# *Bacterial consortium and effect of different conditions on cell growth using Naphthalene as a sole carbon source by the bacteria isolated from soil samples collected from oil refineries in Texas*

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**By**

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***Bacterial consortium and effect of different conditions on cell growth using Naphthalene as a sole carbon source by the bacteria isolated from soil samples collected from oil refineries in Texas***

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At whatever position, I am today here because of my husband Nimish Jagtap and family who supported and encouraged me to pursue higher education.

I dedicate this work to my family and to all my near and dear ones.

# *ABSTRACT*

Petrochemical refineries and human activities are responsible for release of carcinogenic compounds known as Polycyclic Aromatic Hydrocarbons (PAHs). Adverse effects of these compounds on human health and ecosystem resulted in an increased awareness, and lead to dramatic increase in research intended to remove PAHs from the ecosystem. In the present study naphthalene was used as a model compound. It was hypothesized that the microorganisms isolated from naphthalene contaminated soil collected from petrochemical refineries in Texas could be utilizing naphthalene as a carbon source. The naphthalene degradation capability of microorganisms could be an indication of their ability to degrade PAHs. In this study the isolated strains were identified as *Enterobacter cloacae* and *Cellulosimicrobium cellulans* based on results from 16S rDNA gene sequencing analysis. Bacterial consortium and effect of different conditions on cell growth using naphthalene as a sole carbon source were studied.

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# List of Abbreviations

| | |
|---|---|
| PAHs | Polycyclic aromatic hydrocarbons |
| BP | Bacillus pumilus |
| EC | Enterobacter cloacae |
| CC | Cellulosimicrobium cellulans |
| AB | Achromobacter xylosoxidans |
| CSM | Carbon supplement medium |
| MSM | Minimal salt medium |
| GSM | Glycerol supplement medium |
| BH medium | Bushnel Haas medium |
| bph | Biphenyl dioxygenase |
| dfa | Dibenzofuran dioxygenase |
| PCR | Polymerase chain reaction |
| rpm | Revolution per minute |
| g | Gravitational acceleration |
| GC-FID | Gas chromatography-flame ionization detector |
| GC-MS | Gas chromatography-Mass spectroscopy |
| HPLC | High performance chromatography |

# 1 Introduction

## 1.1 Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) have received increase in consideration because of their carcinogenic, toxic and genotoxic properties. PAHs have tendency of bioaccumulation in food, and they can be found in air, water, soil and sediment; hence are considered hazardous to human health. PAHs are formed by incomplete combustion process of organic matter such as wood, coal and oil. Natural activities such as volcanoes and forest fire also lead to the production of the PAH contamination in environment. In general, the anthropogenic activities are more responsible for production of PAH (Ghosal D, 2016). PAHs are colorless, white or pale yellow green color fused aromatic compounds. The carcinogenic index of PAH compounds increases with an increasing number of rings. Many PAHs comprise of 'bay-region' and 'K-region' and both allow formation of metabolic bay- and K-region epoxides, which are highly reactive. These highly reactive epoxides also exhibit Carcinogenicity. Therefore, many PAHs are environmental pollutants that can cause serious health problems and genetic defects in humans (Goldman R, 2001). For example, it is well known that tobacco releases PAHs, and a physician John Hill documented high incidence of nasal cancer in tobacco snuff consumers in 1761 (Ghosal D, 2016).

The important mechanism for removal of PAHs from the environment is their biological transformation by bacteria and fungi. In the last few decades, many microbial strains and fungi have been isolated and studied for PAH biodegradation. The known bacterial strains that efficiently degrade PAHs are *Pseudomonas putida*, *P. fluorescens, P. paucimobilis, P. cepacia, Corynebacterium venale, Streptomyces sp., Rhodococcus sp.*, *Mycobacterium sp*., *Oscillatoria sp.*, and *Alcaligenes sp.* Extensive research has been carried out on fungi having naphthalene degradation capability, e.g., *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pleurotus ostreatus, Cunninghamella elegans*, *Penicillium*, and *T. harzianum.* In the microbial degradation of PAH, the dioxygenase incorporates atom of oxygen at two carbon atoms of a benzene ring and forms cis-dihydrodiol. Then dehydrogenases cause the rearomatization of cis-dihydrodiol to form

dihydroxylated intermediates, which undergo in ring cleavage and form TCA cycle intermediate (Sudip K. Samanta, 2002) (Zafar, 2010).

Naphthalene is the simplest PAH and is volatile in nature. It contains two fused benzene rings. Naphthalene is found in coal tar, petroleum and used in various commercial applications like plastics and mothballs. Naphthalene is naturally produced by fungi as an insect repellent. Naphthalene inhibits the mitochondrial respiration. Acute naphthalene poisoning in humans can lead to hemolytic anemia and nephrotoxicity. Toxicity of naphthalene augments with covalent bond formation with the molecules of tissue in liver, kidney and lung (Sudip K. Samanta, 2002).

Biodegradation is the natural way of degrading or breaking down the organic matter into the nutrients or in the simple form that can be used by the bacteria, fungi and algae. Biodegradation is a microbial process which occurs when all the nutrients and physical conditions are favorable for growth of micro-organisms. These physical conditions include the pH, temperature, moisture content, and chemical properties of chemicals. The nutrients such as carbon, nitrogen, phosphorus, magnesium, sulfur, calcium, metals aid the microorganism growth shown in Fig. 1. Optimum growth conditions ultimately help in degradation of many harmful chemicals into less harmful byproducts.

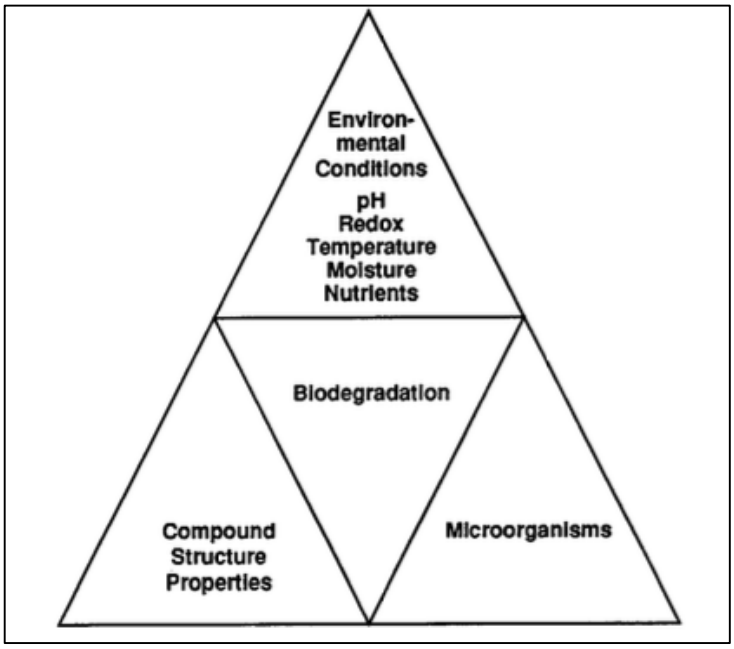



**Fig. 1: Biodegradation triangle (Suthersan, 1999)**

The present study was intended to isolate and identify the Naphthalene degrading bacteria from the PAH contaminated sites in Texas. An additional objective was to study the effect of different environmental conditions on isolated bacterial cell growth when the media is supplemented with Naphthalene as a sole carbon source. This study will provide the optimum condition for cell growth of isolated bacteria and ultimately degradation of Naphthalene.

## *1.2 Objectives*

The objectives of the present study were:

1) Isolation and identification of different strains of bacteria from soil contaminated with naphthalene.
2) Studying the effect of different conditions listed below on cell growth of bacteria:
    - Effect of pH level: 6, 7 and 8
    - Effect of naphthalene concentration: 50, 100, 200 and 400 ppm
    - Effect of solvents used to dissolve naphthalene: Acetone, Ethyl acetate, and Acetonitrile
    - Effect of media: CSM and Bushnell Hass medium
3) Comparison of bacterial consortium growth using naphthalene as an energy source.

# 2 Literature Review

Polycyclic Aromatic Hydrocarbons (PAHs) are composed of multiple aromatic rings which makes them highly hydrophobic in nature and defiant to environmental degradation. They have serious deleterious effects on humans as well as animals (Mastrangelo G, 1996 Nov). PAHs are ubiquitous micro pollutant that are believed to contribute to the development of lung and bladder cancer in humans. Enzymes such as cytochrome P450 are involved in metabolism of PAHs, which forms the reactive metabolites (AA Walsh, 2013). The diol-epoxides formed during metabolic activation of PAHs bind covalently to the DNA resulting in a complex (Adeline Tarantini, 2011). This complex is responsible for the carcinogenic effects (Thamaraiselvan Rengarajan, 2015).

## *2.1 Different PAHs in environment:*

PAHs are made up of two or more benzene ring attached to each other in linear, regular and angular form, which is shown in Fig. 2 (Hussein I. Abdel-Shafy, 2016).

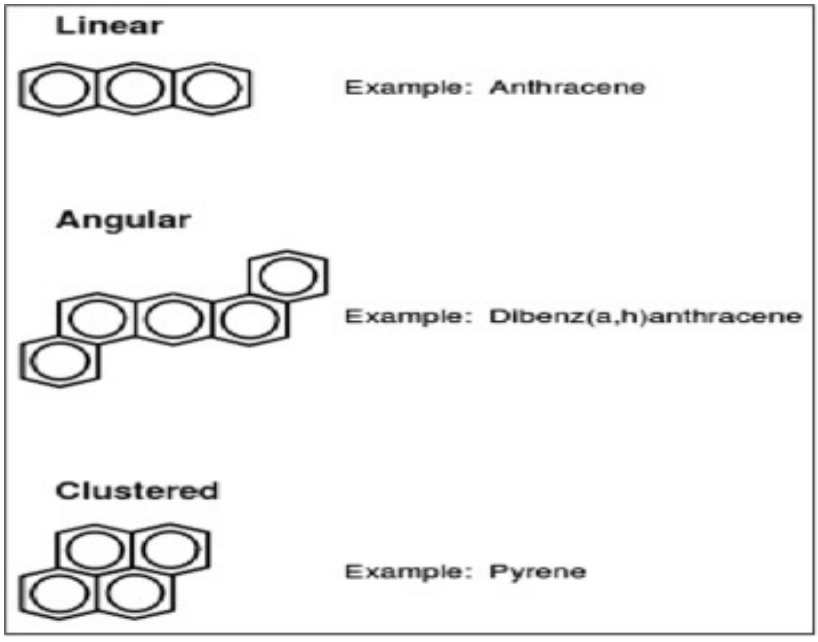



**Fig. 2: Molecular arrangement of the polycyclic aromatic hydrocarbons** (J. Arey, 2003)**,** (D.M. Di Toro, 2000)

PAHs can be classified as small PAHs and large PAHs. Small PAHs contains up to 6 aromatic fused rings and large PAHs contains more than 6 aromatic fused rings. Naphthalene is aromatic hydrocarbon comprising of two 6 member rings which shares the edges. As per the International Agency for Research on Cancer (International Agency for Research on Cancer, 2010), Phenanthrene and Anthracene are the smallest carcinogenic PAH molecules. Small PAH molecules are easily available and hence preponderance of the research has been carried out on these molecules. The most commonly analyzed PAHs compounds are shown in Fig. 3 (Chen Lin, 2010).

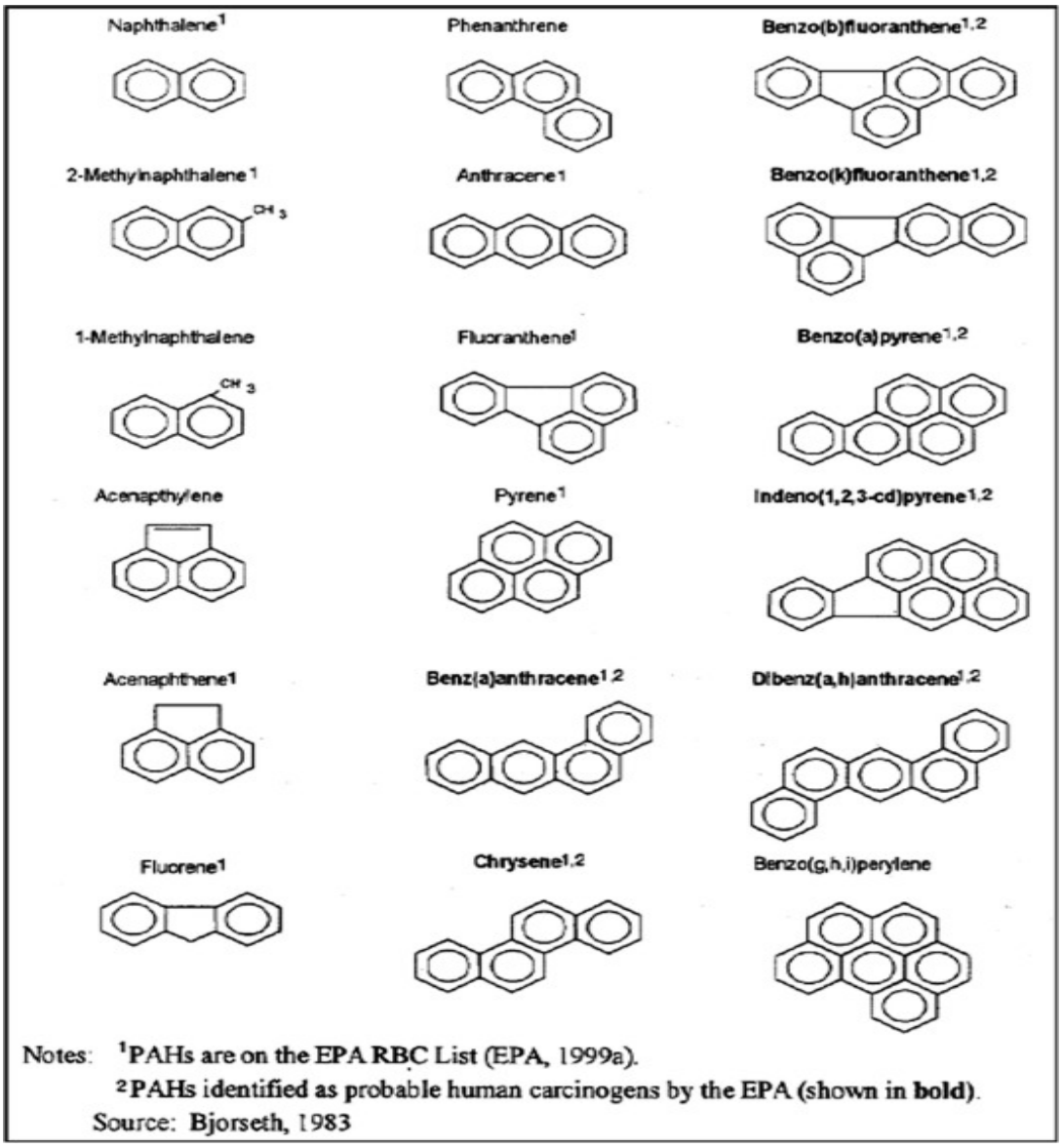



**Fig. 3: Commonly analyzed PAH's compounds** (Alf, 1983)

## *2.2 Sources of contamination of soil with PAHs:*

PAHs are released by the natural sources as well as anthropogenic activities. PAH emission from stationary sources is about 80%, which arises from domestic sources, industrial power generation, and production of coal tar, coke and asphalt (John A. Simon, 2006) (U.S. Department of Health and Human Services., 1995). The PAH sources are summarized in Fig. 4, and are explained briefly below.

### *i. Domestic sources:*

In domestic sources, heating and cooking are principal sources of PAHs. Burning and pyrolysis of gas, garbage, oil, coal, organic substances and wood are the major PAH contributors. Cigarette smoke is also a prime contributor of PAH emission in indoor environment (Byeong-Kyu Lee, 2010) (K Ravindra, 2006).

### *ii. Industrial sources:*

Industrial release of PAH is caused by burning of fuels like gas, oil, and coal. PAHs can also be released during the processing of raw materials such as aluminum and coke, manufacturing/processing of petrochemicals, rubber, cement, bitumen/asphalt, wood, and the process of waste incineration (Byeong-Kyu Lee, 2010).

