Hydrocarbonoclastic Bacteria and the Strategies for Improving Biodegradation

THESIS

Submitted in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

By

Mr. RAJESH PASUMARTHI

Under the Supervision of

Dr. Srikanth Mutnuri

BITS Pilani
Pilani | Dubai | Goa | Hyderabad

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

2016

CERTIFICATE

This is to certify that the thesis entitled "**Hydrocarbonoclastic Bacteria and the Strategies for Improving Biodegradation"** submitted by **Mr. Rajesh Pasumarthi,** ID no. 2010PHXF433G for the award of Ph.D of institute embodies original work done by him under my supervision.

Signature of the Supervisor

Dr. Srikanth Mutnuri

Associate Professor

(Dept. of Biological Sciences)

Date: 22/07/2016

Acknowledgement

It is my pleasure to convey my gratitude to all those who supported and helped me in many ways during the completion of the thesis.

I am greatly indebted to my guide, Prof. Srikanth Mutnuri, Associate Professor, Department of Biological Sciences, BITS Pilani, K K Birla Goa Campus, for his guidance, valuable suggestions, patience, help and constant encouragement. His scientific experience, enthusiasm and consistency have influenced me throughout the successful completion of this research investigation.

I would like thank Prof. B. N. Jain, Vice-Chancellor, Prof. Sasikumar Punnekkat, Director, Prof. K. E. Raman, EX-Director, Late Prof. S. K. Agarwal, also Ex-Director, Prof. P.K Das, Associate Dean, ARD; Prof. S. Bhand, Dean SRCD; Prof. S.D. Manjare, Former-In-Charge RCED; for all their support.

I am thankful to my Doctoral Advisory Committee (DAC) members, Prof. Meenal Kowshik and Prof. Judith Braganca, who is also Head, Department of Biological Sciences, BITS Pialni, K K Birla Goa Campus for their valuable insights, help and guidance throughout my thesis.

My sincere thanks to the faculty members of the Department of Biological Sciences, Dr. Anasuya Ganguly, Prof. Vijayashree Nayak, Dr. Indrani Talukdar, Dr. Malabika Biswas, Prof. Utpal Roy, Prof. Dibakar Chakrabarthy, Dr. Sumit Biswas, Dr. Angshuman Sarkar, Dr. Veeky Baths, Dr. Sukanta Mondol, Dr. Kundan Kumar, Dr. Raviprasad Aduri and Dr. Arnab Banerjee for their cooperation throughout this study.

I am grateful to Dr. Mainak Banerjee, Department of Chemistry, BITS Pilani, K K Birla Goa campus, and his student Mr. Vikash Kumar for their continuous support in synthesis of hydroxy cucurbit[6]uril.

I owe my deepest gratitude to my colleagues Dr. Sivaraman, Dr. Vidhya Prabhudesai, Mr. Meghnath Prabhu, Mrs. Vishnavi Unde, Dr. Gowdaman, Mr. Ram Chavan, Mr. Anant

Yadav, Ms. Anumeha Vats, Mr. Guruprasad Talekar and Ms. Priya Sharma for their help, support, encouragement and motivation throughout this research.

I am deeply indebted to my dear friends and research scholars for their timely help, Mr. Kabilan, Mr. Chandrashekar, Mrs. Bhakti, Mrs. Sruthi, Dr. Vilas, Dr. Raeesh, Mr. Ajay Gosh, Ms. Ramya, Ms. Geethanjali, Mr. Gowtam Krishnan, Ms. Deepthi, Mr. Ram, Mrs. Pallavee, Mrs. Jigni, Mrs. Akrithi, Ms. Nupur, Ms. Mansi, Ms. Ansie, Ms. Kshipra Naik, Mr. Mansoor, Ms. Ketaki, Mr. Ankush, Ms. Priyanka, Ms. Archana, Mr. Rakesh and all the research scholars of the Biological Sciences Department for all the good times and fun, apart from the unconditional support and motivation.

I am grateful to BITS Pilani, K K Birla Goa Campus for providing financial support during this study.

My special thanks to Mr. Mahaling Lamani for his help during collection of sea water. I thank Mrs. Kamna and Mr. Mahadeo Shetkar for their timely help during the study.

Finally, I would not have able to complete this thesis without the understanding and support of my parents and family. Thank you all and I will be forever grateful to you all. Above all, I praise God, the almighty for providing me this opportunity and granting me this capability to proceed successfully.

Rajesh Pasumarthi

Dedicated to My Family

Who valued education above all…

CONTENTS

Chapter	Title	Page No.
1	Introduction and literature review	1-32
2	Isolation of hydrocarbon degrading bacteria and estimating their capabilities to degrade crude oil sediment	33-50
3	Biodegradation of aliphatic hydrocarbons in presence of hydroxy cucurbit[6]uril	51-66
4	Effect of rhamnolipid on biodegradation of non-aqueous phase liquids	67-85
5	Role of horizontal gene transfer in bioremediation	86-99
6	Biostimulation versus Bioaugmentation	100-113
7	Molecular interaction of hydrocarbons to outer membrane protein (TodX) of *Pseudomonas*	114-125
	Summary and conclusion	126-127
	Future Prospects	128
	References	129-156

TABLE OF CONTENTS

Page No.

COVER PAGE …….. I

CERTIFICATE…….. II

ACKNOWLWDGWMENT…….. III

CONTENTS…… VI

ABSTRACT…… XIII

LIST OF TABLES…….. XVI

LIST OF FIGURES..…….. XVII

LIST OF ABBREVATIONS AND SYMBOLS……... XXI

Chapter 1: introduction and literature review

1. Introduction…….1-4

1.1. Literature review…….5-32

1.2. Consequences of oil spills…….....8

1.3. Available cleanup techniques…….8-10

1.3.1. Physical methods……...8-9

1.3.2. Chemical methods…….9

1.3.2. Biological methods…….....9-10

1.4. Biodegradation of hydrocarbons……..10-13

1.4.1. Aliphatic hydrocarbons……...10-11

1.4.2. Aromatic hydrocarbons……...12-13

1.5. Factors affecting Bioremediation……..14-15

1.6. Bioavailabiltiy…….....16-19

1.6.1. Cucurbiturils…….....16-17

1.6.2. Bio surfactants (Rhamnolipids)……..18-19

1.7. Catabolic genes involved in hydrocarbon degradation……...20-24

1.7.1. Alkane hydroxylase……..20-23

1.7.2. Aromatic hydrocarbon dioxygenases…….....23-24

1.8. Horizontal gene transfer of catabolic genes…….24-26

1.9. Biostimulation…….26-27

1.10. Uptake of hydrocarbons……..27-28

1.10.1. Role of outer membrane proteins in hydrocarbon uptake…….....28-30

1.11 Gap in existing research…….....31-32

Chapter 2: Isolation of hydrocarbon degrading bacteria and estimating their capabilities to degrade crude oil sediment.

2. Introduction…….34-35

2.1 Material ……...35

2.1.1. Chemicals…….....35

2.2 Methodology ……35-39

2.2.1. Isolation of bacteria and screening hydrocarbanoclastic capacity……..35-36

2.2.1.1 Enrichment of culture……..35

2.2.1.2. Biodegradation of n-hexadecane as model hydrocarbon……35-36

2.2.2. Analysis of n-hexadecane degradation by Gas chromatography…...36

2.2.3. Identification of Bacteria…….36-37

2.2.3.1. Genomic DNA extraction…….....36

2.3.3.2. Amplification of 16SrDNA…….37

2.2.3.3. Denaturing gradient gel electrophoresis (DGGE)……37

2.2.4. Biodegradation of crude oil…….....38

2.2.4.1. Analysis of crude oil degradation by GC-MS……...38

2.2.5. Quantification of Microbial biomass…….....39

2.3. Results …….....39-48

2.3.1. Enrichment studies……39-41

2.3.2. Biodegradation of crude oil……..42-46

2.3.2.1. n-Alkanes…….....42-44

2.3.2.2. Poly aromatic hydrocarbons……....45-46

2.3.3. Growth of culture in crude oil……47

2.3.4. DGGE of the culture during experiment……...48

2.4. Discussion……49-50

2.5. Conclusion……...50

Chapter 3: Biodegradation of aliphatic hydrocarbons in presence of hydroxy cucurbit[6]uril

3. Introduction……..52-53

3.1. Materials……...53

3.1.1. Chemicals……53

3.2. Methodology…….....53-55

3.2.1. Synthesis of hydroxy CB[6], NMR and ESI-MS…….....53

3.2.2. Test for toxicity of hydroxy CB[6] on animal cell lines…….....54

3.2.3. Enrichment culture…….....54

3.2.4. Amplification of 16SrDNA and DGGE……...54

3.2.5. Biodegradation studies……..54-55

3.2.5.1. Microcosm…….....54-55

3.2.5.2. Analysis of hydrocarbons by Gas chromatography…….55

3.2.6. Quantification of Microbial biomass……..55

3.3. Results and Discussion…….56-66

3.3.1. Synthesis of hydroxy CB[6], NMR and ESI-MS…….56-57

3.3.2. Toxicity test…….58

3.3.3. DGGE……...59

3.3.4. Biodegradation studies……...60-65

3.3.4.1. Effect of hydroxy CB[6] on individual hydrocarbons……...60-62

3.3.4.2. Effect of hydroxy CB[6] on mixed hydrocarbons……..63-65

3.4. Discussion ……65-66

3.5. Conclusion……66

Chapter 4: Effect of Rhamnolipid on biodegradation of non aqueous phase liquids (NAPL)

4. Introduction……..68-69

4.1. Materials……...70

4.1.1. Chemicals and Microorganisms……70

4.2. Methodology……70-72

4.2.1. Biosurfactant Production……..70

4.2.2. Extraction of biosurfactant……...70-71

4.2.3. Estimation of biosurfactant……...71

4.2.4. Preparation of Model NAPL…….....71

4.2.5. Microcosm studies……..71-72

4.2.6. Cell surface hydrophobicity test (BATH assay)……..72

4.2.7. Quantification of Microbial biomass…….....72

4.2.8. 16SrDNA amplification and DGGE……72

4.3. Results…….....73-82

4.3.1. Biodegradation of hexadecane in presence of rhamnolipid……...73

4.3.2. Biodegradation of NAPL in presence of rhamnolipid……...74-77

4.3.3. Rhamnolipid estimation……....77-78

4.3.4. Cell surface hydrophobicity (CSH)…….79-80

4.3.5. Protein biomass…….80-81

4.3.6. DGGE……....82

4.4. Discussion……....83-85

4.5. Conclusion……...85

Chapter 5: Role of horizontal gene transfer in bioremediation

5. Introduction……87-88

5.1. Materials and Methods…….....89-91

5.1.1. Catabolic gene profile of consortium……...89

5.1.2. Collection of sea water and selection of culture…….....90

5.1.3. Microcosm studies…….....90

5.1.4. Gas chromatograph for residual diesel……..91

5.1.5. DNA extraction and DGGE…….....91

5.1.6. Different Parameters (pH, temperature, OD, N, P)……..91

5.2. Results…….....92-97

5.2.1. Catabolic gene profile of consortium…….....92

5.2.2. Collection of seawater and selection of bacteria……..93-94

5.2.3. Biodegradation of Diesel…….....94-95

5.2.4. Different parameters……..96

5.2.5. DGGE during diesel degradation……..97

5.3. Discussion……..98-99

5.4. Conclusion…….....99

Chapter 6: Biostimulation versus Bioaugmentation

6. Introduction……...101-102

6.1. Materials and Methods……102-104

6.1.1. Collection of seawater and selection of culture……102

6.1.2. NAPL design……....102

6.1.3. Microcosm studies……....102

6.1.4. Gas chromatography……...103

6.1.5. DNA extraction and DGGE……...103

6.1.6. Total Protein, Nitrogen and Phosphorus…….....103-104

6.2. Results……...104-111

6.2.1. Collection of seawater and selection of bacteria……..104

6.2.2. Biodegradation of NAPL……105-108

6.2.3. Total protein estimation……..109

6.2.4. DGGE……..110-111

6.3. Discussion……112-113

6.4. Conclusion……113

Chapter 7: Molecular interaction of hydrocarbons to outer membrane protein (TodX) of *Pseudomonas*

7. Introduction……...115-116

7.1. Methodology…….116-117

7.1.1. Multiple sequence alignment…….....116

7.1.2. Molecular docking……..116-117

7.2. Results……..118-123

7.2.1. Molecular interactions……...118-123

7.3. Discussion……124-125

7.4. Conclusion……...125

Summary and conclusions……...126-127

Future prospects……...128

References…….129-156

Appendices ……...157-158

List of publications…… Appendix 1

List of conferences and workshops……. Appendix 2

A Brief Biography of Supervisor……. Appendix 3

A Brief Biography of student…… Appendix 4

ABSTRACT

Marine oil spills either by accidental spillage or intentional disposal oil are a serious concern to Goa tourism sector. The spilled oil turns into tarballs due to physical, biological weathering and gets transported to beaches by ocean currents. Tarballs were collected from the beaches and subjected to incineration resulting in air pollution. It was proved that bioremediation is an ecofriendly technique involving hydrocarbon degrading bacteria. There are different problems faced during bioremediation of marine oil spills. For instance bioavailability of hydrocarbons was limited due to their hydrophobic nature, limitation of nutrients questioning the survival of employed bacteria, limited knowledge about hydrocarbon transport into the bacteria.

Hydrocarbon degrading bacteria was isolated using diesel oil as sole carbon source from Velsao beach, Goa. Denaturing gradient gel electrophoresis (DGGE) was performed for the enriched culture and it was found that *Pseudomonas aeruginosa* (AEBBITS1) and *Escherichia fergusonii* (AEBBITS2) were present. Microcosm study was performed for 45 days to analyze the capabilities of bacteria to degrade crude oil sediment (tarball from Velsao beach). Based on GC-MS results it was found that alkanes were degraded extensively (98% to 66%) compared to Polyaromatic hydrocarbons (PAH). It was also found that the degradation of PAHs was decreased with increase in ring number and the alkylated PAHs were less degraded compared to unalkylated ones. DGGE was performed for the microcosm setup and it was evident from the result that both AEBBITS1 and AEBBITS2 have survived till last day of the experiment.

Hydroxy cucurbit[6]uril (Hydroxy CB[6]) was a cyclic polymer with a hydrophobic inner cavity. It was reported that cucurbiturils are potential hydrophobic drug delivery agents and also their host-guest interaction with hydrocarbons was reported. Degradation of aliphatic hydrocarbons (Tetradecane, Hexadecane, Octadecane) in presence of Hydroxy cucurbit[6]uril was studied. Microcosms were setup using two different consortiums i.e., Halo (AEBBITS1 + AEBBITS2) and Non Halo. Halo is the culture in presence of 3% NaCl and Non Halo is without NaCl. It was found that the degradation of tetradecane and hexadecane in microcosms supplied with Hydroxy CB[6] was more compared to the microcosms without Hydroxy CB[6]. There was no considerable difference in degradation of octadecane by

addition of Hydroxy CB[6]. These hydrocarbons were used as mixture (1:1:1) and it was found that the degradation of all three hydrocarbons was increased (tetradecane-13%, hexadecane-15%, octadecane-8%) by Halo but not Non Halo. This might be due to increased solubility of Hydroxy CB[6] in presence of NaCl.

Taking the process of synthesis of Hydroxy CB[6] into account and also the limitation of cavity size to accommodate different hydrocarbons, Rhamnolipid was used to increase the bioavailability of both aliphatic and aromatic hydrocarbons. Rhamnolipid is a biosurfactant with amphiphilic nature and was reported earlier to increase the hydrocarbon degradation by increasing cell surface hydrophobicity (CSH). The degradation of Non aqueous phase liquid (NAPL) by Halo (AEBBITS1 + AEBBITS2) in presence of rhamnolipid (100 ppm) and rhamnolipid producing bacteria (*Pseudomonas*-AS7) was studied. It was found that the degradation of different hydrocarbons present in NAPL (Naphthalene, Phenanthrene, Tetradecane, Hexadecane, Octadecane) was increased by addition of rhamnolipid. The CSH of Halo was increased in presence of rhamnolipid compared to Halo and AS7. By 120 hours it was found that the amount of rhamnolipid in microcosm inoculated with AS7 was 147.24 mg/L whereas in microcosms inoculated with Halo it was (73.5 mg/L). Based on DGGE result it was found that the synergistic relation between AEBBITS1 and AEBBITS2 was disturbed by addition of AS7.

Bioaugmentation is addition of hydrocarbon degrading bacteria to the contaminated site. Horizontal gene transfer of catabolic genes to the indigenous bacteria would be an alternative in case of failed bioaugmentation (low survival rate of used bacteria). The reliability of HGT of catabolic genes to indigenous bacteria during marine bioaugmentation has not been investigated. In present study HGT was found to be unreliable as there was no change in community of hydrocarbon degrading bacteria by addition of AEBBITS1. On the other hand addition of nitrogen and phosphorus to bioaugmentation resulted in high degradation of NAPL by 96 hours of incubation.

Studying the uptake mechanism of the hydrocarbon would help in designing a better bioavailability strategy for better bioremediation. Molecular interaction of known hydrocarbon transporter protein from Pseudomonas (TodX) with different hydrocarbon was studied using AUTODOCK. It was found that all the used hydrocarbons interact with the

interior hatch domain and S3 kink of the beta barrel protein. It was proposed based on the crystal structure of the protein that the hydrocarbons go into the barrel and pass into cytoplasm through the lateral opening created by S3 kink. The binging positions of the hydrocarbon near to hatch domain and S3 kink support the same theory of transport. It was also observed that the aminoacids interacting with hydrocarbon are conserved among different hydrocarbon transporter proteins (TmoX, Tbux etc.).

