscope at 100X after gram staining. Six strains were cocci while seven were bacilli in shape. Strains SMM5 and SMM9 were in the form of pairs whereas strains SMM2, SMM3, SMM7, SMM11 and SMM12 were in group arrangement. Remaining isolates were present as single.

## 4.3 Biochemical Characterization

Biochemical characterization was performed using different agars for growth of bacteria and different chemicals like tetra-methyl-p-phenyenediamine dihydrochloride (TMPD) and hydrogen peroxide for oxidase and catalase test respectively. The biochemical characterization is listed in Table 4.4.

**Table 4.4: Biochemical Characterization of Isolated Bacteria**

| Bacterial Strains | EMB Agar Test | Simmon's Agar Test | MacConkey Agar Test | Catalase Test | Oxidase Test |
|---|---|---|---|---|---|
| **SMM1** | Positive | Negative | Positive | Positive | Positive |
| **SMM2** | Positive | Negative | Positive | Positive | Positive |
| **SMM3** | Positive | Negative | Positive | Positive | Positive |
| **SMM4** | Positive | Positive | Positive | Positive | Positive |
| **SMM5** | Negative | Positive | Positive | Positive | Positive |
| **SMM6** | Negative | Positive | Positive | Positive | Negative |
| **SMM7** | Positive | Negative | Negative | Negative | Negative |
| **SMM8** | Negative | Positive | Positive | Negative | Negative |
| **SMM9** | Positive | Negative | Positive | Negative | Negative |
| **SMM10** | Positive | Positive | Positive | Negative | Negative |
| **SMM11** | Negative | Positive | Positive | Negative | Negative |
| **SMM12** | Negative | Positive | Positive | Negative | Negative |
| **SMM13** | Negative | Positive | Negative | Negative | Negative |

For EMB agar, 53 % strains showed positive while 46 % showed negative response. Out of thirteen strains, only 8 strains showed growth on Simmon's agar. In case of MacConkey agar, eleven strains showed positive while two showed negative response. Isolated strains SMM1 - SMM6 showed positive whereas SMM7- SMM13 showed negative response to catalase test. Selected bacterial isolates showed weak catalase production, only SMM1 - SMM5 exhibited strong catalase production.

## 4.4 Screening of Potential Toluene Degrading Bacterial Strains

Continuous exposure to increasing concentration of the toxic compounds to the microbes which can competently degrade these compounds is a frequently used enrichment technique (Masakorala *et al.*, 2013). In the present study, same technique was used to isolate toluene degrading bacteria. The ability of isolated strains to survive in toluene enriched environment was measured by calculating their biomass in terms of optical density at 600 nm wavelength in acclimatization process. All the thirteen strains were subjected to enrichment with mineral salt medium and toluene as a sole carbon source. Mazzeo and coworkers (2010) found that biodegradation occurs as a result of consortium of different microorganisms which includes algae, bacteria and fungi etc. However, present study consisted of changes in microbial community only.

The results indicating optical density of thirteen isolated strains in response to 15 mg/L of toluene are being presented in Figure 4.1. Out of thirteen strains namely SMM1 - SMM13, only nine isolates SMM1- SMM5, SMM7, SMM10, SMM12 and SMM13 showed growth at this concentration with maximum optical density of 0.53. These acclimatized strains were further grown on mineral salt medium containing 30 mg/L of toluene as shown in Figure 4.2. Out of these nine isolates, only seven showed prominent growth with maximum optical density of 0.65. These

isolates were SMM1 – SMM5 and SMM12 – SMM13. The other two isolates were unable to grow on toluene. These seven acclimatized strains were further subjected to 45 mg/L of toluene as shown in Figure 4.3. Only five isolates, SMM1 – SMM5 showed efficient growth with maximum optical density of 0.7. These five strains were exposed to 60 mg/L of toluene as represented in Figure 4.4. Out of five strains, only one strain namely SMM5 showed growth even less than initial value of optical density i.e. 0.3 and other four showed prominent growth with maximum optical density of 0.87. These four strains were further subjected to increased toluene concentration of 100 mg/L as shown in Figure 4.5. For 100 mg/L of toluene, all the 4 isolates showed poor growth with decreasing trend of optical density due to the substrate inhibition effect. At higher concentration of substrate, the bacteria experienced toxicity effect that inhibit the microbial activity. Mathur and Majumder (2014) also reported that at high initial concentrations (> 200 mg/L of toluene), there was relatively less degradation rate of the substrate. So 60 mg/L of toluene was selected as optimum concentration for biodegradation studies at bench scale and strains SMM1 – SMM4 were selected as potential toluene degraders.