PAHs are also used as an intermediate compound in Pharmaceutical, agricultural, photographic products, thermosetting plastic, lubricating material and in chemical industries. The general applications of some PAHs are (N.E. Kaminski, 2008):

- Acenaphthene: manufacture of pigments, dyes, plastics, pesticides and pharmaceuticals.
- Anthracene: diluent for wood preservatives and manufacture of dyes and pigments.
- Fluoranthene: manufacture of agrochemicals, dyes and pharmaceuticals.
- Fluorene: manufacture of pharmaceuticals, pigments, dyes, pesticides and thermoset plastics.
- Phenanthrene: manufacture of resins and pesticides.
- Pyrene: manufacture of pigments

***iii. Mobile sources:***

Emission from the mobile sources such as automobiles, railways, ships, aircrafts contains PAHs that are formed by three means: 1) synthesis from smaller molecules and aromatic compounds in fuel; 2) storage in engine deposits and in fuel; and 3) pyrolysis of lubricants (Byeong-Kyu Lee, 2010) (Baek SO, 1991).

***iv. Agricultural sources:***

Open burning of biomass such as wood, straw, moorland heather, stubble under suboptimum combustion conditions leads to the emission of the PAHs. Concentration of PAHs in these emissions depends on various factors such as wood type, combustion temperature and moisture content in the biomass (Hao Lu, 2009).

***v. Natural sources:***

Volcanic eruptions, accidental burning of forests and moorlands and the decay of organic matter leads to the emission of PAHs in environment. The amount of released PAH is affected by the temperature, humidity, fuel characteristics and wind (Byeong-Kyu Lee, 2010).

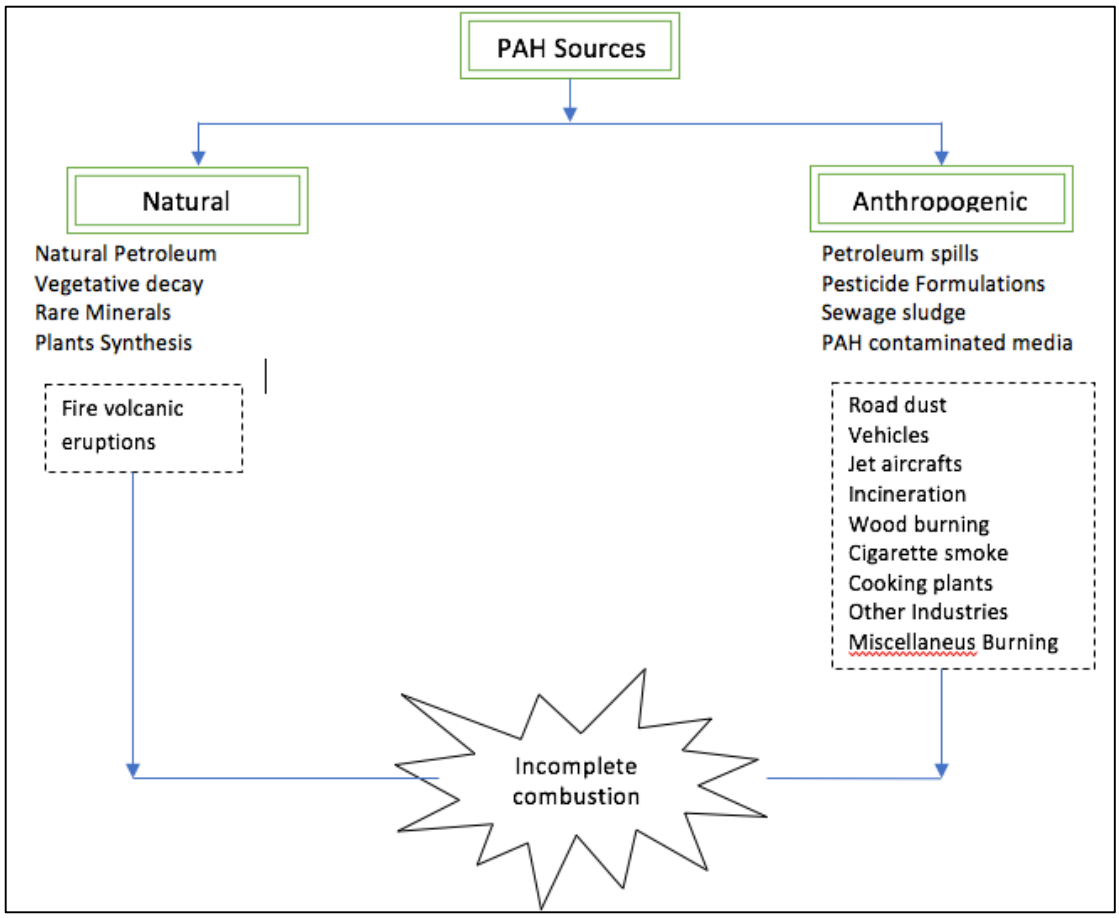



**Fig. 4: Natural and anthropogenic sources of PAHs** (Hussein I. Abdel-Shafy, 2016)**.**

## *2.3 PAH behavior in environment:*

Anthropogenic activity causes distribution of PAH in air, terrestrial and aquatic environment. PAHs are usually found in two phases: vapor and solid phase. Vapor pressure variability of different PAH compounds determines whether the PAH compound eventually remains in vapor or solid phase. The lower vapor pressure PAHs that have higher molecular weight, e.g., benzo (a) pyrene have tendency to deposit on solids; whereas the higher vapor pressure compounds with lower molecular weight such as naphthalene remain in vapor state (Hussein I. Abdel-Shafy, 2016) (Suess, 1976) (Wang, 2013).

## *2.4 Effects of PAHs:*

PAHs have toxic effects on aquatic life, birds, plants and human. PAHs are more toxic in presence of UV light. Humans absorb PAH from ingestion, inhalation, skin contact, and plant absorbs it through the roots from soil. It has been reported that some plants possess chemicals which protect them from toxic effects of PAHs, and some plants produce PAHs that act as growth hormones (Abhilash PC, 2009).

PAH compounds are of paramount concern because of their carcinogenic effects on human. The most health effects in humans are caused by ingestion and inhalation of PAH compounds. Mixed function oxidase system is responsible for the metabolism of PAHs. The first step is PAH epoxidation, which combines with glutathione. This polar complex is then removed from the body. The PAH compounds which do not form complex with glutathione get converted into diols and phenols, and form complex with glucuronic or sulfuric acid and are eliminated through urine or feces (Laura Campoa, 2010).

## *2.5 Adverse health effects on human:*

### *i. Acute and chronic effects:*

The effects of PAHs on human health depend on the length and route of contact, the concentration of PAHs and the relative toxicity of the PAHs. Preexisting disease state and age also affects impact of PAH. Short term effects are skin irritation, inflammation, allergic reaction (American Conference of Governmental Industrial Hygienists, 2005) (IPCS (International Programme On Chemical Safety), 2010). Chronic effects cause liver and kidney damage, asthma, lung function related abnormalities and skin inflammation. Large amount of ingestion or inhalation of naphthalene causes the breakdown of red blood cells (Thamaraiselvan Rengarajan, 2015) (Hussein I. Abdel-Shafy, 2016).

### *ii. Carcinogenicity:*

Many laboratory studies have revealed that when animals get exposed to high amount of PAH for long period it develops lung, stomach, skin cancer. PAH compounds that are classified as the most possible human carcinogen by the International Agency for Research on Cancer and US EPA are benz(a) anthracene, benzo(a)Pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene,

chrysene, dibenz(ah)anthracen, and indeno(1,2,3-cd) pyrene. The main concern about these PAH compounds is their reactive metabolites like epoxide and dihydrodiols. These reactive metabolites form DNA adducts responsible for mutations, tumors, developmental malformations by initiation of biochemical disruption and cell damage process (The United States Environmental Protection Agency, 1984) (International Agency For Research On Cancer, 1987). These PAH-DNA adducts can be removed by nucleotide excision repair. If the adducts remain unrepaired that may cause a permanent mutation (Konstantin Kropachev, 2013).

***iii. Immunogenicity:***

It has been observed in laboratory studies that mice exposed to PAH carcinogens suppressed their humoral immunity and cell mediated immunity. Numerous *in vitro* studies have demonstrated that PAHs can induce T and B cells apoptosis (Jun Gao, 2014).

PAHs from extraterrestrial space in presence of low temperature and UV get converted into organic molecules, which is considered to be the basis for primitive life on earth. PAHs formed by the anthropogenic activity forms epoxides and causes mutagenesis. This is shown in Fig. 5.

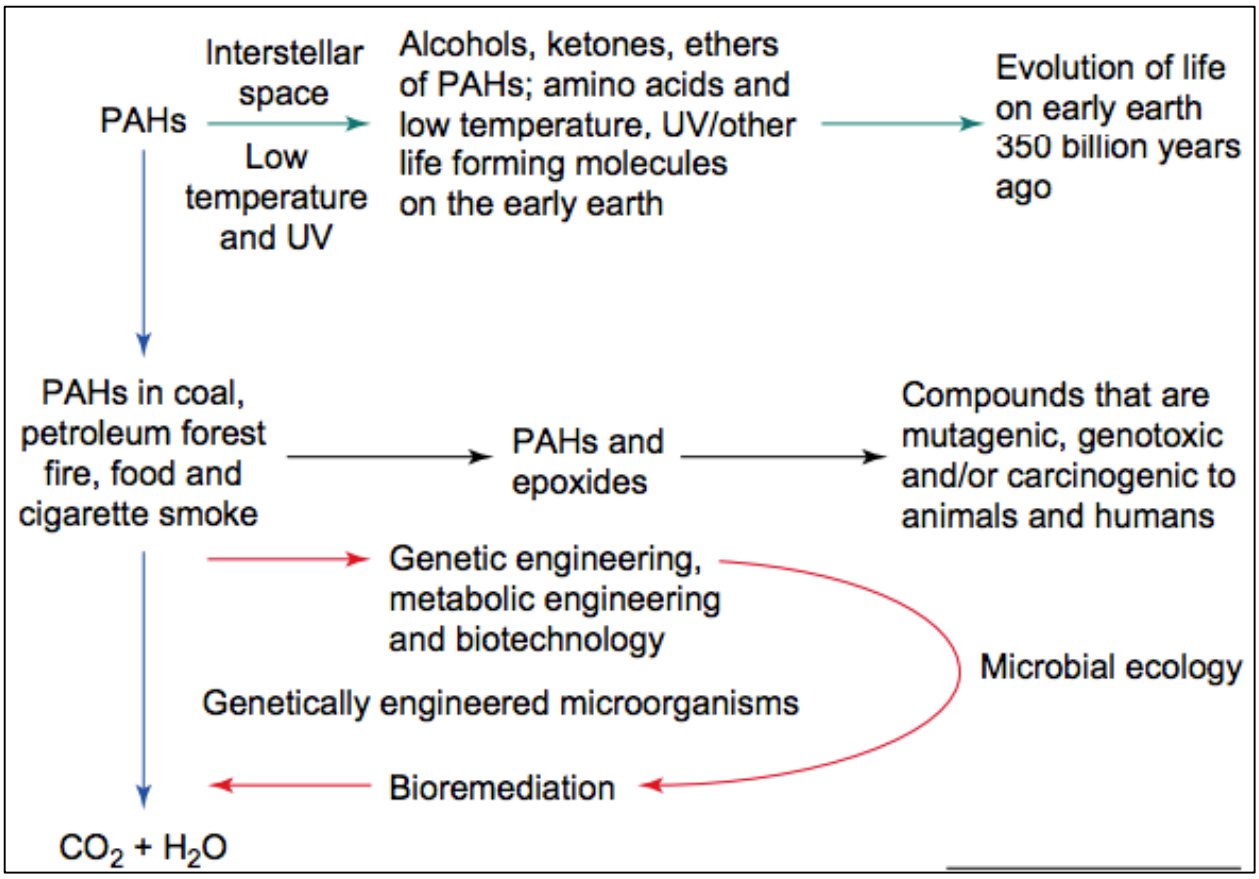



**Fig. 5: Fate, toxicity and remediation of PAHs from the environment** (Sudip K. Samanta, 2002)

### *2.6 Naphthalene:*

Naphthalene is a bicyclic aromatic hydrocarbon classified as a major pollutant. Naphthalene (Fig. 6) is the first member of PAH group with low molecular weight and high vapor pressure, which usually remains in vapor phase. Naphthalene, as a first and structurally simple member of PAH, is usually used as a model compound for PAH degradation study.

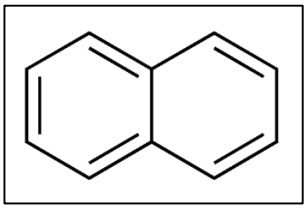


**Fig. 6: Naphthalene structure**

## *2.7 Naphthalene degrading bacteria and pathways:*

Naphthalene degrading bacteria are easy to isolate because of their simple nature and most soluble amongst PAH compounds. There are many bacteria having ability to degrade naphthalene, and they mostly are gram negative bacteria. Most of these bacteria belong to the genus *Pseudomonas, Mycobacterium, Corynebacterium, Aeromonas, Rhodococcus, and Bacillus, Alcaligenes* (A. Mrozik, 2003).

The proposed pathway for the degradation of Naphthalene by *Streptomyces griseous* is shown in Fig. 7, in which Naphthalene undergoes biocatalytic hydroxylation and aromatic ring reduction. 1- Naphthol and 1- Tetralone are the intermediate products during Naphthalene degradation.

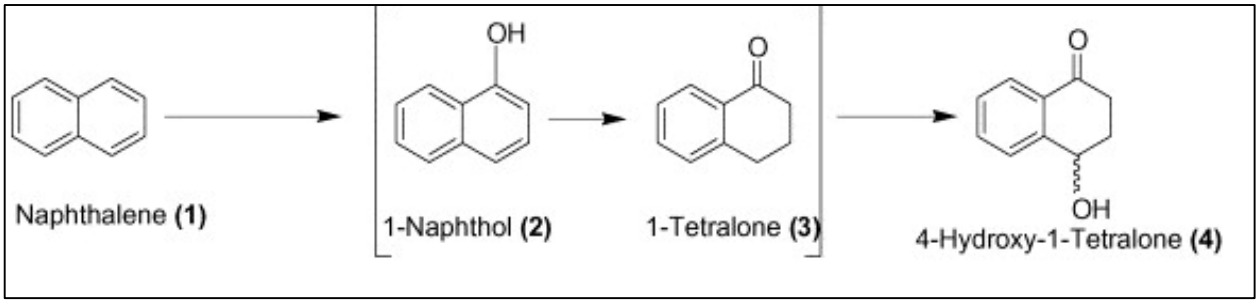



**Fig. 7: Proposed pathway for the degradation of naphthalene by *Streptomyces griseous*** (SR Gopishetty, 2007)

The proposed naphthalene degradation pathway by genus *Pseudomonas* is shown in Fig. 8. In this pathway naphthalene deoxygenases from *Pseudomonas* responsible for oxidation of naphthalene by incorporating the molecular oxygen into aromatic molecule. Upon further degradation, it results in salicylic acid. The salicylic acid is further degraded by either gentisic acid or catechol pathway.

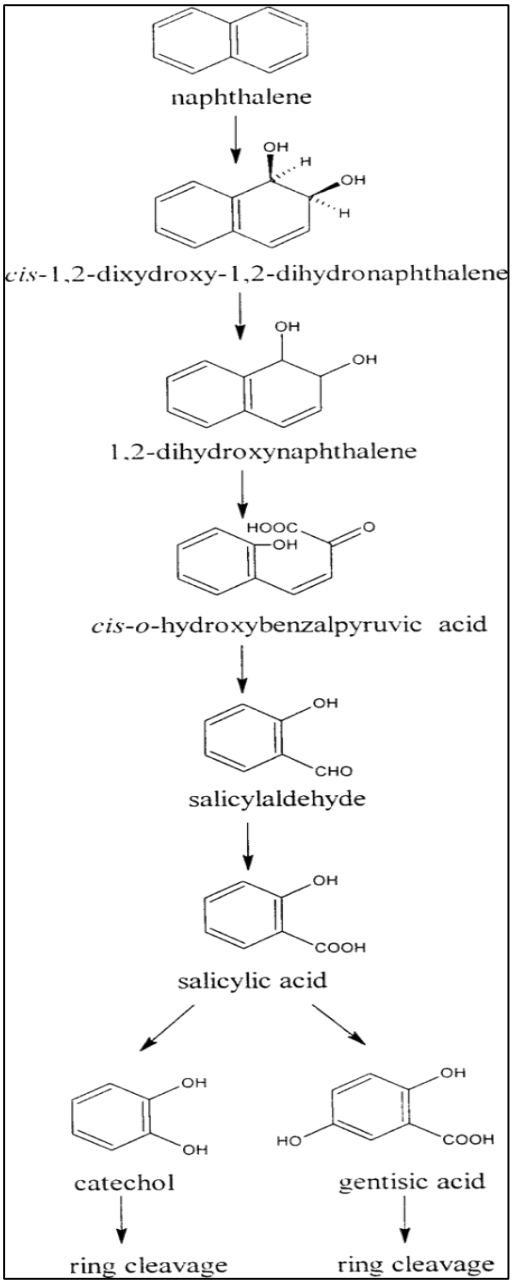



**Fig. 8: The proposed pathway naphthalene degradation by some bacteria from the genus *Pseudomonas*** (A. Mrozik, 2003)**.**

# 3 Material and methods

## 3.1 Selection of Sampling site:

The sampling sites were selected using TOXMAP: TRI and Superfund Environmental Maps (https://toxmap.nlm.nih.gov/toxmap/). TOXMAP is a geographic information system that helps to explore the data with visual projection and map about superfund sites.