LIST OF TABLES

Table	Title	Page no.
Chapter 1		
1a	List of major oil spills around the Globe	**6**
1b	List of major oil spills around in India	**7**
Chapter 2		
2a	Degradation of alkanes	**43**
2b	Degradation of polyaromatic hydrocarbons	**46**
Chapter 5		
5a	PCR program for different catabolic genes	**89**
5b	Amplification of different catabolic genes of the consortium	**92**
5c	Different parameters on day 7, 14, 21 of the experiment	**96**
Chapter 6		
6a	Amount of nitrogen and phosphorus in different microcosm setups on first and last day of experiment	**104**
Chapter 7		
7a	Multiple sequence alignment (interaction of aminoacids with different hydrocarbons near hatch domain)	**122**
7b	Multiple sequence alignment (interaction of aminoacids with different hydrocarbons near S3 kink)	**123**

LIST OF FIGURES

Figure	Title	Page no.
Chapter 1		
1	Map of Velsao beach, Goa	**3**
1.1	Aerobic alkane degradation pathway by terminal and subterminal oxidation	**11**
1.2	Aerobic naphthalene degradation pathway	**12**
1.3	Different factors affecting bioremediation	**15**
1.4	Schematic diagram of action of alkane hydroxylase system	**20**
1.5	Multicomponent Naphthalene dioxygenase system	**24**
Chapter 2		
2.1	Degradation of n-hexadecane as model hydrocarbon	**40**
2.2	DGGE and Phylogenetic analysis	**41**
2.3	Abundance of alkanes of abiotic and biotic samples representing degradation	**44**
2.4	Total protein biomass during degradation of crude oil	**47**
2.5	DGGE of crude oil degradation experiment	**48**
Chapter 3		
3.1	ESI-MS of hydroxy cucurbit[6]uril	**56**
3.2	^{1}H NMR spectrum of hydroxy cucurbit[6]uril	**57**

3.3	^{13}C NMR spectrum of hydroxy cucurbit[6]uril	**57**
3.4	Microscopic picture of toxicity test using hydroxy CB[6] against animal cell lines	**58**
3.5	DGGE of consortiums (Halo and Non halo)	**59**
3.6	Tetradecane degradation as sole carbon source	**60**
3.7	Hexadecane degradation as sole carbon source	**61**
3.8	Octadecane degradation as sole carbon source	**62**
3.9	Degradation of tetradecane in a mixture	**63**
3.10	Degradation of hexadecane in a mixture	**64**
3.11	Degradation of octadecane in a mixture	**64**
3.12	Protein biomass during degradation of mixture of hydrocarbons	**65**
Chapter 4		
4.1	Degradation of hexadecane as sole carbon source	**73**
4.2	Degradation of naphthalene present in NAPL	**75**
4.3	Degradation of phenanthrene present in NAPL	**75**
4.4.	Degradation of tetradecane present in NAPL	**76**
4.5	Degradation of hexadecane present in NAPL	**76**

4.6	Degradation of octadecane present in NAPL	**77**
4.7	Estimation of rhamnolipid during hexadecane degradation as sole carbon source	**78**
4.8	Estimation of rhamnolipid during NAPL degradation	**78**
4.9	Cell surface hydrophobicity during hexadecane degradation as sole carbon source	**79**
4.10	Cell surface hydrophobicity during NAPL degradation	**80**
4.11	Total protein biomass during hexadecane degradation as sole carbon source	**81**
4.12	Total protein biomass during NAPL degradation	**81**
4.13	DGGE of the co-culture during hexadecane degradation as sole carbon source	**82**
Chapter 5		
5.1	Microcosm setup for diesel degradation and HGT	**90**
5.2	Growth of AEBBITS 1 and AEBBITS 2 using diesel as sole carbon source	**93**
5.3	Agarose gel electrophoresis of *alk*B and NDO genes from AEBBITS 1	**94**
5.4	Degradation of diesel in microcosm (seawater+diesel)	**95**

5.5	Degradation of diesel in microcosm (seawater+diesel+AEBBITS 1)	**95**
5.6	DGGE during biodegradation of diesel	**97**
Chapter 6		
6.1	Degradation of tetradecane in different microcosms	**106**
6.2	Degradation of hexadecane in different microcosms	**106**
6.3	Degradation of octadecane in different microcosms	**107**
6.4	Degradation of naphthalene in different microcosms	**108**
6.5	Degradation of phenanthrene in different microcosms	**108**
6.6	Protein biomass during NAPL degradation	**109**
6.7	DGGE gel representing diversity of NAPL enriched bacteria	**110**
6.8	Phylogenetic analysis of different bacteria from DGGE	**111**
Chapter 7		
7.1	Interaction of aliphatic hydrocarbons to TodX	**119**
7.2	Interaction of hydrocarbons to S3 kink	**120**
7.3	TodX interaction with Benzene and Toluene near hatch domain	**121**
7.4	TodX interaction with Benzene and Toluene near S3 kink	**121**

List of Abbreviations and Symbols

PAH- Polyaromatic hydrocarbons

BH- Bushnell Haas medium

NAPL- Non aqueous phase liquid

Halo- Consortium in medium having 3% NaCl

Non halo- Consortium in medium without 3% NaCl

GC- Gas chromatography

GC-MS- Gas Chromatography coupled with Mass Spectrometry

OD- Optical density

µl- micrometer

ml- millileter

Hrs- hours

g/L- Grams/ Liters

MSA- Multiple sequence alignment

PCR- Polymerase chain reaction

EtBr- Ethidium bromide

% - Percentage

Rl- Rhmanolipid

Naug – Natural augmentation

Bst- Biostimulation

Baug- Bioaugmentation

Bst Baug- Biostimulated Bioaugmentation

N- Nitrogen

P- Phosphorus

COD- Chemical oxygen demand

OMP- Outer membrane protein

Gly- Glycine

Thr- Threonine

Ser- Serine

Val- Valine

Leu- Leucine

Glu A- Glutamic acid

Tyr- Tyrosine

Arg- Arginine

Glutm- Glutamine

Phe- Phenylalanine

CB[6]- Cucurbit[6]uril

CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

INTRODUCTION

Environmental pollution caused by petroleum hydrocarbon is of great concern because hydrocarbons are ubiquitous environmental pollutants with carcinogenic and mutagenic potential (Pashin et al., 1979, Masiol et al., 2012). According to U.S energy information administration, about 90.1 million barrels of petroleum and other liquid products were produced per day in 2013. It has been estimated that about 63% of this was transported by marine means. There are different choke points for oil transport around the world among which the Strait of Hormuz and Strait of Malacca are known for their highest volume transit. Strait of Hormuz and Strait of Malacca are located on west and east coast of Indian peninsular region respectively and are well known as accident prone for their narrow routes. Marine oil spills has took every ones attention after the deep water horizon in the Mexican gulf. Accidental oil spills and frequent illegal disposal of oily waste into the sea results in serious damage to marine life. The marine ecosystem is being affected by oil spills due to the presence of toxic organic compounds in the oil, which also affects human health through biomagnification (Dasgupta *et al*., 2013, D'Adamo et al., 1997). There are different methods to cleanup oil spills; they are broadly classified into mechanical, chemical, physical and biological methods of containment. The mechanical containment process includes booms and skimmers which blocks the movement of spilled oil to the shores and removes the oil using boats. The physical recovery involves the evaporation, oxidation, pressure washing, bulldozing etc. Chemicals like dispersing agents are used to stop oil reaching shore and other sensitive areas.

As a result of physical and chemical weathering of oil after spillage, the lighter components of oil will evaporate leaving the denser ones to deposit as thick oil. The condensed thick oil deposits at the bottom of sea or sticks to rocks and sand. The so formed pieces of weathered oil are called tarballs which gets washed away onto coasts due to ocean currents and high tides. According to National Institute of Oceanography (NIO), Goa, the appearance of tarballs along the west coast of India is an annual phenomenon (Dhargalkar et al., 1997). The tarballs from Goan beaches are collected by beach management and sent to oil refinery for incineration, but the incineration process involves disadvantages like expensiveness, lack of

facilities in Goa and more over long term storage of waste etc. Incineration of tarballs or crude oil sediments results in oil smoke. This oil will be inhaled (as volatile organic compounds) causing chemical poisoning called hydrocarbon pneumonia (Salako et al., 2012).

Bioremediation is a better alternative for treating hazardous contaminants as there is no deteriorating effect on the environment. It is less expensive compared to other techniques. Bioremediation can be defined as the natural ability of living microorganisms to reduce or remove or transform organic and inorganic pollutants (Valdi et al., 2001). There are different microorganisms that could degrade hydrocarbons (Saadoun et al., 2002, Head et al., 2006). Bioaugmentation is a process of bioremediation in which a potential hydrocarbon degrading bacteria will be introduced to the contaminated site. Although the process of bioaugmentation is successful, it has been criticized for its failures as the bacteria used in the process might not be able to survive in the field environment. Horizontal gene transfer (HGT) can help in overcoming this situation by the transfer of respective catabolic genes to the indigenous microbial population (Halvorson et al., 1985). Apart from this the success of bioremediation depends on the availability of nutrients, availability of hydrocarbons to the microorganisms

(Maier et al., 2000) and understanding how these hydrophobic compounds i.e., hydrocarbons are being transported into the bacterial cells. Therefore the objectives of this study are

1. To isolate hydrocarbon degrading bacteria from the Goan coast and to characterize the hydrocarbon degrading capabilities of the isolates.
2. To increase the bioavailability of hydrocarbons for enhancing biodegradation.
3. To study the process of bioremediation in seawater and observe the chances of natural horizontal gene transfer.
4. To investigate bioaugmentation versus biostimulation in improving bioremediation.
5. To understand the role of outer membrane proteins in hydrocarbon uptake by bacteria.

1.1 REVIEW OF LITERATURE

A conventional oil production based on geochemical knowledge states that oil is produced from source rock which is formed from decayed organisms. The proteins, cellulose, lignin etc., breaks down into simpler amino acids and sugars which are further degraded. It has been observed that fats remain undegraded and it has been stated by Kenneth Peters (1993) that molecules in oil and living organism have same back bone structures. The organic matter was buried in great depths of high temperatures resulting in the gradual conversion of organic matter to insoluble organic matter known as kerogen which forms the source rock. A source rock can be defined as organic rich rock capable of generating petroleum by exposing it to sufficient heat and pressure. More than 3 billion tons of oil has been produced per year and about half of this is being transported by marine means (Harayama et al., 1999). In the first quarter of 2014 Russia, Saudi Arabia, United States, China, Canada, Iraq, Iran, United Arab Emirates, Kuwait, Mexico stands as top ten producers of oil according to U.S energy information administration. During exploration, production, refining and transportation of oil, leaks and accidental spills occur and it has been estimated that about 60,000 metric tons seeps every year (Kvenvolden et al., 2003).

Oil spill not only disturbs the marine ecosystem but also human health and has social impact (Aguilera et al., 2010). When there is an incident of oil spill, the impact will be predicted based on its location. Spills that occur close to shore and human population have great impact and are reported to be more expensive to clean (Ektin et al., 1999). According to Kontovas et al., (2010) the cost of cleaning up oil spills has been divided into three categories i.e., removal, socioeconomic losses, environmental losses. It has also been stated by Kontovas et al. that the cost of an oil spill is very difficult quantity to estimate involving different factors like type of oil, location of the spill, amount of oil spilled and spillage rate, weather and sea conditions at the time of the spill etc. There are several oil spills around the globe and also Indian coast, which have leaked huge amounts of oil into the marine environment. Some of the oil spills are listed in table 1a and 1b.

TABLE 1a: LIST OF MAJOR OIL SPILLS AROUND THE GLOBE					
S.no	Place	Year	Ship/tanker/driller	Spill	Amount of spilled oil (Tons)
1.	Gulf of Mexico	2010	Tanker/ Crude oil	*Deepwater Horizon*	650,000
2.	Nova Scotia, Canada	1988	Tanker/ Crude oil	*Odyssey* spill	132,000
3.	Prince William Sound, AL, USA	1989	Tanker/ Crude oil	*Exxon Valdez*	104,000
4.	Shetland, UK	1993	Tanker/ light Crude oil	*Braer*	85,000
5.	Southwest Wales, UK	1996	Tanker/ light Crude oil	*Sea Empress*	72,000
6.	Karachi, Pakistan	2003	Tanker/ light Crude oil	*Tasman Spirit*	36,000
7.	Northwestern Spain	2002	Tanker/ heavy fuel oil	*Prestige*	62,000
8.	Bay of Biscay, France	1999	Tanker/ fuel oil	*Erika*	25,000
9.	Daesan, South Korea	2007	Tanker/ Crude oil	*Hebei Spirit*	10,800
10.	Western Honshu, Japan	1997	Tanker/ Crude oil	*Nakhodka*	6,000

TABLE 1b: LIST OF MAJOR OIL SPILLS IN INDIA				
s.no	Place	Date	Ship/tanker/driller	Amount of spilled oil (T)
1	795 nm SW of Mumbai	1989	MT Puppy	5,500/ diesel oil
2	Mumbai High	14-11-1991	MT Zakir Hussain	40,000/ crude oil
3	45 nm west of kochi	2-4-1992	MT Homi Bhabha	1000/ crude oil
4	Off Nicobar islands	21-1-1993	Maersk Navigator	40,000
5	Mumbai high	17-5-1993	BHN riser pipe rupture	6,000
6	Off sacromento pt.	12-5-1994	Innovative -1	1,600
7	Vizag	14-9-1997	HPC refinery	Naphtha, diesel petrol
8	Hogy river	10-7-2001	MV Lucnam	1,305/ diesel oil
9	Mumbai harbor	9-5-2003	MT UPCO - III	2,000/ naphtha
10	Near A&N islands	14-8-2006	MV Bright Artemis & MV Amar	4,500
11	South Gujarat & Maharashtra	6-8-2009	Not known	200/ oil debris
12	Panna offshore near Panna SBM	20-7-2010	PMJ joint venture	80
13	Mumbai	7-8-2010	MV chitra	600

1.2 Consequences of Oil Spills

Oil spills in marine environment effects the marine life in different ways as the toxicity of oil depends on the composition or source of oil and the place of incidence. It has been reported that marine oil spill has disastrous effect on seawater birds, corals, mammals, fishes etc. (Teal et al., 1984, Piatt et al., 1990). Formation of oil slick after spillage reduces the levels of dissolved oxygen and in turn affects the aerobic beings in the sea and also reduces the rate of photosynthesis as the oil slick hampers light penetration into water (Onwurah et al., 2007). The toxicity of polycyclic aromatic hydrocarbons (PAH) increases with increase in ring number and toxicity in specific to aquatic organisms was reported by Heinzetal et al., (1999). Biomagnification of hydrocarbons through food chain leads to deposition in humans. It has been observed that intestinal absorption rate of PAHs is very high due to their high solubility in lipids (Bamforth and Singleton et al., 2005). The anticoagulant potency of hydrocarbons was reported by Onwurash et al., (2002). Benzene, Toluene, ethylbenzene and Xylene (BTEX) are known for their toxic nature and many studies have been conducted that proves the same. Polyaromatic hydrocarbon are known for their carcinogenic nature and Benzo[a]Pyrene is most potent carcinogen, therefore announced by US environmental protection agency as priority pollutant (Juhasz et al., 2000). The epoxides formed from PAHs by the action of Cytochrome P450 monooxygenase forms covalent adducts with DNA resulting in tumour (Harvey 1996).

1.3 Available Clean up Techniques

There are different physical, chemical, biological cleanup techniques available for cleaning oil spills and the usage mainly depends on various factors like location, type of oil, weather, sea conditions, amount of spillage etc.

1.3.1 Physical methods

Physical methods are used to control the trajectory or movement of oil in the sea using different physical barriers without affecting the chemical characteristics of oil. Booms, Skimmers, absorbent materials are different barriers used (Fingas et al., 2011, Vergetis et al., 2002). Booms are type of equipment used to prevent the spreading of oil by blocking its movement. There are three types of booms available i.e., fencing booms, curtain booms and fire resistant booms. Skimmers are the devices equipped with disks, belts, drums and brushes

(Larson 2010, Hammoud 2001) used to recover oil from water. There are weir, oleophilic, suction skimmers available (Jensen et al., 1995, Ventikos et al., 2004). Adsorbent materials facilitate the conversion of oil to semisolid phase which in turn results in easy recovery (Adebajo et al., 2003). Natural organic adsorbents like peat moss, kapok, sawdust, vegetable fibers, milk weed, and straw are available (Karakasi et al 2005). Inorganic adsorbents like clay, glass wool, vermiculate are available (Holakoo 2001). Synthetic adsorbents are widely used commercial sorbent materials. In the incident of oil spill usually booms take the first position to contain oil and skimmers play the recovery action and finally adsorbent material will be applied to remove the left overs.

1.3.2 Chemical methods

Dispersants and Solidifiers are the two broad classification of chemical agents used. Application of chemical methods changes the chemical characteristics of oil. Dispersants are surfactants that breakdown oil into small drops which results in easy degradability by increasing the surface area of oil. Addition of dispersants was proved to be less costly than physical methods (Holakoo et al., 2001) but they have the disadvantage of foul smell and drinking water contamination (NRC 1989). Solidifiers are granular materials that react with oil and change it to solid state followed by recovery in combination of booms and skimmers. Solidifiers have not been used extensively because of their relative high cost compared to dispersants (Fingas et al., 1995).

Ten criteria like cost, efficiency, time, impact on wildlife, reliability, level of difficulty, oil recovery, weather, effect on chemical characteristic of oil and need for further treatment were taken into consideration in a comparative study done by Dave and Ghaly (2010). They have concluded that effective response for marine oil spill should start with mechanical methods followed by application of dispersants and finally bioremediation.

1.3.3 Biological methods

Bioremediation

Bioremediation is natural process taken up by the microorganisms to degrade or metabolize pollutants and restore the pristine environment. It has been reported that hydrocarbon degrading microorganisms are present in marine, fresh water, soil ecosystems. *Pseudomonas,*

Achromotobacter, Arthrobacter, Alcalygenes, Bacillus, Nocardia, Flavabacter are highly reported bacteria for hydrocarbon degradation (Halassanshahian et. al., 2013). *Pseudomonas, Psudidomarina maritima, Marinobacter, Vibrio, Halomonas, Microbulbifer, Microbacterium* are reported to be hydrocarbon degrading bacteria present in beach sands of Gulf of Mexico (Kostka et al., 2011). Straight, branched, cyclic alkanes, aromatics are readily degradable probably due to low molecular weight but polyaromatic hydrocarbons (PAHs) are slightly or slowly degradable due to their less water solubility. The order of degradation has been described by various peoples as linear alkanes> branched alkanes> small aromatics> cyclic alkanes> PAHs (Zhang et al., 2011).