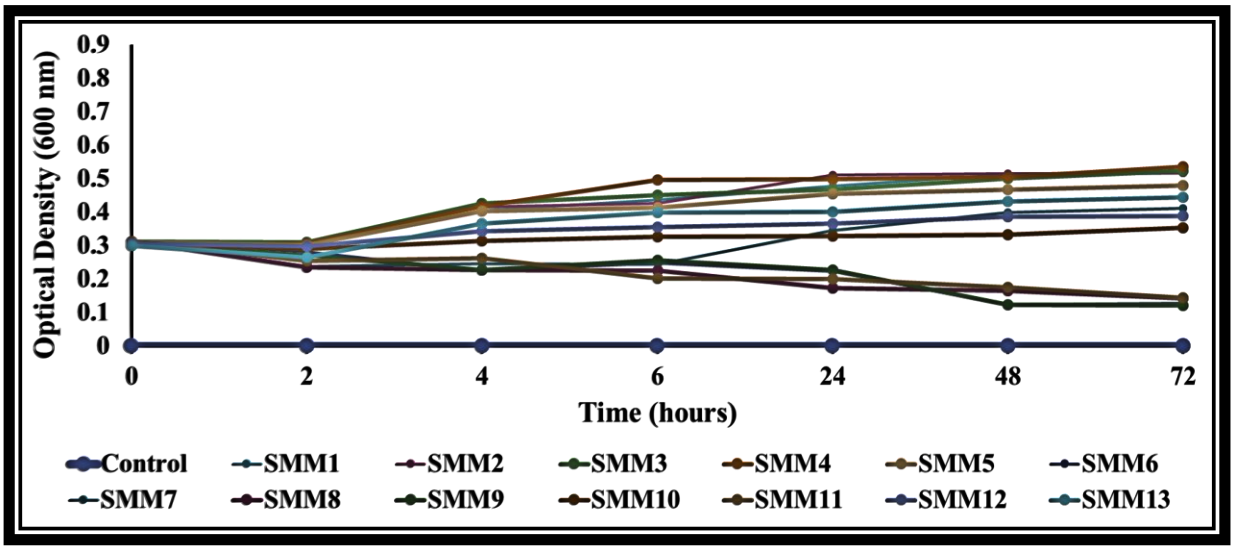


**Figure 4.1: Growth of Bacterial Strains (Toluene = 15 mg/L)**

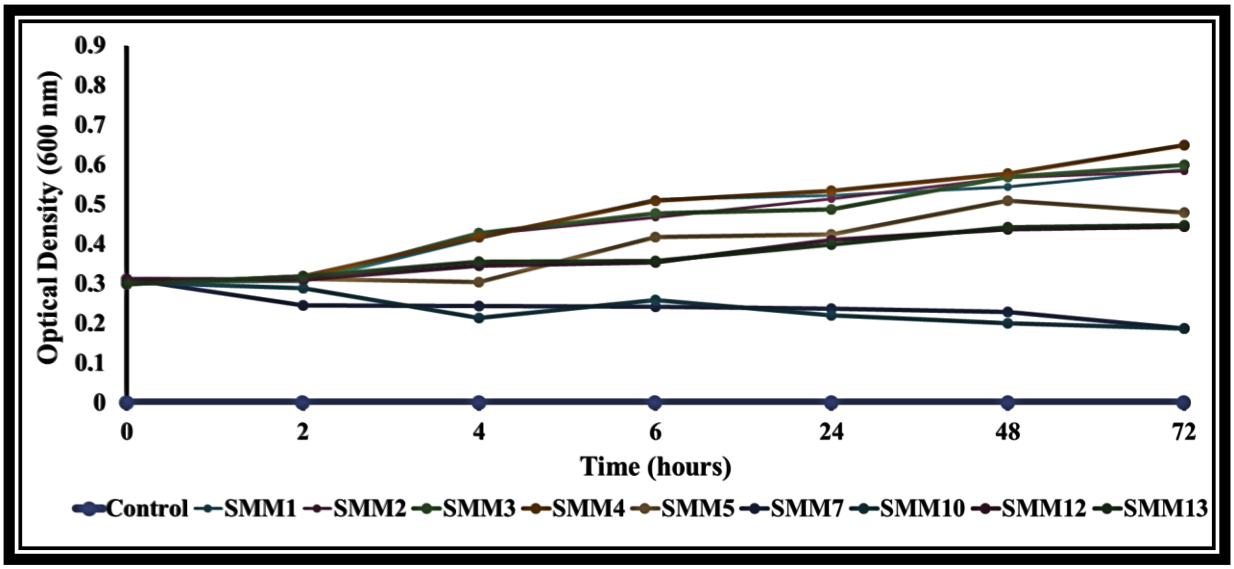


**Figure 4.2: Growth of Bacterial Strains (Toluene = 30 mg/L)**

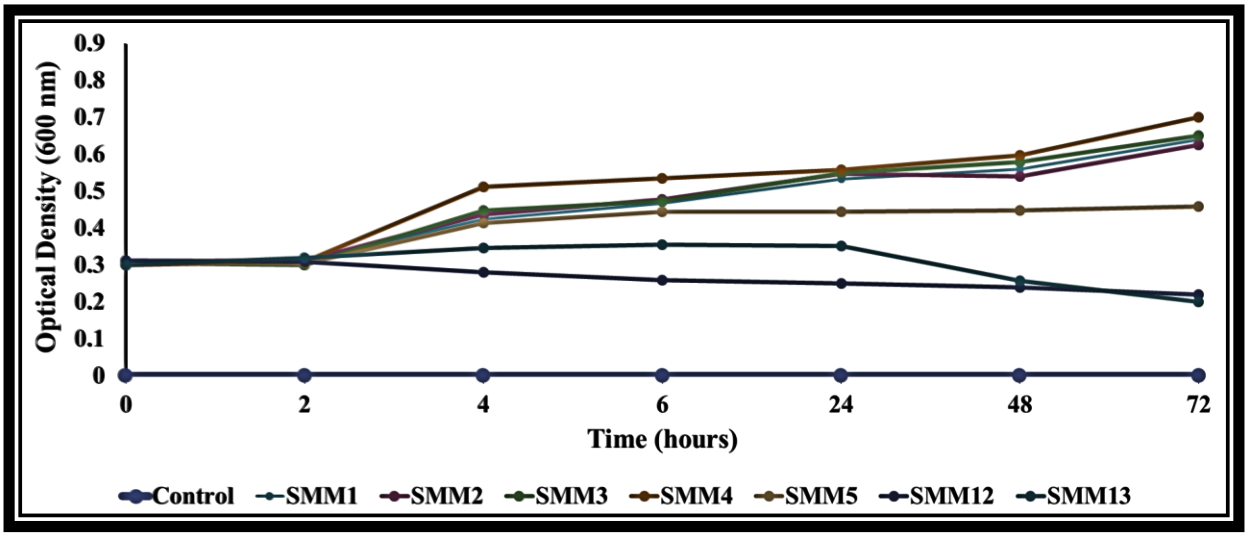


**Figure 4.3: Growth of Bacterial Strains (Toluene = 45 mg/L)**

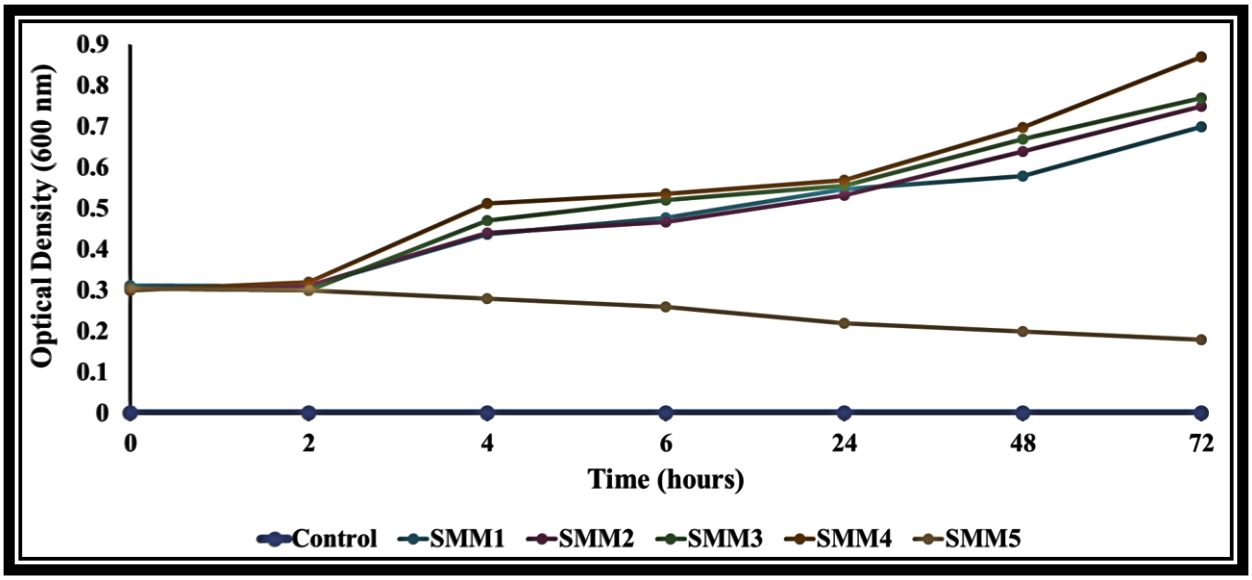


**Figure 4.4: Growth of Bacterial Strains (Toluene = 60 mg/L)**

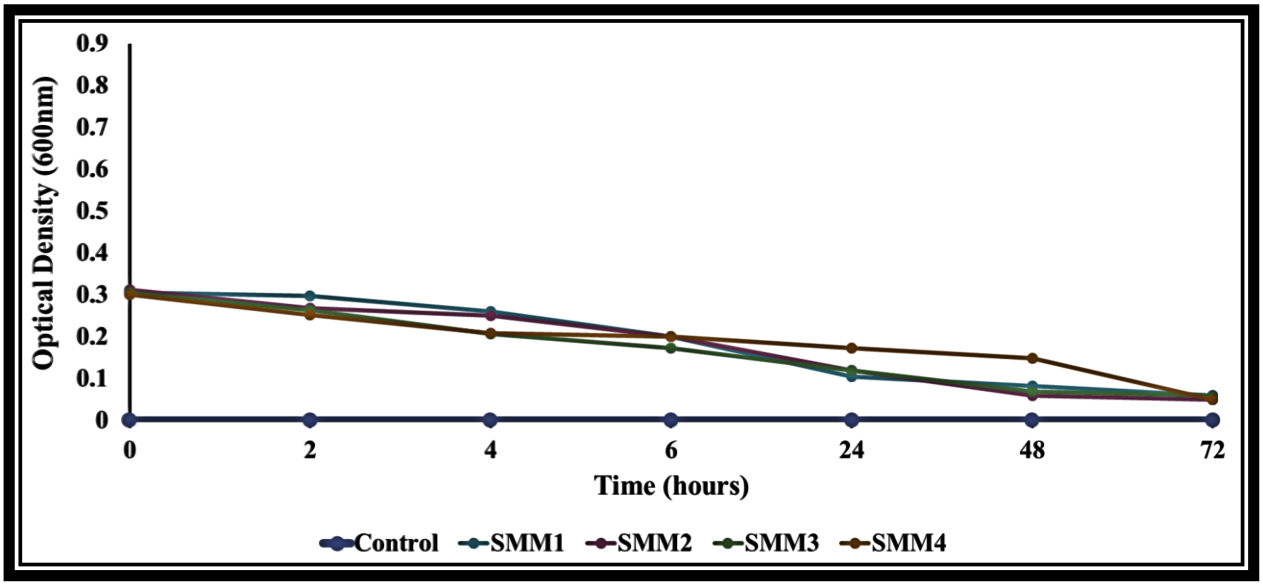


**Figure 4.5: Growth of Bacterial Strains (Toluene = 100 mg/L)**

## 4.5 Molecular Characterization

### 4.5.1 16S rRNA Gene Sequencing of Potential Toluene Degraders

The strains were screened and noise was removed manually. Strains were identified through BLAST search (Althushul, 1997) available at National Center for Biotechnology Information (NCBI) databases revealing up to 99 % similarity to different bacterial species. For identification of species, a limit of 97 % was marketed. A phylogenetic tree, constructed through MEGA 4 program demonstrates the phylogenetic relatedness and linkage among identified strains, shown in Figure 4.6. Isolated species along with their accession numbers are presented in Table 4.5.

**Table 4.5: Predominant Bacterial Isolates for Toluene Degradation**

| Predominant Species | Accession Numbers |
|---|---|
| **1.** *Ochrobactrum anthropi* | KT583131 |
| **2.** *Ochrobactrum intermedium* | KT583132 |
| **3.** *Enterobacter cloacae* | KT583133 |
| **4.** *Pseudomonas aeruginosa* | KT583134 |