Soil samples were collected from the two superfund sites which were explored using the Toxmap site. The selected locations are shown in Fig. 9 and Fig. 10, wherein the sites which release naphthalene (pounds/yr.) in environment are shown. Valero (Texas city) and Exxon Mobile (Baytown) refineries in Texas were selected for soil sample collection. These sites were selected because they release high amount of naphthalene as shown by red color in Fig. 9 and Fig. 10.

### i. *Valero Refinery located in Texas city*


**Fig. 9: Naphthalene release per year Texas city**

### *ii. Exxon Mobil Refinery in Baytown*


**Fig. 10: Naphthalene release per year Baytown**

Soil sample were collected 5-20 cm below the surface. The samples were transferred to the sterile tubes, and were transported to the laboratory for further study.

## *3.2 Isolation of Naphthalene degrading bacteria by enrichment culture:*

The collected samples of soil were immediately used for isolation of Naphthalene degrading bacteria. 1 gm of soil was added to the 5ml of Luria broth (LB), and the mixture was incubated at 37 $^0$C at 200 rpm for overnight duration. The isolation study was carried out after 24 hrs. using two different media: 1) Minimal salt medium (MSM), 2) Carbon supplement media (CSM).

1. Minimal salt medium (MSM): 2 gm $NH_4Cl$, 0.4 gm $KH_2PO_4$, 2 gm $CaCl_2$ and 0.2 gm of $FeSO_4$ were added in 1000 ml of water and the pH was adjusted to 7. The medium was autoclaved at 121 $^0$C for 30 min.
2. Carbon supplement medium (CSM): 2 ml of 1M NTA, 1 ml 20% w/v $MgSO_4$ $7H_2O$ were added in 950 ml of water and the pH was adjusted to 7. The medium was autoclaved at 121$^0$C for

30 min, and sterile 1ml 4% w/v $CaNO_3$, 1 ml 0.5%w/v $FeSO_4$ $6H_2O$ and 50 ml of phosphate buffer was added to it.

Both the media were supplemented with the naphthalene as the only carbon and energy source. 5ml of MSM and CSM media was supplemented with the 100, 300, 500, 1000 ppm of naphthalene dissolved in acetone. The inoculum was incubated for a week at 37 $^0$C at 200 rpm. Each week 0.2 ml of inoculum was added into the respective fresh MSM and CSM media for further sub-culturing and incubation. Each week before sub-culturing 0.2 ml of culture was added into the LB to confirm that the microorganisms were growing and were using Naphthalene. After a series of four further sub-culturing and incubation processes MSM, CSM and GSM (glycerol supplemented media) agar plate supplemented with the 100, 300, 500, 1000 ppm of naphthalene were streaked with the respective 0.1 ml of culture. The plates were incubated for a week and observed for the growth of the bacteria. Then single colony from the plate was grown in LB media. The glycerol stocks were prepared of isolates with the significant growth and were stored at -80 $^0$C.

### *3.3 Molecular identification of Naphthalene degrading bacteria:*

The isolated bacterial strains were subjected to DNA extraction using Qiagen DNAeasy DNA Blood and tissue kit. The bacterial 16S rDNA gene fragment was amplified by the polymerase chain reaction. The universal 16S rDNA PCR forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and universal reverse primer 1492R (5'-ACGGCTACCTTGTTACGACTT-3') were used for gene amplification.

The amplification was performed in a thermocycler. Temperature program used was PCR: 94 $^0$C for 3 min, 94 $^0$C for 0.5 min, 52 $^0$C for 0.5 min, 72 $^0$C for 1.5 min, 30 cycles, and final extension at 72 $^0$C for 10 min. The amplified PCR product was then subjected to the gel electrophoresis for confirmation of amplification of gene of interest. The product was sent to the Epoch Life Science, Inc. laboratory for sequencing. The 16S rDNA gene sequence results were compared with sequences in the GenBank database using BLASTN (available at http://blast.ncbi. nlm.nih.gov) to discover their approximate phylogenetic relationship.

### *3.4 Study the effect of different parameters on degradation of Naphthalene:*

In this study, *Bacillus pumilus* was used as a standard microorganism to compare the naphthalene degradation capacity of isolated *Enterobacter cloacae and Cellulosimicrobium cellulans.* These parameters were maintained for all the tests, except the individual parameter to be studies was varied as described in Sections 3.4.1 through 3.4.4. For example, in studying effect of pH in Section 3.4.1, the following standard parameters were maintained except the pH, which was varied. The standard parameters for study were:

Media: CSM

Media pH: 7

Temperature: $30^0$C

RPM: 200 rpm

Naphthalene concentration: 50 ppm

Solvent used to dissolve naphthalene: Acetone

### *3.4.1 Effect of pH:*

CSM media was prepared and adjusted to different pH levels 6, 7 and 8 using acetic acid and potassium hydroxide. They were supplemented with 50 ppm of naphthalene dissolved in acetone as a sole carbon source. The tubes were inoculated with microorganisms *Bacillus pumilus, Enterobacter cloacae* and *Cellulosimicrobium cellulans.* Tubes were incubated at 30 $^0$C at 200 rpm for 7 days. The control tube containing media of pH levels 6, 7 and 8 were supplemented with 50 ppm naphthalene, but without inoculum, were also kept for incubation. Samples were collected every 24 hrs., and were analyzed for cell growth study.

### *3.4.2 Effect of different concentration of Naphthalene:*

CSM media with pH 7 was prepared and supplemented with different initial concentration of naphthalene dissolved in acetone e.g., 50, 100, 200, 400 ppm. The tubes were inoculated with microorganisms, and were incubated at 30 $^0$C at 200 rpm for 7 days. The control tube containing CSM media supplemented with 50, 100, 200, 400 ppm of naphthalene without inoculum were also kept for incubation. Samples were collected every 24 hrs., and were analyzed for cell growth study.

### *3.4.3 Effect of media:*

Two-different media of pH 7, i.e., CSM and Bushnell Haas, were used for study. Both media were supplemented with 50 ppm of naphthalene dissolved in acetone as a sole carbon source. The tubes were inoculated with the microorganisms, and the control tubes were prepared without addition of microorganisms. Tubes were incubated under standard conditions listed above, and the samples were collected every 24 hr., and were analyzed for cell growth study.

### *3.4.4 Effect of solvent used to dissolve Naphthalene:*

In this study the different solvents such as acetone, ethyl acetate and acetonitrile were used to dissolve naphthalene. The CSM media with pH 7 was prepared and supplemented 50 ppm naphthalene dissolved in acetone, ethyl acetate, acetonitrile. The tubes were inoculated with microorganisms and control tubes were prepared without addition of microorganism. Study was carried out for 7 days, and the samples were collected every 24 hrs. for cell growth study analysis.

### *3.4.5 Consortium study:*

This study involved growth of Consortium of two bacteria using naphthalene as an energy source. The CSM media with pH7 was prepared and supplemented with 50 ppm naphthalene dissolved in acetone, and was incubated at 30 $^{0}$C at 200 rpm for 7 days. For this study *Bacillus pumilus (BP), Enterobacter cloacae (EC), Cellulosimicrobium cellulans (CC), Achromobacter xylosoxidans (AB)* were used. The combinations were prepared in 1:1 ratio as follows:

a) BP with EC
b) EC with CC
c) BP with AB
d) EC with AB
e) BP with CC
f) CC with AB

Study was carried out for 7 days, and samples were collected every 24 hrs. and were analyzed for cell growth study.

### *3.5 Naphthalene extraction, quantification:*

A sample of 10 ml from the study of effect of pH on cell growth using naphthalene as a carbon source was taken for the determination of residual naphthalene. For naphthalene extraction, 2 ml hexane was added into the flask and incubated at 37 $^0$C and 200 rpm for 1 hr. The content was then centrifuged at an acceleration level of 5000g for 10 min, and the extract was dried using anhydrous sodium sulfate to remove the aqueous phase. Residual naphthalene in the extract was analyzed using UV- spectrophotometer at 315 nm (data not shown) (Vilas Patel, 2012).

### *3.6 Detection of Dioxygenase gene in isolated bacteria:*

Presence of dioxygenase gene was detected by PCR amplification in the isolated *Enterobacter cloacae* (EC*), Cellulosimicrobium cellulans* (CC). Primers used in this study were Biphenyl dioxygenase (bph) and Dibenzofuran dioxygenase (dfa), and are listed in Table 1.

**Table 1: List of primers used for detection of Dioxygenase**

| PRIMER | SEQUENCE |
|---|---|
| **bphA1 F** | 5’ CTGGAGACCTACCTCGGTGA 3’ |
| **bphA1 R** | 5’ ACGTAGCCCCTTCTGGATCT 3’ |
| **bphA2 F** | 5’ CTTGGGCACGAGAGTCATGT 3’ |
| **bphA2 R** | 5’ TGGGCAGGAATGAACAGGTC 3’ |
| **dfa1 F** | 5’ GAAGTTCCGGATCAAGGCCA 3’ |
| **dfa1 R** | 5’ TCTGCCAGACCTCGATCTCA 3’ |
| **dfa2 F** | 5’ GTATCTGGACCTGGCCTTCG 3’ |
| **dfa2 R** | 5’ ATGAGTTCGAGCCAGTAGCG 3’ |

The amplification was performed in a thermocycler. Temperature program used for PCR was: 94 $^0$C for 3 min, 94 $^0$C for 0.5 min, 52 $^0$C for 0.5 min, 72 $^0$C for 1.5 min, 30 cycles, and final extension at 72 $^0$C for 10 min. The amplified PCR product was then subjected to the gel electrophoresis for separation. 1 % agarose gel was used with Ethidium bromide as a staining agent. Gel was visualized under UV light and the results were recorded.

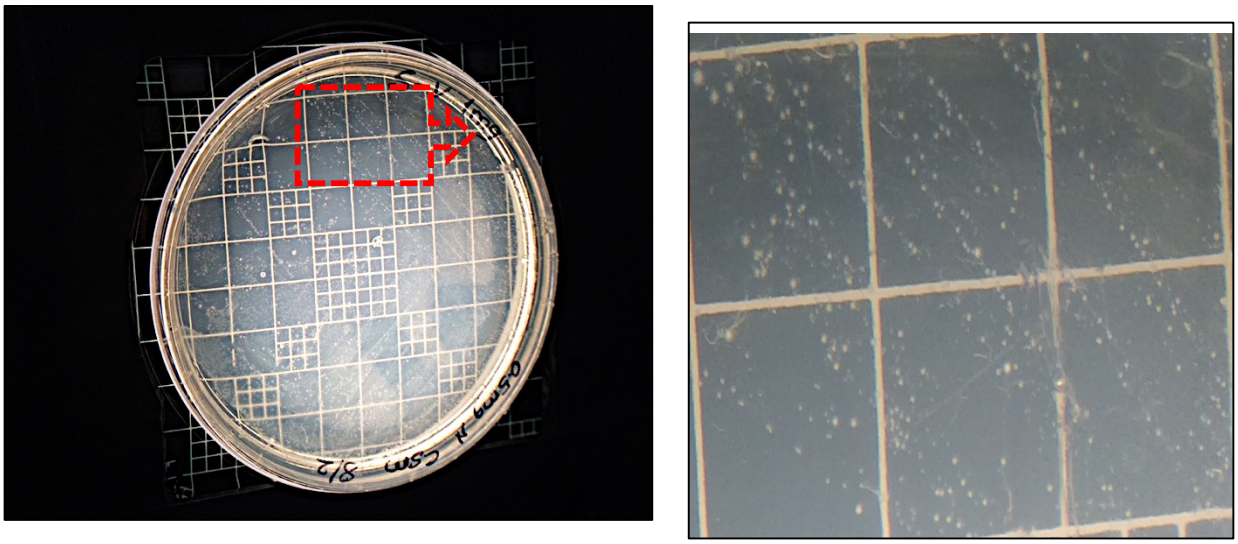
# 4 Result and Discussion

## *4.1 Isolation of Naphthalene degrading bacteria by enrichment culture:*

In the first set of isolation with the use of MSM (Minimal Salt Medium) as an isolation media, naphthalene degrading bacteria was not observed. This may be because insufficient nutrients in the media did not support the bacterial growth in presence of naphthalene as a carbon source.

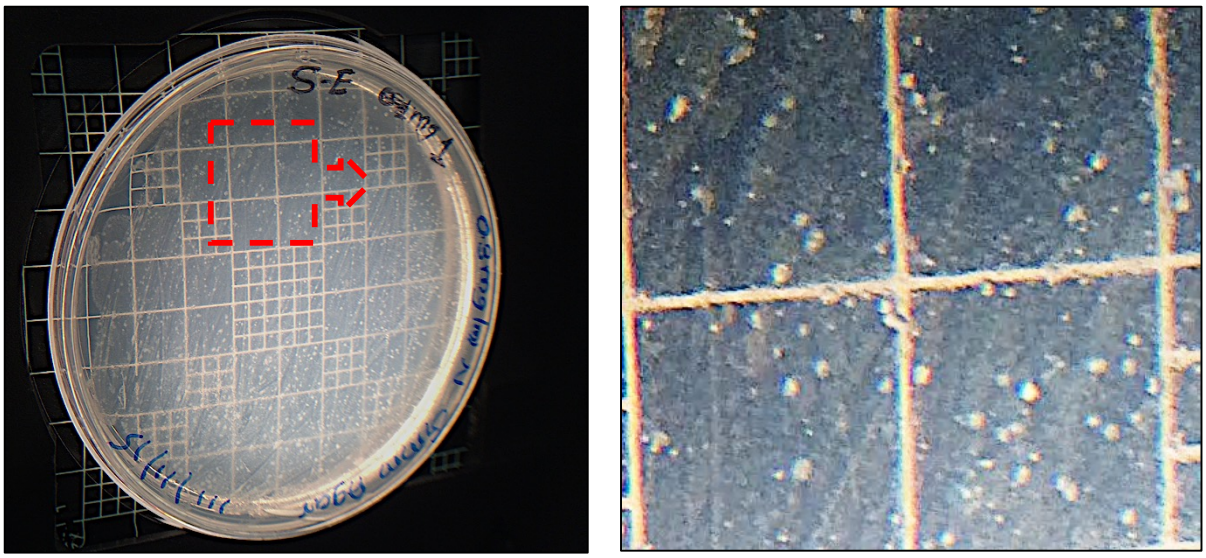
The second set of study using CSM (Carbon Supplement Medium) as an isolation media was observed to have supported growth of the naphthalene degrading bacteria. The cultures after 5 weeks of isolation procedure were plated on the CSM and GSM agar plates. The growth of bacteria was detected in the culture with 1000 ppm naphthalene (sole carbon source) obtained from Valero site. This culture was then streaked on CSM agar plate having 500 ppm naphthalene, wherein the bacteria growth was detected as well.

For the sample obtained from Exxon Mobil site the growth was observed in cultures with 300, 500, 1000 ppm naphthalene on CSM and GSM agar plate. The photographs of the bacterial growth on these agar plates are shown Fig. 11 through Fig. 14 along with a zoomed region for isolated bacterial colonies.



