# KINETICS OF BENZENE DEGRADATION USING SELECTED BACTERIAL STRAINS

**BY**

**SALMAN AHMED**

**NUST201362300MSCEE65213F**

**Institute of Environmental Sciences and Engineering (IESE)**

**School of Civil and Environmental Engineering (SCEE)**

**National University of Sciences and Technology (NUST)**

**Islamabad, Pakistan**

**(2016)**

This is certified that the contents and form of the thesis entitled

# KINETICS OF BENZENE DEGRADATION USING SELECTED BACTERIAL STRAINS

Submitted by

**SALMAN AHMED**

Has been found satisfactory for the requirements of the degree of

Master of Science in Environmental Science

Supervisor: _____
**Dr. Muhammad Arshad**
Associate Professor,
IESE, SCEE, NUST

Member: _____
**Dr. Imran Hashmi**
Professor /Associate Dean,
IESE, SCEE, NUST

Member: _____
**Dr. Sher Jamal Khan**
Associate Professor,
IESE, SCEE, NUST

**This thesis is dedicated to My Parents**

For their endless affection, support and

encouragement

## ACKNOWLEDGEMENTS

Alhamdulillah, all praises to Almighty Allah for the strength and His blessings to complete this degree and achieving success in MS degree program in spite of many challenges faced.

My deepest gratitude goes first to my supervisor **Dr. Muhammad Arshad**, who expertly guided me through my research. His unwavering enthusiasm kept me constantly engaged with my research. His valuable suggestions and help throughout my research work made me able to do my work. **Dr. Muhammad Arshad** has always been my source of inspiration as I moved towards the completion of my research and my master's degree journey at NUST.

I would like to thank my committee members **Prof. Dr. Imran Hashmi** and **Dr. Sher Jamal Khan** for guiding and helping me throughout my research.

I am obliged to have **Ms. Aneela Iqbal** (PhD Candidate at IESE) as a mentor in the laboratory. Her selfless guidance in every step of my research brought me to the place where I am today.

The IESE Lab personnel, especially **Mr. Sanaullah** was very accommodative to all my queries during the experimental work and I am grateful to him for this cooperation. I would also like to acknowledge **Mr. Muhammad Saad Bin Zahid**, who provided me respective bacterial strains for my research work.

Lastly, I am thankful to my friends and well-wishers for their support throughout my research and everyone who helped me directly and indirectly when required.

***Salman Ahmed***

## Table of Contents

# LIST OF ABBREVIATIONS

| | |
|---|---|
| mg/L | Milligram per Liter |
| BTEX | Benzene Toluene Ethylbenzene Xylene |
| EPA | Environmental Protection Agency |
| NESHAP | National Emissions Standards for Hazardous Air Pollutants |
| NEQS | National Environmental Quality Standards |
| PAHs | Polycyclic Aromatic Hydrocarbons |
| EC | Electrical Conductivity |
| rRNA | Ribosomal Ribonucleic Acid |
| NCBI | National Center for Biotechnology Information |
| OD | Optical Density |
| CFU/mL | Colony forming units per milliliter |

# List of Figures

**List of Figures** **Page No.**

# List of Tables

# Abstract

Monoaromatic hydrocarbons such as benzene have been found in wastewaters as well as sludge generated from petroleum industries. Benzene is categorized as a carcinogen. Biodegradation has been found to be an important environmental remediation technique for water and soil associated benzene. In this technique, microorganisms utilize pollutant as a source of carbon and energy. The objectives of present work were to study benzene degradation using selected strains along with effects of salinity and pH on degradation efficiency. Three different bacterial strains used for this study which were already isolated from *Cannabis sativa* and identified on the basis of 16S rRNA sequences. The GenBank accession numbers assigned for *Pseudomonas aeruginosa, Bacillus cereus, Acinetobacter junii* were KM520129, KM520130, and KM520131. Growth kinetics were observed using batch reactions at different substrate concentrations. The strains were used for degradation using M9 media at 250, 500, 750 and 1000 mg/L benzene concentrations. Bacterial colonies were counted in terms of colony forming units per milliliter (CFU/mL). Biomass was determined using dry weight method. Maximum specific growth rate for *Pseudomonas aeruginosa, Bacillus cereus* and *Acinetobacter junii* were found to be 0.177, 0.135 and 0.105 $h^{-1}$. Significant degradation was observed at 500 mg/L for all bacterial strains. *Pseudomonas aeruginosa, Bacillus cereus,* and *Acinetobacter junii* degraded 77, 81, and 74% benzene, respectively. Degradation efficiency was maximum at neutral pH for all the three strains. Results regarding effect of salinity showed that *Pseudomonas aeruginosa* and *Bacillus cereus* degraded 47 and 58% respectively at 0.5% salinity while *Acinetobacter junii* showed 65% degradation efficiency at 3.0% salinity.

# Chapter 1

# Introduction

## 1.1 Background:

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 that include short, medium, and long chain aliphatics such as alkanes and alkenes. It also include aromatics (e.g. benzene, toluene, ethyl benzene and xylene) and polycyclic aromatic hydrocarbons (PAHs) such as naphthalene and pyrene. Oil spills that occur during discharge from accidents of tanks or ships, oil refineries, shore pipelines and off shore oil production causes an irreversible damage to ecosystem. If hydrocarbons enter the food-chain, they can be dangerous since few of them are persistent (Perelo, 2010).Contamination of soil and groundwater by petroleum hydrocarbon such as benzene is a common environmental problem. Mobility rate of hydrocarbons in soil –water systems are linked to their low octanol water partition coefficient and a preferential water transport thereby contaminating the groundwater (Sarkar et al., 2013).

## 1.2 Benzene

Benzene is used on large scale and it is produced from natural and anthropogenic processes. Due to its toxicity as well as relative solubility, it represents a significant threat to human health and is currently known as one of the most important contaminants. Exposure to 1 mg/L of benzene can cause reduction in blood cell count which ultimately results in hematoxicity. Level of benzene in rural atmosphere is less as compared to urban areas.

Benzene is a common component of fossil fuel such as gasoline. It is also classified as an important raw chemical. It is commonly used as solvent and chemical intermediate. Benzene is also recognized

as an anthropogenic contaminant in aqueous environment. Compared with other hydrocarbons, it is highly water-soluble having saturation level of 24 mM at optimum temperature 25°C. According to USEPA, it has been classified as a human carcinogen of Group A. Chemically, benzene is stable under typical environmental conditions, because the compound is stabilized by the aromatic ring system (π-electron system) without any potentially reactive substituent. USEPA states that the maximum contaminant goal of benzene in drinking water is 0 mg/L. However, the acceptable limit in drinking water is 0.005 mg/L while it is 0.14 mg/L in treated industrial wastewater.

### 1.3 Sources of Benzene

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 the soil through industrial discharge and sand leakage of gasoline from underground storage tanks. Benzene from the air can get deposited on the ground through rain or snow. However, benzene breaks down slowly in soil and water. Proximity to gas stations, petroleum refining processes, hazardous waste sites or petrochemical manufacturing sites result in higher levels of benzene exposure. Other than inhalation, people can be exposed to benzene via drinking water, food and beverages.

### 1.4 Biodegradation of Benzene

Biodegradation has emerged as an important cost effective and environmental friendly technique for remediation of such compounds. The potential to degrade organic pollutants varies among different microbial groups and also depends on specific dose (Megharaj et al., 2011). A large number of benzene degrading microorganisms have been identified. These microorganisms can utilize the hydrocarbons as sole carbon source for their metabolic activities as well as energy requirement. Most important principle of bioremediation includes destruction of hazardous

contaminants or transformation into less harmful products. There is no single bacterial strain which has ability to degrade all components in hydrocarbon (Ali, 2012)

Benzene-degrading organisms consists of mono- or dioxygenases which introduce molecular oxygen to yield cis-benzene dihydrodiol or phenol, which are further oxidized to catechol (Tao et al., 2004). The aromatic ring of catechol is cleaved by further dioxygenases in ortho- or meta-position. It is a well-known fact that specific bacterial culture is capable to degrade the organic compounds if they are provided the best optimum environmental conditions for their growth and metabolism.

### 1.5 Microbial Growth and Kinetic Models

Many researchers have presented various microbial growth and biodegradation kinetic models. Such models emphasize on prediction of certain chemicals that persist at a specific time period, estimation of the time required to lessen the concentration of chemical, time required for a chemical to reach desired concentration (e.g. a case of aquifer, soil or surface water) and design of bioremediation setup whether *in situ* or *ex situ* which is used to remove chemical contaminant to a designed concentration. Besides this, it may also be used to forecast the amount of biomass produced which is achievable at a specified time period. A study was performed to investigate the degradation kinetics of benzene, toluene and phenol. The study concluded that toluene and benzene enhance the biodegradation of phenol whereas phenol did not affect the biodegradation of toluene and benzene significantly. It was also noticed that there was no significant interaction among benzene and toluene. Monod model was employed to represent the data in this low substrate concentration (Abuhamed et al., 2004)

Monod model has widely been used to obtain kinetic parameters using single substrate consumption and data of biomass concentration. The equation for the model is as follows:

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{\mu_{max} S}{K_S + S}$$

In this model, the specific growth rate ($\mu$) continues to increase strongly increase for a low substrate concentration and slowly for a high substrate concentration until bacteria reached at a saturation level. This rate has two limiting forms. During high substrate concentration, Monod equation reduces to a zeroth order dependence on substrate concentration. During high substrate concentration, Monod equation reduces to a first order dependence and is linear.

Value of maximum specific growth rate $\mu_{max}$ ($h^{-1}$) varies with the type of microorganism. Its value cannot be negative since it is the maximum rate. Similarly, values for half saturation constant $K_S$ (mg/L) depends on the nature of a substrate.

Here, X represents the microbial cell. S is the initial concentration of substrate, and t stands for time, respectively. If the initial microbial cell concentration was represented by $X_o$, then the microbial cell concentration (X) at time (t) will have the following equation as given below:

$$\mathbf{X = X_o \mu^{et}}$$

## 1.6 Objectives

The objectives of the research work were:

i. To study the growth kinetics of *Pseudomonas aeruginosa, Bacillus cereus,* and *Acinetobacter junii* during benzene degradation

ii. To determine the influence of pH and salinity on benzene degradation efficiency

## 1.7 Scope of Study

The present work would help to understand the microbial growth patterns and biodegradation potential of selected bacterial strains over regular time intervals by change in pH and salinity levels. Removal of benzene along with microbial growth study can be helpful in designing of a bioremediation prototype. Bioremediation tanks could be employed for removal of contaminant in different industries like petroleum, oil and rubber, etc.

# Chapter 2

# Literature Review

## 2.1 BTEX Compounds

Mono aromatic hydrocarbons which include benzene, toluene, ethylbenzene, and xylene (BTEX) are thought to be major contaminants in environment due to leakage from accidental spills and underground storage tanks. They are produced in megatons per year as bulk of chemicals for industrial usage such as solvent, starting materials for pesticides, plastics, and synthetic fibers. Because of their toxicity and carcinogenicity, BTEX compounds are listed among top priority pollutants by United States Environmental Protection Agency. BTEX compounds are of great public concern due to their solubility in water, volatility, toxicity and migration abilities (Mukherjee and Bordoloi, 2012). Remediation of such chemicals are quite difficult because of their relatively soluble in water and rapidly diffusion once they were introduced into aquifer.

## 2.2 Benzene

Benzene is known to be most hazardous compound out of all BTEX compounds because it is a human carcinogen. Benzene is an organic chemical compound having formula $C_6H_6$. It is a colorless volatile liquid and flammable in nature. Benzene is believed to be a carcinogen for all exposures. It is also a risk factor for leukemia and lymphomas (US EPA, 2002). The trade name for benzene are benzol 90, pyrobenzol, polystream etc. Benzene is a stable, water miscible, highly mobile and poisonous hydrocarbon. In vitro tests of benzene showed that it has no mutagenic action, but it is capable to induce breaks and segregation in chromosome segregations (Bird et al., 2005)

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

| Property | Standard Value |
|---|---|
| Molecular weight | 78.11 g/mole |
| Melting point | 5.5 °C |
| Boiling point | 80.1 °C |
| Density | 0.879 g/ml |
| Vapor Pressure at 25 °C | 0.13 atm |
| Flash point (Closed cup) | -11.1°C |
| Solubility in water at 25°C | 1.8 g/l |
| Conversions at 25°C | 1mg/L= 313 ppm |

Source: USEPA

## 2.3 Biodegradation

Since BTEX concentration in groundwater is increasing, there has been a demand for more efficient methods to remove or minimize the damages caused by these chemicals (Mazzeo et al., 2010). Recent development in Environmental Biotechnology have made bioremediation as an environmental friendly technique for removing contaminants. There is a significant amount of data regarding hydrocarbon biodegradation in marine environments (Head et al., 2006). Microbial biodegradation has been considered as an important process for reduction of leaking contaminant concentration. The technique of bioremediation has gained more popularity and encouragement as potentially effective approach for hydrocarbon remediation. Successful degradation of benzene by microorganism in an aerobic environment has been reported. Majority of hydrocarbons are degraded within anaerobic zone which implies that they are degraded by anaerobic bacteria

Degradation of BTEX in soil augmented with BTEX adapted anaerobic microbial consortia appeared to be a feasible method for remediation of BTEX compounds (Dou et al., 2008)

### 2.3.1 Microbes in Biodegradation

Microbial growth on substrate mixture is a key subject to study in the field of bioremediation. Many bacterial isolates capable of degradation pollutants have been isolated. The ability of microorganisms to degrade contaminated hydrocarbon was first demonstrated by Gray and Thornton in 1928. Bacteria may degrade variety of hydrocarbons including aromatic compounds under aerobic as well as anaerobic conditions (You et al., 2013). Several studies have been done in order to find best microorganisms for degradation. Changes in microbial population occur when they were subjected to targeted compounds. This could be due to interactions between microbial populations. BTEX, when released into the environment affect the function and structure of microbial communities. Research has shown that most potential bacteria for degradation have been isolated from oil contaminated sites.

### 2.3.2 Microbial Enzymes in Bioremediation

The detoxification of organic compounds by various bacteria through oxidative coupling is mediated with oxireductases. Microbes gain energy by biochemical reaction mediated by these chemical enzymes to cleave chemical bonds and to help the transfer of electron from donor to acceptor. During such chemical reactions, the toxic products are finally oxidized to harmless products (Karigar and Rao, 2011). A study on aromatic hydrocarbon degradation in environmental samples revealed the potential use and distribution of *bamA* as a biomarker (Matsen et al., 2013)

## 2.4 Metabolic Pathway of Benzene Degradation

Most important step in degradation of aromatic hydrocarbons is the overcoming of resonance energy that stabilizes their ring structure. It also involves the attack by oxygenase that hydroxylate and finally cleave the ring with the help of activated molecular ring (Fuchs et al., 2011).

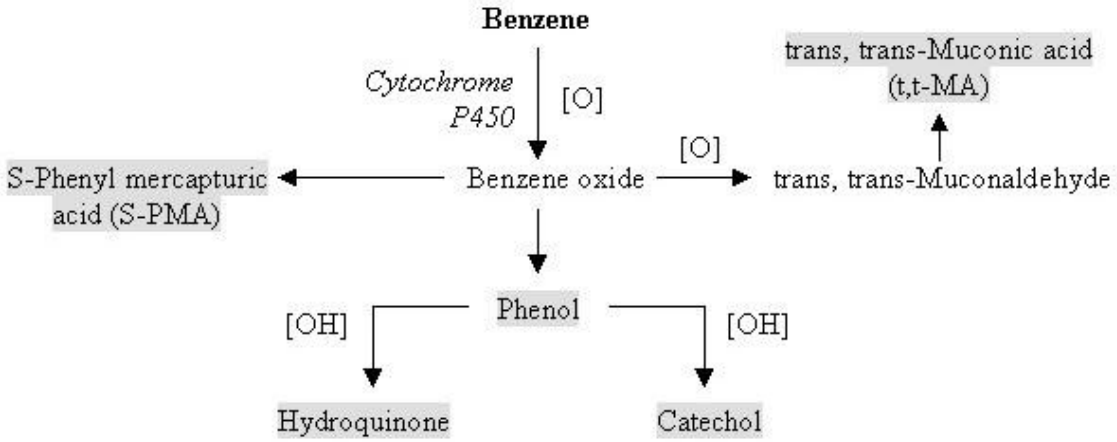



Figure 1. Schematic presentation of metabolic pathways of benzene degradation. Shaded metabolites are important biomarkers of benzene exposure (Snyder, 2007)

## 2.5 Microbial Growth

Microbial growth is usually studied as a population. Microbial cells divide by binary fission in which a single individual cell divides into daughter cells. Binary fission includes increase in cell mass, replication of chromosome and cell division into two daughter cells.

When bacteria were grown in a close system or batch culture, the population always exhibit growth dynamics. Fig 2.1 showed the typical microbial growth curve. There are four phases of microbial growth namely Lag phase, Exponential or Logarithmic Phase, Stationary Phase, and Decline or Death Phase. Detailed explanation of these four phases are as under:

### 2.5.1 Lag Phase:

In 1949, Jacob Monod described first phase of bacterial growth i.e. lag phase as the process of equilibration that was controlled by regulatory mechanism. After inoculation of cells into a suitable medium, the population remains apparently unchanged. During this phase, the cells may be growing in mass or volume, synthesizing enzyme, proteins, RNA as well as increase in metabolic activity.