Marine environment is the largest habitat accounting for more than 90% of total biosphere of planet earth. The microorganisms present in marine ecosystems do more than 50% of global primary production and nutrient cycling (Lauro et al., 2009). Marine environment is known for its adverse and sudden changes in temperature, pH, water currents, salinity etc. So, the bacteria inhabiting marine ecosystems are suitably adaptable for the fluctuations in the environmental conditions. Many marine bacteria are reported for their potential to remove heavy metals, hydrocarbons and production of biosurfactants (Rainbow 1995, Mannerat and Phetrong 2007). To study the diversity of marine bacteria, metagenomics studies like denaturing gradient gel electrophoresis, restriction fragment length polymorphism are employed. *Acinetobacter, Micrococcus, Planococcus, Rhodococcus, Methylobacterium, Nocardia* etc., are well known for their capability to degrade oil in marine environment (Sakalle and Raj kumar 2009). It was stated by Piskorska et al., (2007) that mainly six categories of bacteria namely α, β, and γ Proteobacteria, Actinobacteria, Bacilli, and Flavobacteria are present in Indian Ocean. Mukherji et al (2004) has isolated hydrocarbon degrading bacteria from Arabian Sea sediment.

1.4 Biodegradation of hydrocarbons

1.4.1 Aliphatic hydrocarbons

Alkanes are saturate form of hydrocarbons and are available in different types like linear, cyclic, branched alkanes. They are up to 50% of crude oil and are known for their chemically inert nature. Enzymes like monooxygenases or hydroxylases are required to activate alkanes by incorporating oxygen either by terminal or subterminal oxidation. The inert nature of

hydrocarbons has overcome by these enzymes by generating reactive oxygen species. As a result of oxidation of terminal methyl group a primary alcohol was produced which was converted to aldehyde by aldehyde dehydrogenase. The so formed product was converted into fatty acid and further converted to acetyl-coA by β-oxidation of fatty acids. ω-oxidation is the process where both the terminal methyl groups are oxidized resulting in formation of ω-hydroxy fattyacid which was further converted into dicarboxylic acid by β-oxidation. The subterminal oxidation of n-alkanes generates a secondary alcohol which was later converted to corresponding ketone followed by oxidation to render an ester. An enzyme called easterase hydrolyses the product to

alcohol and fattyacid. Compared to linear alkanes the degradation of branched or cyclic alkanes was less examined and it has been reported that it may involve ω-oxidation pathway (Rojo 2009). Unsaturated alkenes are oxidized at the saturated end of the hydrocarbon chain and then further turn into fatty acids. The complex branching like tertiary butyl groups in branched chains hinders the degradative enzyme action. In case of cyclic alkanes the absence of terminal methyl group for oxidative attack results in resistance for degradation but a few bacteria are known for their capabilities to degrade branched and cyclic alkanes (Rojo 2009) (Fig 1.1).

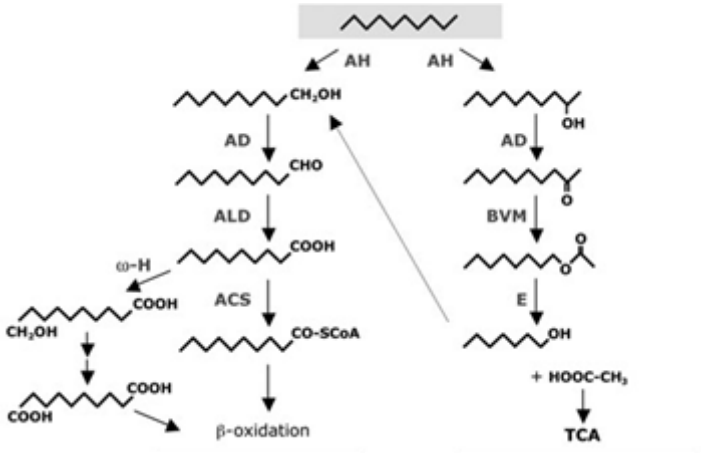

Fig1.1: Aerobic alkane degradation pathway by terminal and subterminal oxidation. (Rojo et al., 2009)

1.4.2 Aromatic hydrocarbons

Aromatic hydrocarbons are organic molecules having aromatic ring made of carbon and hydrogen. Polyaromatic hydrocarbons are type of aromatic hydrocarbons having more than one aromatic ring leading to formation of linear, angular cluster arrangements based on the fusion of aromatic rings. The biodegradation of polyaromatic hydrocarbon is of great interest due to their structural complexity. The first step of incorporating oxygen into the aromatic ring is common for all aromatic hydrocarbons. The degradation pattern of different hydrocarbons was extensively reviewed and it has been stated that the *cis*-dihydrodiols formed by the action of mono or dioxygenases are further oxidized to aromatic dihydroxy compounds (catechol) and then go through ortho or meta cleavage pathways. As a result, the precursors of tricarboxylic acid cycle are formed. For instance Naphthalene degradation is extensively studied for its simple structure and comparatively high solubility. Naphthalene is oxidized by naphthalene dioxygenase to cis-1,2dihydroxy-1,2-dihydronaphthalene which is later converted to 1,2-dihyroxynaphthalene by naphthalene(+)-*cis*-dihydrodiol dehydrogenase. 1,2-dihyroxynaphthalene is converted to *cis*-2-hydroxybenzal pyruvate and then to salicylate and pyruvate. Salicylate is oxidized to catechol by salicalate hydroxylase and further process is carried out by ortho or meta fission. All polyaromatic hydrocarbons follow a similar pattern of degradation involving oxygenase enzymes but the speed of reaction and intermediates or byproduct production depends on the number of aromatic rings and also the bacteria involved (Mrozik et al., 2003, Seo et al., 2009) (Fig 1.2).

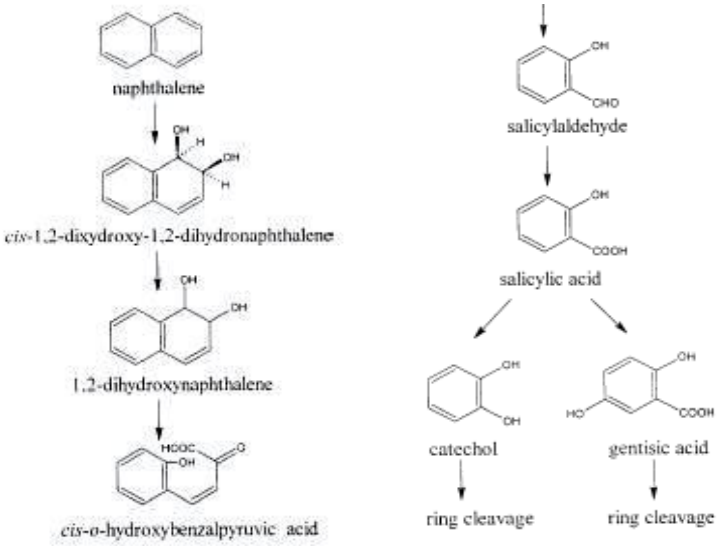

Fig 1.2: Aerobic Naphthalene degradation pathway (Rojo et al., 2009)

From the above mentioned broad scheme of aerobic metabolism any type of hydrocarbon degradation can be divided into four steps as described by Fritsche and Hofrichter (2000) –

1. Process for contact between microbial cells and the hydrocarbons.
2. The oxidative intracellular attack on hydrocarbons (incorporation of oxygen into hydrocarbons).
3. The peripheral degradation pathways leading to the formation of intermediate compounds of TCA cycle.
4. Biosynthesis of cell biomass.

Bioremediation is divided into three types 1) Natural attenuation 2) Bioaugmentation 3) Biostimulation. Natural attenuation is the ability of the local or indigenous bacteria to degrade oil. Bioaugmentation is addition of hydrocarbon degrading bacteria to the contaminated site and biostimulation is stimulating the growth of hydrocarbon degrading bacteria by supplying extra required nutrients. It was proposed that bioremediation was non-invasive and relatively less costly (April et al., 2000). It has been estimated by Etkin et al., (2000) that bioremediation is the cheapest available cleanup technique. It has been estimated bioremediation is 18.9, 9.2, 4.6, 2.5 times less costly than manual cleaning, skimming (mechanical removal), dispersants, insitu burning respectively. The degradation of hydrocarbons is tough as they are apolar, less chemically reactive and with no functional groups. In aerobic biodegradation Oxygenases and Peroxidases play a key role in first step by incorporating oxygen (Das et al., 2011). Several factors that influence degradation of hydrocarbons in marine environment have been extensively reviewed and stated that type and combination of bacteria, bioavailability of substrate, catabolic genes, field survivability of used bacteria, nutrient availability etc., plays an important role in success of bioremediation.

It has been reported in several instances that mixed bacterial cultures are recommended as no single bacteria will be able to degrade all the components of oil and mixed cultures showed effective degradation compared to pure cultures due to complexity of oil (Sugiura et al., 1997). When compared to pure culture, mixed bacterial culture has the advantage of synergistic relation between bacteria leading to complete degradation of oil (Mukred et al., 2008). Rahman et al., (2002) reported that maximum degradation of crude oil was done by mixed bacterial consortium proving the efficiency of consortium.

1.5 Factors affecting Bioremediation (Bioaugmentation)

The viability of bacteria during bioaugmentation (bioremediation) depends on abiotic factors like temperature, pH, salinity, physical state of hydrocarbons, oxygen availability etc., and biotic factors like hydrocarbanoclastic capabilities, catabolic genes, nutrient availability etc. The physical state of hydrocarbons play a key role in kinetics of degradation as their degradation would be more in liquid state compared to solid state. For instance *Pseudomonas* sp. utilizes diphenylmethane at 30°C but not at 20°C due to its availability as liquid state at higher temperature. Temperature influences the physical state of substrate and also the optimal activity of microorganisms (Atlas et al., 1981). Psychrotrophic, Mesophilic, Thermophilic bacteria are known for their capabilities to degrade hydrocarbons. Hydrocarbon degradation is influenced by degradation of specific bacteria according to the seasonal temperature. Atlas and Bartha (1973) found that number of low temperature hydrocarbon degrading bacteria increased during winter season in Raritan bay, New Jersey. Marine bacteria get continuous exposure to change in temperature but they manage to survive by various ways like changing the physical location, chemotaxis, differentiation into viable but not culturable state etc. (Jannasch et al., 1984). Acidification of ocean by accumulation of CO_2 results in lowering of pH but not less than 6. Tackeuchi et al., (2001) showed that marine bacteria are better adapted to pH variations compared to other terrestrial and aquatic microorganisms. Oxygen is a key requirement for aerobic degradation of hydrocarbons as the initial microbial attack on hydrocarbons starts with incorporation of oxygen into hydrocarbon nucleus. Oxygen availability is not a rate limiting factor in the case of marine oil spills (Hassanshahian et al., 2003). Nutrient availability also plays an important role in survival of hydrocarbon degrading microorganisms during bioremediation. Nitrogen, Phosphorus, Sulphur and iron etc. holds a critical role whose deficiency leads to low degradation rate. It has been observed that marine ecosystem is often deficient in these nutrients because of competitive consumption by other non-hydrocarbon degrading bacteria and phytoplankton etc. So biostimulation is opted in nutrient deficient contaminated sites (Lebaron et al., 1999). The success of bioremediation depends on the above mentioned environmental factors of which only addition of nutrients is feasible. The presence of catabolic genes for degradation of specific hydrocarbons has importance as the capabilities of the microorganism depends directly on combination of these genes. The success of bioremediation also depends on the

genetic adaptability of the microorganisms present in the contaminated site by amplification (selective enrichment), gene transfer and mutation of genes involved in hydrocarbon metabolism (Leahy et al., 1990). In the situation of failed bioaugmentation the transfer of catabolic genes from the introduced bacteria to the indigenous bacteria might lead to degradation of hydrocarbons at higher rate as the recipients are already acclimatized to the local environmental conditions. Bioaugmentation strategies uniformly suffer from a lack of information regarding the stability of introduced genes and corresponding physiological traits in the environment.

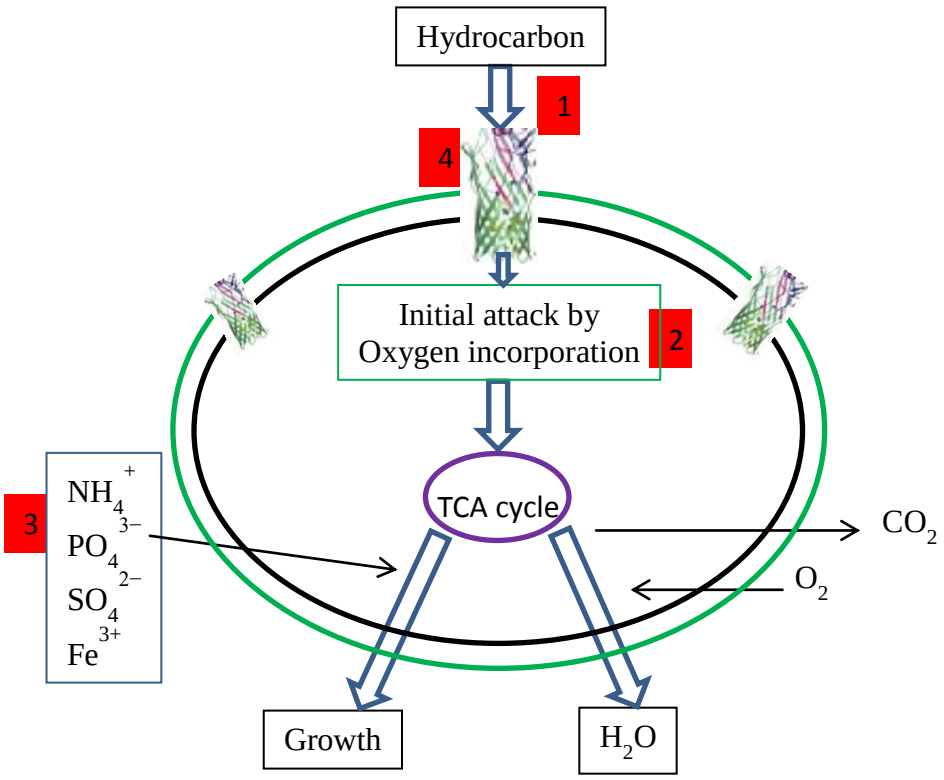

Fig 1.3: Different factors affecting bioremediation

1- Bioavailability

2- Catabolic genes (Horizontal gene transfer)

3- Nutrient availability (Biostimulation)

4- Uptake of hydrocarbons (Role of outer membrane proteins)

1.6 Bioavailability

Biodegradation of hydrocarbon is mainly limited by their bioavailability as they are nonpolar in nature and so their movement from non-aqueous to liquid phase is very slow (Brusseau et al., 1998). Bioavailability can be increased by two methods- 1) increasing the mass transfer of hydrocarbons into aqueous phase 2) bringing bacteria close to hydrocarbons by increasing cell surface hydrophobicity. Several chemical and bio-surfactants are used to increase bioavailability. Cyclodextrins are oligosaccharide molecules in cyclic shape with a hydrophilic surface and a hydrophobic inner cavity. Cyclodextrins are known for their pharmaceutical application to increase the solubility of less soluble drugs. Cyclodextrins forms complex with drug, hiding hydrophobic functionality of drug in the inner hydrophobic cavity. The so formed drug-cyclodextrin complex will be soluble in water due to the exposed hydrophilic hydroxyl groups to the external environment (Tiwari et al., 2010). It was reported by Valle et al., (2004) that cyclodextrins form inclusion complexes with other hydrophobic compounds like hydrocarbons and aromatic dyes etc. Badri et al., (2000) has shown increased hydrocarbon degradation in presence of β-cyclodextrin by soil microorganism. Sivaraman et al., (2010) has compared the effect of α, β, γ cyclodextrins and proved that degradation is more in presence of β-cyclodextrins. Tergitol-NP10 and Tween 80 were found to increase bioavailability of phenanathrene and fluoranthene during degradation by *Pseudomonas stutzeri* and *Spingomonas* strain respectively. It has been reported that cucurbiturils can bind to hydrocarbon but their role in hydrocarbon degradation is yet unknown (Florea and Nau, 2011).

1.6.1 Cucurbiturils (Hydroxy cucurbit[6]uril)

Glycouril and formaldehyde are condensed under acidic conditions (H_2SO_4) and the so formed amorphous cyclic polymer was characterized and named as cucurbituril. Cucurbituril contains n-glycouril units doubly linked by methylene linkers (Behrand et al., Mock et al.,). The name suggests its molecular shape i.e., pumpkin shape (belongs to Cucurbitaceae). There are different homologs of cucurbiturils (CB[n]) based on the number of glycourils present in the polymer i.e., CB[5], CB[6], CB[7], CB[8] etc. Cucurbit[6]uril structure was first reported by Mock and coworkers in 1981 (Freeman et al., 1981) and later in the year of 2000 structures of other homologs were reported by Kim et al., (2000). As the glycouril

number increases creating different homologs, the cavity size also increase. The diameter of the internal cavity increases from ~4.4 to 8.8Å for CB[5] to CB[8]. Cucurbiturils resembles cyclodextrins in molecular shape and in terms of cavity size CB[6], CB[7], CB[8] are analogous to α, β, γ cyclodextrins. The internal cavity diameter of CB[5], CB[6], CB[7], CB[8] is 4.4, 5.5, 7.7, 8.8 Å respectively, the cavity volume is 82, 164, 279, 479 $Å^3$ respectively and height of all homologs is 9.1 Å (Marquez et al., 2004). The cavity volume of CB[6] is almost equivalent to α cyclodextrins i.e., 174 $Å^3$. CB[6] is crystalline compound without colour, insoluble in water and organic solvents but soluble in mineral acids, carboxylic acids, aqueous solutions with alkaline salts. The host guest complexation of cucurbiturils depends on the outer portals and inner hydrophobic or nonpolar cavity. It has been reported that CB[n]s binds to positively charged molecules at carboxylated rims i.e., the two carbonyl portals and neutral molecules accommodated into the internal cavity. The host-guest complexation of CB[n]s includes some drug molecules which facilitate chemical and physical stability, improved solubility and controlled release of drug. Some drugs like Paracetamol, memantine, cisplatin, prilocaine, coumarin, sanguinarine stands as best examples for CB[n]-drug interaction studies (McInnes et al., 2010, Wheate et al., et al., 2006, Wang et al., 2009) and cyclodextrins were also known for their importance in drug delivery. Florea and Nau (2011) have reported strong binding of cucurbiturils to hydrocarbons by fluorescent dye displacement method. Svec et al., 2012 reported the binding of pyridine to CB[6] and CB[7]. Gaseous hydrocarbons can fit into CB[5], Aliphatic and aromatics without substitute will fit into CB[6], aromatics with substitutes can fit into CB[7], a pair of aromatic compounds can fit into CB[8] (Svec et al., 2012). Svec et al., (2012) showed the role of CB[n]s size in binding to pyridine molecule using H^1NMR studies and showed aliphatic part of pyridine interacted with CB[6] but CB[7] hosted aromatic part leaving the aliphatic tail outside. As it has been clearly reported CB[6] binds to hydrocarbons the problem of solubility in water should be addressed and its role in hydrocarbon degradation can also be explored.