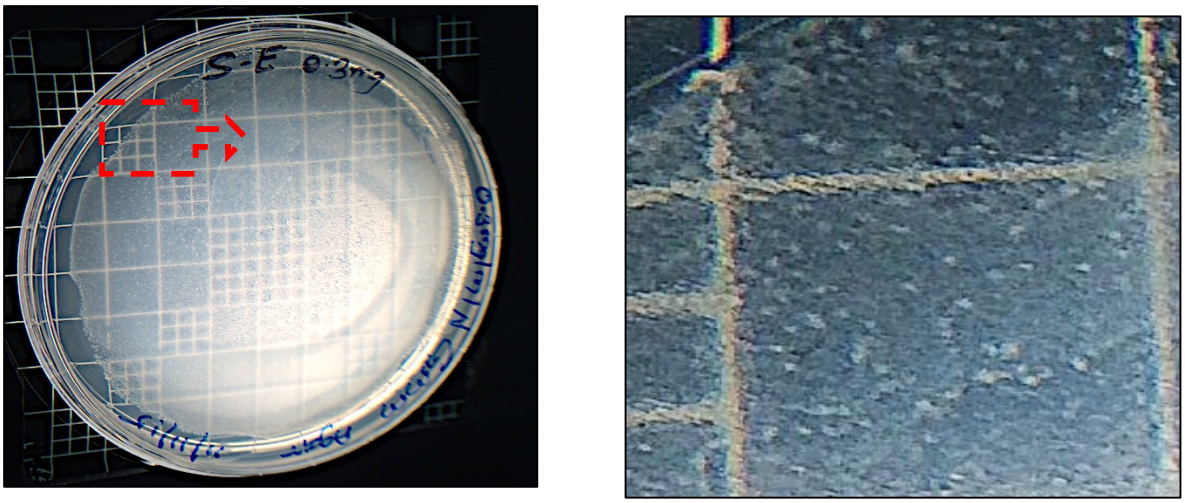
**(A)** **(B)**

**Fig. 11: Valero site, microbial growth from culture isolated with 1000 ppm Naphthalene (A) Cell growth all over the plate; (B) Zoomed region from (A) showing isolated bacterial colonies**



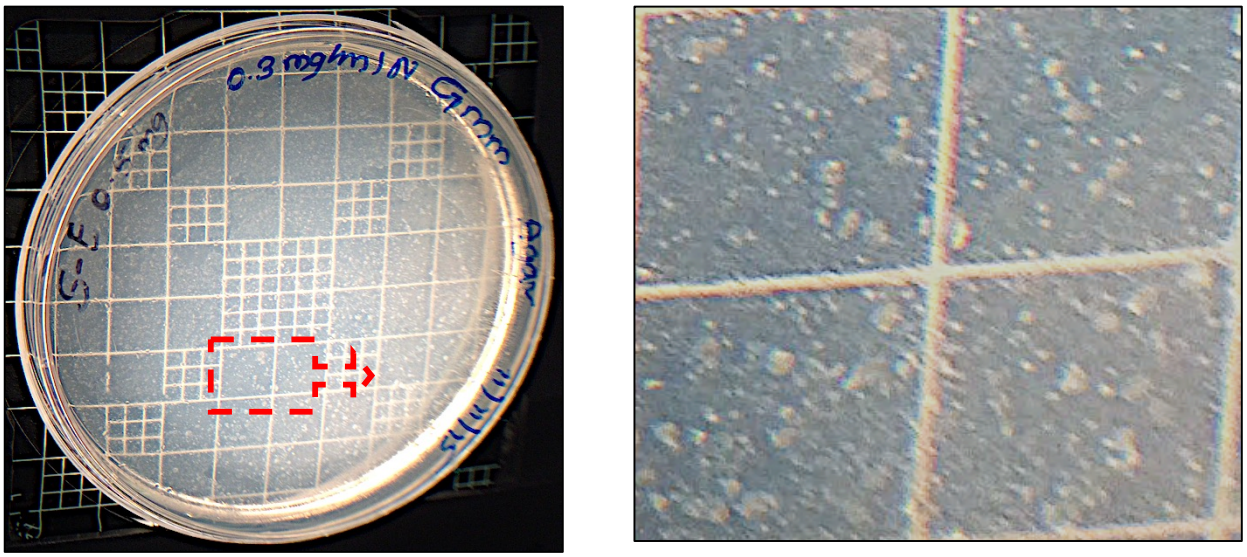
**(A)** **(B)**

**Fig. 12**: **Exxon Mobil site, microbial growth from culture isolated with 1000 ppm aphthalene (A) Cell growth all over the plate; (B) Zoomed region from (A) showing isolated bacterial colonies**



(A) (B)

**Fig. 13: Exxon Mobil site, microbial growth from culture isolated with 1000 ppm naphthalene (A) Cell growth all over the plate; (B) Zoomed region from (A) showing isolated bacterial colonies**



(A) (B)

**Fig. 14: Exxon Mobil site, microbial growth from culture isolated with 500 ppm naphthalene (A) Cell growth all over the plate; (B) Zoomed region from (A) showing isolated bacterial colonies**

### *4.2 Molecular identification of Naphthalene degrading bacteria:*

In gel electrophoresis of 16S rDNA PCR, the only bacteria isolated using 1000 ppm naphthalene from Valero site, and 500, 1000 ppm naphthalene from Exxon Mobil site showed the presence of DNA. Approximately 1500 bp. sized-fragment of 16S rDNA gene of each isolate was sequenced.

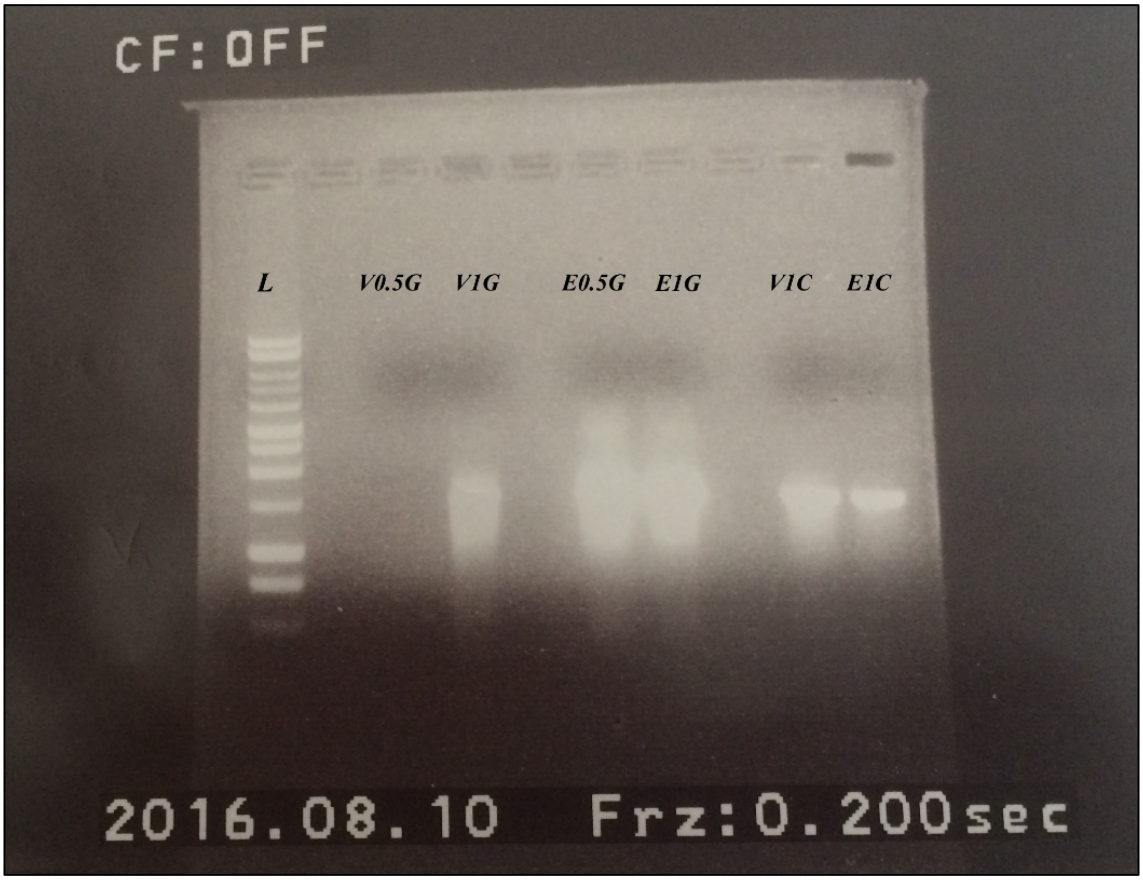



**Fig. 15: Gel Electrophoresis Results**

The sequencing results when searched in the GenBank database at the National Center for Biotechnology Information (NCBI) sequences showed 99.9 % similarity with the *Enterobacter cloacae* for Valero sample. The samples from the Exxon Mobil site showed 99.9 % similarity with both *Enterobacter cloacae* and *Cellulosimicrobium cellulans*.

### *4.3 Study the effect of different parameters on degradation of Naphthalene:*

This study was done in two sets. First set of study was carried out for 3 days and readings were taken at every 24 hrs. These readings displayed an optical density of 0.002 at 600 nm concluding

that the initial concentration of cells for study was low. It has also been reported that if initial cell inoculum concentration is low, the lag phase is longer resulting in a lower biodegradation rate (Chen Lin, 2010). Therefore, in the second set of study initial cell concentration was increased to optical density in the range of 0.2-0.25 at 600 nm. The study was carried out for 7 days, and cell growth readings were taken every 24 hrs.

For an easy comparison of the results of study set 1 and set 2, all graphs were plotted using the same y-axis scale. Because of the low initial concentration of cell inoculum, most of the graphs for set 1 did not show significant growth compared to set 2 study results. Additionally, all graphs were plotted with 95 % of confidence interval error bars.

### *4.3.1 Effect of pH:*

It has been known that initial pH of the culture medium affects the microbial and enzyme activities (J.W.C. Wong, 2002). Therefore, the study was carried out using culture mediums with different initial pH 6, 7 and 8. Yellow coloration was observed in samples containing CC and EC for all the pH values studied after 24 hrs. of incubation period. Samples containing EC showed a dark-yellow color, samples with CC displayed a pale-yellow color, whereas sample containing BP did not exhibit any yellow coloration. These results are shown in Fig. 16. It was concluded that the yellow color of the media might be due to production of phenolic compound through breakdown of naphthalene by bacteria EC and CC. Per Reference (Pumphrey GM, 2007), the yellow color is likely to be due to the phenolic compound **1,2-naphthoquinone**. The degradation path involving 1,2-naphthoquinone is shown in Fig. 17.

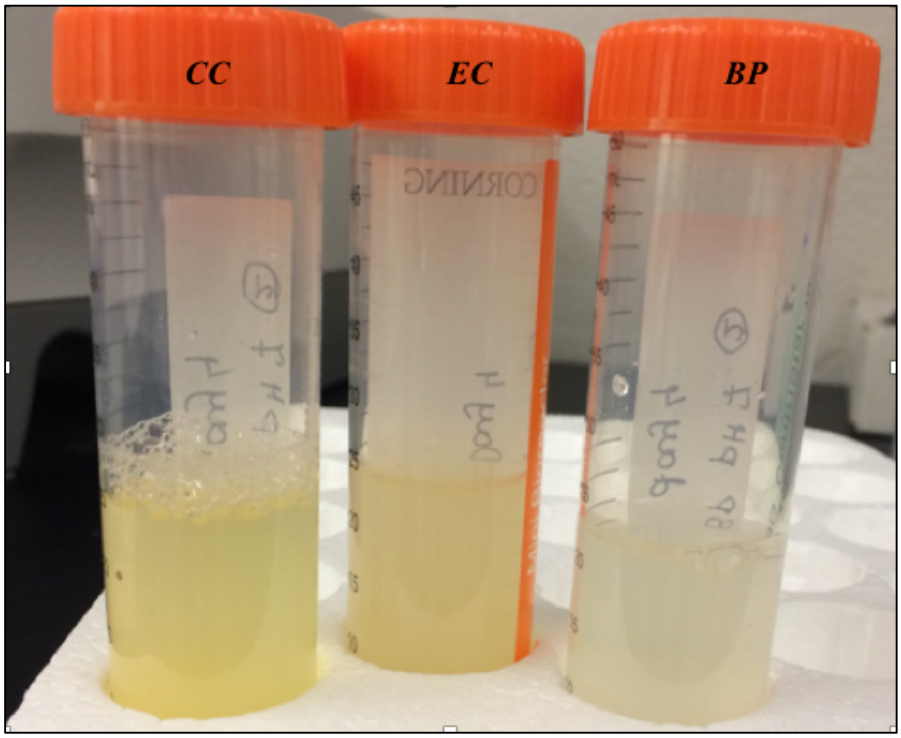



**Fig. 16: Development of yellow coloration in bacterial culture**

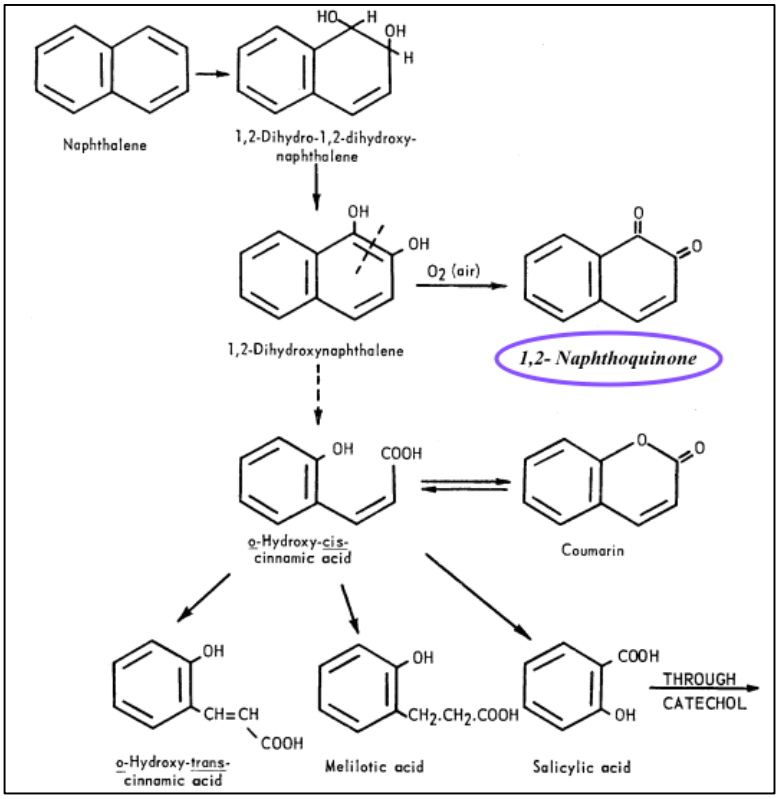



**Fig. 17: Degradation of Naphthalene by soil bacteria** (Martin H. Rogoff, 1962)

***a) pH 6***

In the first set (Fig. 18) of study CC showed more growth than EC and BP on day 1, but CC growth was decreased on day 2. It was contemplated that the growth of BP, EC and CC was very low since the initial inoculum concentration was low.

In the second set (Fig. 19) of extended study, BP showed a continuous decline in cells. EC cells were constant till day 3, increased on day 4, declined on day 5, and remained constant till day 7. CC showed increase in cells on day 1, but exhibited a gradual decline thereafter.

From the results shown in Fig. 18 and 19 it can be concluded that BP was not able to utilize Naphthalene as a sole carbon source. In initial period EC bacteria tried to survive in Naphthalene containing medium, and grew using naphthalene as a carbon source. However, EC could not survive after the products formed due to naphthalene breakdown. CC cell growth on day 1 was maximum compared to other days, indicating that CC consumed naphthalene as a carbon source. But from the error bars shown in Fig. 19, CC got significant amount of variation in two samples. To remove this variation more number of samples needed to be used for the study.