Lag phase depends on various factors such as inoculum size, enzyme synthesis and time for substrate to metabolize. Lag phase is a poorly understood phase of bacterial growth because of lack of data about the basic and core molecular processes during the growth. The functioning during a bacterial lag phase has become unknown concept.

### 2.5.2 Logarithmic or Exponential Phase

During logarithmic or exponential phase of bacterial growth, all cells are dividing by binary fission. The cells grow by geometric progression. Cell growth in this phase depends upon the composition of medium and incubation time. The rate of exponential growth of a bacterial culture is termed as doubling time or generation time. This phase represents the optimum population of bacteria.

Exponential growth involves multiple rounds of DNA synthesis, along with transcription and translation to form larger cells. It also involves gene regulatory processes which could be understood by network interference approach (Faith et al., 2007).

### 2.5.3 Stationary Phase

Stationary phase depicts the pause of cell division (Navarro et al., 2010). During this phase, bacterial populations levels out. Population has been stopped growing or dividing. In pharmaceutical and fermentation industries, bacterial growth is mostly kept at stationary phase.

As the bacteria enters into stationary phase, there exists around 80% reduction in protein synthesis compared with bacterial cells in exponential phase (Reeve et al., 1984). Stationary phase occurs mostly due to inhibitory products or depletion of nutrients. During this phase, some bacteria (antibiotics) produce secondary metabolites which are produced after active stage of growth.

### 2.5.4 Death or Decline Phase

Decline or Death phase include accumulation of dead cells. During this phase, the number of viable bacterial population declines geometrically. This phase could be termed as the reverse of growth during logarithmic phase. Spore formation may persist beyond this phase of growth and it may initiate if favorable conditions are provided to culture.

(Finkel, 2006) reported the death phase as the phase of bacterial growth where cells loose viability. Number of cells decreases rapidly during this phase of growth.

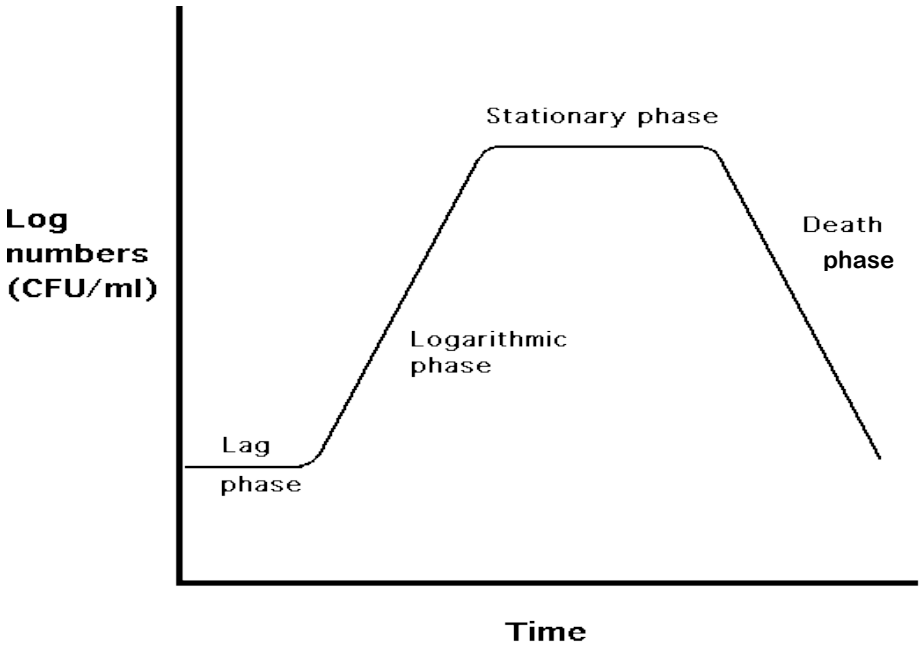



Figure 2. Typical Microbial Growth Curve

Source: http://www.foodsci.uoguelph.ca

## 2.6 Influence of Environmental Factors on Microbial Growth

Microbial growth is affected by physical and chemical nature of their surroundings instead of variations in nutrient levels and nutrient limitation. For successful cultivation of microbes, it is necessary to have proper supply of nutrients and to maintain proper environmental conditions. Growth and death rate of microbes are greatly influenced by environmental factors such as temperature, pH, oxygen requirement and pressure.

### 2.6.1 Temperature

Microorganisms are very sensitive to temperature. Temperature varies with environmental conditions. Temperature influences the rate of reaction as well as protein structure and enzymatic activity. On the basis of temperature, it could be classified into three categories:

Thermophiles are those which show optimum growth at 55°C. They often have maximum growth at 65°C while a few can grow at 100 °C or higher. Majority of thermophiles belong to prokaryotes. Hyperthermophiles are the once that have optimum growth ranging from 80-113°C. Mesophilic are those microorganisms which grow best in moderate temperature. Mesophilic don't need too hot or too cold temperature i.e. between 20-45°C. Psychrophilic are microorganisms that show maximum growth 15°C.However, they can also grow at 0°C. The maximum temperature for growth of Psychrophilic microorganisms is approximately 20°C.

### 2.6.2 pH

Most bacteria can grow best in an environment with a narrow pH range between pH 6.5 and 7.5. Acidophiles could grow at pH values below 4.0. Some bacteria can survive at pH 1.0. Alkalinophilic bacteria can grow in pH that ranges from 9.0 to 10.0. Most could not grow in solutions whose pH values are below neutral. During bacterial growth, organic acids are released into the medium, which lower its pH and so restrict with or totally inhibit growth. Common media

ingredients that include peptones and amino acids have a small buffering effect. An external buffer is mostly desirable in bacteriological media to neutralize the acids in order to maintain the correct pH. Phosphate salts are the common buffers because they buffer in the typical growth range of bacteria. They are non-toxic and they can also provide a source of phosphorus which is an essential nutrient element. High phosphate concentration has a major drawback because of its severe nutrient limitation caused by the precipitation of insoluble metal phosphates in the medium.

## 2.7 Measurement of Microbial Growth

There are three different ways to measure microbial growth. These are as under:

i. Viable counts
ii. Microscopic counts
iii. Turbidimetric methods

### 2.7.1 Viable Counts measurement

Microbial growth has been expressed in terms of colony forming units (CFU) as a direct measurement of growth. Viable count method is quite difficult and laborious work. Two main methods for viable count are spread plate count and pour plate method. When evaluating parameters of microbial growth, population growth data are only derived from the viable counts. The rate of increase of specific growth rate could not be measured from optical density.

### 2.7.2 Microscopic counts

These could be evaluated by microscopic observations. Results are considerable unreliable in this type of measurement. It cannot distinguish between living and dead cells. Those cell suspensions having count greater than $10^6$ are very hard to count. Motile cells need to immobilize. There is also a chance of error regarding debris to be considered as a living cell.

### 2.7.3 Turbidimetric methods

Optical density could be described as indirect measurement of microbial growth. Absorbance measurement in optical density is relatively easy, inexpensive and fast method of measurement. (Dalgaard and Koutsoumanis, 2001) suggested that only those cells that have high cell densities shall have the possibility of absorbance measurement. This is mostly measured by UV visible spectrophotometer.

## 2.8 Microbial Growth Kinetics

Batch culture occurs in a closed system that contains an initial amount of substrate. The inoculated microorganism will pass through different growth phases. During exponential phase, cell number increase exponentially at a constant maximum rate. In mathematical terms, we may write it:

$$\frac{dX}{dt} = \mu X$$

Where X is the concentration of microbial biomass, t is the time (hours), and μ is the specific growth rate (per hour). If we integrate between $t_0$ and $t_1$ when concentrations of cells are $X_o$ and $X_t$ we obtain the equation as under:

$$Xt = Xo\ e^{\mu t}$$

Where $X_{o\ is}$ the original biomass concertation, $X_{t\ is}$ the biomass concentration after time t and e is the base of natural logarithm. On taking natural logarithm, the equation becomes:

$$lnXt = lnXo + \mu t$$

Using an equation, a plot of natural logarithm of cell number versus time was drawn and specific growth rate as slope of the line was calculated from the following equation:

$$\mu = slope = \frac{\ln(Xt - Xo)}{t2 - t1}$$

During exponential phase, nutrients are in excess amount. At this specific stage, microorganism are growing at their peak until they achieved their maximum specific growth rate.

## 2.9 Monod Model

Monod model is a microbiological model that is used to evaluate the biodegradation process. Decrease in growth rate and cessation of growth due to limitation of substrate may be described by μ and the residual growth limiting substrate. This relationship is given by Monod model, which describes the microbial growth kinetics during biodegradation, particularly in batch culture. It consists of three parameters which consists of specific growth rate, saturation constant and yield coefficient. These parameters could be termed as a criteria for biodegradability of organic pollutants (Blok, 1994).

$$\mu = \mu_{max}\, S / K_s + S$$

Where,

$\mu_m$ = Maximum specific growth rate

**S** = Substrate concentration

$K_s$ = Half saturation constant

To evaluate the biodegradation rates of organic contaminants, different researchers have developed mathematical complex models that combine sorption and transport with various reaction terms which include substrate utilization, bacterial growth, decay, and the utilization of electron acceptors. During such cases of microbial growth kinetics, Monod function could be used to describe the utilization of substrate and electron acceptors (Oya et al., 1998)

# Chapter 3

# Materials and Methodology

## Methodology

### 3.1 Washing and Sterilization of Glassware:

All the glassware 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 sterilized glassware was oven dried at 105 °C for 2 h.

### 3.2 Selection of Bacterial Strains:

Three different bacterial strains were used for this study. Bacterial strains namely *Pseudomonas aeruginosa*, *Bacillus cereus, and Acinetobacter junii* with NCBI accession numbers KM520129, KM520130 and KM520131, respectively were obtained from Environmental Biotechnology Research Laboratory, Institute of Environmental Science and Engineering, SCEE, NUST, Islamabad, Pakistan. The data of these isolated bacterial strains used for this study could be accessed online on NCBI website (http://www.ncbi.nlm.nih.gov/).

Conical flasks containing nutrient agar were used to make nutrient agar plates. The flasks were autoclaved at temperature of 121 °C, 15 psi pressure for 15 minutes. Plates of nutrient agar were made in sterile conditions of laminar flow hood. These petri plates were then incubated for 24 h at 37 °C. All strains were streaked on nutrient agar plates to get fresh bacterial culture.

#### 3.2.1 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a blue green bacteria which is also known as opportunistic pathogen. It is a major cause of nosocomial infection. It tends to people with immunodeficiency. It normally resides in soil, marshes and coastal sea shores. Sharma and co-workers (2014) found that *Pseudomonas diminuta*, *Pseudomonas aeruginosa* and *Pseudomonas putida* can be utilized for

effective degradation of organophosphate at optimized conditions of 37ºC and pH 8.5. *Pseudomonas spp.* are capable of using nitrate instead of oxygen as final electron acceptor. The strain has capability to biodegrade pollutants in wastewater. Peter et al. (2014) found that bacteria present in rhizosphere of tomato and cabbage could degrade variety of organic compounds are they were identified as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus magaterium*.

**Pseudomonas aeruginosa strain SAR-1 16S ribosomal RNA gene, partial sequence**

```
LOCUS       KM520129                826 bp    DNA     linear   BCT 17-DEC-2014
DEFINITION  Pseudomonas aeruginosa strain SAR-1 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   KM520129
VERSION     KM520129.1  GI:732550292
KEYWORDS    .
SOURCE      Pseudomonas aeruginosa
  ORGANISM  Pseudomonas aeruginosa
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 826)
  AUTHORS   Zahid,M.S., Iqbal,A. and Arshad,M.
  TITLE     Isolation and identification of rhizospheric bacteria from
            contaminated soil irrigated by petroleum refinery wastewater
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 826)
  AUTHORS   Zahid,M.S., Iqbal,A. and Arshad,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-SEP-2014) Institute of Environmental Sciences &
            Engeering, School of Civil & Environmental Engineering, National
            University of Sciences & Engineering, Sector H-12, Islamabad 44000,
            Pakistan
```

**Figure 3.1 *Pseudomonas aeruginosa* (KM520129)**

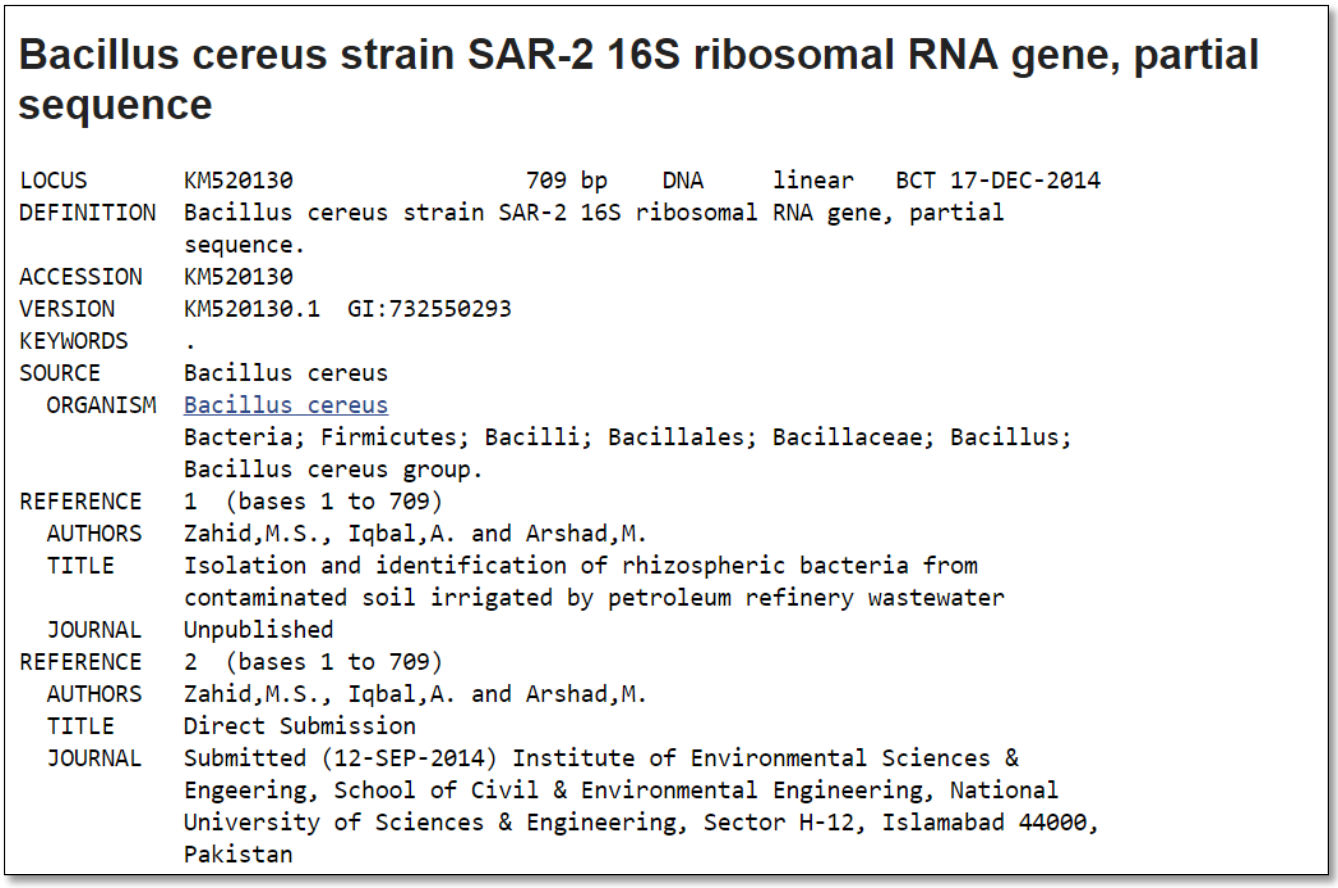
Source: NCBI Databank (http://www.ncbi.nlm.nih.gov)

### 3.2.2 *Bacillus cereus*

*Bacillus cereus* is a large, rod shaped, gram-positive, facultative aerobic, and endospore forming bacterium. *B. cereus* was first successfully isolated in 1969 from a case of fatal pneumonia in a male patient. Comparison of 16s rRNA showed that *Bacillus cereus* was related to *Bacillus anthracis*. Although they have similar characteristics, but they are unique as *B. cereus* is motile While *B. anthracis* is non-hemolytic.

*B. cereus* is a mesophilic bacterium which mostly grows at temperatures between 20°-40°C, and is capable of adapting to an extensive range of environmental conditions. It is distributed commonly in nature. Mostly, it is found in soil as a saprophytic organism. *B. cereus* is also a major contributor to insect's microflora, derive nutrients from its host, and lives in rhizosphere of some plants. *B. cereus* can spread easily in many types of foods such as eggs, meat, and dairy products. It is known for food-borne intoxications due to its secretion of emetic toxins and enterotoxins. Food poisoning mostly occurs when food is left without refrigeration for several hours.

Vilian et al. (2006) reported the presence of *B. cereus* in rhizosphere of plants as well as in microflora of insects. It is also known as human pathogen, responsible for causing infections in human.