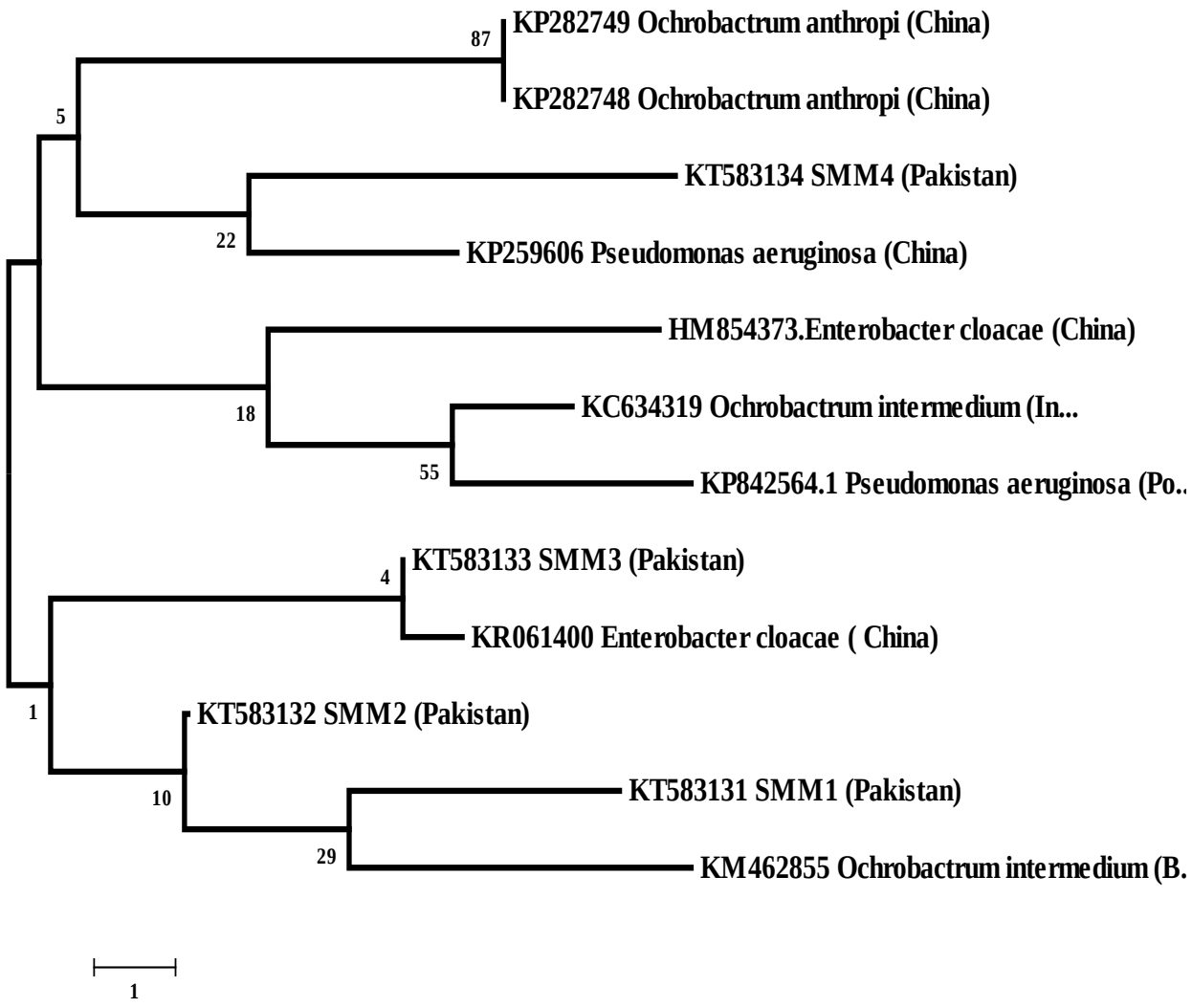


**Figure 4.6: Phylogenetic Tree Showing Linkage between Bacterial Strains**

Strains SMM1-SMM4 were identified as *Ochrobactrum anthropi, Ochrobactrum intermedium, Enterobacter cloacae and Pseudomonas aeruginosa* respectively. In the above figure, horizontal lines are called branches that indicate the amount of genomic change. These lines represent evolutionary changes among identified strains over time**.** Larger the branch length in horizontal dimension, larger the amount of change. The bar line present at the left corner of the tree provides the scale for this evolutionary change. In the above phylogenetic tree, the bar line with numerical

value of 1 demonstrates the branch length that indicates a genomic change of 1. The vertical lines are used to construct the phylogenetic tree and they simply elaborate the connection as well as irrelevancy between different branches. In a phylogenetic tree, the tips of the branches are called taxa that represent the descendants (the species that have been identified). The central point of the horizontal line joining the two branches is called node that is representative of mutual ancestor of descendants.

62.9 % toluene removal efficiency was achieved by *Ochrobactrum anthropi* after 72 hours (Figure 4.6). There was a direct relation between optical density (1.1) and % degradation of toluene.

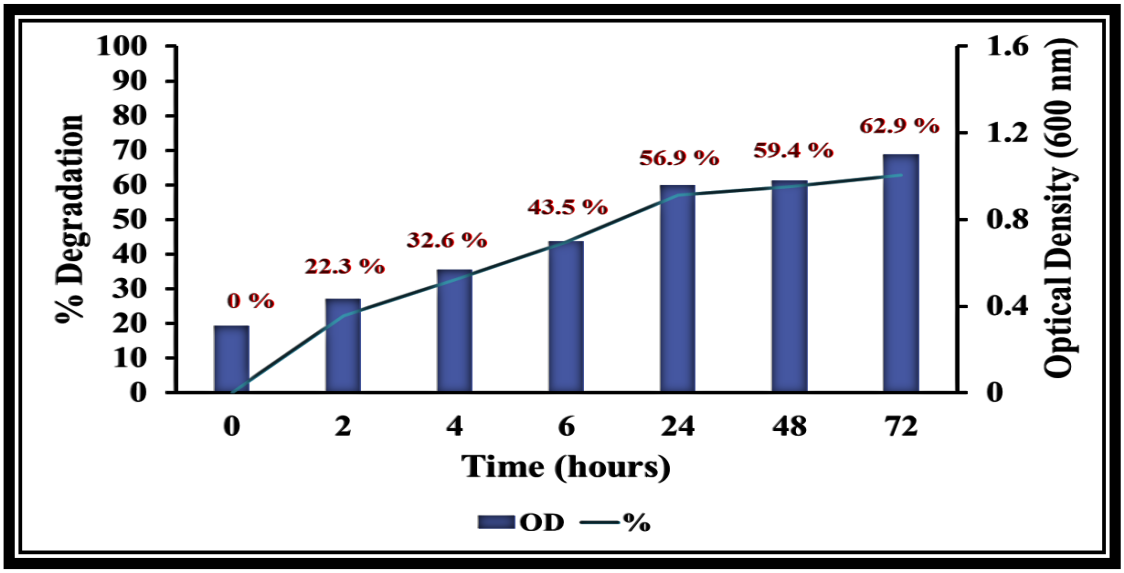


**Figure 4.7: Rate of % Degradation of Toluene by *Ochrobactrum anthropi***

Similar results were reported when Bhattacharya and coworkers (2015) studied the biodegradation of waste lubricants and found that *Ochrobactrum anthropi* degraded 60 % of lubricant oil. The same bacterial culture *Ochrobactrum anthropi* was reported to enhance the degradation of oily

sludge. The bacterium degraded commercial oil (having toluene concentration of 84.90 µg/kg) after 3 days (Mariano *et al*., 2007).