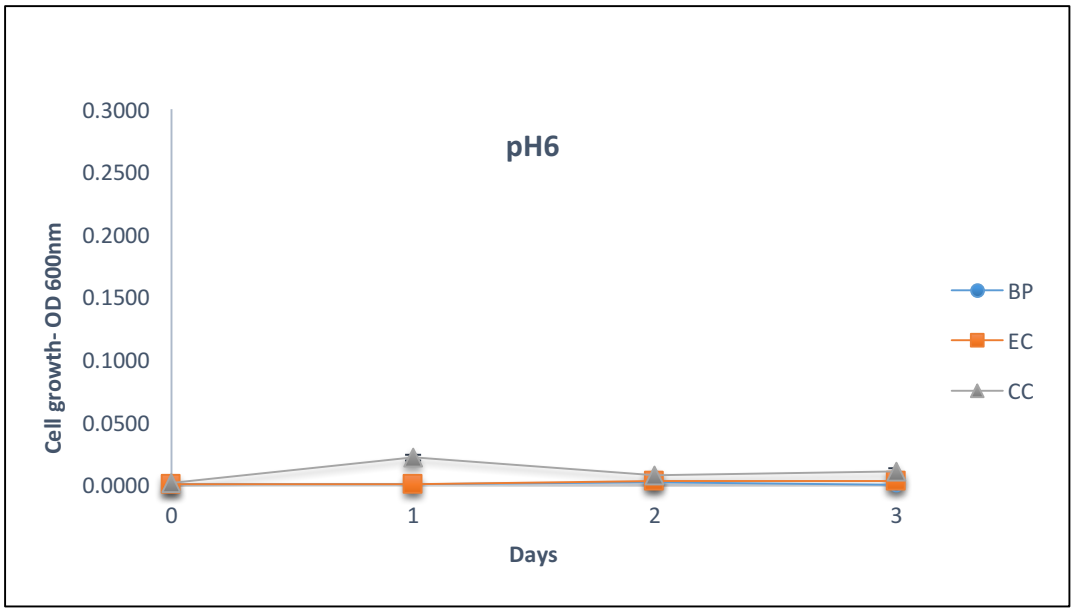



**Fig. 18: Effect of pH 6 (I$^{st}$ set)**

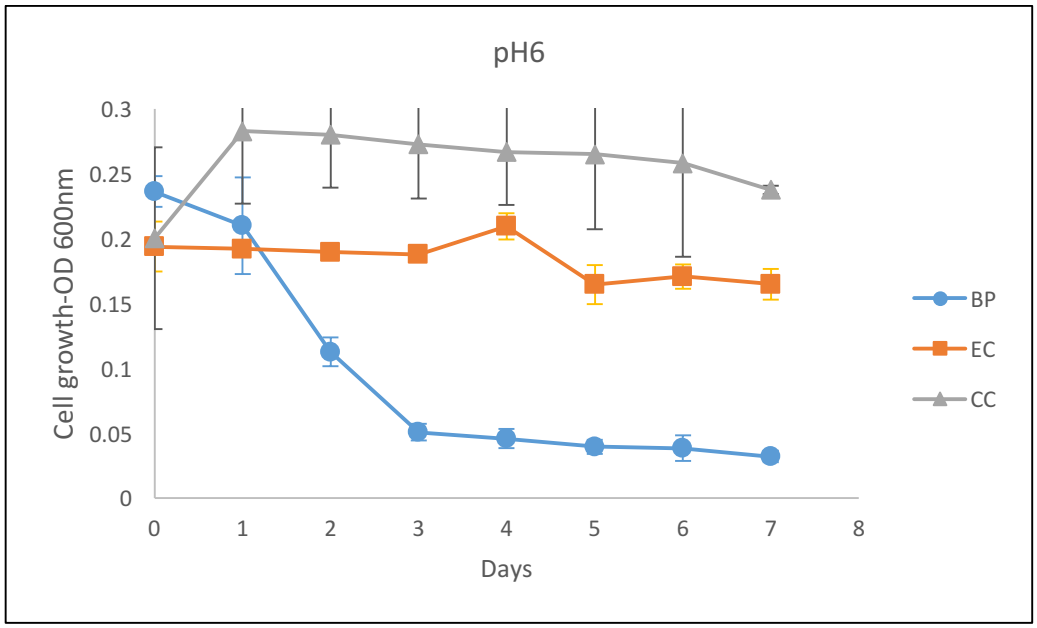



**Fig. 19: Effect of pH 6 (II$^{nd}$ set)**

***b) <u>pH 7</u>***

In set 1 (Fig. 20) it can be observed that the pH 7 CSM media for set 1 in all three microorganisms BP, EC and CC exhibited negligible growth.

In set 2 (Fig 21) BP showed a significant decline in cells during first three days, and did not show any noticeable change thereafter. EC showed decline in cells till day 3, increase in cells on days 4-5, and again decline after day 6. Cell growth reading for EC on day 5 showing significant variation. CC showed the growth at day 1, but started to gradually decline in cells after day 1.

From Fig. 20 and Fig. 21 it can be deduced that BP was not using naphthalene as a carbon source at CSM medium pH 7. EC showed adaption to the growth environment after day 3, therefore, it is likely that EC started using naphthalene after day 3. CC could have started using naphthalene as a carbon source from day 1.

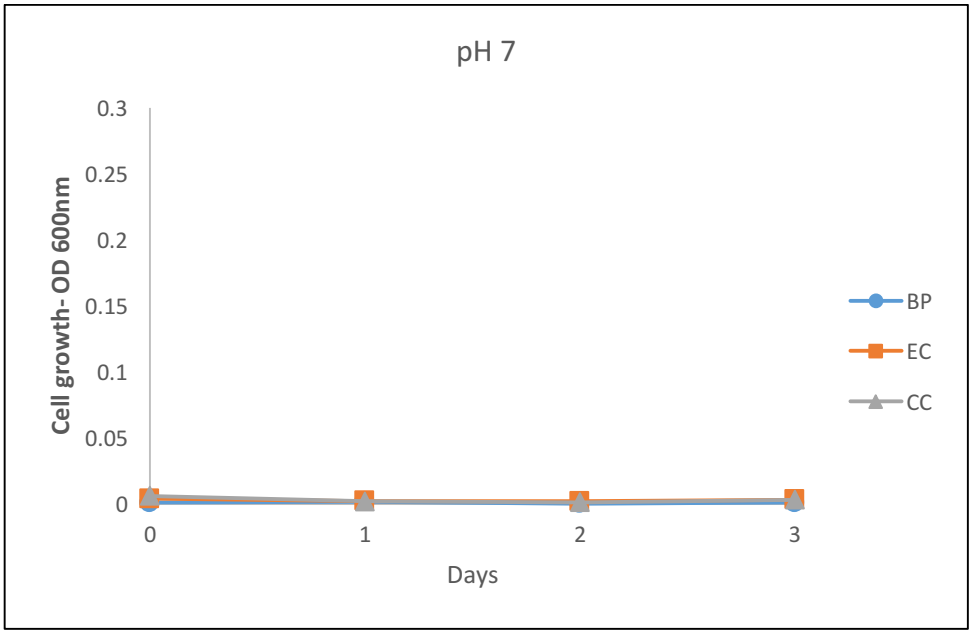



**Fig. 20: Effect of pH 7 (I$^{st}$ set)**

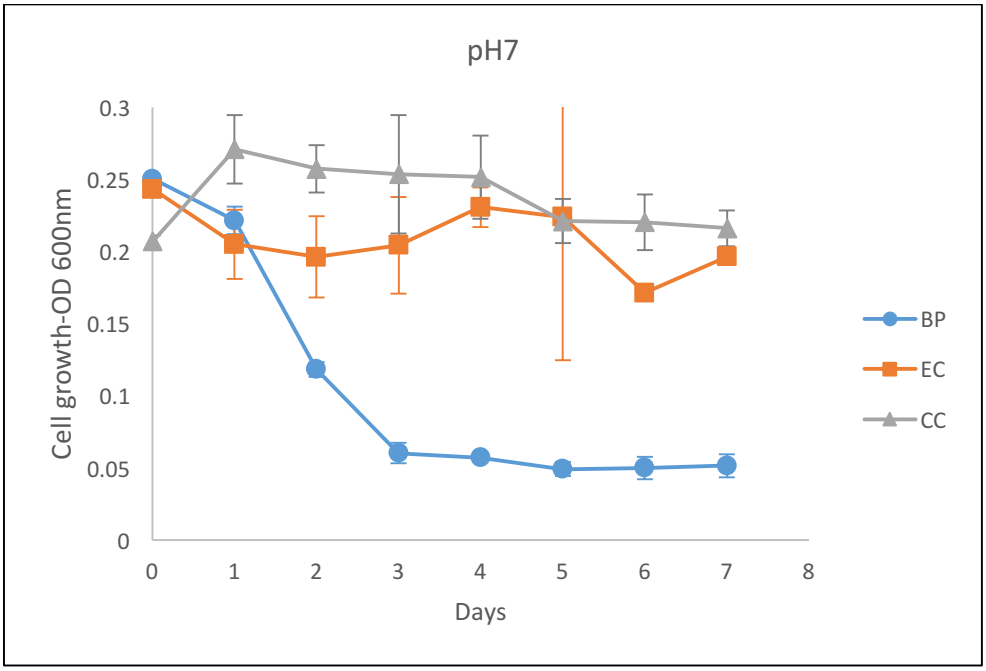



**Fig. 21: Effect of pH 7 (II$^{nd}$ set)**

***c) pH 8***

In the first set (Fig.22) BP and CC did not show any significant growth. EC sowed the growth on day 1 but due to the significant amount of variation in two reading cell growth at day 1 may not be substantial.

In set 2 (Fig. 23), BP exhibited substantial decline in cell growth during first 3 days. EC showed a gradual increase in cell growth with the maximum growth occurring on day 4, and decline in growth after day 5. CC exhibited maximum growth on day 1, and a gradual decline after day 2.

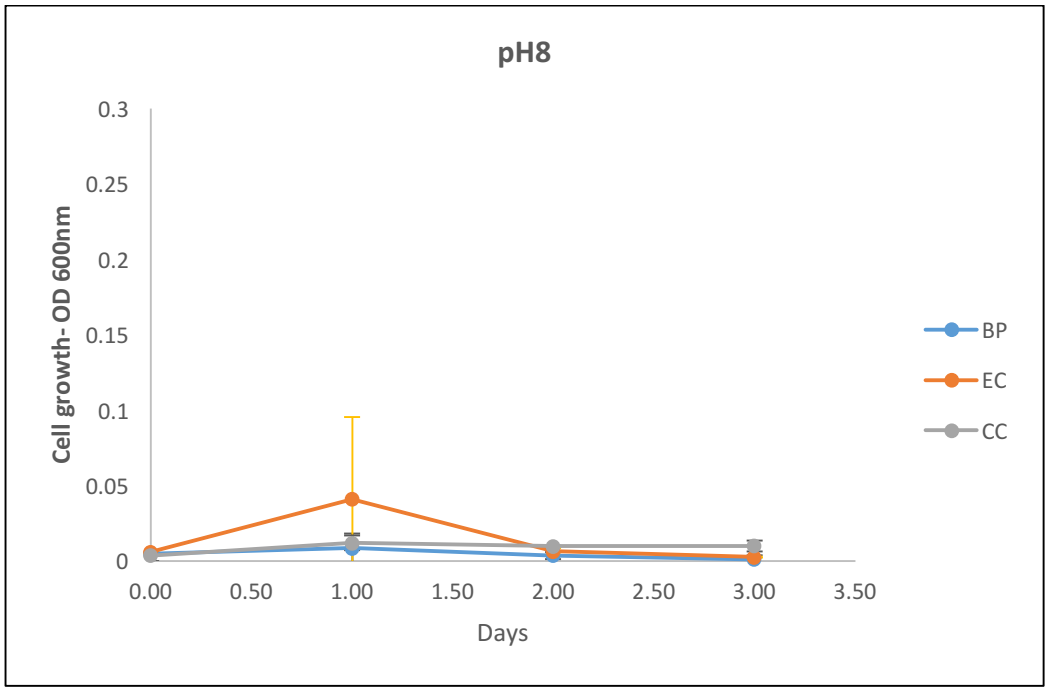



**Fig. 22: Effect of pH 8 (I$^{st}$ set)**

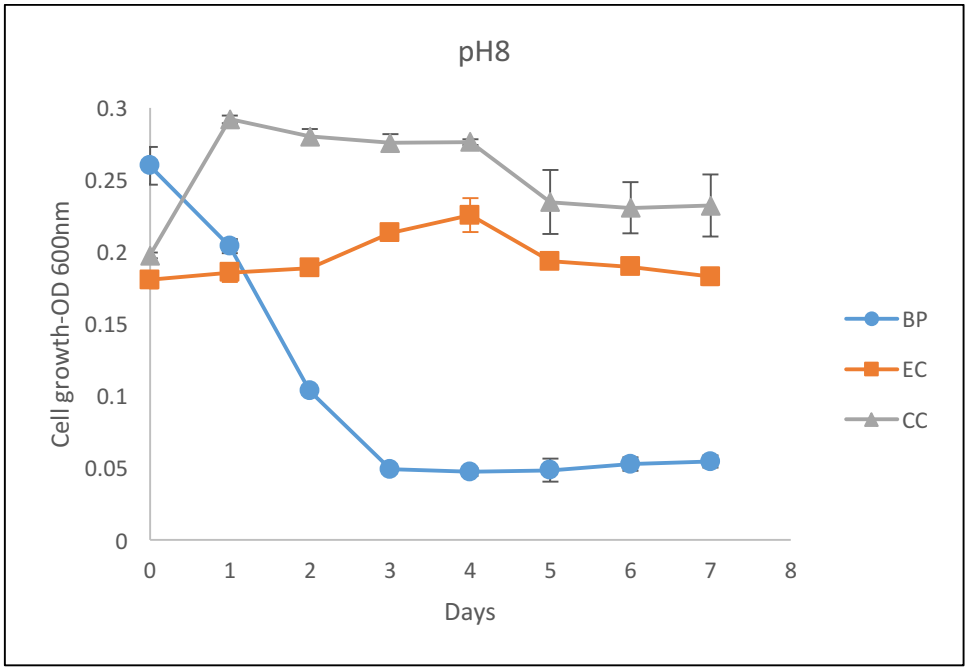



**Fig. 23: Effect of pH 8 (II$^{nd}$ set)**

From these results, it can be concluded that BP may not be using naphthalene as a carbon source thereby resulting a decrease in cells.

Irrespective of the pH level, EC exhibited maximum growth on day 4. This implies that EC adapted to the media environment, and started using naphthalene. The decline in cells after day 4 suggests that EC successfully degraded naphthalene, but could not degrade the metabolites formed after breakdown of naphthalene.

CC exhibited significant growth on day 1 for all pH levels, but decrease in cell growth after day 1. This indicates that CC is degrading naphthalene, but was not able to survive due to inhibitory and toxic effect of accumulated metabolites.

To summarize, the results indicated that medium pH level did not affect the cell growth in presence of Naphthalene as an energy source.

The quantitative study of naphthalene degradation was carried out for all above samples but are not reported here.

### *4.3.2 Effect of different concentration of Naphthalene:*

Effect of different initial concentrations of naphthalene on individual strain was studied to assess the strain capacity to tolerate different concentration of naphthalene.

***a) Concentration 50 ppm of Naphthalene***

In set 1 (Fig. 24), no significant growth was observed for all three microorganisms BP, EC and CC. In set 2 (Fig. 25), BP exhibited substantial decline in cells in first 3 days. EC showed gradual decrease as well as increase in cell growth, and the maximum cell growth was observed on dat 4. As can be seen in the error bar for EC in Fig. 25, the cell growth reading was showing significant variation on day 5. Day 5 cell growth reading may not be a conclusive result. CC showed an initial increase, and a gradual decline thereafter.