1.6.2 Biosurfactants

Biosurfactants are surface active and structurally diverse metabolites produced by microorganisms. Most of the biosurfactants are lipids and they are classified as Glycolipids, phospholipids, lipoproteins, natural lipids, fatty acids, lipopolysaccharides etc. (Parkinson et al., 1985). Biosurfactants are amphipathic molecules having a hydrophilic and a hydrophobic domain for the reason they facilitate the transport of hydrocarbons into the cells. They have characteristics of surface active agents like enrichment at interfaces, lowering interfacial tension, forming micelles. Biosurfactants are more efficient than chemical surfactants in increasing the bioavailability of hydrocarbons (Ron et al., 2002). Biosurfactants are divided into low molecular weight and high molecular weight compounds. Low molecular weight compounds are usually glycolipids which contain a carbohydrate head and fatty acid tail and were known to decrease the interfacial tension between oil and water. Rhamnolipids, Trehalose lipids, Sophorose lipids are some of the examples of low molecular weight biosurfactants. The high molecular weight bacterial surfactants are emulsifiers composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins, or complex of above mentioned polymers. An emulsan from *Acinetobacter*, a surface active extracellular polysaccharide protein complex from *Acinetobacter calcoaceticus*, Alasan an anionic polysaccharide from *Acinetobacter radioresistents* are some of the examples of high molecular weight biosurfactants (Taylor et al., 1961, Novan et al., 1995). This high molecular weight biosurfactants are substrate specific, some works for only aliphatics and some works for aromatics (Ron et al., 2002).

Rhamnolipids

Rhamnolipids are glycolipids composed of β-hydroxyl fatty acid and rhamnose sugar produced by bacteria like *Pseudomonas aeruginosa* usually during stationary phase of growth. It has been reported that rhamnolipid production is induced by quorum sensing under nitrogen depleted conditions (Manresa et al., 1991). Chemically they consists rhamnose sugar molecule and β-hydroxyalkanoic acid and they were reported to have over 60 homologues produced by different bacteria. *P. plantarii, P. putida, P. chlororaphis, P. fluorescens, Burkholderia mallei, B. pseudomallei, B. thailandenis,* are known to synthesize rhamnolipids (Sekhon Randhawa et al., 2014, Toribio et al., 2010). *Acinetobacter calcoaceticus,*

Enterobacter asburiae, *Enterobacter hormaechei*, *Pantoea stewarti* are isolated from a biodiesel facility and found to produce rhamnolipids (Roony et al., 2009).

It has been reported in many occasions that rhamnolipids can increase the bioavailability of hydrocarbons therefore enhancing their biodegradability and also known for their environmental friendly nature as they are biodegradable. It has been observed that biodegradation of crude oil by *Pseudomonas aeruginosa* was increased by 58% when supplied with 1g/L glycerol or 0.22g/L rhamnolipid. It has been explained by the authors that there was no rhamnolipid production when crude oil oil was used as substrate and glycerol was used to induce rhamnolipid production (Guo-lang et al., 2005). Abalos et al., (2004) has conducted a study to see the effect of rhamnolipids on biodegradation of Casablanca crude oil by a microbial consortium. They have found that biodegradation of total petroleum hydrocarbons have been increased from 32 to 61% in 10 days of incubation time. They also found that the degradation of some alkylated PAHs have been increased from 9 to 44%. There were several reports proving the improved degradation of various hydrocarbons like n-alkanes, polyaromatic hydrocarbons in presence of rhamnolipids. Using a mathematical model, the effect of mono and dirhamnolipids on dissolution, bioavailability, biodegradation of phenanthrene has been studied. It was found that both monorhamnolipid and dirhamnolipid has increased biodegradation of phenanthrene. The authors have concluded that effect of surfactant on hydrocarbon degradation depends on solubilizing capacity of surfactant and bioavailability of hydrocarbons (Zhang et al., 1997). In some cases the increased solubilization of hydrocarbons by rhamnolipids cannot guarantee increase in degradation (Mata-Sandoval et al., 2001) and rhamnolipids are also known for their antimicrobial properties against bacteria as well as some fungal species. Rhmanolipids removes the lipids from the cell membranes and increase their cell surface hydrophobicity (CSH) but the changes in cell surface depend on the amount of surfactant used, carbon sources available and the type of bacteria etc. The co-culturing of biosurfactant producing bacteria with hydrocarbon degrading bacteria has shown better degradation of various hydrocarbons but the antimicrobial activity of rhamnolipids should be taken into consideration before usage.

1.7 Catabolic genes involved in hydrocarbon degradation

1.7.1 Alkane hydroxylases

Methanotrophs assimilate methane by the action of a membrane bound monooxygenases which were classified as soluble methane monooxygenase and particulate monooxygenase (Lieberman and Rosenzweig 2004). Butane monooxygenase from *Pseudomonas butanovora* was studied in detail and found to hydroxylate C_2-C_9 alkanes (Dubbels et al., 2007). Propane monooxygenase from *Gordonia* sp. TY-5 and *Mycobacterium* sp. TY-6 are known to oxidize propane at the subterminal position. Propane monooxygenase from *Pseudomonas* sp. TY-7 oxidize at both terminal and subterminal position (Kotani et al., 2003, 2006, 2007).

The *alk*B family of alkane hydroxylases is integral membrane non-haem diiron monooxygenases. They hydroxylate alkanes at terminal position which is the first step in alkane degradation process. The enzyme *alk*B require the support of two soluble electron transfer proteins. Rubredoxin reductase (FAD dependent) transfers the electrons from NADH to rubredoxin and then the electrons are transferred to *alk*B (Fig 1.4). Alkane hydroalxylases contain eight hydrophobic sequences which span in the cell membrane and seem to be conserved in all the alkane degrading bacteria. The organization of the genes coding for alkane hydroxylase, rubredoxin reductase and rubredoxin varies among bacteria. In most of the strains rubredoxin reductase is not located close to alkane hydroxylase whereas rubredoxin is located downstream to alkane hydroxylase gene (van Beilen et al., 2003).

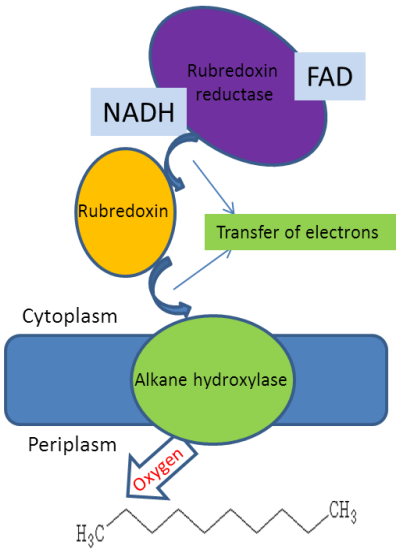

Fig 1.4: Schematic diagram of action of alkane hydroxylase system

Till now more than 60 homologues of *alk*B are known to be present in different gram positive and gram negative bacteria but their sequence is highly diverse. A novel oxygenase from *Acinetobacter* sp. Strain M-1 was reported by Maeng et al., (1996) which could act on alkanes ranging from C_{10} to C_{30}, alkenes ranging from C_{12} to C_{20}, aromatic substituted with alkyl groups but not branched alkanes. This enzyme is about 134 kDa and requires Cu^{2+} for its activity. Van Beilen et al., (1998) discovered Alkane hydroxylase system in *Pseudomonas aeruginosa* strain which was able to grow on n-octane and based on PCR and sequencing results it was found that the enzymes are identical to alkane hydroxylase of *P. oleovorans* GPo1. Marin et al., (2001) have reported the presence of alkane hydroxylase gene in *Burkholderia cepia* RR10 and that the transcription of gene was induced in presence of alkanes ranging from C_{12} to C_{30}. The group reported that the transcription rate of the gene was increased by five folds in stationary phase compared to exponential phase and the expression is under catabolite repression in presence of organic acids and sugars. The *alk*B present in OCT plasmid of *Pseudomonas putida* GPo1 was well characterized and it could oxidize propane, n-butane and C_5-C_{13} alkanes (van Beilen et al., 2005). *Pseudomonas putida* Gpo1 and *Acinetobacter* have only one alkane hydroxylase but some bacteria have more than one alkane hydroxylase. Van Beilen et al., (2004) have characterized two alkane hydroxylase genes (*alk*B1 and *alk*B2) from *Alcanivorax borkumensis* a marine bacterium. These two enzymes have an overlapped substrate range between C_5-C_{16}. The *alk*B2 of *Alcanivorax borkumensis* showed 65% similarity to *alk*B2 alkane hydroxylase of *Pseudomonas aeruginosa*. Marin et al., (2003) have discovered the two alkane hydroxylases from *Pseudomonas aeruginosa* i.e. *alk*B1 and *alk*B2 and their differential expression at late exponential and exponential phase respectively. The genes for rubredoxin and rubredoxin reductase are expressed at constant level irrespective of alkane's presence but *alk*B1 and *alk*B2 are expressed only in the presence of C_{10}-C_{22}/C_{24} alkanes.

Metagenomics studies have been employed to study the distribution of alkane degrading bacteria in the environmental samples. Jurelevicius et al., (2013) suggested that the use of combination of *alk*B primers to study the distribution of *alk*B genes is a better approach to analyze the distribution of alkane degrading bacteria. They have used a combination of primers as the nucleotide sequence encoding the *alk*B enzymes are varying in different bacteria although they share a sequence homology to some extent. The number of *alk*B genes

in a contaminated site can be a potential scale of indicator for determining the extent of bioremediation occurring. The increase in hydrocarbon concentration or sudden addition of hydrocarbons to an environment results increase in gene copy number of the catabolic genes. Smits et al., (1999) have selected 24 strains that have been reported to grow on n-alkanes like n-dodecane, n-hexadecane, and n-octadecane. The group has used PCR and southern blotting techniques to analyze the diversity of alkane hydroxylase systems in both Gram positive and Gram negative strains and they have reported that the first, second and third histidine boxes present in the amplicons are conserved in all sequences. They also have designed degenerative primers based on the conserved region of first histidine box. Glebler et al., (2013) has reported the sequence type of *alk*B in distinct bacterial taxa using terminal restriction fragment length polymorphism. They have selected 50 stains out of 400 isolates degrading hexadecane. Based on 16S rDNA they found that they belong to Alpha, Beta, Gama *Proteobacteria*. Fifty six distinct *alk*B homologs were obtained out of which 38 are similar to *Actinobacteria* strains, 8 are similar to *Proteobacteri*a and remaining are similar to environmental clone libraries of *Pseudovibrio* sp. strain JE062.

Due to anthropogenic and other natural sources alkanes are widely available in marine environments which could be reason for presence of alkane degrading bacteria in uncontaminated areas or sites devoid of oil spills. Diversity and abundance of *alk*B genes in sediments from hydrocarbon seep areas of Timor sea has been studied by Wasmud et al., (2009) stating that the copy number of *alk*B increased in the sediments which were exposed directly to active seepage. Their real time PCR experiment did not show any positive correlation between hydrocarbon degradation and copy number of genes but the group has justified the results showing the very low levels of hydrocarbons present in the sediments. A microcosm study was conducted by Paisse et al., (2011) using polluted sediments. They have used TRFLP to study the diversity and expression of *alk*B genes and found that there was no correlation between the expression of *alk*B and bacterial response to oil contamination.

Nie et al., (2014) have used 3,979 microbial genomes and 137 metagenomes from terrestrial, fresh water, marine environment to find out the diversity of alkane hydroxylases. They have reported that out of 369 *alk*B containing genomes 73 have multiple copies of *alk*B gene with a sequence similarity ranging from 27 to 99.7%. The distribution of *alk*B gene using metagenomes have been studied and reported that Proteobacteria are 51.6%, Bacteroidetes

are 13.4% and cyanobacteria are 12%. In addition the phylogenies of *alk*B were compared to phylogenies of 16S rDNA resulted in speculating the hint for HGT between some bacteria.

OCT plasmid has been well characterized by Harder et al., (1985) stating that OCT plasmid harbors alkane oxidation and mercury resistance genes. They have used stock *P. putida* PpG6 culture ($PpG6_{ICG}$ and $PpG6_{JAS}$ were designated based on the plasmid they host), octane was used as carbon source and mercury vapors for resistance studies. They have observed that OCT plasmid has both alkane oxidation and mercury resistance genes and they have observed low frequency of plasmid transfer (Harder et al., 1985). The expression of alkane oxidation genes of OCT plasmid present in *P. putida* was studied by Dinamarca et al., (2003) and found that two signals i.e., the concentration of carbon source and oxygen availability regulates the expression. The genes coding for the enzymes involved in OCT plasmid regulated alkane degradation were grouped as *alk*BFGHJKL and *alk*ST. The *alk*BFGHJKL is for terminal oxidation of alkanes and *alk*ST is for transcriptional regulation of first cluster. In presence of alkanes, *alk*S induces the expression of *alk*BFGHJKL via promoter *palk*B. *Pseudomonas maltophilia* hosts OCT plasmid and the expression of alkBA genes encoding alkane hydroxylase and rubredoxin reductase was studied by Lee et al., (1996). The authors stated that the *alk*BAC is the cluster present in the plasmid which encodes for alkane hydroxylase, rubredoxin reductase and alcohol dehydrogenase. The *alk*R region positively regulates the expression in presence of n-octane. The authors have cloned the *alk*BA fragment of cluster into *E.coli* and found that it is expressing as the alcohol dehydrogenase and other genes involved in oxidation of fatty acid are harbored in the chromosomal DNA. Therefore it has been concluded by Lee et al., (1996) that *alk*BA is enough for the recipient bacteria to survive on n-alkanes if it has other necessary genes for further degradation downstream.

1.7.2 Aromatic hydrocarbon dioxygenases

These enzymes belong to the family of Rieske non-heme iron oxygenases. Usually they have broad substrate range. Dihydroxylation is the primary step in degradation of aromatic hydrocarbons by oxidizing them to vicinal arene *cis*-diols. All these enzymes need support of one or two electron transport proteins (Gibson et al., 2000).

Naphthalene dioxygenase is the enzyme responsible for oxidation of naphthalene. Naphthalene dioxygenase from *Pseudomonas* sp. Strain 9816 was well characterized. It was multicomponent enzyme system having α3β3 hexamer. NADH dependent FAD reductase and Ferredoxin are involved in the electron transport (Kauppi et al., 1998) (Fig 1.5). In *Pseudomonads* cis-naphthalene dihydrodiol will be formed by addition of two oxygen atoms at 1 and 2 positions. *nahAC* is the subunit that incorporates oxygen into naphthalene. *nahAA* and *nahAB* are the subunits that transfer electrons to *nahAC*.

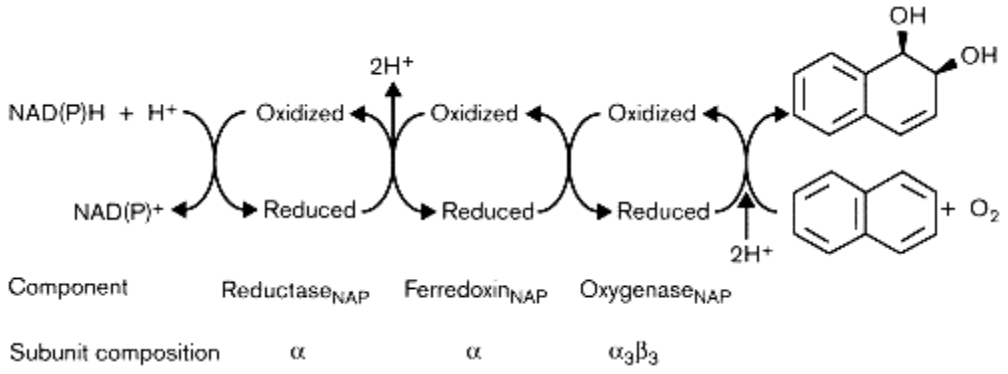

Fig 1.5: The multicomponent Naphthalene dioxygenase system. FAD reductase is coded by gene *nahAA*, Ferredoxin is coded by gene *nahAB*, and Oxygenase is coded by *nahAC*

1.8 Horizontal Gene Transfer of Catabolic Genes

Horizontal gene transfer (HGT) is transfer of genes between closely or distantly related organisms without reproduction in a unidirectional way. HGT is different from intentional gene transfer, transient gene transfer, intragenomic gene transfer, vertical gene transfer. Intentional gene transfer is by human intervention of transferring the genes to recipients. Transient gene transfer can be either intentional or unintentional transfer of genes from parents to offspring but doesn't perpetuate in offspring. Intragenomic gene transfer is movement of genetic material from one location to other location of same genome. Vertical gene transfer is transfer of genes from parents to offspring by reproduction. HGT helps the bacteria to adapt to selection pressure prevailing in the inhabitant environment. For instance the development of antibiotic resistance in bacteria is an example for the adaptation of bacteria by horizontal gene transfer (Tschäpe 1994, Mazel and Davies, 1999). Transformation (uptake of naked DNA), Transduction (mediated by bacteriophages) and Conjugation (by means of plasmids or conjugative elements) are the three types of process of

HGT in bacteria (Syvanen 1994). Mobile genetic elements like plasmids, bacteriophages, transposons, integrons etc., play in important role in HGT. During bioaugmentation the introduced bacteria might fail due to abiotic or biotic stress which can be overcome by horizontal gene transfer of respective catabolic genes into well-established and competitive indigenous bacteria. The catabolic genes encoding degradation of xenobiotics are often present in plasmids or transposons (Tsuda et al., 1999). HGT is affected by many factors like temperature, pH, presence of pollutant and nutrient availability etc. It has been observed in many studies that stress created by the pollutants on the respective environment results in increase of gene transfer frequency. *Alcaligenes xylosoxidans* hosting 2,4-dichloropropionate (DCPA) degrading plasmid was introduced into soil and the transfer of plasmid was observed only in the presence of 2,4-dichloropropionate. In a study related to 2,4-D contaminated soil, the transfer of plasmid hosting 2,4-D degradation gene (pJP4) was transferred from *Ralstonia eutropha* to *Variovorax paradoxus* only in the presence of 2,4-D and the rate of plasmid (pJP4) transfer to bacteria like *Pseudomonas* and *Burkholderia* has increased with increase in pollutant concentration. It has been explained by Top et al., (2002) that the high number of transconjugants observed in presence of pollutant is may be due to proliferation of transconjugants but not due to increased conjugation efficiency. Nutrient availability is a major factor that influences HGT as it directly affects the number and activities of donor and recipient bacteria (van Elsas et al., 2000).