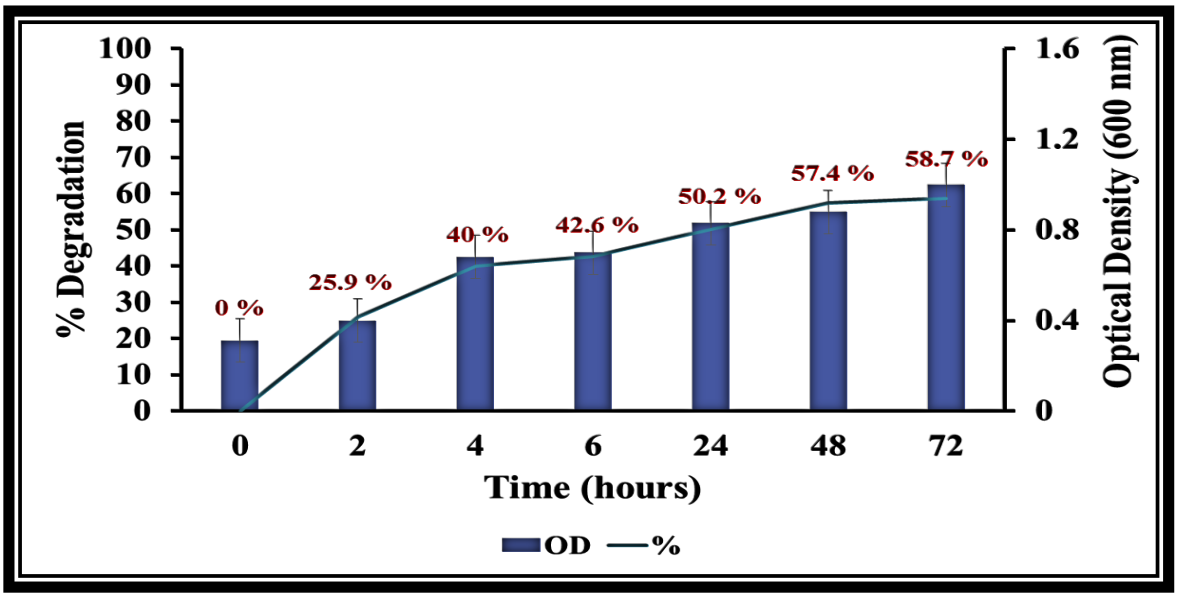


**Figure 4.8: Rate of % Degradation of Toluene by *Ochrobactrum intermedium***

*Ochrobactrum intermedium* showed 58.7 % degradation efficiency of toluene with optical density of 1 (Figure 4.7). This removal efficiency coincides with the findings of Hassanshahian and coworkers (2013) who studied bioremediation of hydrocarbon rich wastewater refinery effluent and found that *Ochrobactrum intermedium* degraded 60 % of total petroleum hydrocarbons. *Ochrobactrum intermedium* completely degraded petroleum hydrocarbons within period of 225 days with continuous feeding of microbes (Gargouri *et al*., 2011). A study of continuous stirred tank reactor using *Ochrobactrum intermedium* removed 97.5 % of TPHs (Ishak *et al*., 2012).

*Enterobacter cloacae* degraded toluene with removal efficiency of 72.1 % after 72 hours with optical density of 1.2. Ahmed and coworkers (2014) studied biodegradation of crude oil contaminated water and found that *Enterobacter cloacae* degraded 70 % of total petroleum

hydrocarbon. pH range 6.6 - 7 and temperature range 31-35 °C were the optimum conditions for maximum biodegradation.