**Bacillus cereus strain SAR-2 16S ribosomal RNA gene, partial sequence**

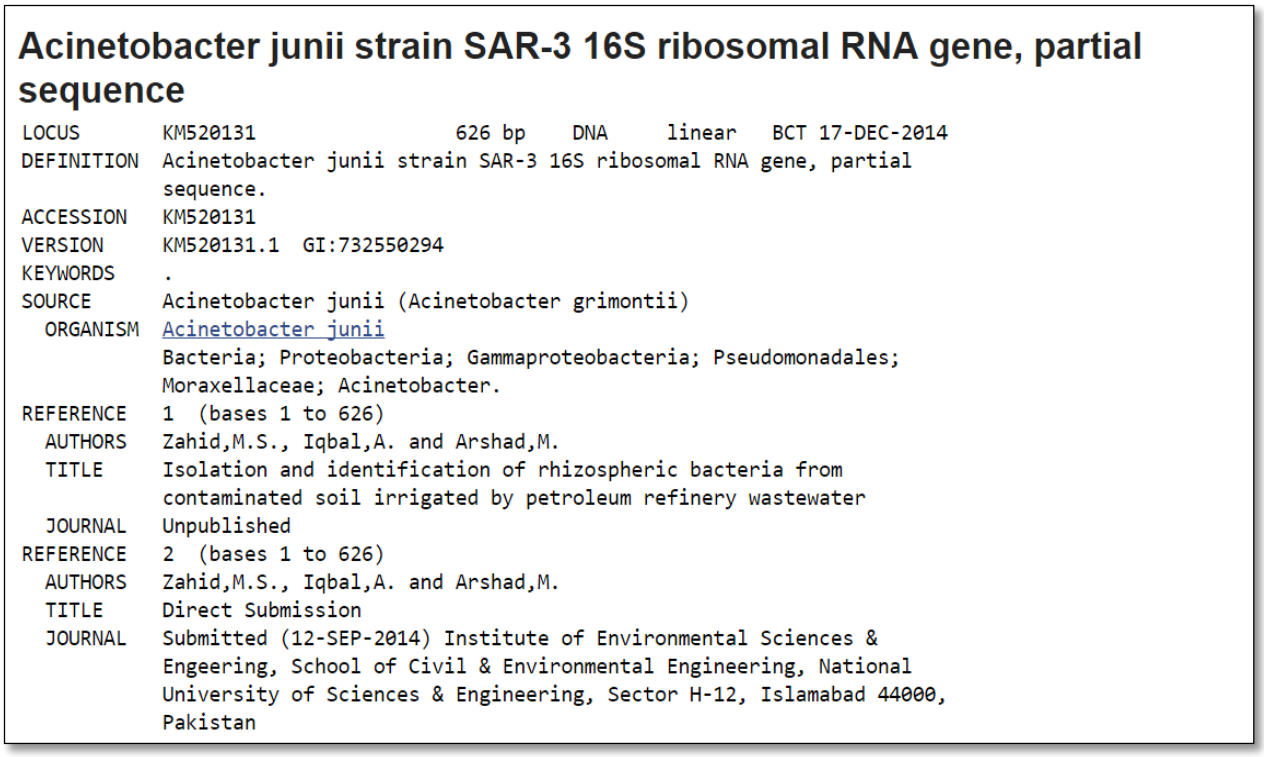
```
LOCUS       KM520130                709 bp    DNA     linear   BCT 17-DEC-2014
DEFINITION  Bacillus cereus strain SAR-2 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   KM520130
VERSION     KM520130.1  GI:732550293
KEYWORDS    .
SOURCE      Bacillus cereus
  ORGANISM  Bacillus cereus
            Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus;
            Bacillus cereus group.
REFERENCE   1  (bases 1 to 709)
  AUTHORS   Zahid,M.S., Iqbal,A. and Arshad,M.
  TITLE     Isolation and identification of rhizospheric bacteria from
            contaminated soil irrigated by petroleum refinery wastewater
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 709)
  AUTHORS   Zahid,M.S., Iqbal,A. and Arshad,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-SEP-2014) Institute of Environmental Sciences &
            Engeering, School of Civil & Environmental Engineering, National
            University of Sciences & Engineering, Sector H-12, Islamabad 44000,
            Pakistan
```

**Figure 3.2. *Bacillus cereus* (520130)**

Source: NCBI Databank (http://www.ncbi.nlm.nih.gov)

### 3.2.3 *Acinetobacter junii*

*Acinetobacter spp.* are known as emerging nosocomial pathogens. One of the most important strain of this genus is *Acinetobacter junii* which is a rare cause of disease in humans. *A. junii* was associated mainly with microorganisms present in preterm infants as well as pediatric oncologic patients. *Acinetobacter junii* is classified genomospecies 5 of genus with reference to DN-DNA hybridization (Bouvet et al., 1986). It has also been reported that members of this can also cause nosocomial infections (Villers et al., 1996)



**Acinetobacter junii strain SAR-3 16S ribosomal RNA gene, partial sequence**

```
LOCUS       KM520131                626 bp    DNA     linear   BCT 17-DEC-2014
DEFINITION  Acinetobacter junii strain SAR-3 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   KM520131
VERSION     KM520131.1  GI:732550294
KEYWORDS    .
SOURCE      Acinetobacter junii (Acinetobacter grimontii)
  ORGANISM  Acinetobacter junii
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Moraxellaceae; Acinetobacter.
REFERENCE   1  (bases 1 to 626)
  AUTHORS   Zahid,M.S., Iqbal,A. and Arshad,M.
  TITLE     Isolation and identification of rhizospheric bacteria from
            contaminated soil irrigated by petroleum refinery wastewater
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 626)
  AUTHORS   Zahid,M.S., Iqbal,A. and Arshad,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-SEP-2014) Institute of Environmental Sciences &
            Engeering, School of Civil & Environmental Engineering, National
            University of Sciences & Engineering, Sector H-12, Islamabad 44000,
            Pakistan
```

**Figure 3.3. *Acinetobacter junii* (KM520131)**

Source: NCBI Databank (http://www.ncbi.nlm.nih.gov)

### 3.3 Isolation of bacterial strains

Benzene degrading indigenous microorganisms were isolated from the soil near Attock Oil Refinery (Pvt) Ltd. Procedure for isolation starts with collection of soil samples. Soil samples were then brushed off from the roots of *Cannabis sativa*. 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 (Zahid et al., 2015)

## 3.4 Morphological Characterization

Cell morphology of selected strains was determined by performing standard procedures. Morphological characterization of three bacterial strains is listed in Table 3.1

**Table 3.1: Morphological characterization of bacterial isolates**

| Bacterial Isolates | Gram stain | Shape | Arrangement | Motility |
|---|---|---|---|---|
| ***Pseudomonas aeruginosa*** | Negative | Cocci | Chain | + |
| ***Bacillus cereus*** | Positive | Bacilli | Single | + |
| ***Acinetobacter junii*** | Negative | Cocci | Group | + |

## 3.5 Minimal media preparation

All enrichment of these strains were done in M9 minimal medium that contain the following constituents per liter of distilled water: $Na_2HPO_4$, 64g; $KH_2PO_4$, 15 g; NaCl, 2.5 g; $NH_4Cl$, 5.0 g. The minimal medium M9 was prepared and autoclaved at 121 °C, and 15 psi pressure for 15 min.

## 3.6 Biodegradation Experiments

Biodegradation experiments were conducted in conical flasks. Each 250 mL conical flask contain 140 mL distilled water, 60 mL M9 media, 3 mL bacterial inoculum and different concentrations of benzene (250, 500, 750, 1000 mg/L) added as a single carbon source to study the microbial growth patterns during biodegradation and percentage benzene degradation.

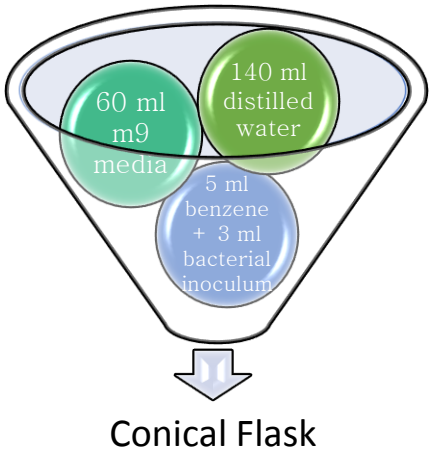



**Figure 3.4. Experimental Design for Biodegradation**

### 3.6.1 Benzene used for Biodegradation 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.

### 3.6.2 Concentrations of Benzene

Different concentrations of analytical grade benzene were prepared. This include the concentrations ranging from 250 mg/L to 1000 mg/L. A total of four concentrations were used in this experiment. Concentrations levels include 250, 500, 750 and 1000 mg/L. All solutions were prepared using 1000 ml stock solution. 5 ml benzene of each concentration was added in the conical flask containing biodegradation media i.e. M9 media and distilled water.

### 3.6.3 Preparation of Inocula

The inocula for the biodegradation experiment were prepared by growing bacterial cultures on nutrient agar plates for 24 h at 37 °C. Significant amount of autoclaved distilled were added on the fresh culture plate. Distilled water was spreaded using spreader. Serial dilution was performed. Inoclua used for degradation experiment was picked from the fourth to eight dilution.

### 3.6.4 Benzene Degradation in Orbital Shaker

For a batch experiment (3 days), the degradation potential of selected bacterial strains were determined using flask shaker. 140 mL distilled water, 60 mL M9 media, different benzene concentrations (5ml), inoculated with 3 mL of bacterial strains were added in 250 mL conical flask. These were then and incubated on orbital shaker at ambient temperature at 120-180 rpm for 72 hours (Zahid et al., 2015)

### 3.6.5 Sampling Procedure

For the first day, 5 mL samples were extracted from experiment flask after 2, 4, 6 hour 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 Visible Spectrophotometer.

## 3.7 Analytical Procedure

### 3.7.1 UV Spectrophotometer

Benzene shows absorbance at 254 nm wavelength. 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. Decrease in benzene concentration was further calculated.

## 3.8 Serial Dilution Technique

Serial dilution technique was followed to estimate the microbial counts. Eight sterilized test tubes were marked as $10^{-1}$ to $10^{-8}$. Nine milliliter water is added in each test tube. All test tubes were autoclaved at 121 °C.

A significant amount of distilled autoclaved water is placed on each agar plate with bacterial strain. Now, this bacterial inoculum were mixed with the help of spreader. One mL of this inoculum was

added in the test tube marked as $10^{-1}$. One milliliter of inoculum 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.

200µL samples were taken from $10^{-5}$ to $10^{-8}$ dilution and was spread on nutrient agar plates and the plates were incubated at 37 °C for 24 hours. This was done for all four different substrate concentrations. The bacterial colonies were counted with the help of Colony Counter and reported as Colony forming Unit (CFU/mL). CFU/mL has been calculated by following formula

$$\text{CFU/mL} = \frac{\textbf{\textit{Number of Colonies x Dilution Factor}}}{\textbf{\textit{Volume plated (ml)}}}$$

### 3.9 Biomass determination

For this purpose, empty Eppendorf were weighed and autoclaved. Dry weight was noted. Samples were taken from flask at regular time intervals of 0, 2, 4, 6, 24, 48 and 72 hours. Centrifugation of samples in these Eppendorf was done in a centrifuge at 5000 pm. The supernatant was discarded. Pellets of bacteria were remained at the bottom of Eppendorf. Now, the wet weight with pellet was noted down. Biomass has been calculated by using following equation:

$$\textbf{Biomass determination} = Wet\ Weight\ (mg) - Dry\ Weight(mg)$$

### 3.10 Determination of kinetic parameters

When substrate biodegradation is related to growth of bacteria, Monod type kinetics could be helpful in determination of various parameters (Monod, 1949). Based on the growth pattern of different bacterial strains, the specific growth rate (µ) was obtained for each initial substrate concentration (S) using the following relationship given in Eq. (1):

$$\mu = \ln(X_2 - X_1)/(t_2 - t_1) \text{ ---------- } (1)$$

Under ideal (non-limiting growth conditions, the growth could only be altered by changing temperature. Growth increase with increasing in temperature.

For growth kinetics, mathematical modelling was performed and Monod equation was used.

$$\mu = \mu_{max} S / K_s + S$$

### 3.11 Studies regarding effect of pH and salinity during benzene degradation

Different pH studies were performed only at benzene concentration where bacterial strain showed the best degradation. For this purpose, 250 mL conical flask containing 140 mL distilled water, 60 mL M9 media were prepared.

### 3.11.1 pH

Different pH levels were adjusted in flasks to pH 5, 6, 7, 8, and 9. Different benzene concentrations were added along with bacterial inoculum under laminar flow. Degradation potential was determined using UV visible spectrophotometer. Degradation was checked at regular time intervals of 0, 2, 4, 6, 24, 48, and 72 hours by taking samples from flask on orbital shaker.

### 3.11.2 Salinity

Different salinity levels of 0.5, 1.5, 3, and 5% were adjusted in 250 mL conical flasks in order to check the effect of salinity on benzene degradation. Same procedure was repeated to check degradation as in case of pH studies.

**Chapter 4**

# RESULTS AND DISCUSSIONS

## 4.1 Biomass Production

### 4.1.1 Biomass production of *Pseudomonas aeruginosa*

Results regarding biomass production for *Pseudomonas aeruginosa* are shown in Figure 4.1. It is evident from the figure that biomass at 1000 mg/L substrate concentration is more as compared to all other concentrations. Exponential increase in biomass has been observed after 4$^{th}$ hour, followed by approximate same amount of biomass production at 6$^{th}$ and 24$^{th}$ hours. Bordoloi and Konwar (2008) also reported the highest biomass of *Pseudomonas aeruginosa* in media containing glucose and n-hexadecane as substrates. Decline starts after 24$^{th}$ hour. At 48$^{th}$ and 72$^{nd}$ hour, there is very less biomass. This depicts that bacterial growth has been decreased to a greater extent which showed the death of bacteria. Greatest number of rhamnnolipids and highest biomass production of *Pseudomonas aeruginosa* was observed after variation in glycerol as carbon source from 1 to 3% v/v (Anna et al., 2002)

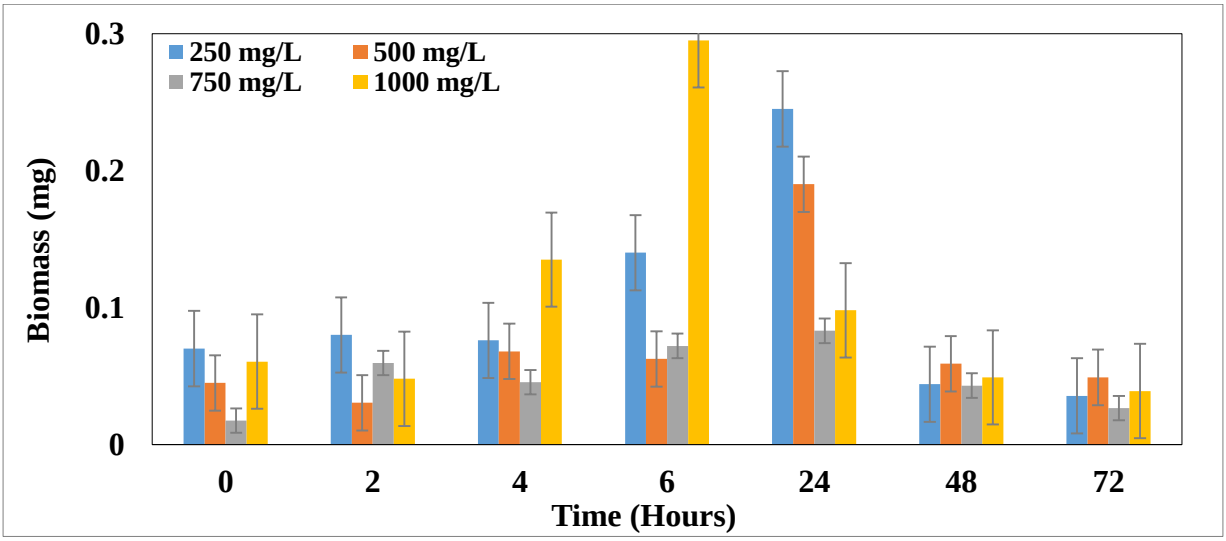



**Figure 4.1 Biomass production of *P. Aeruginosa* at different substrate concentrations**

### 4.1.2 Biomass production of *Bacillus cereus*

Results regarding biomass production of *Bacillus cereus* have been shown in Figure 4.2. It has been observed that biomass is increasing with time. There is almost same amount of biomass produced during different substrate concentrations. However, there is exponential growth starting from 24$^{th}$ hour to 48$^{th}$ hour. In this case, *Bacillus cereus* consumed the substrate in logarithmic phase of growth. At these hours, biomass production has increased while just after the 48$^{th}$ hour, there is a fast decline towards bacterial biomass. Growth after the exponential phase fluctuated and starts declining which showed the death phase of bacteria. Infrared spectroscopy results of *B. cereus* cell mainly contains carboxyl, hydroxyl, phosphate, amino and amide functional groups. Best isotherm for adsorption of metals on *B. cereus* biomass was Langmiur isotherm (Pan et al., 2007)

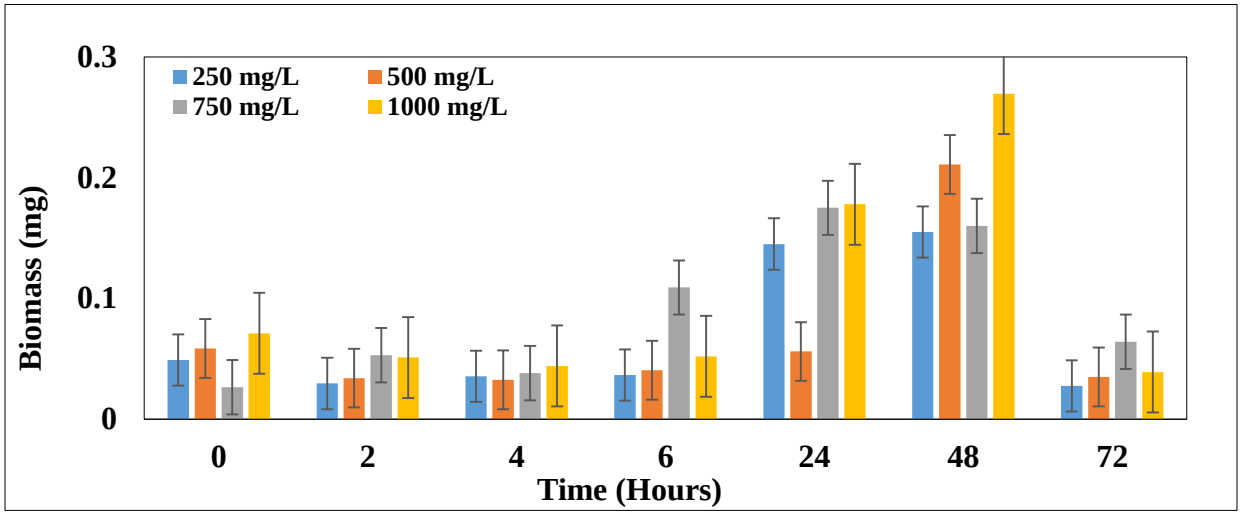



**Figure 4.2 Biomass production of *Bacillus cereus* at different substrate concentration**

### 4.1.3 Biomass production of *Acinetobacter junii*

Results regrading biomass production of *Acinetobacter junii* have been shown in Figure 4.3. There is a significant increase in amount of biomass produced at 6$^{th}$ hour which continues till 24$^{th}$ hour.