Retrospective and mechanistic are the two classical approaches used to study horizontal gene transfer in polluted sites. Retrospective approach is to study the catabolic genes present in different bacteria present in the contaminated site, analyze the similarity and indirectly conclude the possibility of horizontal gene transfer. Mechanistic approach is to directly monitor the transfer of catabolic genes from donor to recipient. Mechanistic method can be done in four different ways of altering donor and recipient- 1) defined donor and recipient 2) unknown donor from environment and defined recipient 3) donor and recipient from field 4) defined donor mingled with undefined naturally occurring bacteria. The fourth model represents the strategy associated with bioaugmentation (Stuart-Keil et al., 1998). In many retrospective experiments it has been proved that *alk*B and NDO have undergone horizontal gene transfer between different bacteria of contaminated sites. It has been reported using TRFLP and DGGE that diverse *alk*B and NDO genes available. Naphthalene dioxygenase

was studied widely for its presence in chromosomal and plasmid DNA. Alkane hydroxylase genes are reported to be present mostly in chromosomal DNA except OCT plasmid having *alk*B gene. Very few mechanistic studies have reported the transfer of naphthalene dioxygenase gene to indigenous bacteria of contaminated site. Mechanistic studies to observe the transfer of *alk*B have not been done.

1.9 Biostimulation

As discussed earlier nutrient availability is one of the important factors affecting bioremediation. The addition of nutrients to support the growth of bacteria during pollutant degradation process is called biostimulation. During an oil spill there might be indigenous bacteria capable of degrading oil but limited in their activity due to low nutrient availability. There were different and extensive studies done till date to prove the importance of nutrients in bioremediation of oil contamination in marine environment. The beaches contaminated during Exxon Valdez oil spill were subjected to nutrient addition and achieved better bioremediation. Coulon et al., (2006) have supported the concept of biostimulation by reporting that hydrocarbon degradation by indigenous bacteria has increased by addition of nutrients. Carbon, nitrogen and phosphorus (C: N: P) ratio should be maintained as 100:10:1 as suggested by Hoeppel et al., (1992). The nutrients used in biostimualtion are broadly classified as water soluble, slow release and oleophilic nutrients.

Water soluble biostimulants are inorganic mineral salts like KNO_3, NH_3NO_3, and $NaNO_3$ etc. These mineral salts are generally sprayed or applied as granules in the contaminated site and found to be successful. For example garden fertilizer (23:2=N: P) was used successfully in Exxon Valdez oil spill. The major disadvantage with water soluble fertilizers is washing out by wave action. Periodic addition of these fertilizers may overcome the washout problem. Lee et al., (1987, 1989, 1991) have conducted studies to evaluate the effect of different fertilizers on biodegradation of oil in sandy beaches and found that periodic addition of inorganic fertilizers gave successful results.

Slow release nutrients like paraffin coated $MgNH_4PO_4$, vegetable oil coated calcium phosphate, ammonium phosphate, and ammonium nitrates were used in Prince William oilspill. The oil degradation rate was found to be more compared to water soluble nutrients

because they were released slowly from the trapping material like paraffin, vegetable oil. A study at field scale was conducted by Swannell et al., (1999) to compare the efficiency of inorganic nutrients ($NaNO_3$, $KH2PO_4$) and soya oil coated inorganic nutrients (slow release). They have found no difference in efficiency between inorganic nutrients and slow release fertilizers. But they have recommended slow release fertilizers considering cost effectiveness as their application is easy at the site. It was proved that not only supply of slow release fertilizers, type of oil also plays an important role in rate of degradation. A field study was conducted in salt marsh plots by Prince et al., (1999). It was found that medium range crude oil was degraded more by 20% in presence of slow release fertilizers but did not show any effect on degradation of heavier bunker C oil.

Oleophilic fertilizers are nitrogen and phosphorus in oleophilic form. As the oleophilic compounds are readily soluble in oil they stay at oil water interface and enhance the growth of microorganism which intern results in increased degradation. Inipol EAP 22 is a well-known oleophilic fertilizer found to be successful in enhancing the removal of oil. S200 is another type of oleophilic fertilizer used in shore line of Prince William Sound. It has been observed in a microcosm study that S200 enhances degradation at higher rate compared to inorganic fertilizers. Oleophilic fertilizers were found to be better than water soluble fertilizers when used in intertidal zone but show no effect in sublittoral zone.

All three different type of biostimulants used in bioremediation were reviewed for their site specificity, effect on degradation and concluded that oleophilic fertilizers should be used for surface oil contamination, water soluble and slow release fertilizers should be used for subsurface contamination.

1.10 Uptake of hydrocarbons

The uptake of hydrocarbons by bacterial cells is important step in hydrocarbon degradation as the enzymes involved are intracellular. The solubility of hydrocarbons plays an important role in hydrocarbon uptake by bacteria. The water soluble compounds like short chain hydrocarbon and water soluble aromatics are easily taken up due to their high dissolution rates. It has been stated that high dissolution rate results in high growth rate and vice versa. Uptake of pseudosolubilized hydrocarbon droplets has been achieved by production of

biosurfactants. Biosurfactants are emulsifying agents having amphiphilic property and increase the water solubility of hydrocarbons. Some bacteria take up the hydrocarbon by directly attaching to big hydrocarbon drops. Goswami and Singh (1991) have reported the uptake of hexadecane by *Pseudomonas* by directly attaching to the surface of hexadecane. However the uptake mechanism also depends on the type of bacteria. The outer leaflet of outer membrane of gram negative bacteria is composed of lipopolysaccharides made up of lipid and oligosaccharide molecules. Even though it acts as a barrier, uptake of nutrients is essential for survival (Wiener et al., 2011). Once the hydrocarbon comes in contact with the bacterial cell it should be transported into the cell through the cell membrane. This uptake process can be divided into three types- 1) Passive diffusion 2) Passive facilitated diffusion 3) Energy dependent uptake (Whitman et al., 1998, Kim et al., 2002). Many studies have been conducted to determine whether the transmembrane transport of hydrocarbons is energy dependent or independent. The role of energy in hydrocarbon transport is tested using sodium azide. Sodium azide is an inhibitor of the electron flow chain in oxidative phosphorylation. Verdin et al., (2005) has proved that benzo[a]Pyrene uptake by *Fusarium solani* was through passive diffusion as addition of sodium azide did not affect hydrocarbon transport. The transport of Naphthalene into *P.putida* has been proved to be energy independent (Bateman et al., 1986). On the other hand it has been proved that hydrocarbon transport is energy dependent. Sodium azide or 2,4 dinitrophenol reduced the uptake of phenanthrene as they inhibit oxidative phosphorylation (Kallimanis et al., 2007). *Pseudomonas sp*. DG17 has given ^{14}C-labelled octadecane as substrate and found that intracellular ^{14}C-labelled octadecane has been decreased in presence of sodium azide. From the above mentioned experiments it was evident that energy requirement for transmembrane transport of hydrocarbon depends on type of bacteria and also substrate concentration.

1.11 Role of outer membrane proteins in hydrocarbon uptake

Recently the role of outer membrane proteins in hydrocarbon transport has come into lime light after crystallization of two proteins involved in hydrocarbon transport. FadL is the family of outer membrane proteins that have been extensively studied for their capability to transport hydrophobic compounds. With some exceptions most of the outer membrane proteins are β-barrel proteins and some of these proteins are classified as porins which are

said to be involved in nutrient uptake. FadL protein of *E.coli* known for long chain fatty acid transport was crystalized and studied extensively. It is a long barrel of about 50 Å made of 14 antiparallel β strands. There was a hatch domain made of 42 amino acids and the hatch domain is capped by NPA sequence which is conserved among all FadL members. The transport of fatty acids has been speculated by the positon taken by a detergent molecule (C_8E_4) during crystallization. It was observed that a hydrophobic cleft formed between L3 and L4 (loops) was bound to C_8E_4 detergent molecule indicating that this hydrophobic groove is involved in initial binding to fatty acid molecules in extracellular environment. About 15 hydrophobic amino acids at least one from strands of S3 to S10 forms a hydrophobic pocket. Most of these amino acids are found to be conserved among FadL family proteins. The extracellular opening of the hydrophobic pocket is close to hydrophobic grove of L3 and L4 loops. It also has been stated that a conformational change in the hatch domain would lead to the transport of hydrophobic substrate into cytoplasm. So based on the binding of detergent molecules to FadL protein of *E.coli*, it has been proposed that the fatty acid (hydrophobic substrate) will first interact with the extracellular hydrophobic groove and then pass into the hydrophobic pocket and then into pass into the cytoplasm by a conformational change in hatch domain (van den Berg et al., 2004).

Two proteins called TodX and TbuX from *Pseudomonas putida* F1 and *Ralstonia pickettii* PKO1 has been crystalized by Hearn et l., (2008). Both these proteins show 15 to 20% identity with FadL Protein of *E.coli*. They are also identical to hydrocarbon transporter protein TmoX of *Pseudomonas mendocina*. According to X-ray crystal structure of the proteins, they are said to have 14 strands forming a β-barrel. In this study also the position of interaction with detergent molecule C_8E_4 has been studied. It was found that the detergent molecules were bound near the extracellular hydrophobic groove and interior of the barrel. It has been found that, like FadL of *E.coli* these two proteins also have a hatch domain in the interior of the protein barrel. Based on the positons taken by the detergent molecules it was found that the transport of hydrophobic molecules might be like FadL of *E.coli*. The substrate transport assay proved that these proteins are substrate specific and could not transport oleic acid. It has been explained by the authors that molecules like toluene and benzene can easily fit into the narrowest constriction of the barrel and these proteins can play a role in hydrocarbon transport.

Another protein called ompW has been crystallized and it has been found that it is also a β-barrel transmembrane protein involved in hydrophobic substrate transport. This protein has been isolated from *Pseudomonas aeruginosa*. It was found that ompW has S3 kink like FadL, TodX and TbuX leading to formation of lateral opening. In this study it was found that a hydrophobic detergent molecule was bound near to S3 kink in the crystal structure. The mutated proteins with no S3 kink/lateral opening are unable to transport substrate. This lead to an idea that the hydrophobic substrate will enter the protein barrel and pass to transmembrane through lateral opening (Hearn et al., 2009). However, even the crystal structures of outer membrane proteins and known to bind to hydrophobic substrates their role in hydrocarbon transport is not known clearly.

1.12 Gap in existing research

It has been noticed in many occasions that tarballs turns out to be a major problem for recreational activities in Goa. A few studies were conducted to deal with the problem but a realistic strategy for their removal has not been proposed. As the incineration process that is presently under use is not a recommendable procedure taking air pollution into consideration. It already has been proved that bioremediation is ecofriendly process to remove oil spills. Although many bacteria were reported for their capability to degrade hydrocarbons they also have been criticized for their failure in field environment. The major limitations faced by bioaugmentation are at different levels of degradation and they are: bioavailability of hydrocarbons, transport of hydrocarbon into the cell, single bacteria cannot degrade all fractions of oil, viability of used bacteria. All these problems were addressed in different studies taking single problem as an agenda. The present study brings all these problems on a single platform by isolating hydrocarbon degrading bacteria followed by increasing bioavailability of hydrocarbons. The present study deals to study the reliability of HGT and biostimulation. So bacteria should be isolated from oil contaminated site of Goa. Many synthetic and commercial fertilizers are been known to increase the bioavailability of hydrocarbon and intern increasing their biodegradation rate. Cucurbiturils are known for their strong binding capacity to hydrocarbon and also acts as hydrophobic drug delivery agents. They are cyclic polymers resembling the structure of cyclodextrins. Cyclodextrins are reported for their capacity to increase bioavailability as they have a hydrophobic inner cavity. Cucurbit[6]uril also have inner hydrophobic cavity equivalent to the cavity size of alpha cyclodextrins. The role of Cucurbiturils in hydrocarbon degradation has never been tested. Therefore their role in increasing bioavailability of hydrocarbon should be tested using hydroxy Cucurbit[6]uril due to its high yield and water solubility. As only aliphatic hydrocarbons are found to fit into the cavity of Cucurbit[6]uril, another agent for increasing degradation of both aliphatic and aromatic hydrocarbons should be recommended to bridge up the gap between bacteria and hydrocarbons. There are various biosurfactants known for their role in biodegradation of hydrocarbons and rhamnolipid was reported in various studies. Rhamnolipids are known for their capability to increase bioavailability of hydrocarbons and cell surface hydrophobicity of bacteria. The effect of rhamnolipid on biodegradation of NAPL (representing tarball) should be tested. It has been reported in various studies that

HGT of catabolic genes to indigenous bacteria and biostimualtion are possible solutions for low survival rate of introduced bacteria. But it has been never investigated whether HGT is reliable in stipulated time as the target is to degrade oil as fast as possible. So the present study tries to evaluate HGT versus biostimualtion using Goan seawater as a medium. Even though it was proposed outer membrane proteins play a role in hydrocarbon transport but their interaction with hydrocarbon molecules has not been understood. So, the interaction of hydrocarbon to these outer membrane proteins should be studied. By bringing up different problems associated with bioaugmentaion onto a common platform, the present study may lead to frame a possible strategy of bioremedaiton suitable for marine oil contamination in Goa.

CHAPTER 2

ISOLATION OF HYDROCARBON DEGRADING BACTERIA AND ESTIMATING THEIR CAPABILITIES TO DEGRADE CRUDE OIL SEDIMENT

Publication:

Biodegradation of crude oil by *Pseudomonas aeruginosa* and *Escherichia fergusonii* isolated from the Goan coast (Mar. Poll. Bull. 2013, 76, 276-282).

2. Introduction

India has a main land coastline of 5,500 kms apart from its offshore coastline of 2,500 kms. Backwaters, estuaries, creeks, lagoons and beaches especially from the coastline of Goa are the country's major tourist attractions. The Arabian Sea adjoining Indian peninsular region is the main international tanker route for transport of oil from the Persian Gulf. Strait of Hormuz and Strait of Malacca are the oil choke points of the world which are located on west and east coasts of India and the transport route is narrow enough to consider it as an accident prone zone (Sivadas *et al*., 2008). Accidental crude oil spills and frequent illegal disposal of oily waste into the sea results in serious damage to marine life. The marine ecosystem is being affected by oil spills due to the presence of toxic organic compounds present in the oil which affects human health through biomagnifications (Dasgupta *et al*., 2013). Aliphatic and aromatic hydrocarbons are the two major fractions of crude oil which are reported for their recalcitrant and health hazardous nature (Hidayat and Tachibana, 2012). The major aliphatic fraction in crude oil is easily degraded by bacteria but the fractions with large branched chains are non-degradable and therefore persist in the environment (Hasanuzzaman *et al*., 2007). The degradation of polyaromatic hydrocarbons is difficult due to their complex structures.

Bioremediation is a 'green' alternative for treating hazardous contaminants as there is no deteriorating effect on the environment. It may also be less expensive compared to other techniques. Bioremediation is defined as the natural ability of living microorganisms to reduce / remove / transform organic and inorganic pollutants. The success of bioremediation is dependent on the inherent biodegradability of the pollutant, the accessibility of the pollutant to degrading organisms and the optimization of biological activity (Dua *et al*., 2002). Indigenous bacteria from contaminated sites hold great interest as these bacteria will be more adapted to long exposures to the contaminants compared to genetically modified bacteria having small acclimatization time (Ojo, 2006). Considering the adverse effects of oil contamination on the beaches of Goa and oil spills along the Indian coast, the present study focuses on biodegradation of hydrocarbons present in such crude oil contaminated sediments by naturally occurring bacteria.

The aim of this study is to isolate indigenous bacteria capable of degrading crude oil and estimating their capabilities for a possible solution to clean up the Indian coast. This study will play a role in highlighting bioremediation as a possible tool in developing different strategies and models for removal of hydrocarbons from marine environment.

2.1 Materials

2.1.1 Chemicals

n-hexadecane was obtained from Hi-media, India. Organic solvents like ethyl acetate and dichloromethane were obtained from S.D. fine chemicals, India. Commercially available Bushnell Hass (BH) medium was obtained from Hi-media, India. Na_2Co_3, NaOH, $CuSo_4$ and sodium potassium tartarate were obtained from Merck, India. Folin's Ciocalteau's reagent was obtained from Fisher Scientific, India. All the chemicals and reagents used were of the highest purity available.

2.2. Methodology

2.2.1. Isolation of bacteria and screening of hydrocarbanoclastic capacity

2.2.1.1. Enrichment of culture

Crude oil contaminated sediments from Velsao beach, Goa, India were collected and enriched in BH medium containing 3% NaCl with 0.5% diesel oil as sole carbon source and incubated on a rotary shaker at 110 rpm and 30^0C. The enriched culture was transferred into fresh medium with same substrate at the rate of 5% every five days. This enriched culture was used for degradation studies.