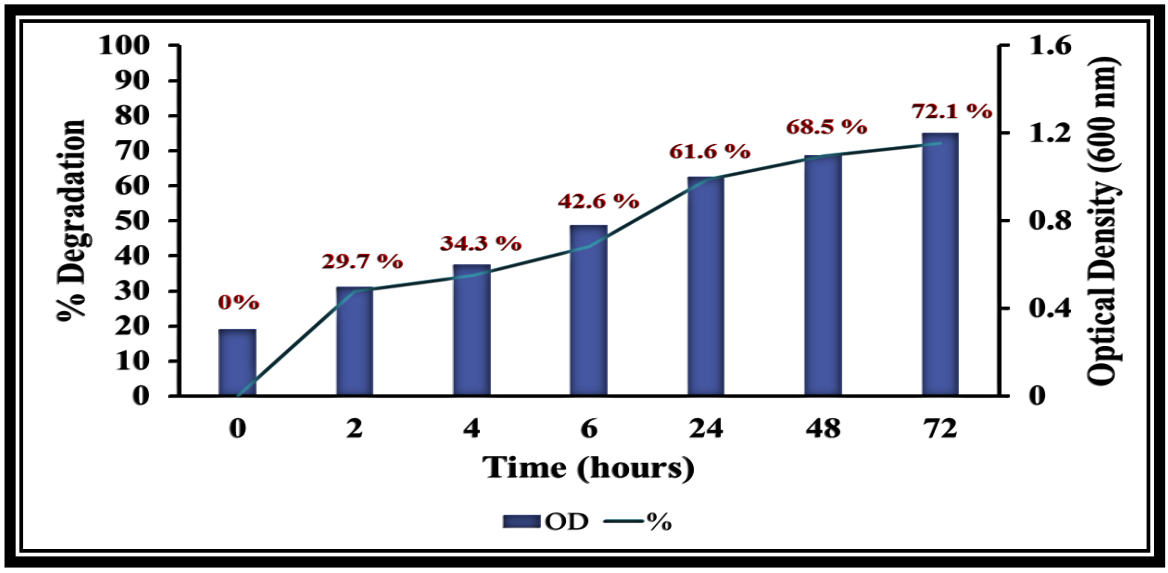


**Figure 4.9: Rate of % Degradation of Toluene by *Enterobacter cloacae***

*Pseudomonas aeruginosa* turned to be efficient in degrading toluene as compared to all other isolates.

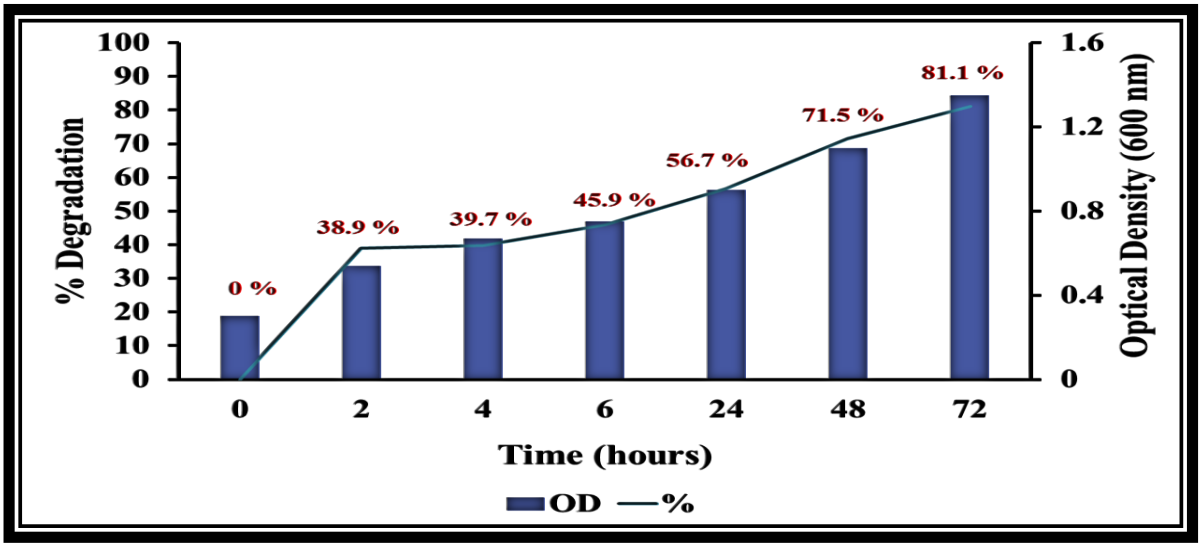


**Figure 4.10: Rate of % Degradation of Toluene by *Pseudomonas aeruginosa***

For 60 mg/L of toluene, *Pseudomonas aeruginosa* metabolized toluene with degradation efficiency of 81.1 % and optical density of 1.35 in 72 hours (Figure 4.9). These findings coincide with the results produced by Lin and coworkers (2010) where 79 % of toluene was degraded by *Pseudomonas aeruginosa*. Men and Cheng (2011) reported that at low initial concentration of 1.19 mg/L, removal efficiency of toluene was observed to be 94.1 %. Removal efficiencies were found to be 93.3 and 92.5 % as the initial concentration was increased from 3.28 to 6.17 mg/L. *Pseudomonas aeruginosa* was found to degrade the highest concentration of engine oil (53.44 %) in 30 days.

Promising results were obtained when consortium of four strains SMM1 - SMM4 was utilized for toluene degradation. 88.4 % toluene removal efficiency was obtained after 72 hours when consortium of *Ochrobactrum anthropi, Ochrobactrum intermedium, Enterobacter cloacae and Pseudomonas aeruginosa* was subjected to 60 mg/L of toluene.