(Al-Wasify and Hamed, 2014) also argued that dry weight (biomass) increases with increase in incubation period. However, a small decrease was observed at 48$^{th}$ hour but it was also similar as the amount produced at 48$^{th}$ hour. After 48$^{th}$ hour, decay in bacterial biomass was noticed which could be the decline in utilization of carbon source. At 72$^{nd}$ hour, very low amount is produced which could be justified by less amount of substrate utilization.

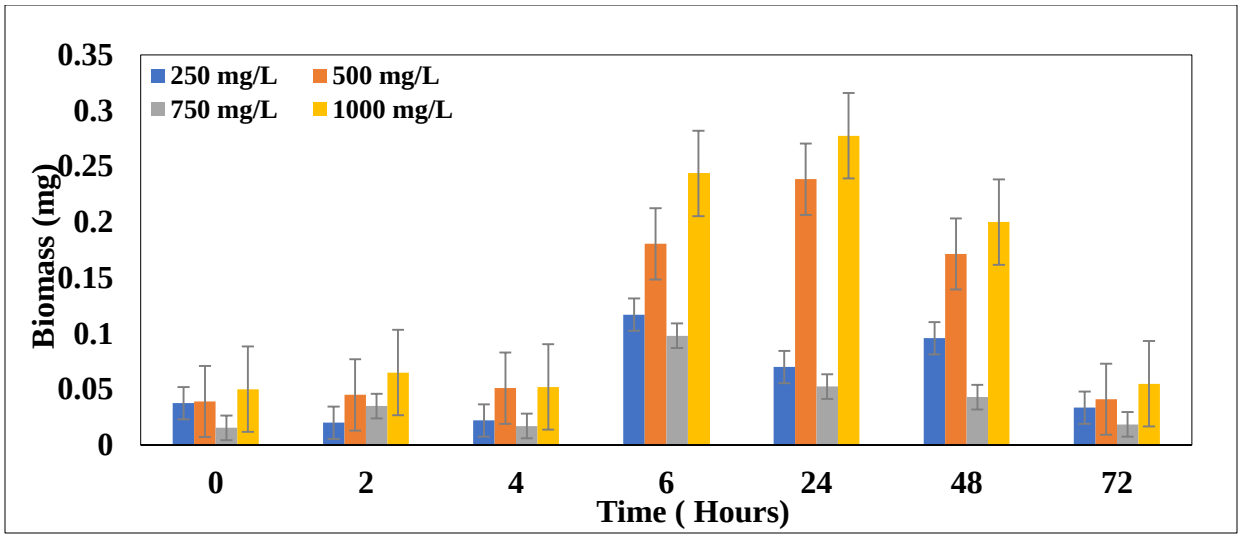



**Figure 4.3 Biomass production of *Acinetobacter junii* at different substrate concentrations**

## 4.2 Bacterial Growth Curves

### 4.2.1 Growth curves of *Pseudomonas aeruginosa* at different benzene concentrations

Growth curve at different benzene concentrations of 250,500,750 and 100 mg/L for *Pseudomonas aeruginosa* has been shown in Figure 4.4 – 4.7.

Fig 4.4 showed the growth of *Pseudomonas aeruginosa* at 250 mg/L. Growth of *P. aeruginosa* starts to increase till 6$^{th}$ hour where maximum CFU count was 1.6x10$^{9}$ CFU/mL. This was termed as lag or exponential phase of bacteria. Starting from 6$^{th}$ to 24$^{th}$ hour is the stationary phase where CFU count was 1.5 x 10$^{8}$ to 2 x 10$^{8}$ CFU/mL. After 24$^{th}$ hour, bacterial growth starts to decline resulting in death or decline phase.

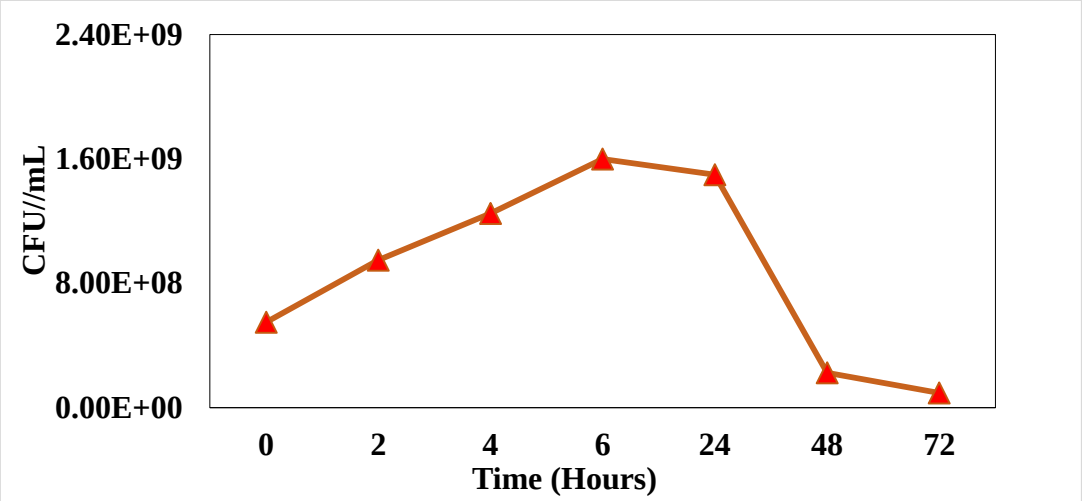



**Figure 4.4 Growth of *Pseudomonas aeruginosa* at 250 mg/L**

Fig 4.5 showed the growth of *Pseudomonas aeruginosa* at 500mg/L. During 0 to 2$^{nd}$ hour, there is a short lag phase observed followed by long exponential phase. Increase in CFU with increase in incubation time clearly depicts the benzene degradation (Mukherjee and Bordoloi, 2012). Maximum CFU count was 1.11x10$^{8}$ CFU/mL. Stationary phase starts from 24$^{th}$ to 48$^{th}$ hour where CFU count reaches 1x10$^{8}$ CFU/mL. Bacterial growth decreases to 9.5 x 10$^{7}$ CFU/mL, resulting in decline phase of bacterial growth.

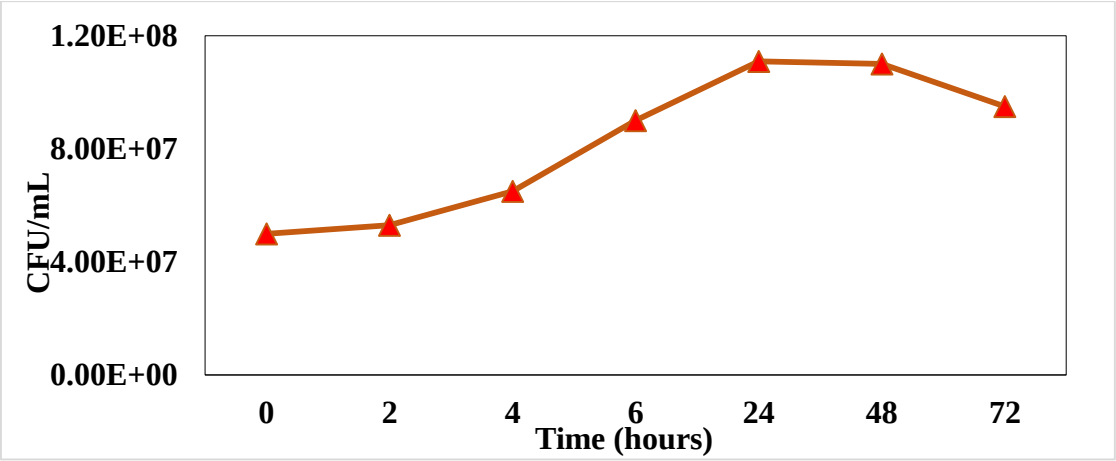



**Figure 4.5 Growth of *Pseudomonas aeruginosa* at 500 mg/L**

Fig 4.6 represents the growth of *Pseudomonas aeruginosa* at 750 mg/L. Maximum CFU count was 1 x $10^8$ CFU/mL at the 4$^{th}$ hour. Cell growth was found to be decreasing with incubation time and increase in benzene concentration. This could be decrease in cytotoxicity in the medium with increase in hydrocarbon concentration (Borah and Yadav, 2014). No stationary phase was observed at this concentration. Death or decline starts just after 4$^{th}$ hour, resulting in 1.8 x $10^7$ CFU/mL.

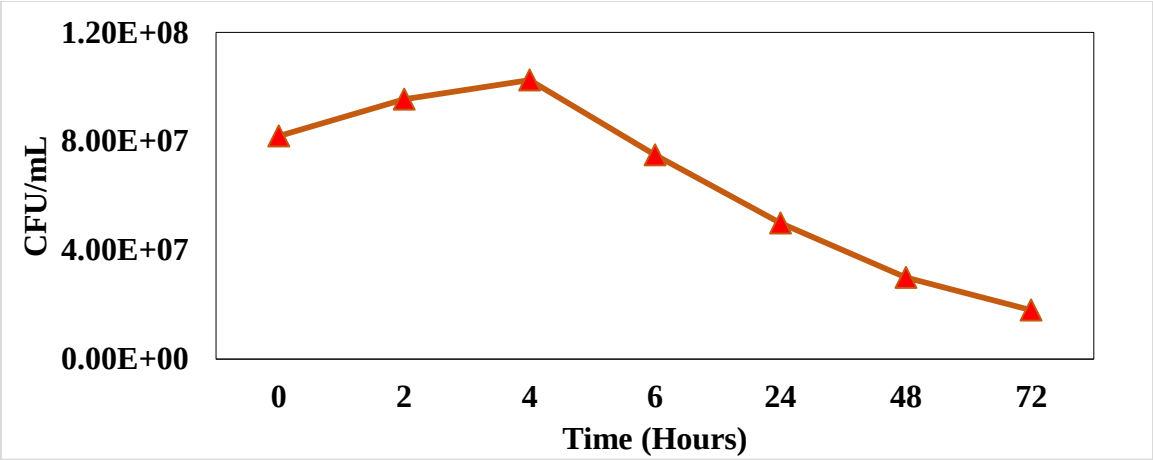



**Figure 4.6 Growth of *Pseudomonas aeruginosa* at 750 mg/L**

Fig 4.7 signifies the growth pattern of *Pseudomonas aeruginosa* at 1000 mg/L benzene concentrations. There was exponential growth observed from 0 to 6$^{th}$ hour. Maximum CFU count was 1.5 x $10^9$ CFU/mL at the 24$^{th}$ hour. Minor increase in growth rate was witnessed at 6 to 24$^{th}$ hour which could be termed as stationary phase. Bacterial growth rapidly declines just after the 24$^{th}$ hour resulting in death phase and reaches 4.9 x $10^7$ CFU/mL which was below the initial CFU count.

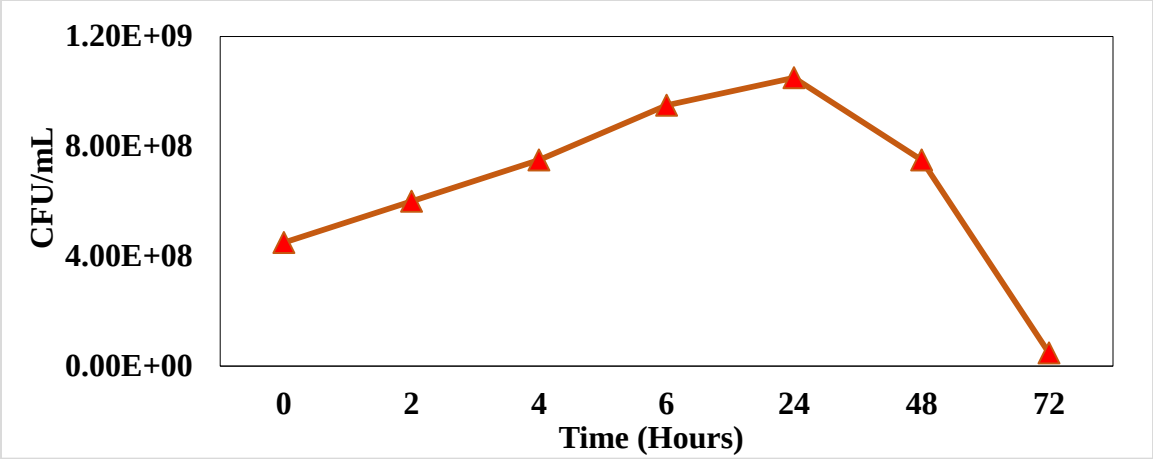



**Figure 4.7 Growth of *Pseudomonas aeruginosa* at 1000 mg/L**

### 4.2.2 Growth curves of *Bacillus cereus* at different benzene concentrations

Fig 4.8 depicts the growth of *Bacillus cereus* towards benzene concentration 250 mg/L. A short lag phase was observed between till 2$^{nd}$ hour followed by rapid increase in growth till 24$^{th}$ hour. Maximum CFU count observed was around 8 x 10$^{8}$ CFU/mL. After 24$^{th}$ hour, very slight decrease in CFU count was noticed and could be termed as stationary phase of *Bacillus cereus*. Followed after 48$^{th}$ hour, bacteria starts to declining and it resulted in death phase.

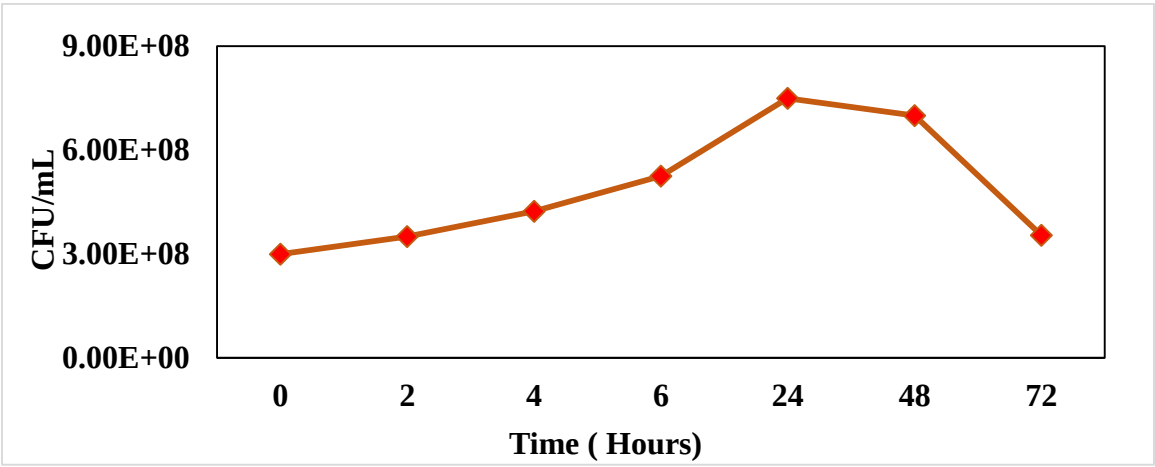



**Figure 4.8 Growth of *Bacillus cereus* at 250 mg/L**

Fig 4.9 shows the growth profile of *Pseudomonas aeruginosa* at 500mg/L. Maximum CFU count was observed as 8 x 108 CFU/mL at 48$^{th}$ hour. A long exponential phase could be observed from 2$^{nd}$ hour to 48$^{th}$ hour. No stationary phase is observed at this concentration. At this specific concentration of 500 mg/L, *Bacillus cereus* utilizes benzene as a carbon source efficiently till 48$^{th}$ hour. A very short death phase has been observed where CFU count reaches 5 x $10^8$ CFU/mL.