2.2.1.2. Biodegradation of n-hexadecane as model hydrocarbon

Biodegradation studies were performed in sterile conical flasks in duplicate using the above enriched culture as inoculum. The cells were harvested at their late log phase by centrifuging the enriched culture at 6300 x g (Eppendorf AG 5810, Germany) for 10 minutes. The pellet was then re-suspended in sterile distilled water which was used as inoculum for the biodegradation experiment. The experimental setup was as follows: 0.5% (v/v) n-hexadecane

was added to the conical flasks containing 20ml sterile BH medium. One milliliter of freshly re-suspended culture was added to the culture flasks and incubated at 100 rpm at room temperature. The amount of biomass and residual hydrocarbon was estimated every 24h for 6 days.

2.2.2. Analysis of n-hexadecane degradation by Gas chromatography

Depletion of hydrocarbon in the microcosm was calculated by measuring amount of residual hydrocarbon left over in the medium. Residual hexadecane was extracted from the whole volume of each flask by adding equal amount of ethyl acetate to the medium. The mixture was stirred vigorously with a magnetic stirrer for 15 minutes. One milliliter of organic phase was sampled out and stored in glass vials at 4^{o}C until gas chromatographic analysis. Samples were analyzed with gas chromatograph equipped with flame ionization detector (GC-FID) (Chemito 2100 model). The injection and detector temperatures were 250^{o}C. The temperature program was 50^{o}C, 5 minute isotherm, 5^{o}C per minute (Sivaraman *et al.*, 2010). BPX5 capillary column (30M x 0.32mm) was used with a carrier gas (Nitrogen) flow rate of 4ml per minute and the ratio of hydrogen (detector) to air was 1: 100.

2.2.3. Identification of Bacteria

2.2.3.1. Genomic DNA extraction

Genomic DNA was extracted from the enriched culture by a modified protocol of Tsai and Rochelle (Rochelle, 2001) as follows: the bacterial culture was centrifuged and the pellet was dissolved in 0.5 ml of 1x TAE buffer. 18µl of 18mg/ml Proteinase K and 2µl of 10% (w/v) sodium dodecyl sulphate were added prior to incubation at 37ºC for 30 min. 100µl of 2% (w/v) sarkosyl was added to the mixture and it was incubated again for 15 minutes at 37ºC. After the addition of 80µl of 5M NaCl, sample was given the shock by freezing at -80ºC and thawing at 65ºC followed by addition of 80µl of 5% cetyltrimethylammonium bromide (CTAB) in 0.7M NaCl and incubated for 30 minutes at 65ºC. Equal amounts of Phenol: chloroform: isoamylalcohol (25:24:1) was added and centrifuged at 12857 x g for 10 minutes. The upper aqueous layer was collected and the DNA was precipitated by addition of isopropanol and overnight incubation at -20^{o}C. The precipitated DNA was pelleted out by centrifugation for 10 minutes followed by washing with 70% ethanol at 12857 x g, 4ºC and dissolved in 1x Tris EDTA (TE) buffer.

2.2.3.2. Amplification of 16S rDNA

16S rDNA genes were amplified using primers 27F and 1492R in a Bio-Rad mini personal thermal cycler. The PCR program is as follows: 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 52ºC for 30seconds, extension at 72^{o}C for 1 minute with an initial denaturation at 94ºC for 5minutes prior to first cycle and final extension for 10 minutes at 72ºC after the last cycle. GC clamping was done using primers 968F-GC and 1492R with the same PCR program except with a change in annealing and extension conditions of 50ºC for 30 seconds and 72ºC for 50 seconds respectively (Székely *et al*., 2009).

2.2.3.3. Denaturing gradient gel electrophoresis (DGGE) and Sequencing

PCR product of 15µl was loaded onto 8% polyacrylamide gel (40% acrylamide, 2% bis acrylamide 37:2:1) with 30 to 60% denaturant (7M urea and 40% formamide) and was run at 60V. 1x Tris-Acetic acid EDTA was used as running buffer. The bands were visualized by silver staining. DNA was eluted from the bands excised from DGGE gel by incubating in distilled water at 4^{o}C overnight (Nikolausz *et al*., 2008) and 1 µl sample was used as template for PCR using primers 968FGC and 1492R as per the program mentioned earlier in section 2.3.2. The PCR product was eluted by gel elution kit by Bangalore GENEI, India and was cloned into a plasmid (pGEM r-Tesay vector) from Promega cloning kit as per manufacturer's protocols and was transformed into bacterial strain JM109 by exposing the cells to calcium chloride and heat shock. Primary identification was done by growing the cells in antibiotic containing Luria-Bertani (LB) medium and secondary identification for transconjugants having the plasmid with cloned gene was done by growing them in LB medium enriched with X-gal and IPTG (Isopropyl β-D-1-thiogalactopyranoside) (Seidman *et al*., 2001), to avoid duplicate banding problem from PCR. Plasmid DNA was extracted from the cells of stationary phase and the 16S rDNA was amplified and was sequenced using an automated DNA sequencer (Applied Biosystems). The obtained sequence was identified by Basic local alignment search tool (BLAST) of National Centre for Biotechnology Information (NCBI) and phylogenetic tree was constructed on the basis of neighbour joining method using software MEGA 5.0. Bootstrap values (expressed as percentage of 1000 replications) greater than 50% were given at nodes.

2.2.4. Biodegradation of crude oil

With respect to crude oil degradation experiments, 18 g of crude oil contaminated sediments was collected from Velsao beach, Goa, India and was dissolved in dichloromethane. 0.5 ml was added to sterile conical flask and dichloromethane was allowed to evaporate in laminar airflow chamber. Twenty milliliter of BH medium with 3% NaCl was added to each flask followed by inoculation of enriched culture which was at log phase at the rate of 5% and incubated on a rotary shaker (100 rpm, room temperature). The residual crude oil was extracted at specific time intervals (0, 2, 5, 15, 25, 35, 45 days) and 1 ml of culture was preserved from each flask every day for DNA extraction and PCR amplification followed by DGGE as per the protocol mentioned earlier (Section 2.3.1-2.3.3).

2.2.4.1. Analysis of crude oil degradation by GC-MS

At specific time intervals, as mentioned earlier the residual crude oil was extracted by adding equal amount of dichloromethane with respect to the amount of medium (Khan *et al*., 2005). The mixture was stirred vigorously on magnetic stirrer for 15 minutes and was centrifuged at 2000 x g for 5 minutes. Lower organic phase was collected carefully using micropipette and was condensed at 28 rpm, 40ºC until the dichloromethane got evaporated. The dried crude oil sediment was collected by adding 1 milliliter of dichloromethane and dehydrated in a column with 2 g anhydrous Na_2SO_4. The oil content in the sample was determined by gravimetry in 0.5 ml of the elute and carefully evaporated until dryness. An aliquot (10 mg) of the elute was cleaned by passing it through 2 g of Al_2O_3 (5% w/w deactivated), concentrated and exchanged to hexane (1.0 ml) by a gentle solvent evaporation under a stream of nitrogen gas. GC–MS analysis was performed using a 7890A gas chromatograph (Agilent Technologies), fitted with a capillary column (J&W Scientific, Folsom, CA, USA) HP-5 MS (30 m _0.25 mm i.d., 0.25 lm film), coupled to a 5975C MS spectrometer equipped with a triple axis detector (Agilent Technologies). Initial column temperature was held for 1 minute at 60^{o}C followed by an initial ramp rate of 15^{o}C per minute to 150^{o}C and then at 6^{o}C per minute to a final temperature of 320^{o}C as reported elsewhere (Jimenez *et al*., 2012). Data were acquired in the full screen mode from 510 to 490 units with 5 minutes of solvent delay and processed by the aligent chemostation software.

2.2.5. Quantification of Microbial biomass

The amount of biomass in the medium was measured by estimation of the protein content using the Lowry method. The cells were centrifuged at 12857 x g for 10 minutes and were re-suspended in 1 ml distilled water and lysed by adding 2 ml of 2.5% sodium dodecyl sulphate in 0.2N Sodium hydroxide to release the proteins (Guerlava *et al*., 1998). The samples were vortexed and incubated in a boiling water bath for 10 minutes. The amount of protein was measured at 740 nm using a UV-visible spectrophotometer (shimadzu UV 2450, Japan). The protein biomass was estimated for all experiment setups.

2.3. Results

2.3.1. Enrichment studies

Enrichment culture has long been the method of choice for isolating bacteria expressing specific phenotypes (Golovlev, 2001) and has been used successfully to isolate bacteria capable of degrading hydrocarbons. There is significant interest in studying and isolating microorganisms present in contaminated environments as means for bioremediation. The crude oil samples were enriched on diesel oil so as to isolate bacteria that could utilize hydrocarbons.

The enrichment culture was evaluated for its ability to degrade hydrocarbons by conducting degradation microcosm studies using n-hexadecane as the model hydrocarbon. Gas chromatographic analysis of microcosm studies reveals degradation of hexadecane. The amount of residual hexadecane in biotic and abiotic samples was shown in Fig. 2.1. Chromatographic analysis of residual hexadecane after inoculation with enriched culture showed about 24% degradation in 24 h and 34% and 43% in 96 h and 120 h respectively (Fig. 2.1). This enrichment culture is highly essential as the culture could be used for successfully treating oil contaminated samples. Harayama *et al*., (2004) emphasize that there is little or no understanding of the vast majority of marine bacteria which will be useful for assessing the fate and effects of the spilled oil. The growth of the culture was monitored by quantifying the protein content of the biomass.

The culture was grown in BH medium enriched with diesel oil. To see different bacteria present in the consortium, DGGE was used and in the process 16S rDNA was also sequenced

to identify the bacteria. Spread plate with diesel oil as sole carbon source can be used but while spreading culture diesel doesn't get mixed and intern effects the separation of bacteria on the plate. So DGGE is opted to see presence of different bacteria.

By partial sequencing (16S rDNA) of the clones of extracted bands from DGGE gel (Fig. 2.2a) and by BLAST (NCBI) results of obtained sequences it was found that *Pseudomonas aeruginosa* (1) and *Escherichia fergusonii* (2) are the two bacteria present in the enrichment culture (Fig. 2.2b). Both the organisms belong to phylum proteobacterium and are classified as halophilic bacteria. The sequences were submitted to DNA database of Japan (DDBJ) and the accession numbers are AB793685.1 and AB793686.1 for *Pseudomonas aeruginosa* and *Escherichia fergusonii* respectively. *Escherichia fergusonii* was also reported for its heavy metal resistance and production of lipopeptide biosurfactant (Sriram *et al*., 2011). *Pseudomonas aeruginosa* was also reported for its capability to degrade hydrocarbons and also for its ability to degrade high molecular weight hydrocarbons like benz[a]anthracene, chrysene, flouranthene and pyrene (Chauhan *et al*., 2008).

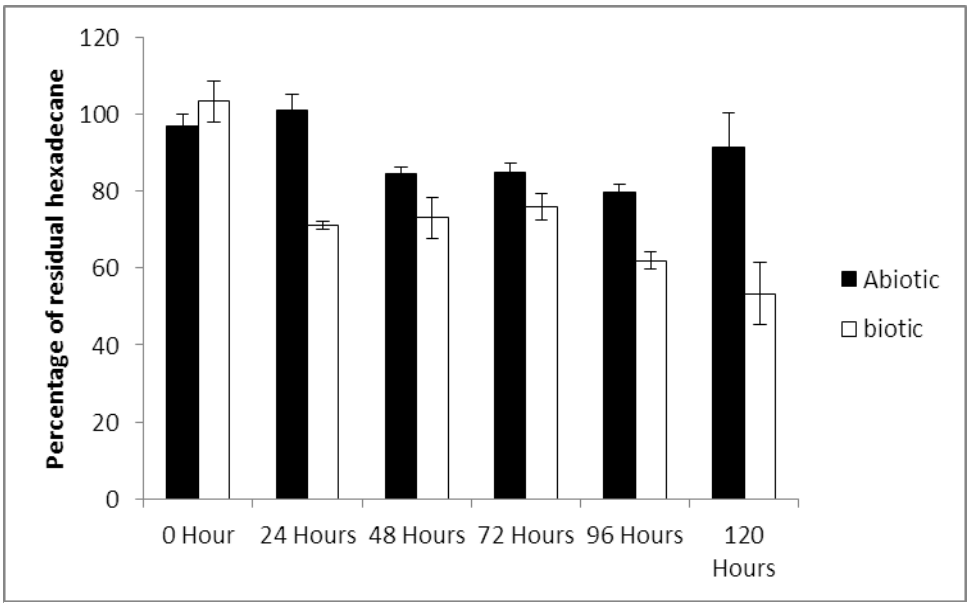

Fig. 2.1: Degradation of n- hexadecane as model hydrocarbon

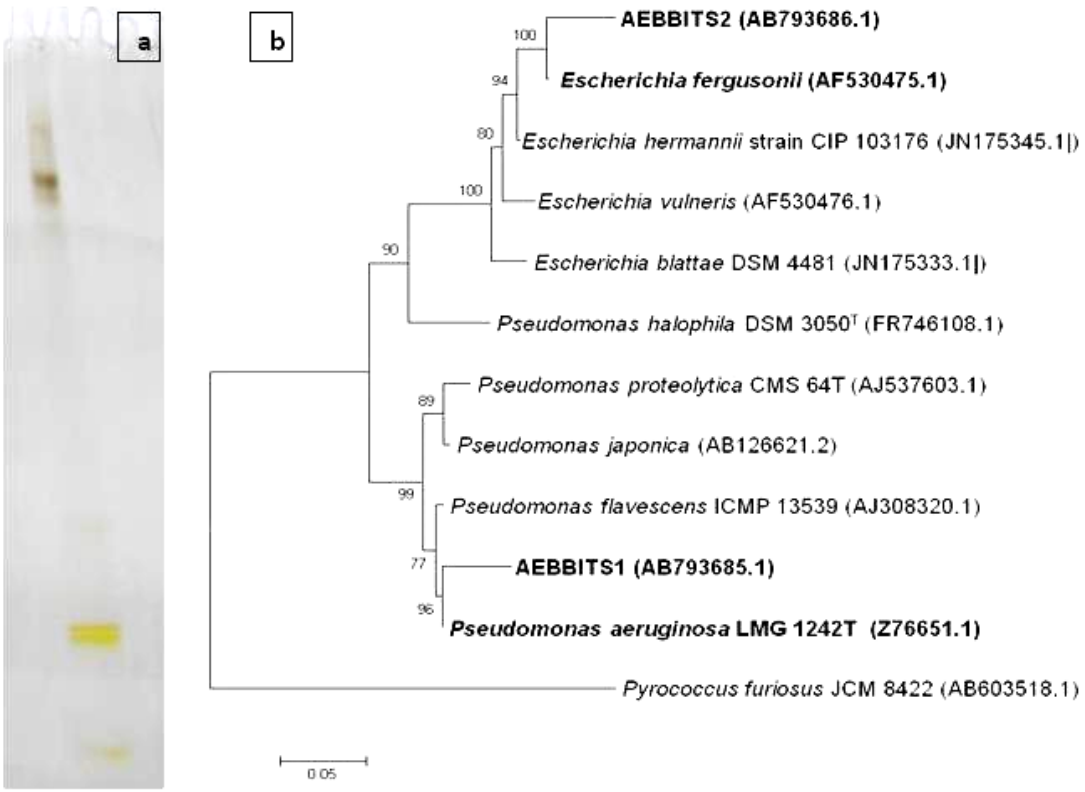

Fig. 2.2: a) DGGE of the culture b) Phylogenetic tree of the organisms, *Pyrococcus furiosus* was used as out group

2.3.2. Biodegradation of crude oil

In a study of oil degradation in soil contaminated by oil refinery it has been reported that hopane can be used as conserved internal biomarker as it showed invariable concentrations after laboratory experiments (Prince *et al*., 1994). The changes in the chemical composition of tar ball using hopane as an internal conservative marker were studied to assess the degradation process (Zakaria *et al*., 2000). Therefore we also used hopane as an internal marker. The relative loss of hydrocarbons in the biotic samples compared to abiotic samples was attributed to hydrocarbanoclastic activity of the culture.

2.3.2.1. n-Alkanes

The crude oil sample was found to have alkanes ranging from C_{12} to C_{42} (m/z 85) indicating that the spillage is of recent occurrence as it contains lighter alkanes. The degradation of alkanes followed specific pattern i.e., the degradation rate decreased as the carbon number increased. Alkanes ranging from C_{12} to C_{33} were highly degraded when compared to alkanes ranging from C_{34} to C_{42} (Table 2a). Most of the lighter alkanes i.e $<C_{25}$ were degraded by day 15 and the heavier ones were degraded by day 45. The reason for this may be that the bacteria might have opted for the lighter ones first (Fig. 2.3) or may be due to decrease in solubility with increase in molecular weight. Isoprenoid alkanes, pristane and phytane chains remained undegraded compared to their reference alkanes C_{17} and C_{18} by day 45 (Fig. 2.3) which supports the studies that reports pristane and phytane degradation will be slow until alkanes are available for bacteria (Seifert and Michael Moldowan, 1979).