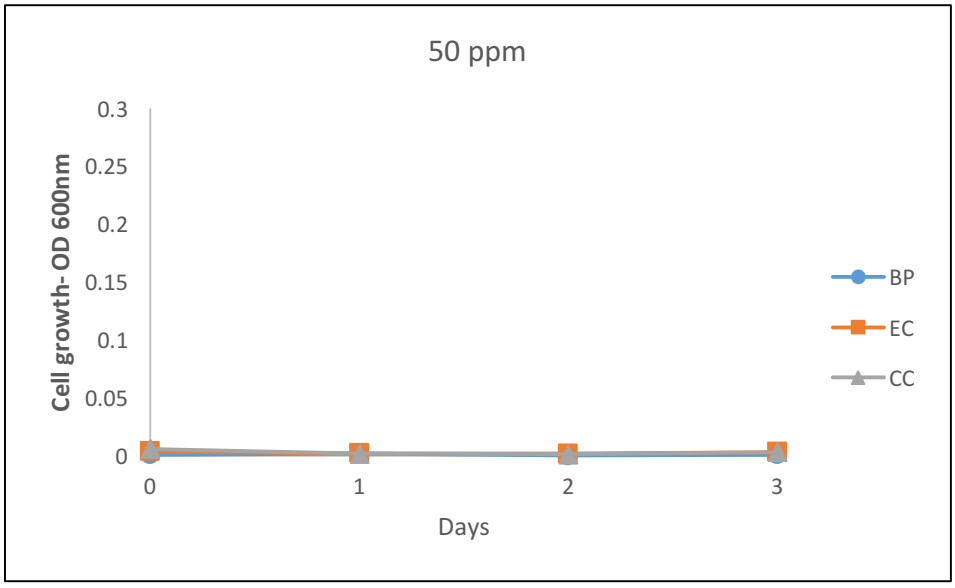



**Fig. 24: Effect of 50 ppm Naphthalene (I$^{st}$ set)**

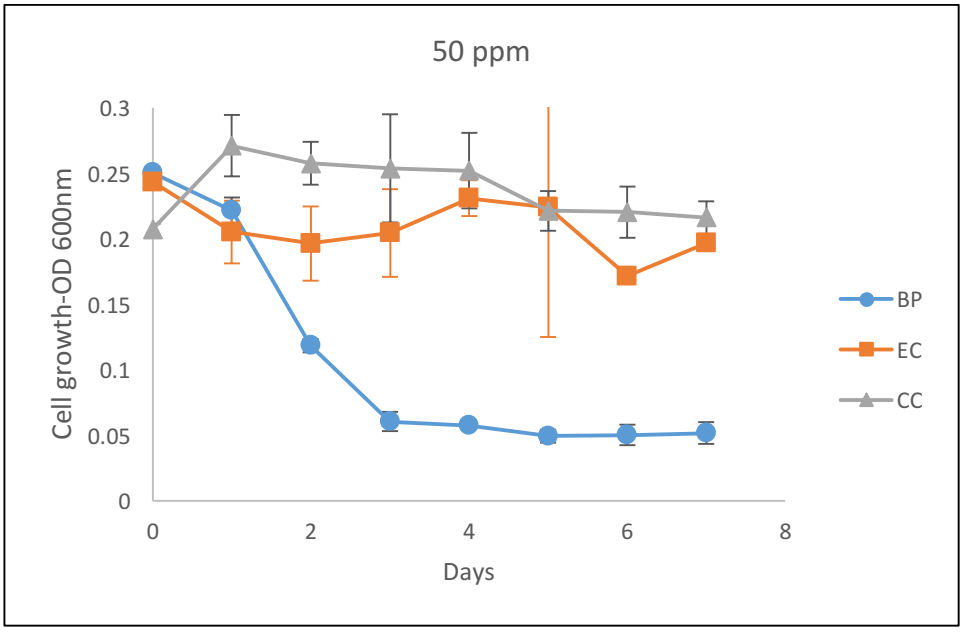



**Fig. 25: Effect of 50 ppm Naphthalene (II$^{nd}$ set)**

***b) <u>Concentration 100 ppm of Naphthalene:</u>***

In set 1 (Fig. 26) for all 3 microorganism BP, EC and CC, there was not significant growth in cells over period of study. In set 2 (Fig. 27), BP showed significant decline in cell growth. EC and CC did not exhibit substantial growth.

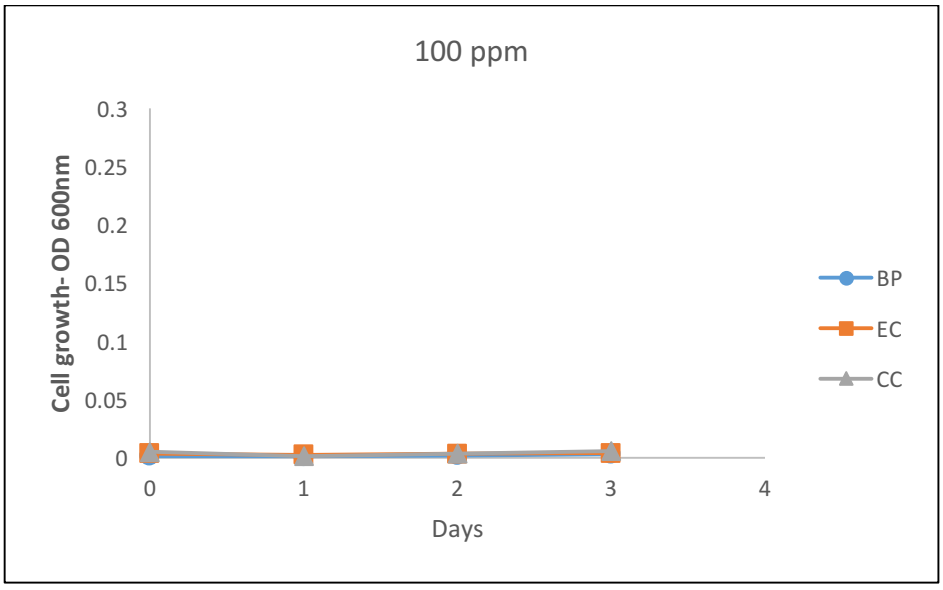



**Fig. 26: Effect of 100 ppm Naphthalene (I$^{st}$ set)**

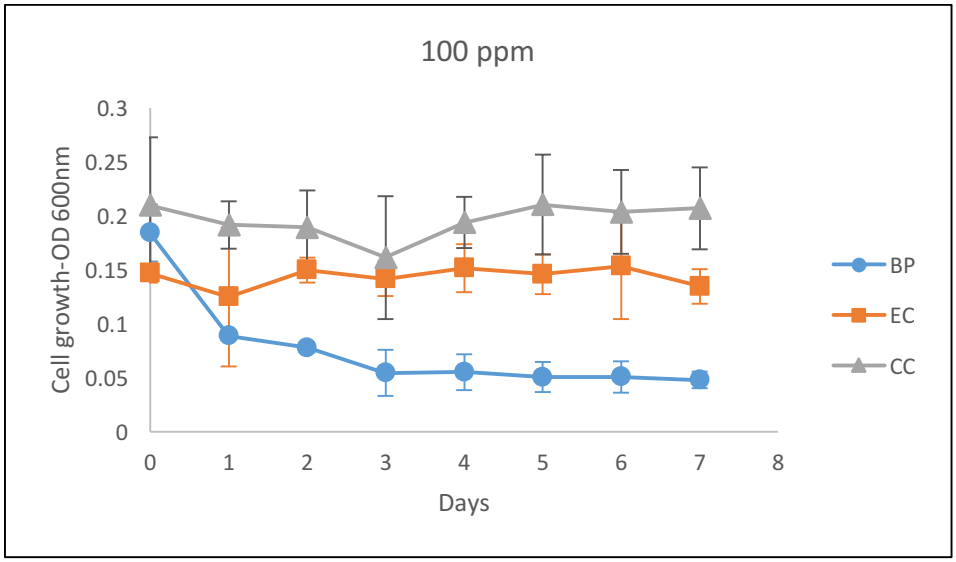



**Fig. 27: Effect of 100 ppm Naphthalene (II$^{nd}$ set)**

***c) <u>Concentration 200 ppm of Naphthalene:</u>***

In set 1 (Fig. 28), there was no substantial growth in cells over the period of study for all 3 microorganisms. In set 2 (Fig. 29), BP exhibited sharp decline on day 1, and gradually declined after day 2. EC and CC showed gradual decline on day 1, and the cell growth remained relatively constant with a minute variation after day 2.

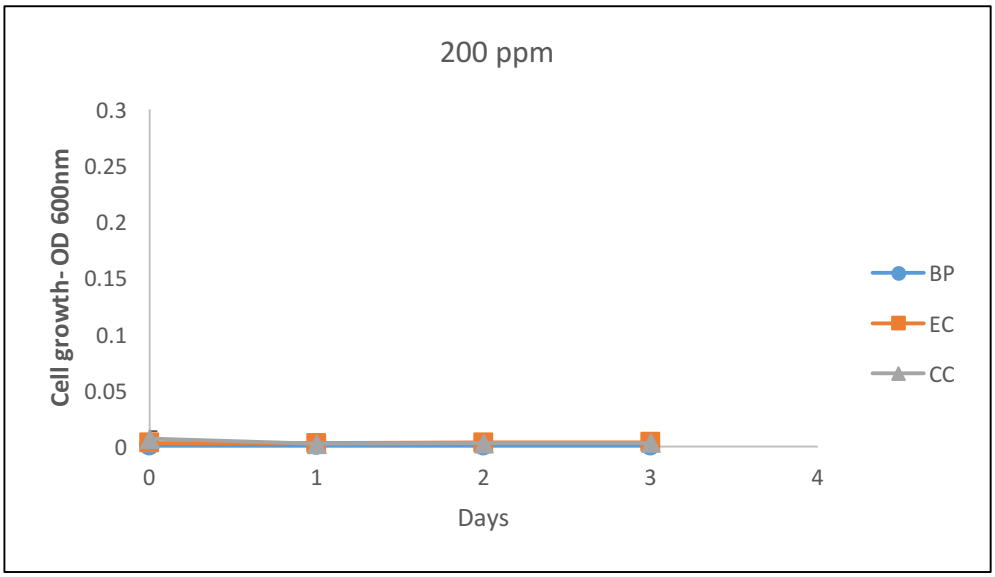



**Fig. 28: Effect of 200 ppm Naphthalene (I$^{st}$ set)**

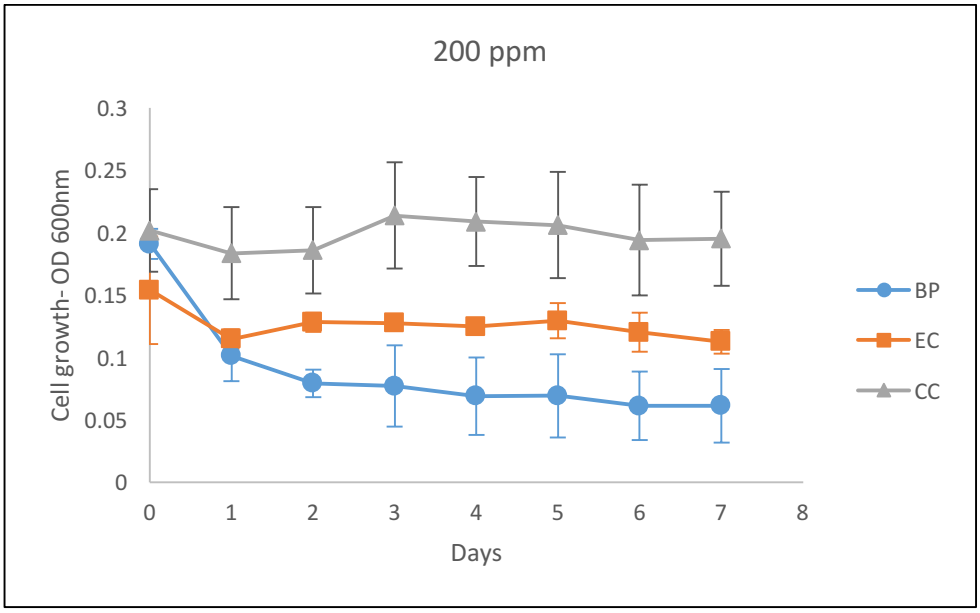



**Fig. 29: Effect of 200 ppm Naphthalene (II$^{nd}$ set)**

***d) Concentration 400 ppm of Naphthalene:***

In set 1 (Fig. 30) for all 3 microorganism BP, EC and CC, there was not substantial growth in cells over period of study. In set 2 (Fig. 31), significant cell growth was not observed for all 3 microorganisms. But the data for EC and CC showed significant variation in readings as can be seen in the error bars. Therefore, this study needs to be done with more number of samples.

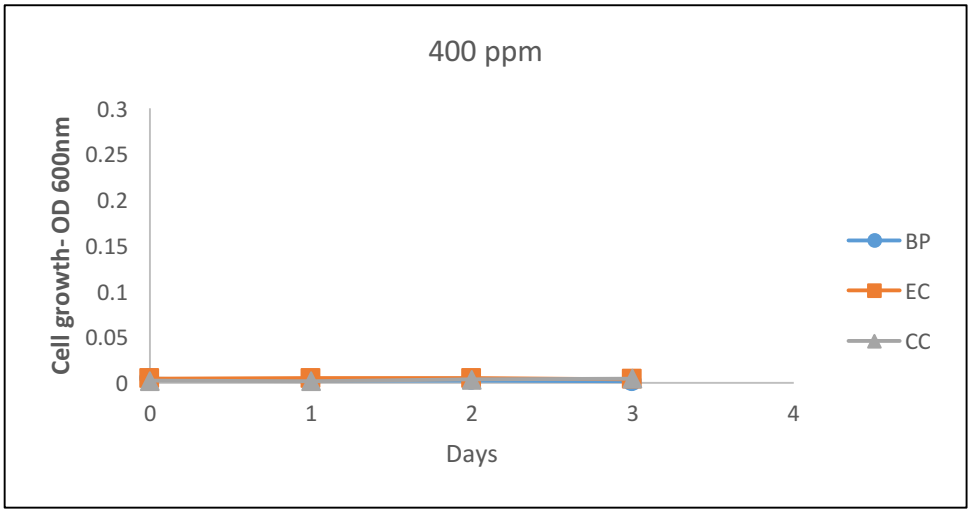



**Fig. 30: Effect of 400 ppm Naphthalene (I$^{st}$ set)**

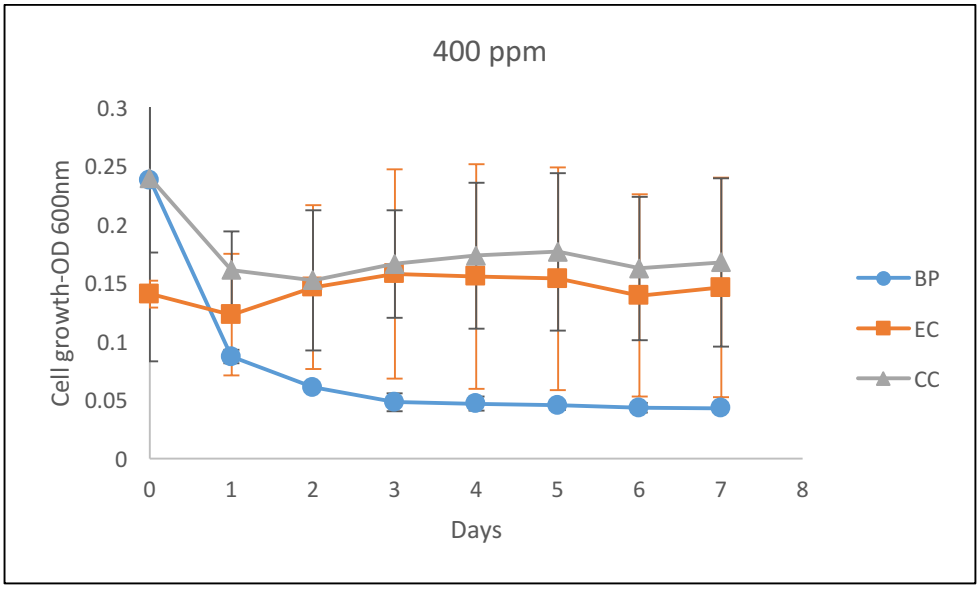



**Fig. 31: Effect of 400 ppm Naphthalene (II$^{nd}$ set)**

From the above results, BP consistently showed a decline in cell growth for all concentrations studied, i.e., 50, 100, 200, 400 ppm. EC and CC could use naphthalene as carbon source at 50 ppm concentration and could tolerate 100, 200, 400 ppm of naphthalene concentration, but was unable to multiply cells. This may be due to lower degradation rate at higher Naphthalene concentration.

### *4.3.3 Effect of media:*

It was noted from the literature study that presence of carbon, nitrogen and phosphorus in different ratios has different effect on cell growth, and ultimately on biodegradation of xenobiotic compounds. In this study two different media, i.e., CSM and Bushnell Haas medium were used. These media were made up of different chemicals which act as nitrogen and phosphorus source.

***a) <u>Bushnell Haas medium:</u>***

In set 1 (Fig. 32), there was no significant growth in cells over period of study for all 3 microorganisms. In set 2 (Fig. 33), there was no significant growth for BP, EC and CC in Bushnell Haas medium, with BP showing a significant decline, and EC and CC exhibiting no change in cell growth. This suggests that the nutrients provided by Bushnell Haas medium are sufficient to keep the bacteria alive, but not optimum for multiplication of cells.