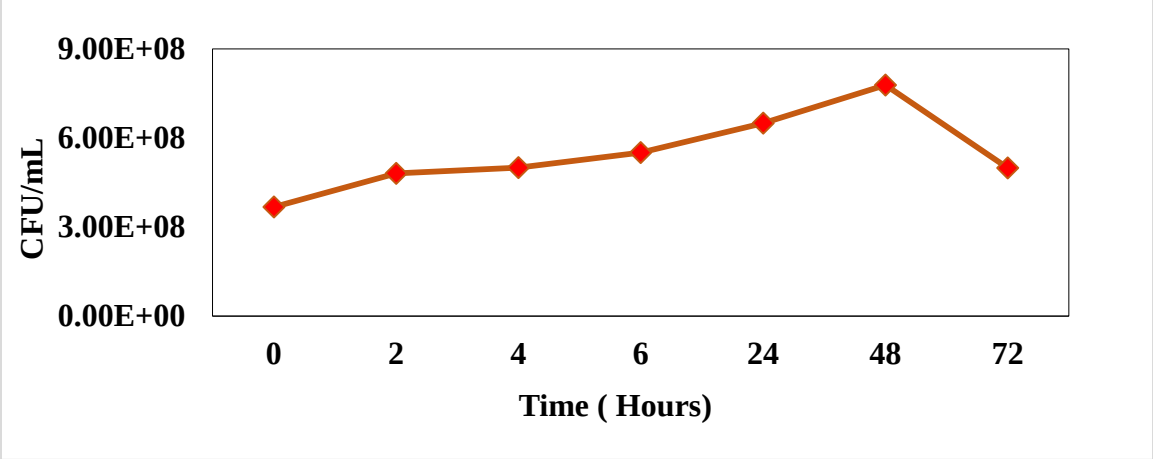



**Figure 4.9 Growth of *Bacillus cereus* at 500 mg/L**

Fig 4.10 showed the growth pattern of *Bacillus cereus* at substrate concentration of 750 mg/L. Small lag phase was observed till 2$^{nd}$ hour. Exponential increase in CFU count started from 2$^{nd}$ to 6$^{th}$ hour and reaches maximum CFU count of 2.8 x $10^8$ CFU/mL Growth starts to decrease slightly after 6$^{th}$ hour but remains almost constant. Bacterial growth from 24$^{th}$ to 48$^{th}$ is almost same. However, rapidly decline in growth started just after 48$^{th}$ hour and reaches 1 x $10^8$ CFU/mL. Ting et al. (1999) reported decrease after initial increase in microbial population.

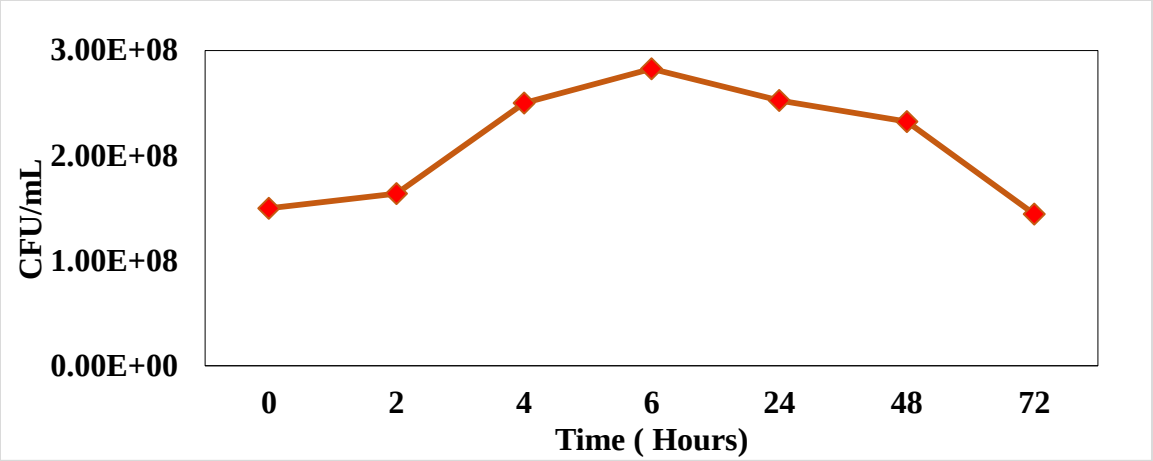



**Figure 4.10 Growth of *Bacillus cereus* at 750 mg/L**

Fig 4.11 represents the growth of *Bacillus cereus* at 1000 mg/L substrate concentration. An exponential increase has been observed from $0^{th}$ hour to $24^{th}$ hour. At $6^{th}$ to $24^{th}$ hour, bacterial count was increased rapidly and reached maximum CFU count of 2.2 x $10^8$ CFU/mL. Bacterial growth follows stationary phase between $24^{th}$ to $48^{th}$ hours. After $48^{th}$ hour, progressive death of cells started, resulting in decline phase.

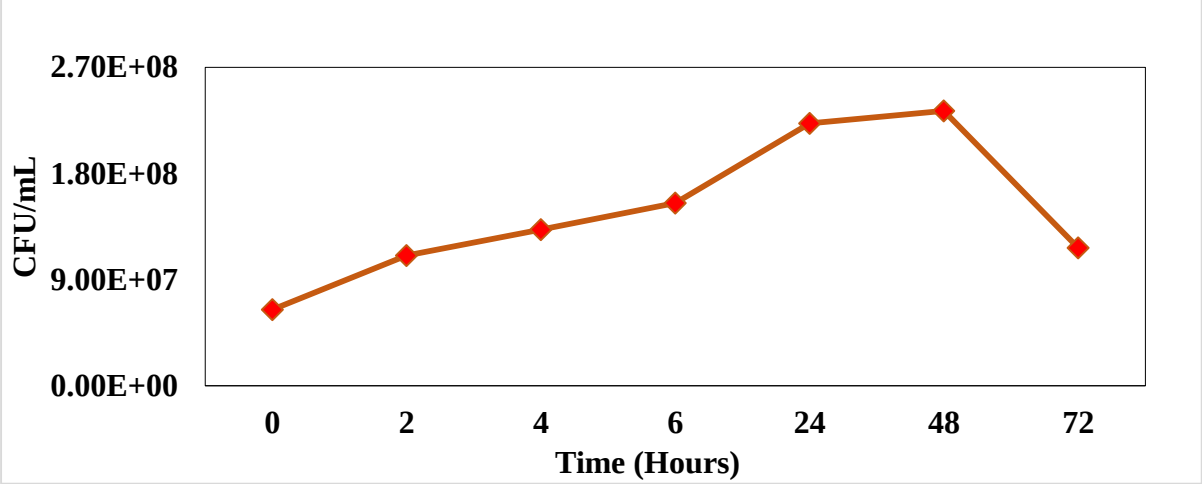



**Figure 4.11 Growth of *Bacillus cereus* at 1000 mg/L**

### 4.2.3 Growth curves of *Acinetobacter junii* at different benzene concentrations

Fig 4.2 represents the growth of *Acinetobacter junii* at 1000 mg/L substrate concentration. *A. junii* showed better growth at 250 mg/L. Many bacteria respond to shorter or longer life time when nutrient levels in environment fluctuate (Young, 2006). Maximum CFU count occurred at 24$^{th}$ and 48$^{th}$ hour which was 5.5 x 10$^{8}$ CFU/mL. Similarly, stationary phase also exist in this range. Bacterial growth tends to decrease just after the 48$^{th}$ hour.

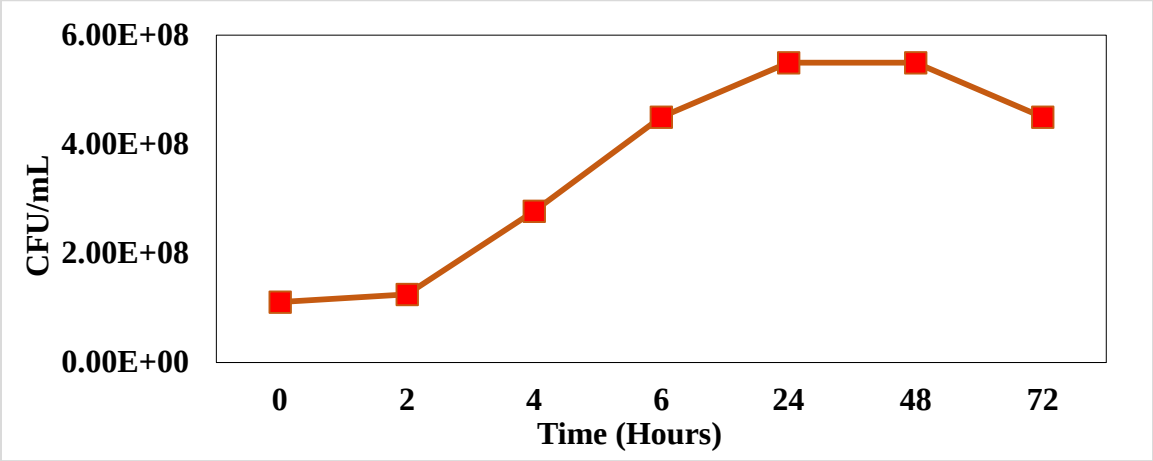



**Figure 4.12 Growth of *Acinetobacter junii* at 250 mg/L**

Fig 4.13 depicts the growth of *Acinetobacter junii* at 500 mg/L concentration. Maximum CFU count occurred at 48$^{th}$ hour which was 3.25 x 10$^{8}$ CFU/mL. There exists a long lag phase with short exponential phase and no stationary phase in the growth curve. *Acinetobacter junii* showed rapid decrease after 48$^{th}$ hour, resulting in CFU count of 2 x 10$^{8}$ CFU/mL.

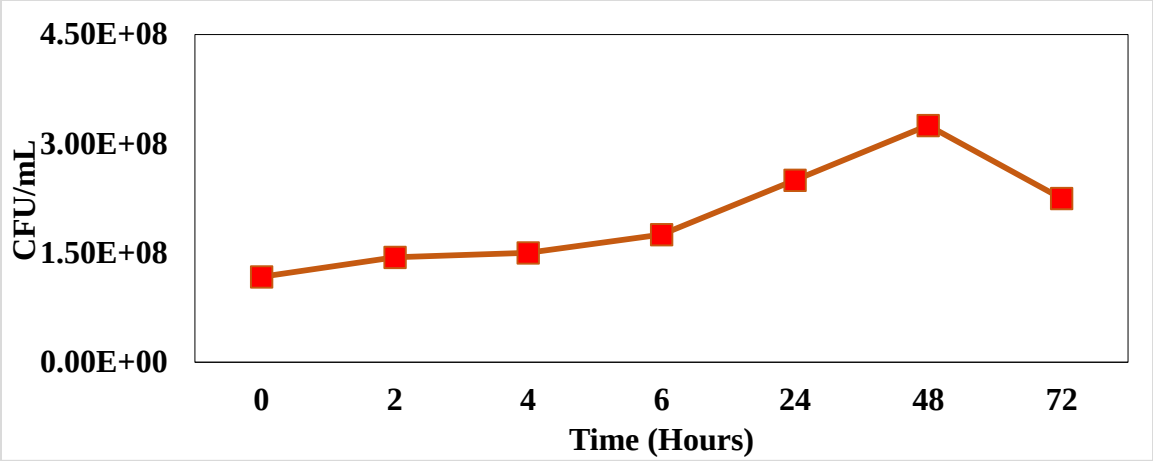



**Figure 4.13 Growth of *Acinetobacter junii* at 500 mg/L**

Fig 4.14 describes the growth rate of *Acinetobacter junii* in response to benzene concentration 750 mg/L. Bacterial growth initially increases till 2$^{nd}$ hour. An exponential increase occurs till 6$^{th}$ hour showing the logarithmic phase. At the end of logarithmic phase, maximum CFU count was observed as 9 x 10$^{7}$ CFU/mL. Bacterial growth showed a slight decrease till 24$^{th}$ hour and rapid decrease till 72$^{nd}$ hour. In this typical growth curve, *A. junii* experienced very short stationary phase.

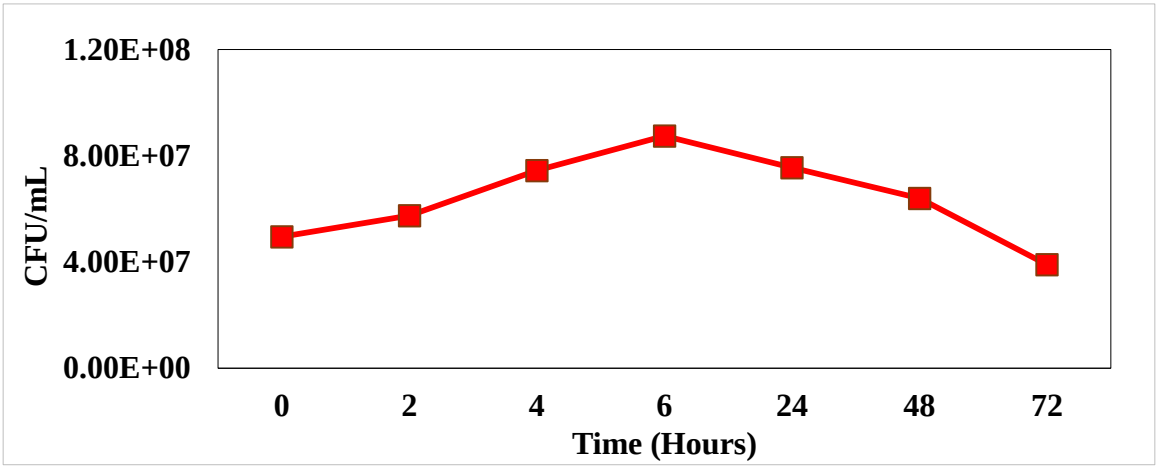



**Figure 4.14 Growth of *Acinetobacter junii* at 750 mg/L**

Fig 4.15 discussed the growth profile of *Acinetobacter junii* at substrate concentration of 1000 mg/L. *A. junii* showed a short lag phase till 2$^{nd}$ hour. After 2$^{nd}$ hour, it started to increases

exponentially till 24$^{th}$ hour where maximum CFU count was observed as 4.4 x 10$^{8}$ CFU/mL. It has been observed that stationary phase exists between 24$^{th}$ to 48$^{th}$ hours. A rapid decline in *A. junii* exists till 72$^{nd}$ hour where CFU count was 2.2 x 10$^{7}$ CFU/mL.

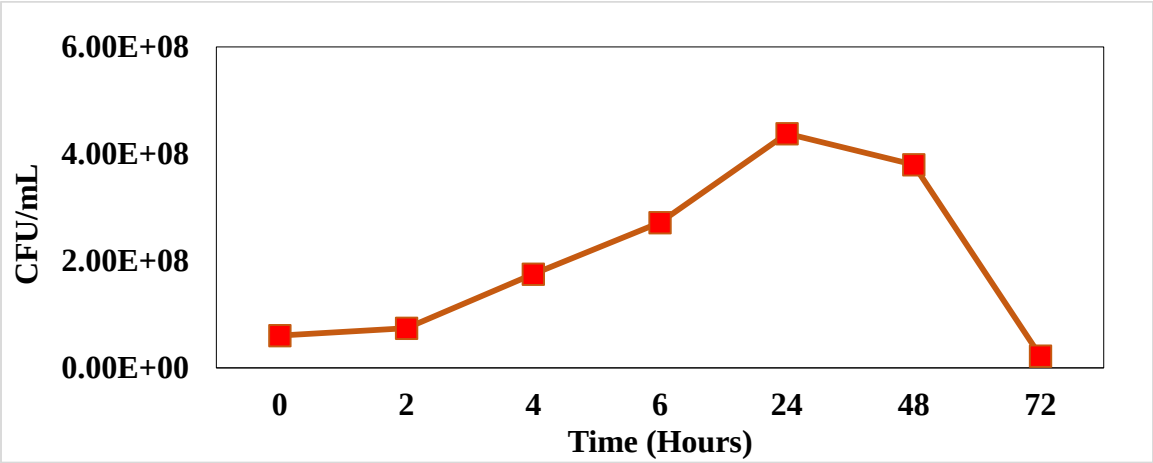



**Figure 4.15 Growth of *Acinetobacter junii* at 1000 mg/L**

### 4.3 Estimation of Monod parameters

Table 4.1 shows the specific growth rate of *Pseudomonas aeruginosa, bacillus cereus* and *Acinetobacter junii.* Maximum specific growth rate was 0.177 for *Pseudomonas aeruginosa*. Maximum specific growth rate ($h^{-1}$) was 0.135 for *Bacillus cereus* while it was 0.105 for *Acinetobacter junii.* The maximum specific growth rate of *Pseudomonas aeruginosa* indicated that the strain has degraded the contaminant in less time. Similarly, specific growth rate was maximum at 250 mg/L concentration for *Pseudomonas aeruginosa* while it was 750 mg/L for both bacterial strains namely *Bacillus cereus* and *Acinetobacter junii*. Half saturation constant ($K_s$) was maximum for *Pseudomonas aeruginosa* which was 0.088. Similarly, half saturation constant ($K_s$) was 0.052 as minimum for *Acinetobacter junii* and 0.067 for *Bacillus cereus.*

**Table 4.1: Monod Parameter Estimation**

| Bacterial Strains | Substrate Concentration (mg/L) | Specific growth Rate ($\mu$) | Maximum Specific Growth Rate ($\mu_{max}$) | Half Saturation Constant ( $K_s$) |
|---|---|---|---|---|
| ***Pseudomonas aeruginosa*** | 250 | 0.177 | 0.177 | 0.088 |
| | 500 | 0.132 | | |
| | 750 | 0.055 | | |
| | 1000 | 0.141 | | |
| ***Bacillus cereus*** | 250 | 0.034 | 0.135 | 0.067 |
| | 500 | 0.010 | | |
| | 750 | 0.135 | | |
| | 1000 | 0.031 | | |
| ***Acinetobacter junii*** | 250 | 0.067 | 0.105 | 0.052 |
| | 500 | 0.015 | | |
| | 750 | 0.105 | | |
| | 1000 | 0.081 | | |

## 4.4 Biodegradation of Benzene:

### 4.4.1 Benzene degradation using *Pseudomonas aeruginosa*

Results related to biodegradation of benzene using *Pseudomonas aeruginosa* at different concentrations of 250, 500, 750 and 1000 mg/L have been presented in Fig 4.16. Maximum percentage degradation was found to be 77% at 500 mg/L. Decrease in benzene concentration at 250, 750 and 1000 mg/L was 63.5, 74, and 69% respectively. Gilbert et al. (2003) reported about detoxification of several organic compounds in the environment that was carried out by enzyme termed as carboxy esterase and these enzymes are found in bacteria such as *Pseudomonas aeruginosa*. Biodegradation rate was decreased with increasing benzene concentration up to 1000 mg/L. The reason for this decrease could be the harsh and stressful conditions to which the bacterial strains are exposed.