Table 2a: Percentage of degradation of alkanes by day 45					
S.no	% of Degradation	Half life	S.no	% of degradation	Half life
C12	98.0	0.15	C28	97.9	1.195
C13	99.3	0.15	C29	97.6	1.553
C14	98.8	0.23	C30	96.4	4.596
C15	97.7	0.43	C31	95.6	-
C16	95.6	1.40	C32	92.0	-
C17	99.3	0.33	C33	86.1	-
C18	91.8	-	C34	76.8	-
C19	97.5	0.82	C35	74.3	-
C20	98.2	0.61	C36	70.7	3.493
C21	98.5	0.54	C37	63.7	1.868
C22	98.6	0.54	C38	60.0	0.944
C23	98.8	0.50	C39	61.3	0.592
C24	96.9	2.11	C40	60.0	0.431
C25	98.1	0.89	C41	60.0	0.327
C26	98.4	0.80	C42	60.0	0.274
C27	98.2	1.00			

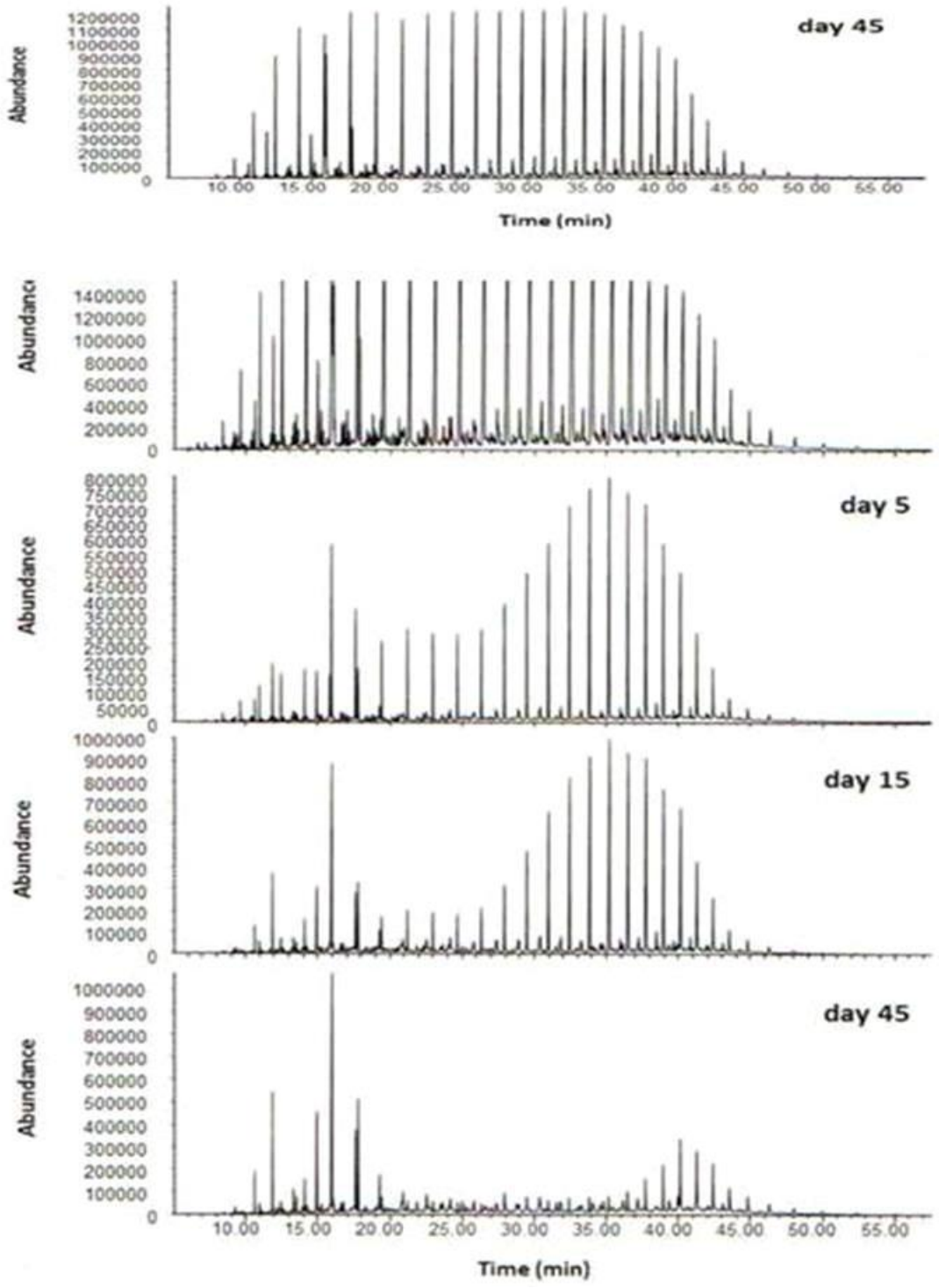

Fig. 2.3a.: Abundance of alkanes (m/z 85) of abiotic samples by day 45

2.3.2.2. Poly aromatic hydrocarbons

It appears that the rate of degradation of anthracene, fluorene, pyrene, naphthalene, fluoranthene, dibenzothiopene and phenanthrene is of first priority for the culture. Two to four ring PAHs like dibenzothiopene, naphthalene, fluorene, anthracene, fluoranthene, phenanthrene, pyrene and benzanthracene were degraded to 47.3%, 76.4%, 78.8%, 82.0%, 70.7%, 33.5%, 78.8% and 20.5% respectively by day 45, whereas four ring PAHs like chrysenes remained undegraded (Table 2b). This mode of degradation based on ring number was may be because of the fact that the increase in size and angularity resulted in increased hydrophobicity (Kanaly and Harayama, 2000). It was observed that chrysene remained undegraded whereas pyrene was degraded, this might be because the solubility of hydrocarbons decreases with the increase in molecular weight, the water solubility of chrysene and pyrene are 0.003 mg/L and 0.145 mg/L respectively (Morozik *et al.*, 2003). The lesser the solubility the lesser the bioavailability (Stucki and Alexander, 1987), so even both chrysene and pyrene are having four aromatic rings chrysene was not degraded may be due to its low water solubility. The degradation rate has decreased with methylation, for example, degradation of pyrene is 78.8% whereas methyl, dimethyl, trimethyl pyrenes was 0% and degradation of 1-methyl naphthalene, 2-methyl naphthalene and 3-methyl naphthalene were 85.5%, 69.5% and 33.2% respectively. The same pattern was observed for dibenzylthiopene with degradation of 47.3% but whereas degradation rates of methyl, dimethy, trimethyl dibenzothiopenes were 8.1%, 8.8% and 13.5% respectively. The decrease in degradation by alkylation was may be due to decrease in solubility by alkylation (Mackay *et al*., 1992). The low degradation rate of PAHs was also may be due to maintenance of culture on medium enriched with diesel oil as sole source of carbon.

Table 2b: Degradation of Poly aromatic hydrocarbons					
PAH	% of degradation	Half life	PAH	% of degradation	Half life
N	76.4	18	DBT	47.3	80
N1	85.5	12	DBT1	8.1	315
N2	69.5	23	DBT2	8.8	374
N3	33.2	74	DBT3	13.5	193
F	78.8	23	FL	70.7	27
F1	13.0	215	Py	78.8	21
F2	6.5	399	Py1	0.0	-
F3	0.0	-	Py2	0.0	-
P	33.5	98	Py3	0.0	498
A	82.0	27	BA	20.5	116
P1	1.1	1082	C	0.0	-
P2	4.9	987	C1	0.0	964
P3	0.0	1771	C2	0.0	1352
			C3	0.0	408

2.3.3. Growth of culture in crude oil

The growth of the culture in crude oil throughout the 45 days experiment was monitored by quantifying the microbial biomass. The culture was in exponential phase till day 2 followed by stationary phase till day 5 and fell into decline phase till day 10 and then it showed again exponential growth till last day of the experiment. This can be due to the utilization of readily available lighter alkanes in the first 2 days followed by probable adaptation period to degrade the heavier alkanes which might have resulted in a decline phase between day 5 and day 10. This conclusion could be arrived at as heavier alkane degradation was low between day 5 and day15 (Fig. 2.4).

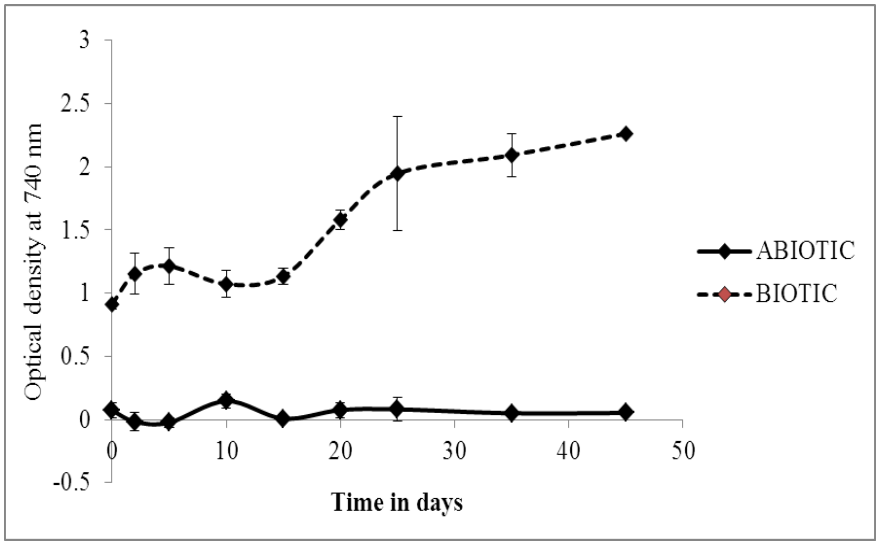

Fig 2.4: Total protein biomass by Lowry method during degradation of crude oil sediment

2.3.4. DGGE of the culture during experiment

The survival of both the strains identified during enrichment culture during crude oil degradation studies was found by DGGE. Based on DGGE gel (Fig. 2.5) it was clear that both the organisms survived till the last day of the experiment and *Escherichia fergusonii* seems to be the dominant strain based on the thickness of the band in DGGE gel, Murray *et al*., (1996) reported that the band intensity in DGGE gel is directly proportional to abundance of DNA from the particular phenotype in the template mixture. However it is not clear which organisms among the two has taken the lead role in biodegradation because the growth profiles cannot reflect the degradation capabilities. The study of growth profiles of hydrocarbonoclastic bacteria isolated from Bight of Bonny, Nigeria revealed that ability of microorganisms to degrade hydrocarbons depends on its vital enzymes but not necessarily on its fastidious growth (Itah and Essien, 2005).

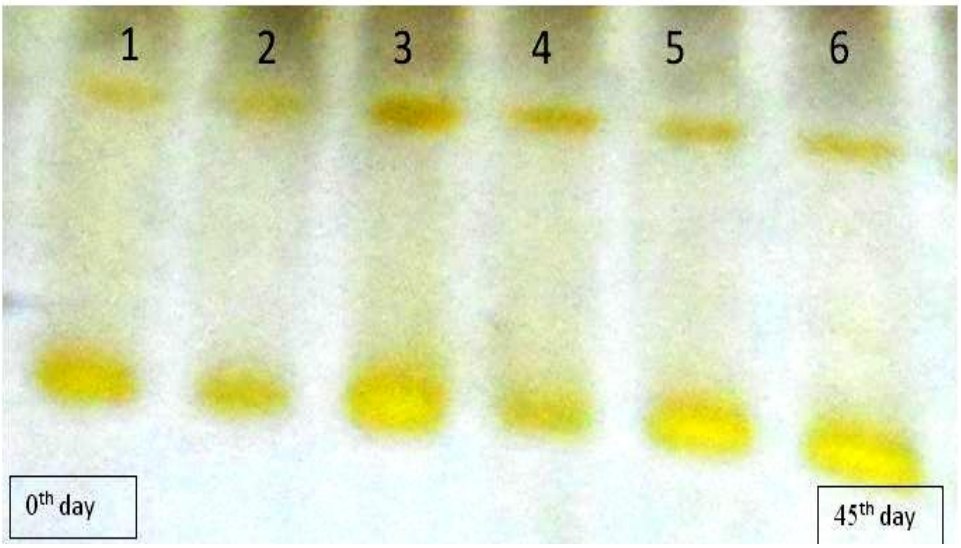

Fig. 2.5: DGGE gel indicating both the stains are alive till day 45. First lane is of day 0 and the last lane is of day 45, in between are day 5, 15, 25, 35.

2.4. Discussion

A Japanese tanker collided with a small Indian tanker in the year 2006 resulting in spillage of 4,500 tons of oil into sea. In the year of 2010 MV MSC Chitra (tanker) collided with MV Khalijia (tanker) near Mumbai resulting in spillage of about 800 tons of oil into sea. The spilled oil undergoes weathering process and turns into blob of semisolid crude oil called tarball. Goa is facing the problem of tarball pollution since 1970's.

In the present study a consortium having two bacteria capable of degrading crude oil sediment (tarball) has been isolated. *Pseudomonas aeruginosa* (AEBBITS1) and *Escherichia fergusonii* (AEBBITS2) are the two bacteria present in the consortium. *Pseudomonas aeruginosa* has been reported in many studies for its capabilities to degrade hydrocarbons. *Escherichia fergusonii* has never been reported to degrade hydrocarbons.

The presence of indigenous bacteria capable of degrading hydrocarbons in Goan coasts was reported earlier. Marine bacteria belonging to genus *Alkaligenes* capable of degrading dibenzothiopene was isolated from Goan coast contaminated with furnace oil (Rodrigues *et al*., 2011) and also *Pseudomonas mendocina* was isolated from bilge oil sample and the organisms was reported to degrade hydrocarbons and to produce exopolysaccharides by Sivaraman *et al.,* (2011). Both the bacteria were known for their hydrocarbanoclatic capabilities but occurrence along the coast of Goa was not yet reported. Salinity is one of the major environmental factors that affect bioremediation, the effect of salinity on biodegradation of oil by an Arabian Sea sediment culture was studied over a salinity range of 0-5% and concluded that there was a decrease in rate of degradation of diesel oil with increase in salinity and significant degradation of diesel oil was observed up to a salt concentration of 3.5% (Mukherji *et al.,* 2004). The two bacterial isolates that we have isolated can degrade crude oil as sole carbon source and had shown preference for aliphatic hydrocarbons under saline conditions (3% NaCl) which mimics the marine environment. Thus these bacterial strains can provide a partial solution to oil contamination in Goan coast.

From the GC-MS results of the microcosm it was evident that alkanes were degraded extensively compared to Polyaromatic hydrocarbons (PAHs). It was also observed that the alkylated PAHs were not degraded but the unalkylated PAHs were degraded. The reason for

high degradation of smaller alkanes followed by higher alkanes and low molecular weight PAHs followed by high molecular PAHs is due to difference in solubility. The bioavailability of hydrocarbons decreases with decrease of solubility and the solubility of hydrocarbon decreases with increase in size and ring number of PAHs. It was evident from the earlier reports that alkylation of PAHs results in decrease of solubility. The degradation of hydrocarbons not only depends on the type of hydrocarbons but also on the molecular capabilities of bacteria to degrade different types of hydrocarbons. In many instances it was reported that usage of consortium is better as a single organism might not be able to degrade different components of oil. The range of alkanes that was degraded by consortium (Halo) was an indication for the consortiums molecular capabilities to degrade wide range of substrates (hydrocarbons). The DGGE of the microcosm samples also proves the good synergistic relation between AEBBITS1 and AEBBITS2 as both the bacteria survived till last day of the experiment.

2.5. Conclusion

The coastline of Goa, India requires an immediate and permanent solution for oil contamination as oil pollution is an annual phenomenon and it affects the tourism sector. *Pseudomonas aeruginosa* and *Escherichia fergusonii* showed great potential in degrading hydrocarbons present in crude oil. Our future work is aimed at conducting pilot scale bioremediation experiments with crude oil contaminated sediments using the above isolated bacterial strains. The bacteria that have been reported earlier have very short range of alkanes as substrate but these bacteria could utilize alkanes ranging from C12 to C42 and also unalkylated aromatic hydrocarbons.

CHAPTER 3

BIODEGRADATION OF ALIPHATIC HYDROCARBONS IN PRESENCE OF HYDROXY CUCURBIT[6]URIL

Publication: Biodegradation of aliphatic hydrocarbons in the presence of hydroxy cucurbit[6]uril.

(Mar. Poll. Bull. 2014, 88, 148-154)

3. Introduction

Aliphatic hydrocarbons are major fraction of petroleum products (Hidayat and Tachibana, 2012). Apart from their toxic nature, hydrocarbons are also known for their hydrophobic property which is the important factor that limits their bioavailability (Wick et al., 2002). Bioavailability of hydrocarbons can be increased either by making the microorganisms access the hydrocarbon directly or bringing the hydrocarbons into the aqueous phase by increasing their solubility (Zhao et al., 2011). Increasing the solubility attains priority as the hydrocarbons can be used by any type of microorganism whereas bringing the microorganism in contact to the hydrocarbon fraction may not be a good option because different organisms have different membrane characteristics (Mohnaty and Mukherji, 2011). It has been already reported that cyclodextrins can increase the bioavailability of aliphatic hydrocarbons by hosting them in their hydrophobic cavity (Sivaraman et al., 2010). Cucurbiturils are cyclic polymers of glycourils which can exhibit similar host-guest chemistry as cyclodextrins. There are different homologues of cucurbiturils i.e., CB[5], CB[6], CB[7], CB[8] etc., among which CB[6] is most popular member of this family because of its ease of synthesis and smooth isolation from the rest of its homologues. This pumpkin shaped compound has a sufficiently large hydrophobic internal cavity of ~5.5 Å which is accessible through two carbonyl fringed hydrophilic portals of ~ 4 Å diameters for strong interaction with the hydrophobic molecules (Mock et al., 1986). Till date, several host-guest binding studies have been reported on cucurbiturils as the host molecule. For instance CB[7] was reported as a drug delivery agent to deliver a cancer drug named oxaloplatin (Kim et al., 2005). It is also known that cucurbiturils can strongly bind to the hydrocarbons. CB[5], CB[6] can hold small gas molecules, aliphatic and unsubstituted aromatic guests and CB[7] can host some substituted aromatic guests. Recently, Nau and co-workers reported a fluorescence dye displacement method utilizing the strong binding behaviour of hydrocarbons to hydrophobic interiors of cucurbit[6]uril (Florea and Nau, 2011). However, the solubility of CB[6] and its further functionalization were the two major concerns until the seminal work of Kim and co-workers to convert CB[6] to hydroxy CB[6] a decade back (2003). Although CB[6] is partly soluble in water, a huge improvement in the solubility has been observed upon polyhydroxylation. In the present study, we have used hydroxy CB[6] instead of CB[6] keeping in mind its increased water solubility. The present study deals with

the improvement or enhancement of bioavailability of aliphatic hydrocarbons by hydroxy CB[6] using the indigenous hydrocarbanoclastic bacteria isolated from a contaminated site. The usage of naturally occurring bacteria would be beneficial as they take less time to acclimatize to the contaminated environments (Ojo, 2006).

3.1. Materials

3.1.1 Chemicals

Commercially available Bushnell Hass (BH) medium, tetradecane, hexadecane, octadecane were obtained from Himedia, India. Sodium bicarbonate, Sodium Chloride, Folin's ceocaltue was obtained from Fisher Scientific, India. Ethyl acetate, Acetone, glyoxal, urea, hydrochloric acid, sulphuric acid and potassium per sulphate were purchased from S D Fine - Chem Limited, Mumbai, India. Milli-Q water was used in all experiments as per requirement.