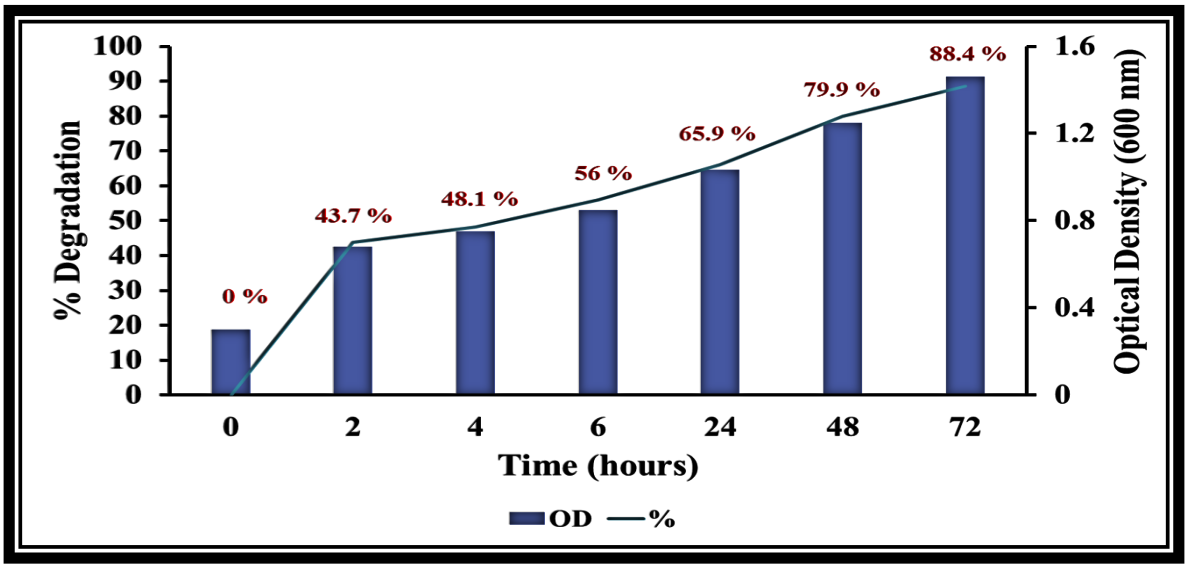


**Figure 4.11: Rate of % Degradation of Toluene by Consortium**

It was observed that there was direct relation between growth and % degradation of toluene. This consortium showed the maximum optical density of 1.46 as compared to individual isolates. Maximum degradation efficiency can be attributed to the fact that microorganisms are able to work co-metabolically thus facilitating each other to use contaminants actively (Horne *et al*., 2002). This study coincides with the biodegradation study conducted by Mukherjee and Bordoloi (2012) where BTX levels of polluted soil were decreased from 40 to 7 g/kg (83 % degradation) after 90 days of treatment with bacterial consortium of *Bacillus subtilis* and *Pseudomonas aeruginosa*. Mnif and coworkers (2009) reported the isolation of different species of thermophilic and mesophilic monoaromatic hydrocarbons degrading microbes from Tunisian oil fields. Amongst these, *Pseudomonas* sp. and *Halomonas* sp. were found to degrade 93-96 % of aliphatic fraction of oil. 100 % degradation of PAHs was found to be done by a bacterial consortium consisting of *Enterobacter cloacae, Ochrobactrum sp.* and *Stenotrophomonas* sp. (Pugazhandi *et al*., 2009).

*Chapter 5*

# CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Conclusions

The conclusions of the present study were:

1. A total of thirteen bacterial species were isolated from petroleum contaminated soil.
2. Out of thirteen isolates, only four (SMM1, SMM2, SMM3 and SMM4) were able to grow on varying concentrations of toluene (15, 30, 45 and 60 mg/L).
3. On the basis of 16S rRNA gene sequencing, the potential isolates for toluene degradation were identified as *Ochrobactrum anthropi*, *Ochrobactrum intermedium*, *Enterobacter cloacae* and *Pseudomonas aeruginosa* respectively.
4. Individual degradation rates of *Ochrobactrum anthropi, Ochrobactrum intermedium, Enterobacter cloacae and Pseudomonas aeruginosa* for 60 mg/L of toluene concentration were recorded to be 62.9, 58.7, 72.1 and 81.1 % after 72 hours. However in case of consortium, the degradation rate of toluene increased and 88.4 % of degradation was achieved in 72 hours.

## 5.2 Recommendations

Following recommendations are suggested:

1. Simultaneous degradation of all BTEX components should be carried out in hollow fiber membrane bioreactor to investigate effect of substrate on each other.
2. Isolated strains should be applied in the field for the treatment of petroleum industry effluents.
3. Enzymatic studies of toluene degrading bacterial strains should be investigated.
4. Biokinetic studies of toluene degradation pathway should be carried out.

# *Chapter 6*

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