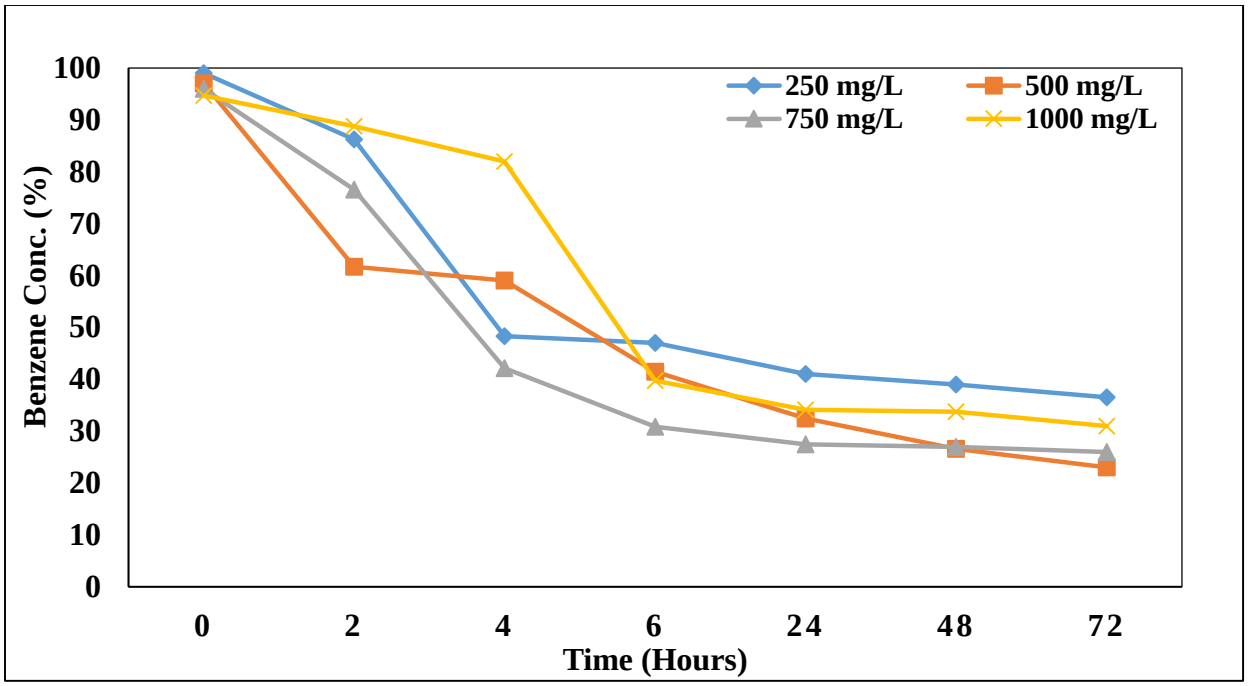



**Figure 4.16 Decrease in Benzene concentration by *Pseudomonas aeruginosa***

### 4.4.2 Benzene degradation using *Bacillus cereus*:

Biodegradation of benzene using *Bacillus cereus* at different concentrations of 250,500,750 and 1000 mg/L has been shown in Fig 4.16. *Bacillus sp*. has been reported as an effective agent for hydrocarbon degradation. It has been clearly observed that maximum degradation was found to be 81% at 500 mg/L. Decrease in benzene concentration for 250, 750 and 1000 mg/L was 70, 72 and 77% respectively. As far as decrease in concentration is concerned, there is very minor difference after increase from 500 to 100 mg/L substrate concentration. The decrease in degradation could be correlated with the enzymatic activity that helped in catabolism of further intermediate compounds (Vermani et al., 1997)

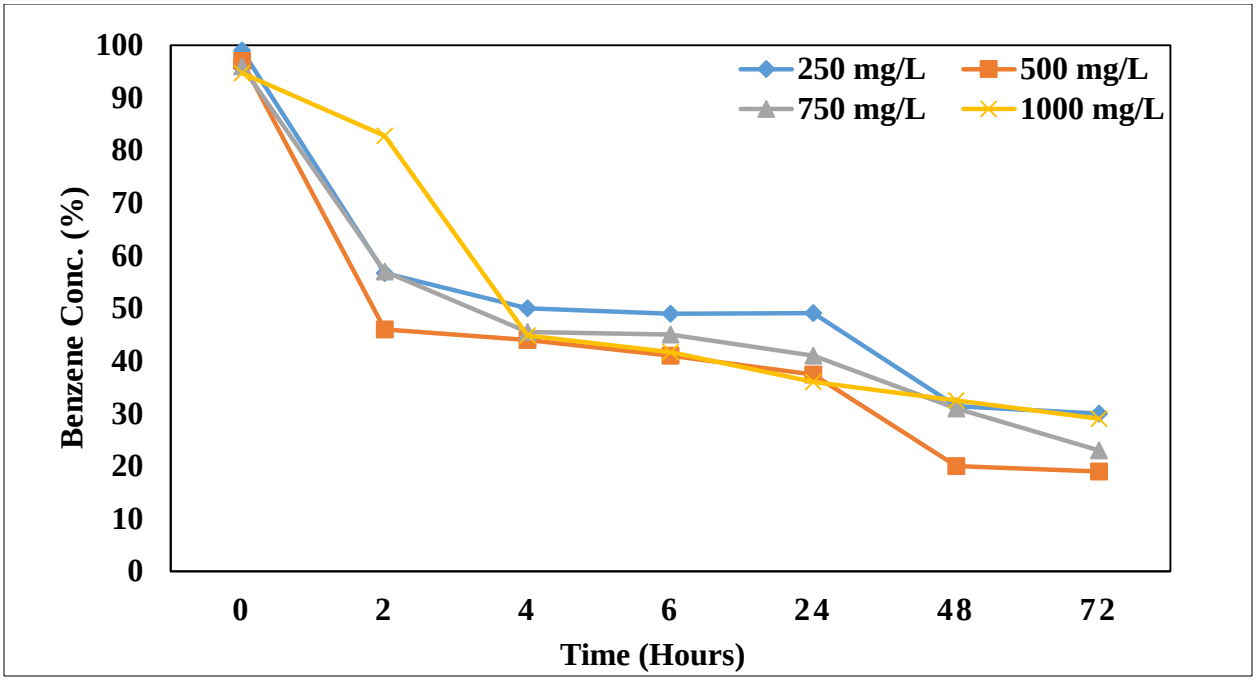



**Figure 4.17 Decrease in Benzene concentration by *Bacillus cereus***

### 4.4.3 Benzene degradation using *Acinetobacter junii*

Benzene biodegradation using *Acinetobacter junii* at four different substrate concentrations have been reported in Fig 4.18. Decrease in benzene concentration was found to be maximum at 74.7% at 500 mg/L whereas degradation percentage was 60.13, 74.7 and 72% at 250, 750 and 1000 mg/L. The reason for less degradation at 750 and 1000 mg/L could be the adverse effects on bacterial growth. Another reason could be the dissolved oxygen, as the increase organic load might decrease the dissolved oxygen availability (Corbitt, 1990). Highest concentration might inhibit the bacterial enzymes involved in biodegradation process (Singh et al., 2013)

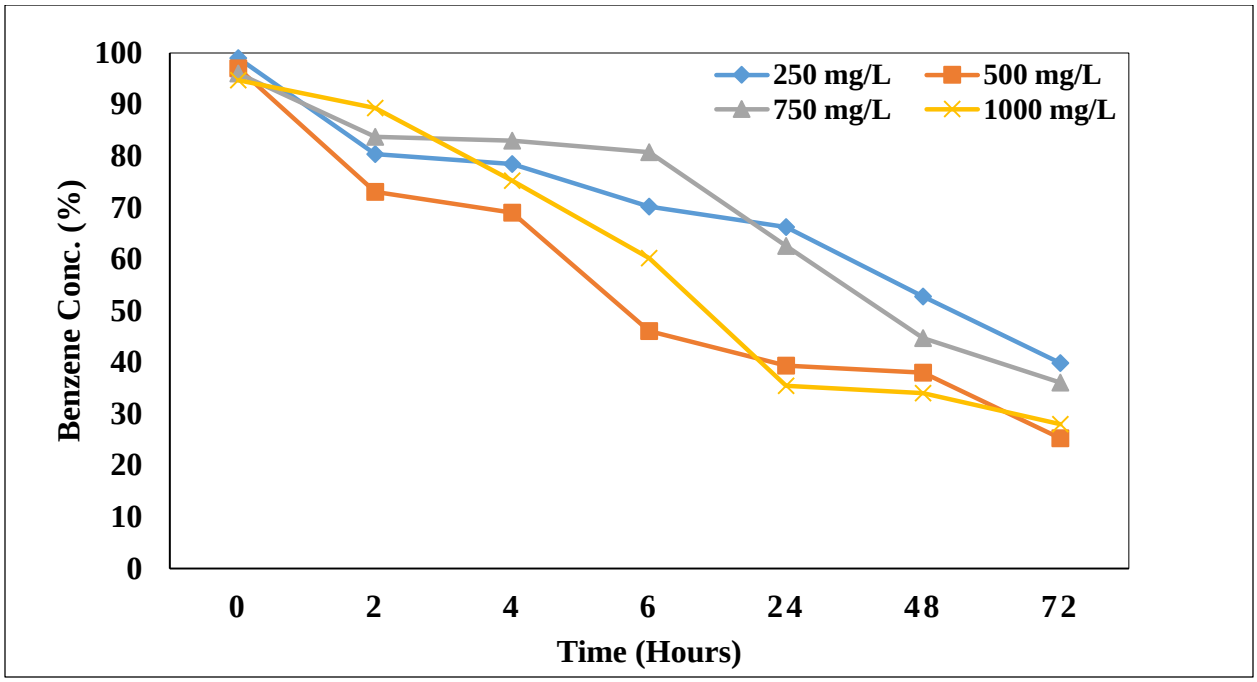



**Figure 4.18 Decrease in Benzene concentration by *Acinetobacter junii***

## 4.5 pH and salinity studies

### 4.5.1 Effect of pH on benzene degradation using *Pseudomonas aeruginosa*

Fig 4.19 illustrates the effect of pH on benzene degradation using *Pseudomonas aeruginosa* at pH 5.0, 6.0, 7.0, 8.0 and 9.0. Benzene degradation percentage was 56% which was maximum at pH 7.0. Similarly, degradation percentage at pH 5, 6, 8, 9 was 29, 34, 44, and 36 %. Hamed et al. (2003) studied the effect of pH on benzene degradation and found higher degradation at pH 6.7. Increase in percentage degradation started after 6$^{th}$ hour. A trend of gradual increase in degradation percentage has been shown by *Pseudomonas aeruginosa* from 24$^{th}$ to 72$^{nd}$ hours. Yuan et al. (2003) investigated the optimum pH of about 7.0 for *Pseudomonas sp.*

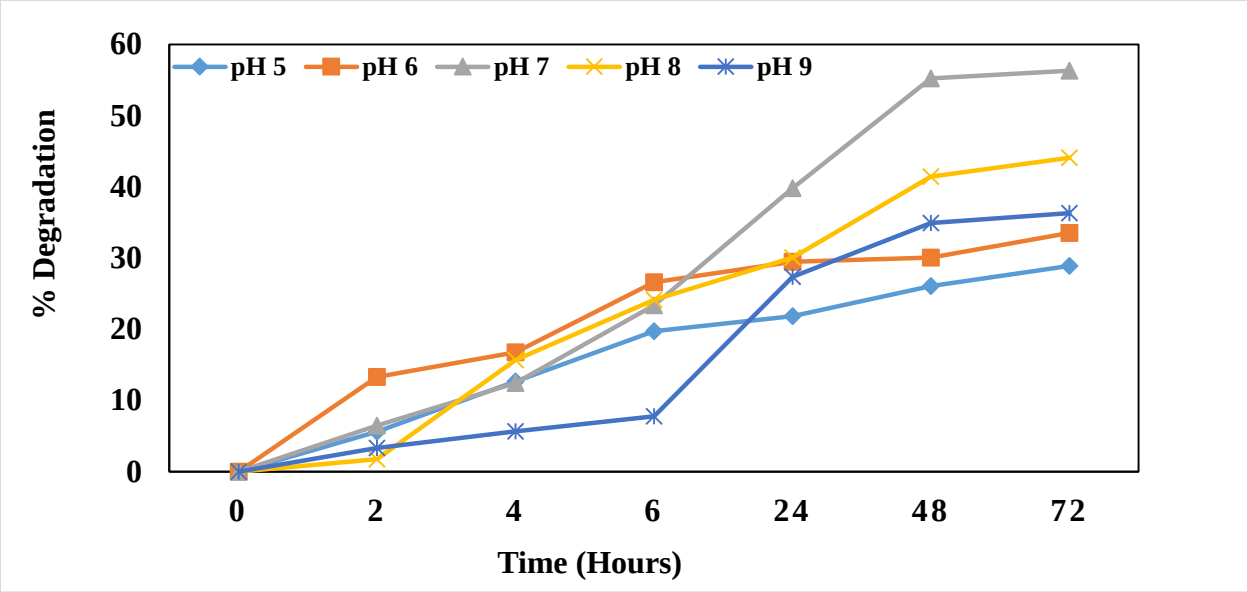



**Figure 4.19 Percentage benzene degradation by *Pseudomonas aeruginosa***

### 4.5.2 Effect of pH on benzene degradation using *Bacillus cereus*

Fig 4.21 demonstrates the effect of pH on benzene degradation using *Bacillus cereus* at pH 5.0, 6.0, 7.0, 8.0 and 9.0. Benzene degradation percentage was 50% which was maximum at pH 7.0. Similarly, degradation percentage at pH 5, 6, 8, 9 was 25, 38, 29, and 39%. Degradation efficiency was significantly declined when pH was less than 7 and greater than 8. (Blum et al., 2009) studied the effect of pH on degradation and found that acidic or alkaline conditions might affect bacterial activity. Rapid increase in percentage degradation started after $6^{th}$ hour at all pH levels.

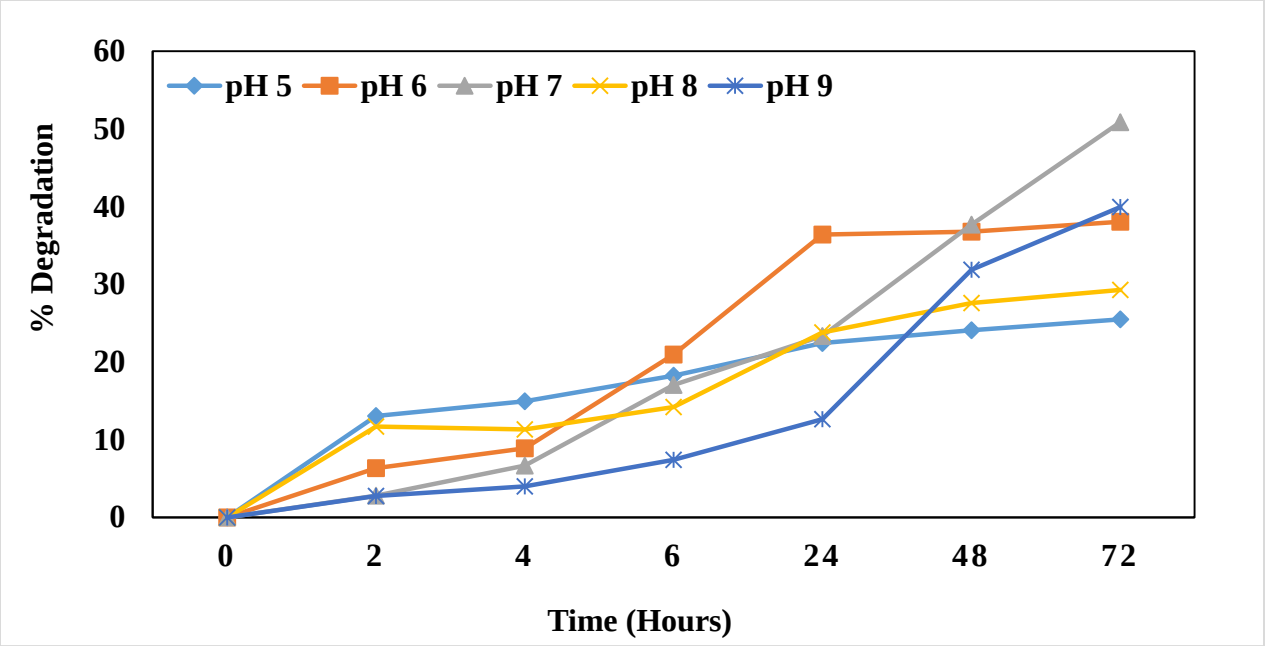



**Figure 4.20 Percentage benzene degradation by *Bacillus cereus***

### 4.5.3 Effect of pH on benzene degradation using *Acinetobacter junii*

Fig 4.21 demonstrates the effect of pH on benzene degradation using *Bacillus cereus* at pH 5.0, 6.0, 7.0, 8.0 and 9.0. Benzene degradation percentage was 69% which was maximum at pH 7.0. Similarly, degradation percentage at pH 5, 6, 8, 9 was 42, 43, 50, and 46%. Garrity et al. (2004) reported the optimum pH for growth around 5 to 8. Ivankovic et al. (2013) also reported that *Acinetobacter junii* did not grow on pH 3.0. Degradation percentage was higher at pH 7.0 followed by lower percent degradation at pH 8.0. Further decrease in degradation efficiency was observed at pH 5, 6, and 9.