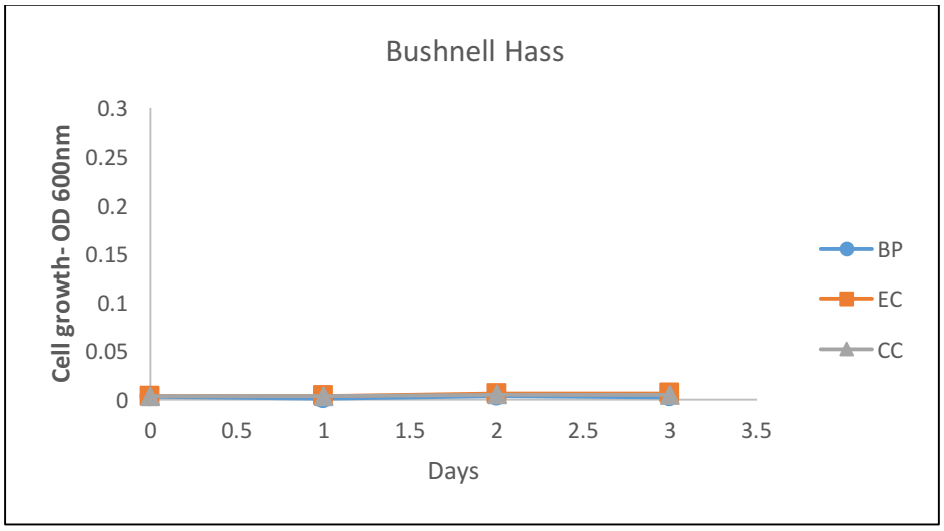



**Fig. 32: Effect of Bushnell Haas medium (I$^{st}$ set)**

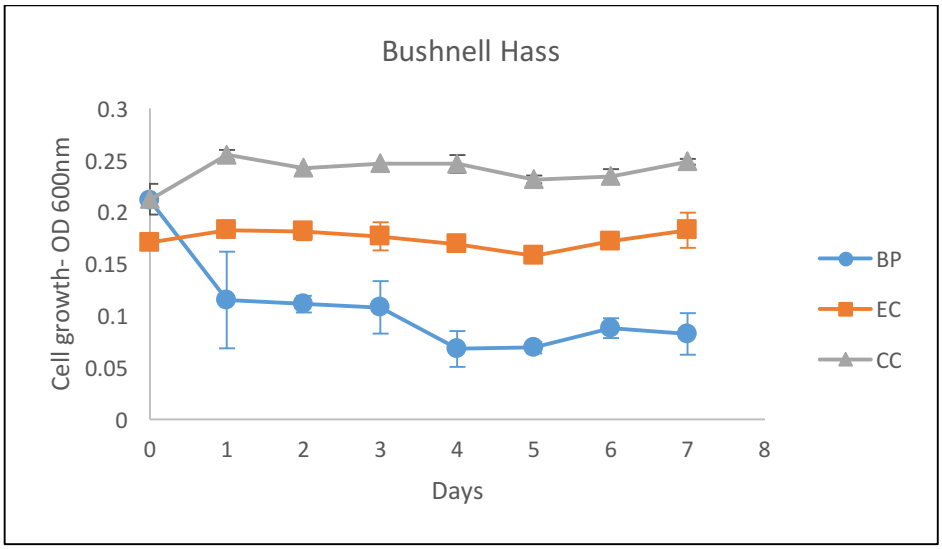



**Fig. 33: Effect of Bushnell Haas medium (II$^{nd}$ set)**

***b) CSM:***

In set 1 (Fig. 34) no significant growth was seen for all 3 microorganisms. In set 2, CSM medium BP showed a decline in cells for all 7 days. EC showed the decline in cells till day 3 and again increase in cells on day 4-5 started declining again day 6 onward. CC showed the growth on day 1, but started to decline in cells thereafter.

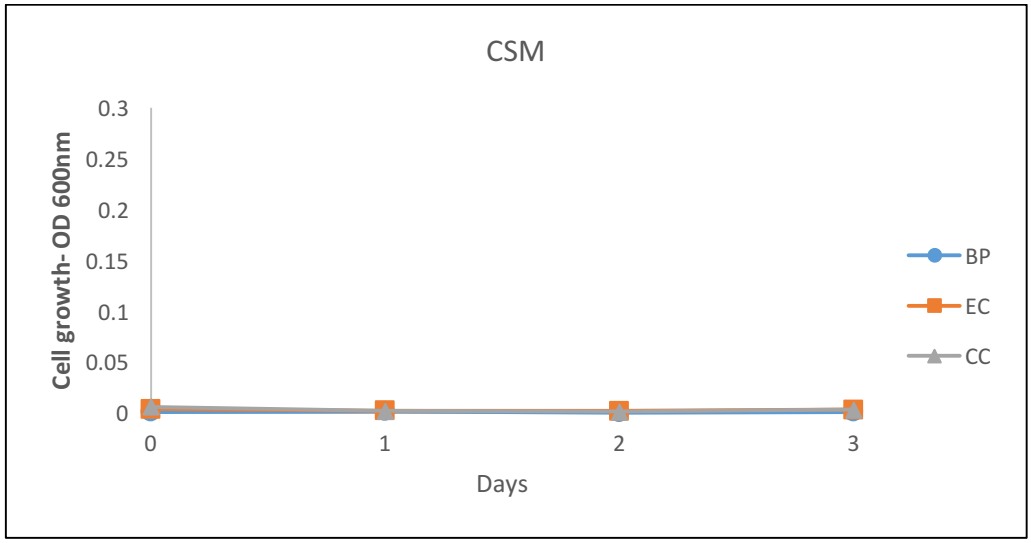



**Fig. 34: Effect of CSM (I$^{st}$ set)**

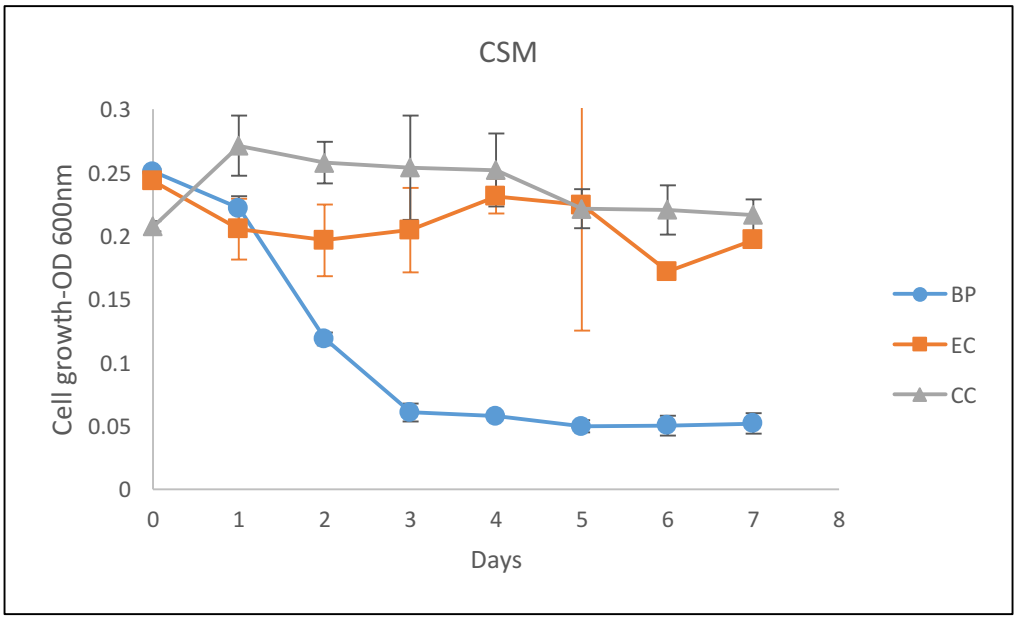



**Fig. 35: Effect of CSM (II$^{nd}$ set)**

From the results for medium used for study, it can be concluded that CSM provided optimum amount of nutrients for cell growth and degradation of naphthalene. In Bushnell Haas, mediums EC and CC were using naphthalene as a carbon source, but the degradation rate is possibly much lower than that for CSM medium. Previous work has shown that different sources of nitrogen and phosphorus exhibit different effects on biodegradation (Semboung Lang, 2016).

### *4.3.4 Effect of solvent used to dissolve Naphthalene:*

It has observed in the literature that, compounds having less solubility in water need to be dissolved first in organic solvent. The organic solvent used for dissolution of naphthalene helps naphthalene to be available for degradation. Use of organic solvent helps bacteria to orient themselves to organic phase due to nonpolar nature of portion of bacterial surface. This phenomenon helps to increase availability of naphthalene for biodegradation (Riser-Roberts, 1998).

***a. Acetone as a solvent:***

In the first set (Fig. 36) there was not a significant growth for BP, EC and CC because of very low initial concentration of cell inoculum. In set 2 (Fig. 37), CC showed the maximum growth on day 1 and a gradual decrease in cells after day 2. EC showed a decline in cells till day 3, an increase in cells on days 4-5, and declined on day 6 onward. BP showed a decline in cells for all 7 days of study. Previously it has been concluded that acetone stimulates naphthalene degradation (Chang, et al., 2015). Results from this study confirms this conclusion.

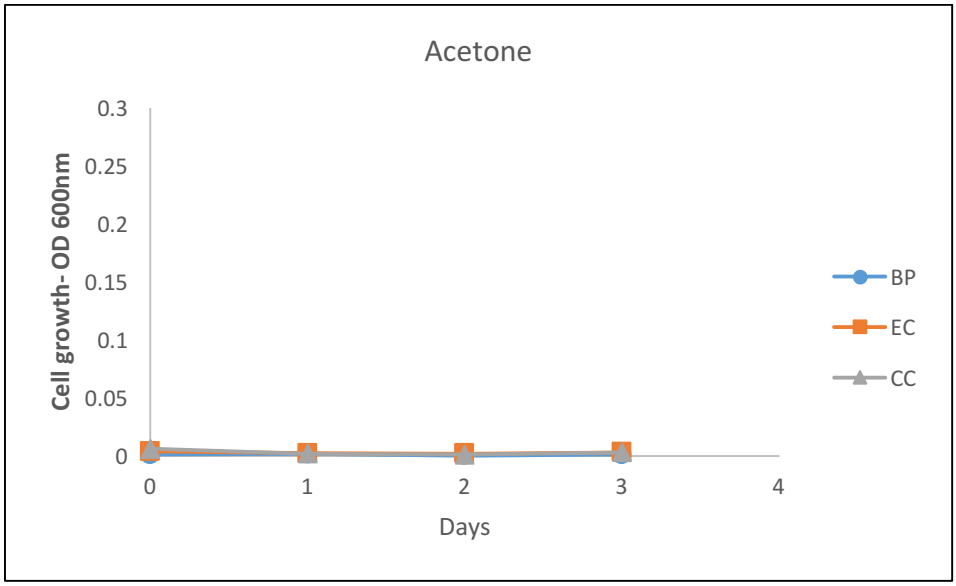



**Fig. 36: Effect of Acetone as a solvent (I$^{st}$ set)**

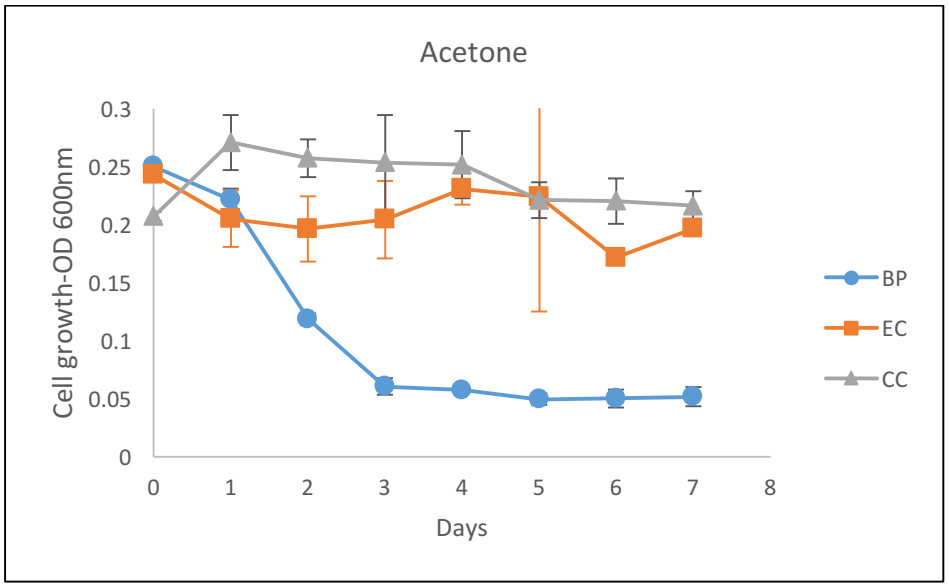



**Fig. 37: Effect of Acetone as a solvent (II$^{nd}$ set)**

***a) <u>Ethyl-acetate as a solvent:</u>***

In set 1 (Fig.38), no significant growth was observed. In set 2 (Fig. 39), BP showed a decrease in cells growth. EC cells sustained for whole study. For CC there was decrease in cells till day 5 but increase in cell on day 6 and again decreases on day 7. Though CC showing growth on day 6 this growth is not significant because of substantial variation in readings.

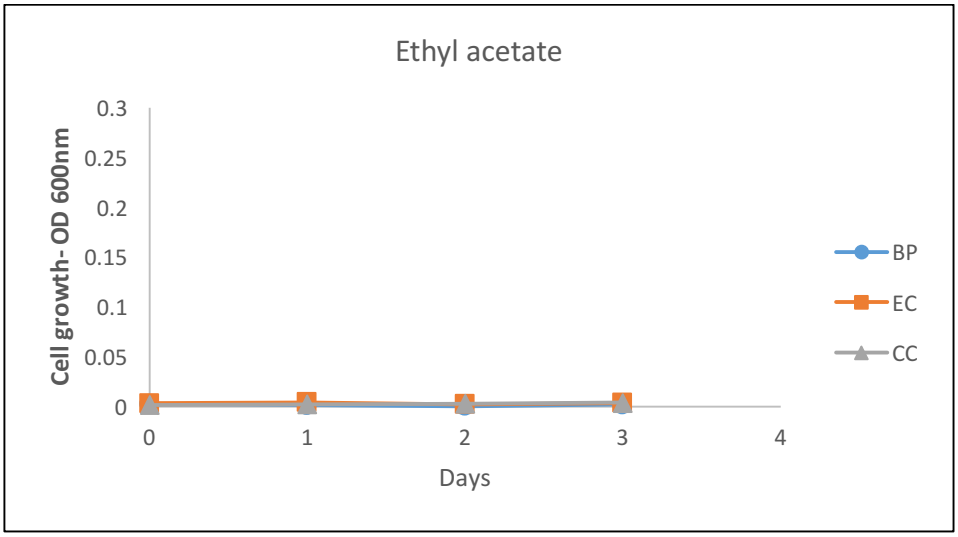



**Fig. 38: Effect of Ethyl acetate as a solvent (I$^{st}$ set)**

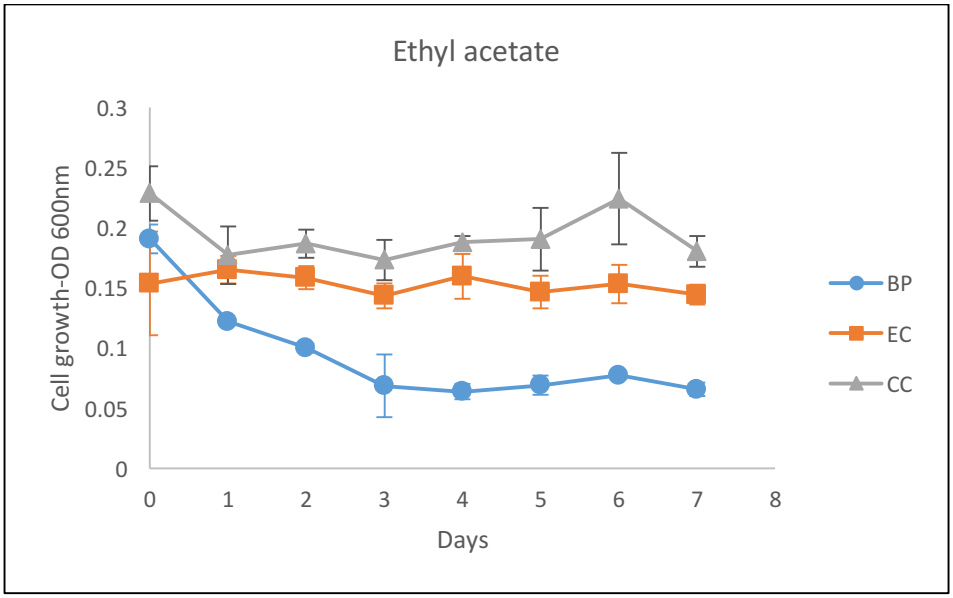



**Fig. 39: Effect of Ethyl acetate as a solvent (II$^{nd}$ set)**

***b) <u>Acetonitrile as a solvent:</u>***

In set 1 (Fig. 40), Acetonitrile was observed to have no significant effect on cell growth of BP, EC, CC. In set 2 (Fig. 41), Acetonitrile as a solvent for naphthalene showed gradual decrease of CC cells, and maximum growth on day 6, a decrease thereafter. For EC, cell growth was observed on day 1, with a gradual decrease till day 4, and again an increase on day 5.