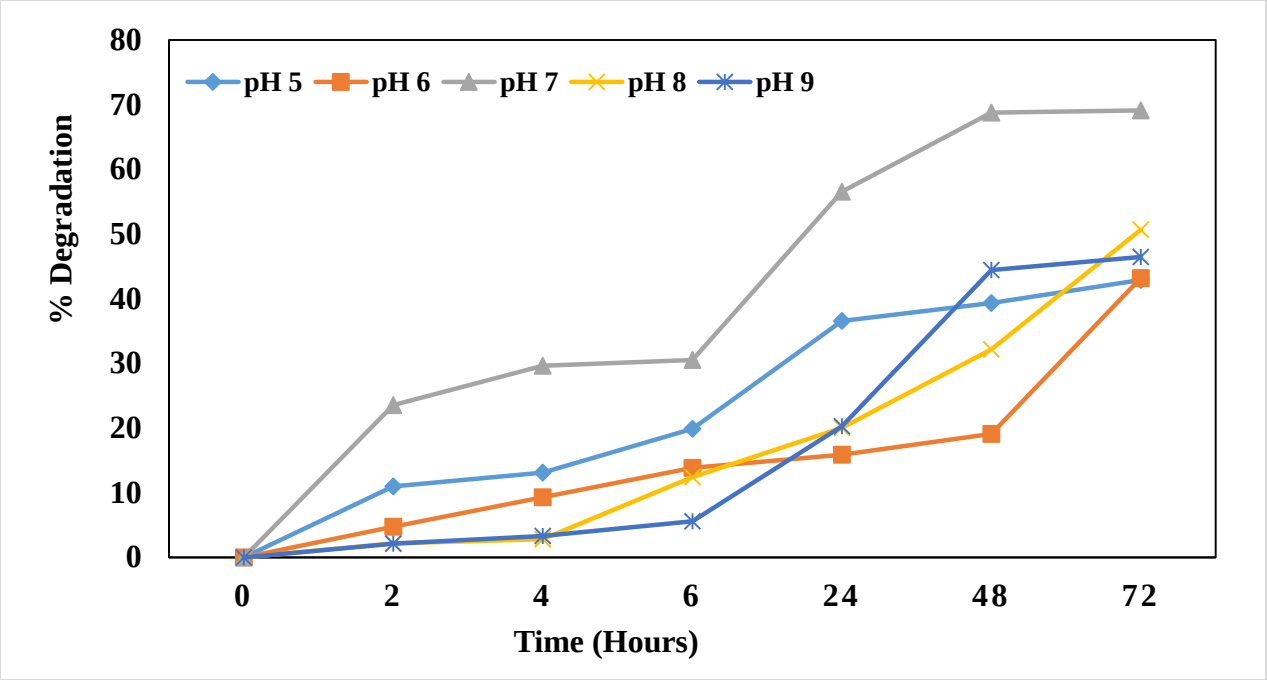



**Figure 4.21 Percentage benzene degradation by *Acinetobacter junii***

## 4.6 Salinity studies

### 4.6.1 Effect on salinity on benzene degradation using *Pseudomonas aeruginosa*

Fig 4.22 shows the percentage degradation using *Pseudomonas aeruginosa* by change in salinity levels. It is evident from the figure that benzene removal efficiency continues to decrease with increase in salinity. Degradation was recorded 47, 32, 35 and 32% at 0.5, 1.5, 3, and 5% salinity. Maximum degradation was found to be 47% at 0.5% salt concentration. Extreme salinity could act as natural barrier for hydrocarbon metabolism (Ward and Brock, 1978)

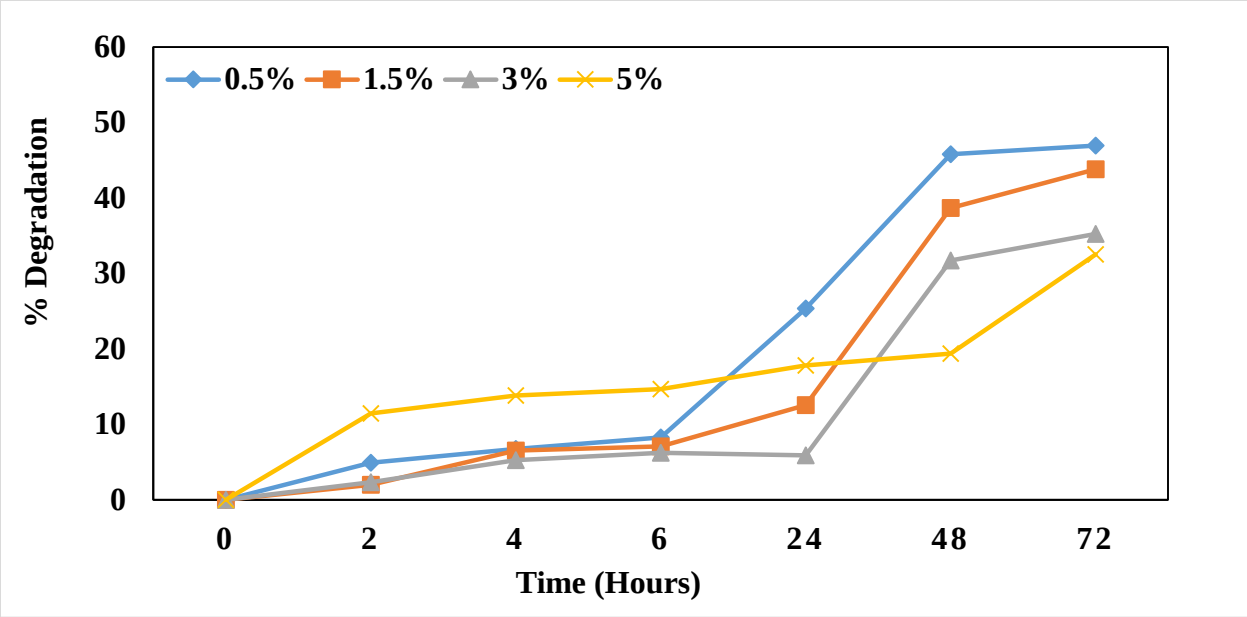



**Figure 4.22 Percentage degradation by change in Salinity using *Pseudomonas aeruginosa***

### 4.6.2 Effect on salinity on benzene degradation using *Bacillus cereus*

Fig 4.23 defines the percentage degradation using *Bacillus cereus* by change in salinity levels. It has been clearly observed in the graph that benzene removal efficiency decreases with increase in salinity. Degradation was recorded at 58, 55, 50 and 41% at 0.5, 1.5, 3, and 5% salinity. Minai-Tehrani et al. (2009) reported a sharp decrease in removal efficiency of polyaromatic hydrocarbons when salt concentration was raised from 1.0 to 5.0 %.

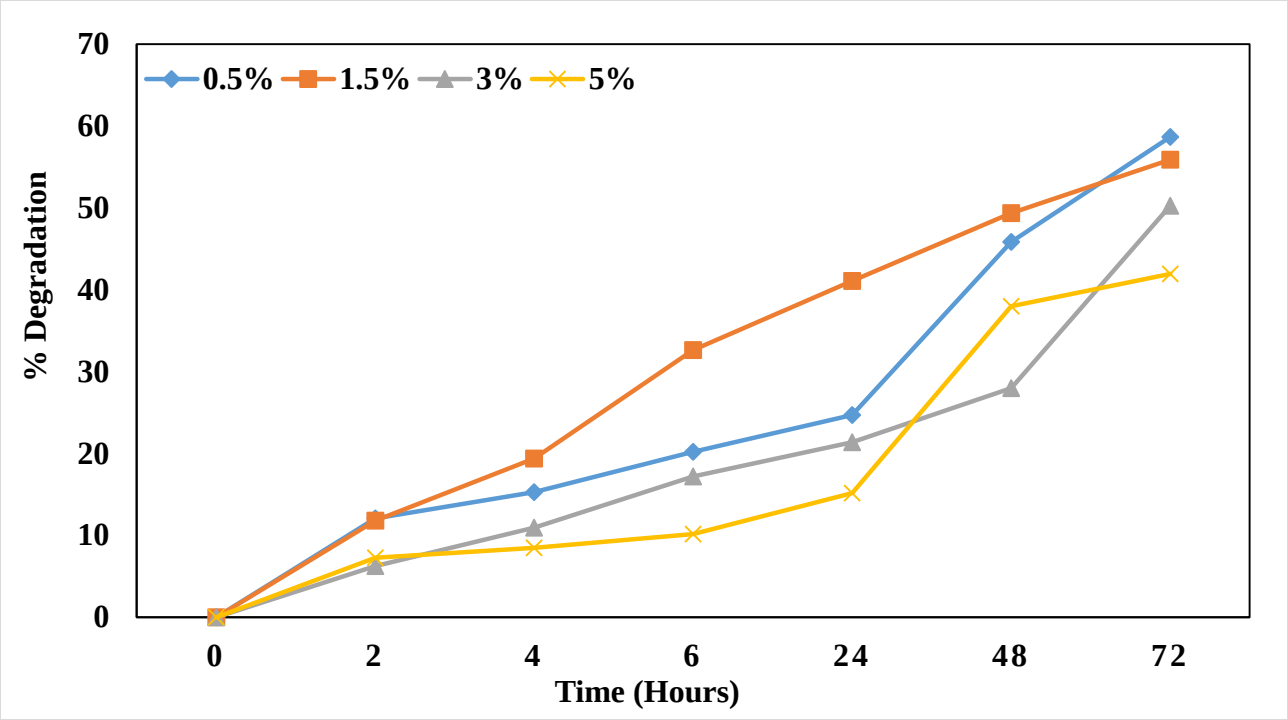



**Figure 4.23 Percentage degradation by change in Salinity using *Bacillus cereus***

### 4.6.3 Effect on salinity on benzene degradation using *Acinetobacter junii*

Fig 4.23 depicts the percentage degradation using *Acinetobacter junii* by change in salinity levels. It has been clearly observed in the graph that benzene removal efficiency decreases with increase in salinity. Degradation was recorded 59, 57, 65 and 50% at 0.5, 1.5, 3, and 5% salinity. Lozupone and Knight (2007) reported that salinity significantly affects the microbial distribution. Degradation increases with increase in salinity but after 3.0%, it started decreasing. Highly salinity might negatively affect the bacterial growth which may have effect on degradation efficiency.

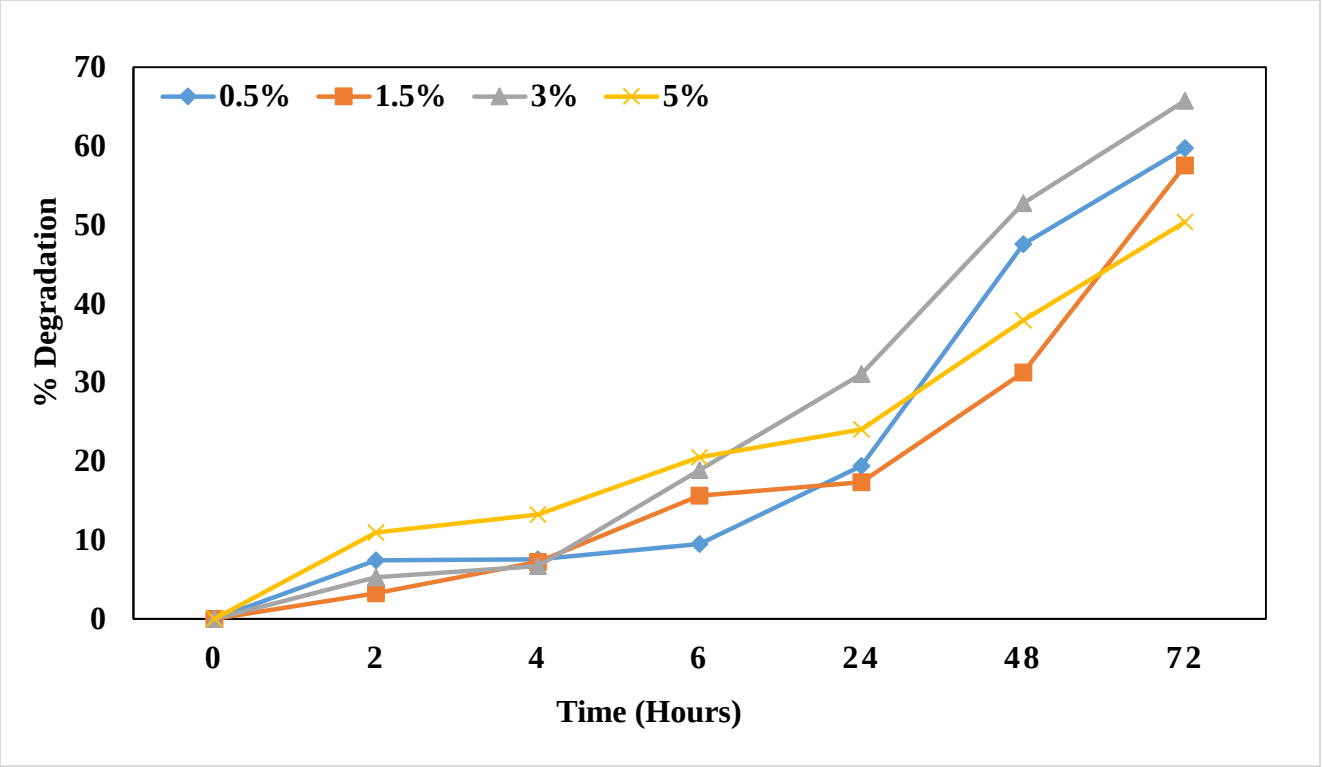



**Figure 4.24 Percentage degradation by change in Salinity using *Acinetobacter junii***

Biodegradation of organic compounds proved to be more efficient when microorganisms in inoculum are preselected and thus becomes potentially more adapted to target pollutants. Based on findings of this research, some of the important factors that must be considered for biodegradation process include time, pH, oxygen, temperature, and amount of nutrients. Consortium of above three selected strains could grow and degrade benzene efficiently.

# Chapter 5

# Conclusions and Recommendations

## 5.1 Conclusions:

i. Bacterial growth in terms of biomass production exhibited decline for *Pseudomonas aeruginosa* and *Acinetobacter junii* after two days where as decay in biomass of *Bacillus cereus* was noticed after 3$^{rd}$ day, showing the prolonged growth. Similarly, result of CFU/mL depicted higher values at 500 mg/L for *Bacillus cereus* as compared to other two strains.

ii. *Pseudomonas aeruginosa, Bacillus cereus and Acinetobacter junii* showed the significant degradation at 500 mg/L and degraded 77, 81 and 74 % benzene.

iii. Maximum specific growth rate ($\mu_{max}$) was found to be above 0.177 hr$^{-1}$ for *Pseudomonas aeruginosa* which indicated the contaminant degradation in less time.

iv. *Pseudomonas aeruginosa* and *Bacillus cereus* showed 47 and 58 % degradation with 0.5% increase in salinity whereas *Acinetobacter junii* showed 65% degradation at with 3% increase in salinity

v. All three bacterial strains showed better degradation efficiency at pH 7.0.

vi. Considering overall performance of the three species, *Acinetobacter junii* appeared to tolerate harsh conditions and showed better degradation efficiencies under stress particularly the salinity.

## 5.2 Recommendations:

This work is the continuity of the previous work and has helped to understand the degradation of benzene in a better way. During the execution of this study, many points were highlighted that can be considered for future work. However, some of the most important recommendations are as under:

i. Expression of various genes results in the production of enzymes responsible for degradation of contaminants. Identification of genes involved in the process can help to ease out the manipulation of biological system in order to improve the efficiency.

ii. Although individual performances are very encouraging for all the three strains. But in field conditions, there is always competition among different organisms for food and energy. Consortium studies can help to understand accumulative performances of the three selected strains and most probably would improve the efficiency of degradation.

## REFERENCES


Abuhamed, T., Bayraktar, E., Mehmetoğlu, T., Mehmetoğlu, Ü. 2004. Kinetics model for growth of *Pseudomonas putida* F1 during benzene, toluene and phenol biodegradation. *Process Biochemistry*, **39**(8), 983-988.

Al-Wasify, R.S., Hamed, S.R. 2014. Bacterial biodegradation of crude oil using local isolates. *International Journal of Bacteriology*, 940-943.

Bird, M.G., Greim, H., Snyder, R., Rice, J.M. 2005. International symposium: Recent advances in benzene toxicity. *Chemico Biological Interactions*, **153-154**, 1-5.

Blum, P., Hunkeler, D., Weede, M., Beyer, C., Grathwohl, P., Morasch, B. 2009. Quantification of biodegradation for o-xylene and naphthalene using first order decay models, Michaelis–Menten kinetics and stable carbon isotopes. *Journal of Contaminant Hydrology*, **105**(3), 118-130.

Borah, D., Yadav, R. 2014. Biodegradation of diesel, crude oil, kerosene and used engine oil by a newly isolated *Bacillus cereus* strain DRDU1 from an automobile engine in liquid culture. *Arabian Journal for Science and Engineering*, **39**(7), 5337-5345.

Bordoloi, N.K., Konwar, B.K. 2008. Microbial surfactant-enhanced mineral oil recovery under laboratory conditions. *Colloids and Surfaces B:Biointerfaces*, **63**(1), 73-82.

Corbitt, R.A. 1990. Standard handbook of environmental engineering.

Daifullah, A.A.M., Girgis, B.S. 2003. Impact of surface characteristics of activated carbon on adsorption of BTEX. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, **214**(1-3), 181-193.

Dalgaard, P., Koutsoumanis, K. 2001. Comparison of maximum specific growth rates and lag times estimated from absorbance and viable count data by different mathematical models. *Journal of Microbiological Methods*, **43**(3), 183-196.

Dou, J., Liu, X., Hu, Z. 2008. Anaerobic BTEX degradation in soil bioaugmented with mixed consortia under nitrate reducing conditions. *Journal of Environmental Sciences*, **20**(5), 585-592.

Faith, J.J., Hayete, B., Thaden, J.T., Mogno, I., Wierzbowski, J., Cottarel, G., Kasif, S., Collins, J.J., Gardner, T.S. 2007. Large-scale mapping and validation of *Escherichia coli* transcriptional regulation from a compendium of expression profiles. *PLoS Biology*, **5**(1), e8. doi: 10.1371/journal.pbio.0050008

Finkel, S.E. 2006. Long-term survival during stationary phase: evolution and the GASP phenotype. *Nature Reviews Microbiology*, **4**(2), 113-120.

Fuchs, G., Boll, M., Heider, J. 2011. Microbial degradation of aromatic compounds — From one strategy to four. *Natire Reviews Microbiology*, **9**(11), 803-816.

Garrity, G.M., Bell, J.A., Lilburn, T.G. 2004. Taxonomic outline of the prokaryotes. Bergey's manual of systematic bacteriology. *Springer, New York, Berlin, Heidelberg*.

Gilbert, E., Walker, A., Keasling, J. 2003. A constructed microbial consortium for biodegradation of the organophosphorus insecticide parathion. *Applied Microbiology and Biotechnology*, **61**(1), 77-81.

Hamed, T.A., Bayraktar, E., Mehmetoğlu, T., Mehmetoğlu, Ü. 2003. Substrate interactions during the biodegradation of benzene, toluene and phenol mixtures. *Process Biochemistry*, **39**(1), 27-35.

Head, I.M., Jones, D.M., Roling, W.F.M. 2006. Marine microorganisms make a meal of oil. *Nature Reviews Microbioogy*, **4**(3), 173-182.

Ivankovic, T., Hrenovic, J., Matonickin-Kepcija, R. 2013. Resistance of bioparticles formed of phosphate-accumulating bacteria and zeolite to harsh environmental conditions. *Biofouling*, **29**(6), 641-649.

Karigar, C.S., Rao, S.S. 2011. Role of Microbial Enzymes in the Bioremediation of Pollutants: A Review. *Enzyme Research*, 1-11. doi:10.4061/2011/805187

Lozupone, C.A., Knight, R. 2007. Global patterns in bacterial diversity. *Proceedings of the National Academy of Sciences*, **104**(27), 11436-11440.

Matsen, J.B., Yang, S., Stein, L.Y., Beck, D.A.C., Kalyuzhanaya, M.G. 2013. Global molecular analyses of methane metabolism in methanotrophic alphaproteobacterium, Methylosinus trichosporium OB3b. Part I: transcriptomic study. *Frontiers in Microbiology*, **4,** 1-16.

Mazzeo, D.E.C., Levy, C.E., Marin-Morales, M.A. 2010. BTEX biodegradation by bacteria from effluents of petroleum refinery. *Science of The Total Environment*, **408**(20), 4334-4340.

Megharaj, M., Ramakrishnan, B., Venkateswarlu, K., Sethunathan, N., Naidu, R. 2011. Bioremediation approaches for organic pollutants: a critical perspective. *Environment International*, **37**(8), 1362-75.

Minai-Tehrani, D., Minoui, S., Herfatmanesh, A. 2009. Effect of salinity on biodegradation of polycyclic aromatic hydrocarbons (PAHs) of heavy crude oil in soil. *Bulletin of Environmental Contamination and Toxicology*, **82**(2), 179-184.

Mukherjee, A.K., Bordoloi, N.K. 2012. Biodegradation of benzene, toluene, and xylene (BTX) in liquid culture and in soil by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains and a

formulated bacterial consortium. *Environmental Science and Pollution Research International*, **19**(8), 3380-8.

Pan, J.H., Liu, R.x., Tang, H.X. 2007. Surface reaction of Bacillus cereus biomass and its biosorption for lead and copper ions. *Journal of Environmental Sciences*, **19**(4), 403-408.

Perelo, L.W. 2010. Review: In situ and bioremediation of organic pollutants in aquatic sediments. *Journal of Hazardous Materials*, **177**(1-3), 81-9.

Reeve, C., Amy, P., Matin, A. 1984. Role of protein synthesis in the survival of carbon-starved Escherichia coli K-12. *Journal of Bacteriology*, **160**(3), 1041-1046.

Santa Anna, L., Sebastian, G., Menezes, E., Alves, T., Santos, A., Pereira Jr, N., Freire, D. 2002. Production of biosurfactants from *Pseudomonas aeruginosa* PA 1 isolated in oil environments. *Brazilian Journal of Chemical Engineering*, **19**(2), 159-166.

Sarkar, P., Rai, A., Ghosh, S. 2013. Degradation of aromatic petroleum hydrocarbons (BTEX) by a Solvent Tolerant Bacterial Consortium. *Journal of Urban and Environmental Engineering*, **7**(2), 274-279.

Singh, B., Kaur, J., Singh, K. 2013. Bioremediation of malathion in soil by mixed Bacillus culture.

Snyder, R. 2007. Benzene's toxicity: a consolidated short review of human and animal studies by HA Khan. *Human & Experimental Toxicology*, **26**(9), 687-696.

Tao, Y., Fishman, A., Bentley, W.E., Wood, T.K. 2004. Oxidation of benzene to phenol, catechol, and 1,2,3-trihydroxybenzene by toluene 4-monooxygenase of *Pseudomonas mendocina* KR1 and toluene 3-monooxygenase of *Ralstonia pickettii* PKO1. *Applied Environmental Microbiology*, **70**(7), 3814-20.

Ting, Y., Hu, H., Tan, H. 1999. Bioremediation of petroleum hydrocarbons in soil microcosms.

Vermani, M., Kelkar, S., Kamat, M. 1997. Studies in polysaccharide production and growth of *Azotobactervinelandii* MTCC 2459, a plant rhizosphere isolate. *Letters in Applied Microbiology*, **24**(5), 379-383.

Ward, D.M., Brock, T. 1978. Hydrocarbon biodegradation in hypersaline environments. *Applied and Environmental Microbiology*, **35**(2), 353-359.

You, Y., Shim, J., Cho, C.H., Ryu, M.H., Shea, P.J., Kamala-Kannan, S., Chae, J.C., Oh, B.T. 2013. Biodegradation of BTEX mixture by *Pseudomonas putida* YNS1 isolated from oil-contaminated soil. *Journal of Basic Microbiology*, **53**(5), 469-75.

Young, K.D. 2006. The selective value of bacterial shape. *Microbiology and Molecular Biology Reviews*, **70**(3), 660-703.

Yuan, S., Chang, S., Chang, B. 2003. Biodegradation of polycyclic aromatic hydrocarbons in sludge. *Bulletin of Environmental Contamination and Toxicology*, **71**(3), 0625-0632.

Zahid, M.S.B., Iqbal, A., Arshad, M. 2015. Benzene degradation with bacterial strains isolated from rhizosphere of *Cannabis sativa* being irrigated with petroleum refinery wastewater. *Desalination and Water Treatment*, 1-6. doi: 10.1080/19443994.2015.1086896

Zulfiqar Ali, M. 2012. Degradation of petroleum hydrocarbons by oil field isolated bacterial consortium. *African Journal of Biotechnology*, **11**(3), 650-658.

Peter, J. K., Masih, H., Kumar, Y., Singh, A. K., & Chaturvedi, S. 2014. Organophosphate pesticide (methyl parathion) degrading bacteria isolated from rhizospheric soil of selected plants and optimization of growth conditions for degradation. *International Journal of Research*, **1**(5), 1-13.

Vilain, S., Luo, Y., Hildreth, M., and Brozel, V. 2006. “Analysis of the Life Cycle of the Soil Saprophyte *Bacillus cereus* in Liquid Soil Extract and in Soil.” *Applied Environmental Microbiology.* **72**(7), 4970–4977.

Villers, D., E. Espaze, M. Coste-Burel, F. Giauffret, E. Ninin, F. Nicolas, and H. Richet. 1998**.** Nosocomial *Acinetobacter baumannii* infections: *microbiological and clinical epidemiolog*y. *Ann. Intern. Med.* 129

Bouvet, P. J., and P. A. Grimont. 1986. Taxonomy of the genus *Acinetobacter* with the recognition of*Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *Intertnational. Journal of Systems Bacteriology*. **36:**228-240

Oya S, Valocchi AJ. 1998. Transport and biodegradation of solutes in stratified aquifers under enhanced in situ bioremediaion conditions. *Water Resources Research.* **34:**3323–3334.

“EPA Regulatory Announcement”. 2012. EPA **420**-F-07-017.

Monod, J. 1949. The growth of bacterial cultures. *Annual Reviews in Microbiology*, **3**(1), 371-394.

# Annexure-I

## Experimental growth data for *Pseudomonas aeruginosa*

| Concentration (mg/L) | Time (hours) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 24 | 48 | 72 |
| 250 | 5.5E+08 | 9.5E+08 | 1.25E+09 | 1.6E+09 | 1.5E+09 | 2.25E+08 | 9.5E+07 |
| 500 | 5.O+07 | 5.3+07 | 6.3+07 | 9.O+07 | 1.11E+08 | 1.1E+08 | 9.5E+07 |
| 750 | 8.2+06 | 9.5+06 | 1.03E+08 | 7.5+07 | 5.O+07 | 3.O+07 | 1.8E+07 |
| 1000 | 4.5E+08 | 6E+08 | 7.5E+08 | 9.5E+08 | 1.05E+09 | 7.5E+08 | 4.9E+07 |

## Experimental growth data for *Bacillus cereus*

| Concentration (mg/L) | Time (hours) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 24 | 48 | 72 |
| 250 | 3E+08 | 4E+08 | 4E+08 | 5E+08 | 8E+08 | 7E+08 | 4E+08 |
| 500 | 4E+08 | 5E+08 | 5E+08 | 6E+08 | 7E+08 | 8E+08 | 5E+08 |
| 750 | 2E+08 | 2E+08 | 3E+08 | 3E+08 | 3E+08 | 2E+08 | 1E+08 |
| 1000 | 6E+07 | 1E+08 | 1E+08 | 2E+08 | 2E+08 | 2E+08 | 1E+08 |

## Experimental growth data for *Acinetobacter junii*

| Concentration (mg/L) | Time (hours) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 24 | 48 | 72 |
| 250 | 1.11E+08 | 1.25E+08 | 2.78E+08 | 4.5E+08 | 5.5E+08 | 5.5E+08 | 4.5E+08 |
| 500 | 1.17E+08 | 1.44E+08 | 1.5E+08 | 1.75E+08 | 2.5E+08 | 3.25E+08 | 2.3E+08 |
| 750 | 4.9+06 | 5.7+06 | 7.4+06 | 8.7+06 | 7.4+07 | 6.4+07 | 3.9E+07 |
| 1000 | 6.0+09 | 7.4+09 | 1.75E+08 | 2.72E+08 | 4.38E+08 | 3.8E+08 | 2.2E+07 |

# Annexure-II

## Determination of Kinetic Parameters

Specific growth rate was determined by the following equation:

$$\mu = slope = \frac{\ln(Xt - Xo)}{t2 - t1}$$

Where,

$X_o$= Growth at 0 hour/ Initial bacterial concentration

$X_t$= Bacterial growth at t hours

$T_2$= Time where growth is maximum (usually at the end of exponential phase)

$T_1$= Time where growth starts ((usually at the end of exponential phase)

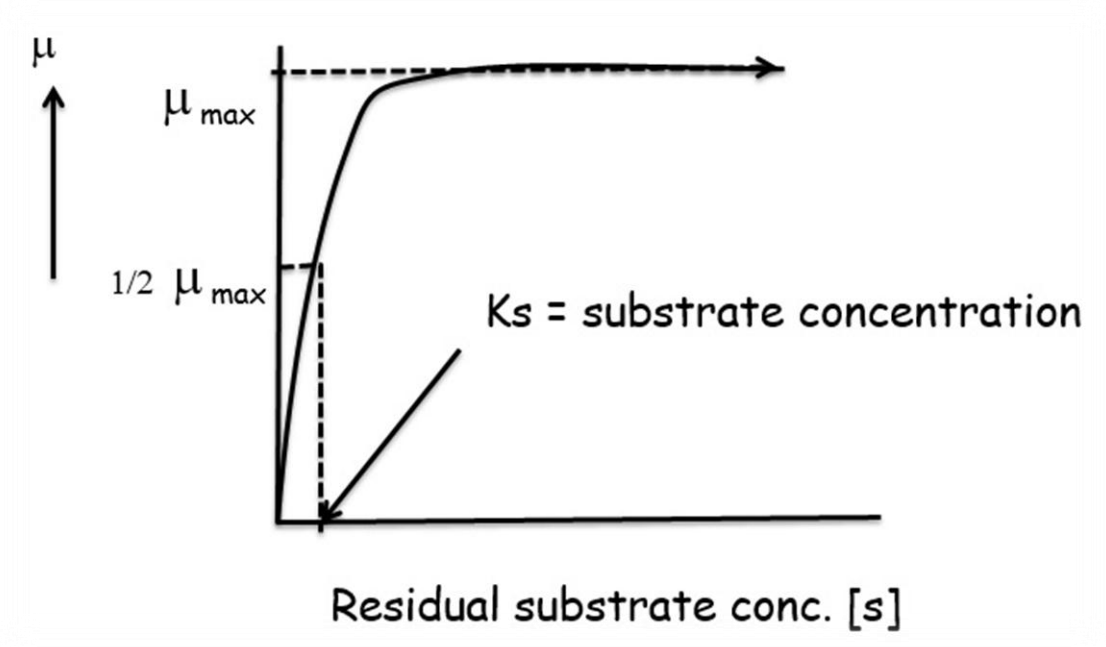
## Batch Culture: Growth Kinetics:

i. During log phase, bacterial growth reaches maximum level
ii. After depletion of substrate (available as carbon source), growth rate starts decreasing and finally ceases

$$\mu = \mu_{max}\, S / K_s + S$$

## Annexure-III

### Graphical Representation to Calculate Kinetic Parameters




## Calculation Procedure:

From the figure of growth curves of all bacterial strains, specific growth rate (μ) has been calculated in the exponential phase at four different benzene concentrations i.e. 250,500,750 and 1000 mg/L.

$$\mu = slope = \frac{\ln(Xt - Xo)}{t2 - t1}$$

## Annexure- IV

### Calculation for *Pseudomonas aeruginosa:*

| Conc.(mg/L) | $X_t$ | $X_o$ | $T_2$ | $T_1$ | μ |
|---|---|---|---|---|---|
| 250 | 5.5+08 | 1.6E+09 | 6 | 0 | 0.177 |
| 500 | 5E+07 | 9E+07 | | | 0.132 |
| 750 | 8.2E+07 | 7.5E+07 | | | 0.055 |
| 1000 | 4.5E+08 | 9.5E+08 | | | 0.141 |

### Calculation for *Bacillus cereus:*

| Conc.(mg/L) | $X_t$ | $X_o$ | $T_2$ | $T_1$ | μ |
|---|---|---|---|---|---|
| 250 | 7.5E+08 | 4E+08 | 24 | 2 | 0.034 |
| 500 | 8E+08 | 5E+08 | 48 | 2 | 0.0105 |
| 750 | 2.8E+08 | 2E+08 | 6 | 2 | 0.135 |
| 1000 | 2.2E+08 | 1E+08 | 24 | 2 | 0.031 |

### Calculation for *Acinetobacter junii:*

| Conc.(mg/L) | $X_t$ | $X_o$ | $T_2$ | $T_1$ | μ |
|---|---|---|---|---|---|
| 250 | 5.5E+08 | 1E+08 | 24 | 2 | 0.06 |
| 500 | 3E+08 | 2E+08 | 48 | 6 | 0.01 |
| 750 | 9E+07 | 6E+07 | 6 | 2 | 0.105 |
| 1000 | 4.4E+08 | 7E+07 | 24 | 2 | 0.0808 |