The inhibitory effect of acetonitrile was observed on CC and EC in initial growth period this is because of the toxic effects of acetonitrile. But in later stage bacteria showed the adaptation to environment and cell growth was observed. Acetonitrile is good source of nitrogen and carbon, because of this showing growth in later phase of incubation. (Rezende, Dias, Monteiro, Carraza, & Linardi, 2003).

From these results, it can be concluded that acetone as solvent to dissolve Naphthalene may be supporting CC and EC for degradation of Naphthalene. Ethyl acetate did not show any positive effect on cell growth. Though Acetonitrile initially showed inhibitory effect on cell grwth, in later period of incubation it supported the cell growth and ultimately naphthalene degradation. Therefore, Acetone and Acetonitrile are found to be equally good for degradation.

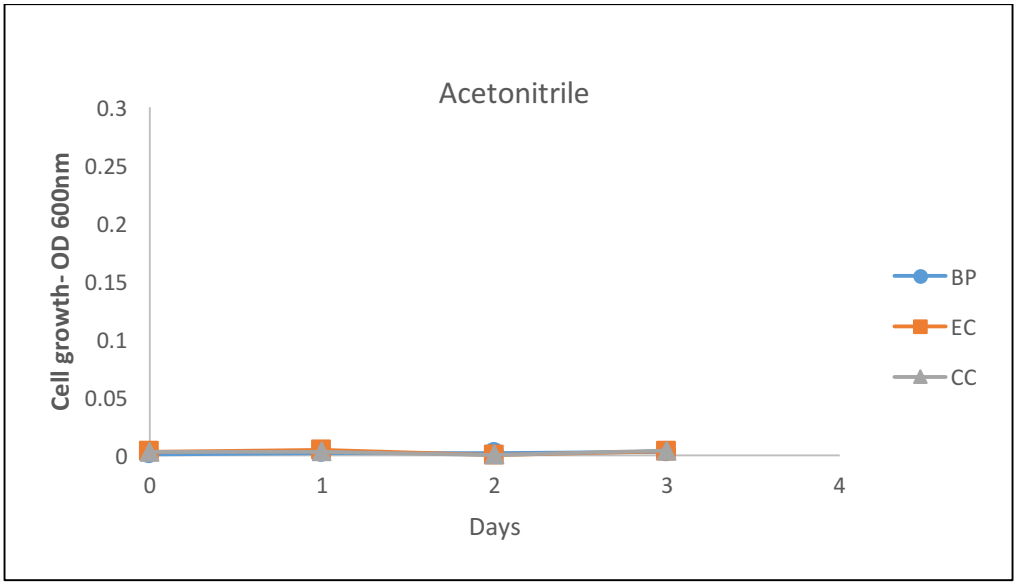



**Fig. 40: Effect of Acetonitrile as a solvent (I$^{st}$ set)**

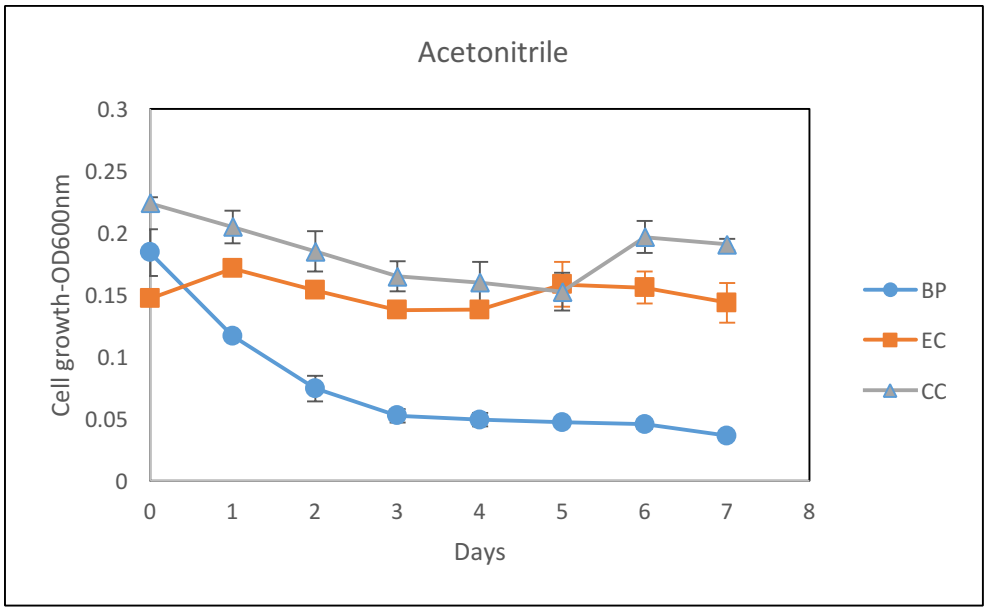



**Fig. 41: Effect of Acetonitrile as a solvent (II$^{nd}$ set)**

In this study, *Bacillus pumilus (BP)* did not show any significant growth and degradation of naphthalene at all used parameters. Bacterial strains *Enterobacter cloacae (EC)* and *Cellulosimicrobium cellulans (CC)* exhibited the optimum results when 50 ppm of naphthalene was dissolved in acetone using CSM media.

## *4.4 Consortium study:*

The details of the consortium study are given in Section 3.4.5. In set 1 (Fig. 42) a negligible growth was seen for CC+AB. This may be due to the initial low concentration of bacterial inoculum. In set 2 study (Fig. 43), it was observed that the consortium of CC+AB and EC+AB showed good results for 7-day study period. Consortium of CC+AB showed continuous growth till day 3, but it decreased on day 4, and again started to increase thereafter. This indicates that consortium degraded the naphthalene till day 3, after which the cell growth decreased because of inhibitory effect of metabolites, but after day 4 started to tolerate the metabolites formed. The consortium EC+AB degraded naphthalene, and may also be utilizing the metabolites formed during Naphthalene degradation.

In conclusion, consortium of CC+AB and EC+AB were efficient to degrade naphthalene compared to individual bacteria. These results imply that AB is contributing to degrade the metabolites formed by naphthalene break down which previously has shown inhibitory effect on EC and CC cell growth study. In a similar study (Reference (Vilas Patel, 2012)), it was reported that the consortium of EC+AB successfully degraded naphthalene.

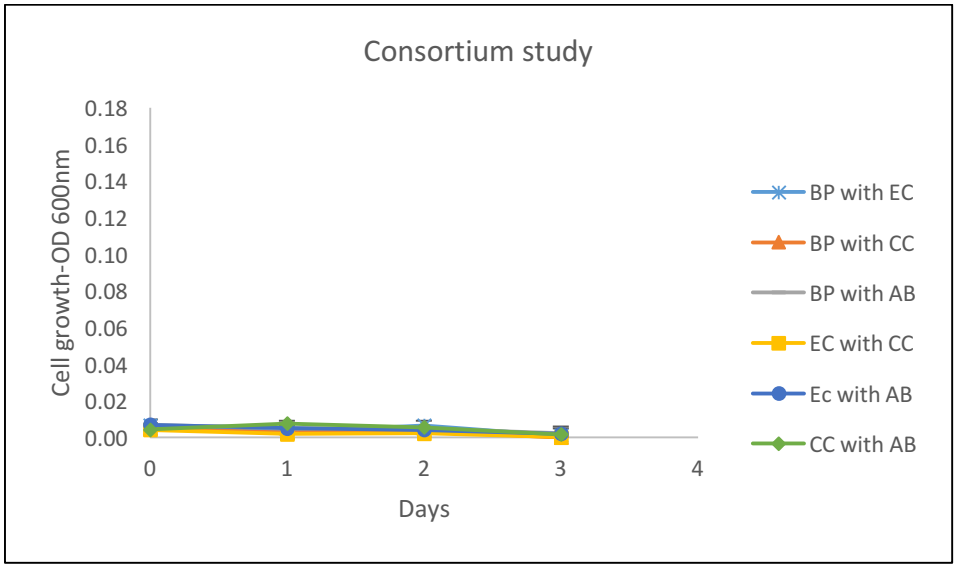



**Fig. 42: Consortium study (I$^{st}$ set)**

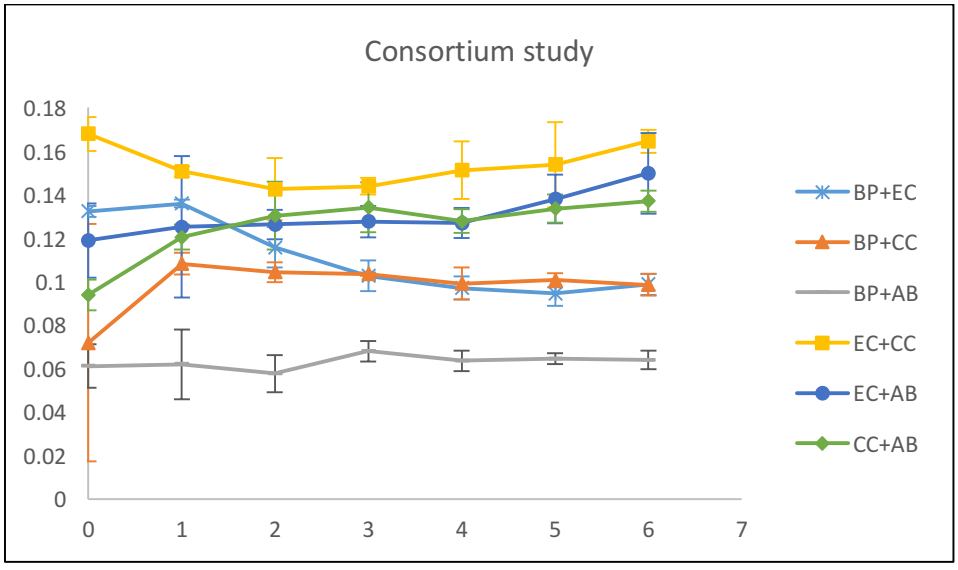



**Fig. 43: Consortium study (II$^{nd}$ set)**

### *4.5 Detection of Dioxygenase gene in isolated bacteria:*

The gel electrophoresis results are shown in Fig. 46. For the Biphenyl dioxygenase primers EC showed the smeared band and seen below 250 bp (marked by an arrow in Fig. 46), which indicated formation of primer dimer. A band was observed with the use of dfa2 primer for EC at approximately above 600 bp., which indicates presence of dioxygenase enzyme in *Enterobacter cloacae*. *Cellulosimicrobium cellulans* showed very light band below 500 bp. This indicates presence of dioxygenase gene but needs a further optimized study using different set of primers. The presence of dioxygenase gene in *Enterobacter cloacae* was confirmed using Dibenzofuran dioxygenase (dfa2) primers.

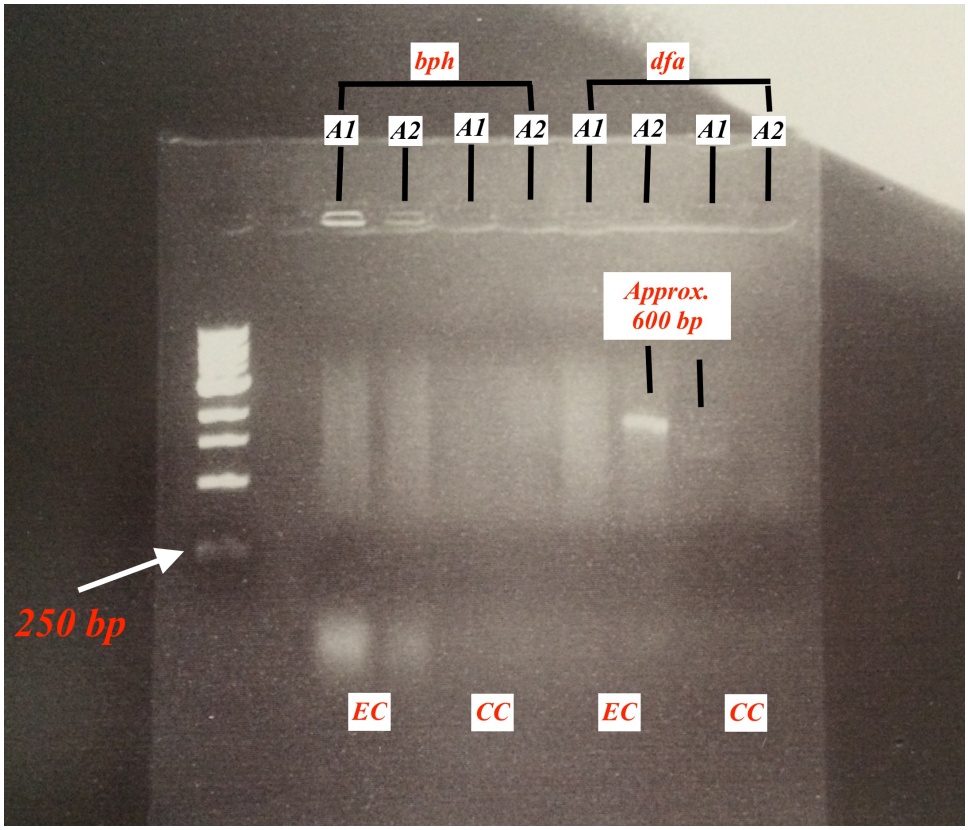



**Fig. 44: Gel electrophoresis results for Dioxygenase gene PCR**

# 5 Conclusions & Future Work

## 5.1 Conclusions

Soil collected from naphthalene contaminated sites (Valero and Exon Mobil oil refineries in Texas) contains the cultured *Enterobacter cloacae* and *Cellulosimicrobium cellulans* which can degrade naphthalene using it as a carbon source. Presence of dioxygenase gene also supported naphthalene degradation capacity of the isolated strains. From the effect of different conditions on cell growth, these bacteria can survive in presence of naphthalene by degrading it but unable to use the metabolites formed from naphthalene degradation. There are possibilities that naphthalene and metabolite accumulation has growth inhibition effect on microorganism. Comparison of consortium of bacterial study concluded that consortium of *Cellulosimicrobium cellulans* + *Achromobacter xylosoxidans,* and *Enterobacter cloacae* + *Achromobacter xylosoxidans* has naphthalene degrading capacity. This consortium can be further used for degradation of other PAH compounds.

## *5.2 Future work:*

1) Quantitative study of Naphthalene degradation using GC-FID or HPLC.

*2)* Identification of Naphthalene degradation pathway by *Enterobacter cloacae* and *Cellulosimicrobium cellulans* using GC-MS.

*3)* Study of degradation of other PAH compound using *Enterobacter cloacae* and *Cellulosimicrobium cellulans.*

*4)* Identification of gene from *Enterobacter cloacae* and *Cellulosimicrobium cellulans* responsible for degradation of PAH compounds.

5) Effect of bio-surfactant producing capacity of *Enterobacter cloacae* on degradation of PAH.

The above studies will give insight about the capacity of bacteria to degrade different PAH compounds. Identification of degradation pathway and enzyme responsible for the degradation will help to construct recombinant gene by recombining the catabolic genes from diverse microorganism in specific host cell to generate distinctive PAH metabolic pathway. Effect of bacterial bio-surfactant producing properties will help in study of bioremediation process as it affects the bioavailability of the PAH compounds for degradation. In summary, the study of above factors will deepen the knowledge and will aid in natural remediation of PAH contaminated environment.

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