3.2 Methodology

3.2.1 Synthesis of hydroxy Cucurbit[6]uril, NMR and ESI-MS

Cucurbit[6]uril was synthesized following the reported procedure by Kim and co-workers (Anthony Day et al., 2001). Hydroxy CB[6] was synthesized by using reported procedure by Kim and co-workers (2003). A slurry of CB[6] (1.0 g, 1.0 mmol) and $K_2S_2O_8$ (3.9 g, 14.4 mmol) in distilled water (50 mL) was taken and nitrogen gas was bubbled through the mixture for degassing. Under the atmosphere of nitrogen the mixture was heated at 85°C for 6 h. After cooling to room temperature, the resulting precipitate was removed by filtration and the filtrate was concentrated to 25 mL under the reduced pressure. Acetone vapour diffusion into the resulting solution gave a white precipitate, which was collected by filtration, washed with acetone and dried in vacuum to give hydroxy CB[6] $[(HO)_{12}CB[6](K_2SO_4)_2]$ (600 mg, 39 %). NMR spectra were recorded on a Bruker 400 MHz spectrometer. Electrospray ionization (ESI) mass spectra were obtained on a Waters Q-TOF micro mass spectrometer.

3.2.2 Test for toxicity of hydroxy CB[6] on animal cell lines

The cell viability of A549 and HeLa cell lines in presence of hydroxy CB[6] was determined by trypan blue cell viability assay. Filter sterilized hydroxy CB[6] was added to the growth medium of the animal cell lines at a concentration of about 2.5 mM along with a control. The cells were incubated for 48 hours and cell viability was checked every 24 hours. Cell morphology was observed using inverted microscope.

3.2.3 Enrichment culture

Even though only 1% of the microbial diversity is cultivable from any contaminated site (Torsvik et al., 2002), enrichment technique has been used successfully for isolating hydrocarbanoclastic bacteria (Golovlev, 2001). The crude oil samples were enriched on diesel oil so as to isolate bacteria that could utilize hydrocarbons. A consortium was isolated from Velsao beach, India, from a crude oil contaminated site by enrichment culture technique using diesel oil as sole carbon source under both halophilic (Halo-3% NaCl) and nonhalophilic (Nonhalo) condition. The cultures have been maintained by continuous sub culturing for every five days in BH medium using diesel oil as sole carbon source. The BH medium used for consortium under halophilic conditions was added with 3% NaCl.

3.2.4 Amplification of 16S rDNA and Denaturing Gradient Gel Electrophoresis (DGGE)

Genomic DNA from both the consortiums i.e., halophilic and non halophilic was extracted using procedure recommended by Paul and Rochelle (2001). 16S rDNA was amplified by nested PCR using BIORAD mini personal thermal cycler as per the protocol mentioned in chapter 2, section 2.3. DGGE was used to identify the number of bacteria present in each consortium. 7.5% (40% acrylamide) with 30% to 60% gradient was used (Nikolausz *et al*., 2008). The gel was stained by silver staining.

3.2.5 Biodegradation Studies

3.2.5.1 Microcosm

The degradation studies were conducted in conical flask with 20 ml BH medium and 0.5% (100ul) of carbon source. Tetradecane, Hexadecane, Octadecane were used as individual carbon source and also as a mixture. The consortia were grown in 1 liter BH medium with diesel oil as sole carbon source and late log phase culture was centrifuged at 8000 rpm for 10

minutes at 4^{o}c. The pellet was washed with sterile distilled water to remove the unused diesel oil and was dissolved in 30 ml of sterile distilled water and 1ml was inoculated into each flask except into abiotic samples. The procedure was followed for both the consortia i.e., Halophilic and Non halophilic. The conical flasks were kept in shaking incubator at 100 rpm, room temperature. Abiotic samples without inoculum and biotic samples with inoculum serve as controls for the flasks with hydroxy CB[6]. The amount of residual hydrocarbon left over in the flask was extracted every 24 hrs till 120 hrs by adding equal amount of ethyl acetate with respect to the amount of medium used. 1ml of the extracted sample was preserved in a glass vial till the time of analysis.

3.2.5.2 Analysis of hydrocarbons by Gas chromatography

The percentage of degradation was calculated assuming the residual hydrocarbon present in the abiotic sample at 0 hours as 100%. Residual hydrocarbon present in each sample was quantified using gas chromatography (CHEMITO) with FID detector and BPx5 model column to determine the rate of degradation of hydrocarbon. Temperature program was 50^{o}C, 5 minutes isotherm, 5^{o}C rise per minute with an injector and detector temperature of 250^{o}C. The carrier gas used was nitrogen with a flow rate of 4ml per minute. Air and hydrogen were used for detection at the ratio of 1:100. The residual hydrocarbon was analyzed up to 120 hours for every 24 hours (Sivaraman et al., 2010). 100 µl of 1% naphthalene was added to each sample as an internal standard before injecting into gas chromatograph (Bardi et al., 2000).

3.2.6. Quantification of Microbial biomass

Total protein was estimated by Lowry method to quantify the amount of biomass. The cells from each flask were centrifuged at 12857 x g for 10 minutes and were re-suspended in 1 ml distilled and were lysed by addition of 2 ml of 2.5% sodium dodecyl sulphate in 0.2N Sodium hydroxide to release the proteins by incubating the samples in a boiling water bath for 10 minutes (Guerlava *et al*., 1998). The blue colour formed was measured at 740 nm using a UV-visible spectrophotometer (shimadzu UV 2450, Japan).

3.3 Results and Discussion

3.3.1 Synthesis of hydroxy CB[6] and NMR, ESI-MS

Hydroxy CB[6] was successfully synthesized using a reported procedure by Kim and co-workers (2003) in descent yield. Due to its good water solubility this macrocyclic molecule can play a major role as a hydrocarbon carrier like cyclodextrins. The data obtained from ^{1}H NMR, ^{13}C NMR and ESI-MS (fig: 3.1, 3.2, 3.3) are in well agreement with the literature values (Jon et al., 2003). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.44 (d, J = 14.8 Hz, 12H), 5.35 (d, J = 14.8 Hz, 12H), 7.20 (bs, 12H) (Fig 3.2); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 40.6, 94.2, 153.1 (Fig 3.3); MS (ESI) m/z: 1227.2 ($M + K^+$), 1305.2 ($M + 3K^+$) (Fig 3.1).

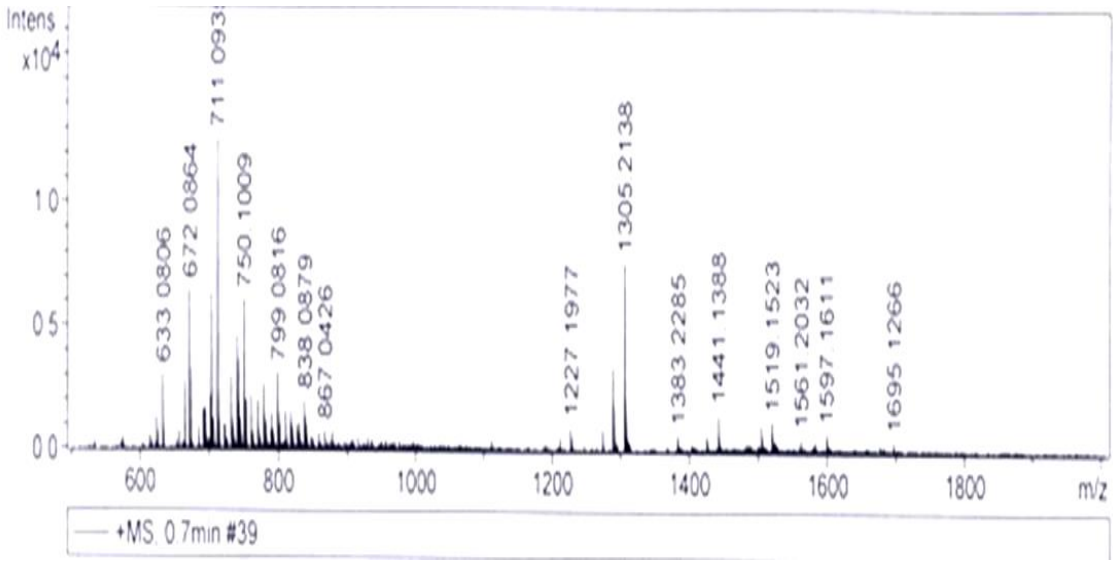

Fig 3.1: ESI-MS of hydroxy Cucurbit[6]uril

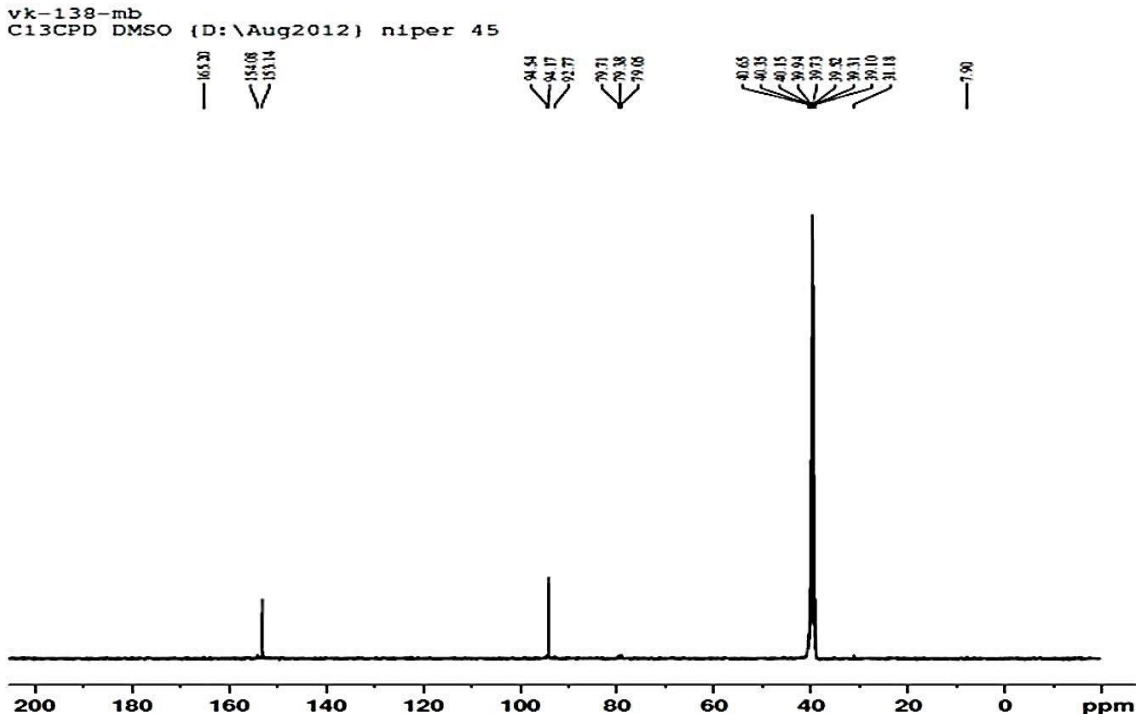

Fig 3.2: ^{1}H NMR spectrum of hydroxy Cucurbit[6]uril

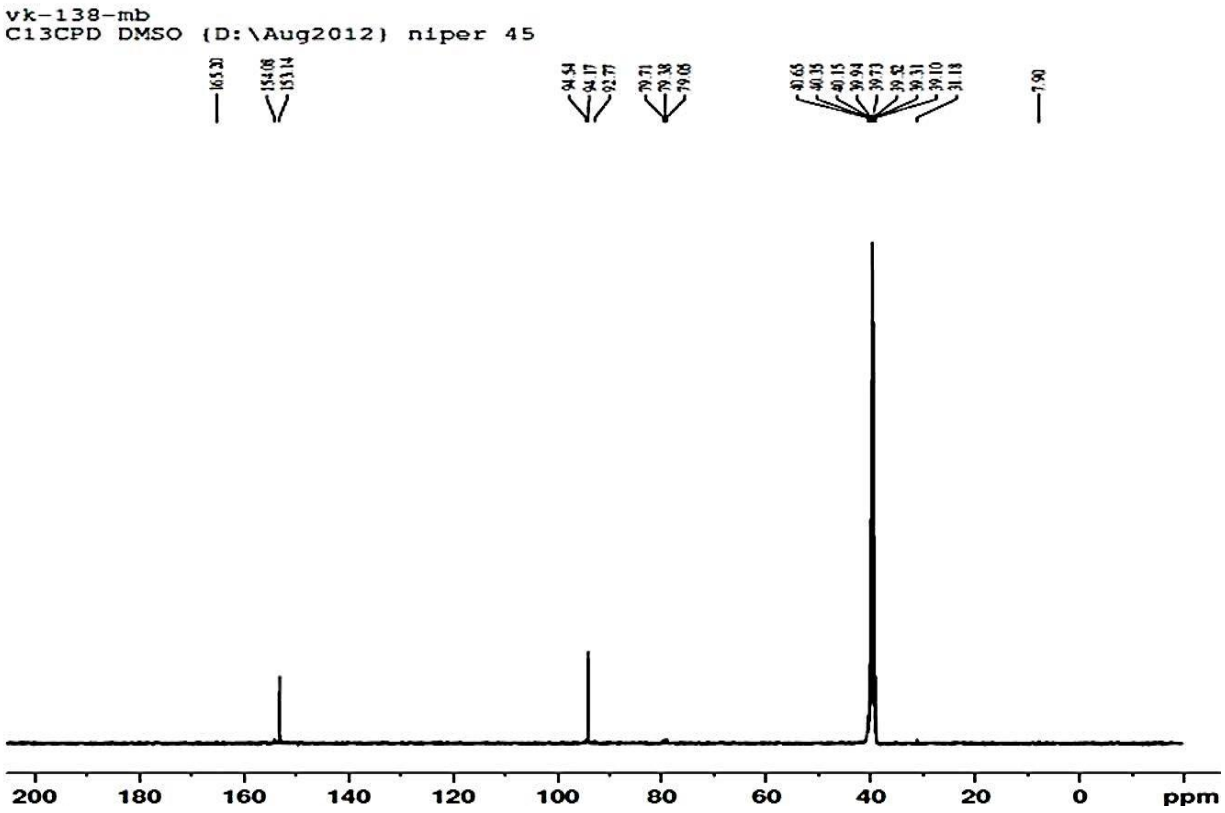

Fig 3.3: ^{13}C NMR spectrum of hydroxy Cucurbit[6]uril

3.3.2 Toxicity Test

It has been reported that *in vitro* and *in vivo* cucurbiturils are inert. Extensive studies have been done stating that CB[7] doesn't show any toxicity against Chinese hamster ovary CHO-K1 cells (0.5mm), human kidney cells (1.0mm), human hepatocyte cells, murine macrophage cells, and human A549 non-small lung cells (Eirc et al., 2012). In this study the toxicity of hydroxy CB[6] against HeLa and A549 was tested. After 12 and 24 hours of incubation the survived cell number was counted and cell morphology was observed using inverted microscope. It was observed that there were no changes in cell number and cell morphology in the experimental setups with both HeLa and A549 cell lines. The percentage of HeLa cells survived in 12 hours control was 91.67% and in test it was 86.09%. The percentage of survival rate at 24 hours was 85.71% and 83.72% respectively. The percentage of viability of A549 cells in 12 hours control was 97.56% and test was 94.83% which was equal to viability of 24 hours samples i.e., 92.68% and 94.73% respectively. The toxicity tests on animal cell lines support the idea of using hydroxy CB[6] on contaminated sites as an enhancer of bioavailability of aliphatic hydrocarbons as it has not showed any toxic effects on the cell lines used (Fig: 3.4).

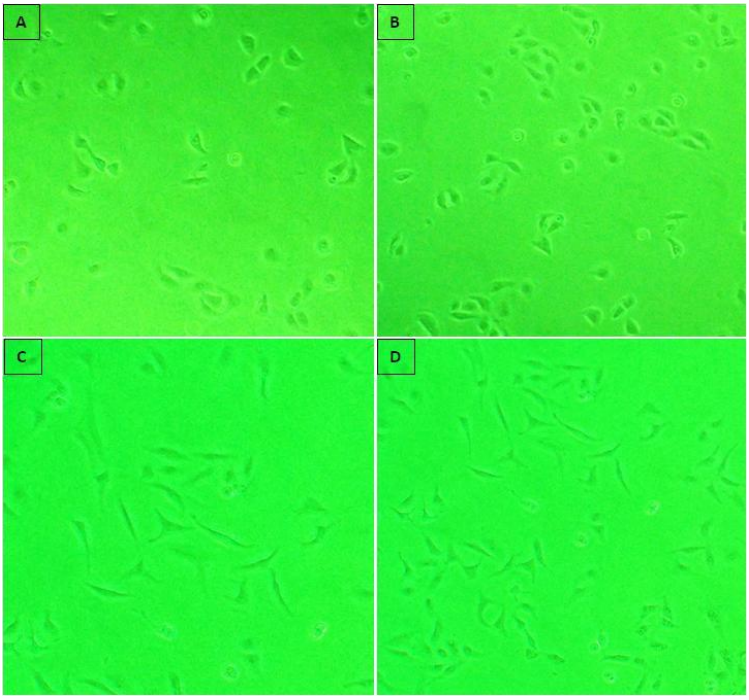

Fig 3.4: Microscopic picture of toxicity test using hydroxy CB[6] against animal cell lines. Picture A is of HeLa control at 24 hrs, B is of HeLa cells supplied with hydroxy CB[6], C is of A549 control at 24 hrs, D is of A549 cells supplied with hydroxy CB[6] at 24 hrs.

3.3.3 Denaturing Gradient Gel electrophoresis (DGGE)

The amplified 16S rDNA products of both the consortia were loaded onto the DGGE. It was found that the Non halo consortium under non halophilic conditions (hereafter represented as non halo) has three different bacterial strains and consortium under halophilic conditions (hereafter represented as halo consortium) has two different bacterial strains. As per the results obtained by sequencing of Halo strains it was identified as *Pseudomonas aeruginosa* (1) and *Escherichia fergusonii* (2). From figure: 3.5 it was very clear that these two strains are present in the Non halo consortium as well.

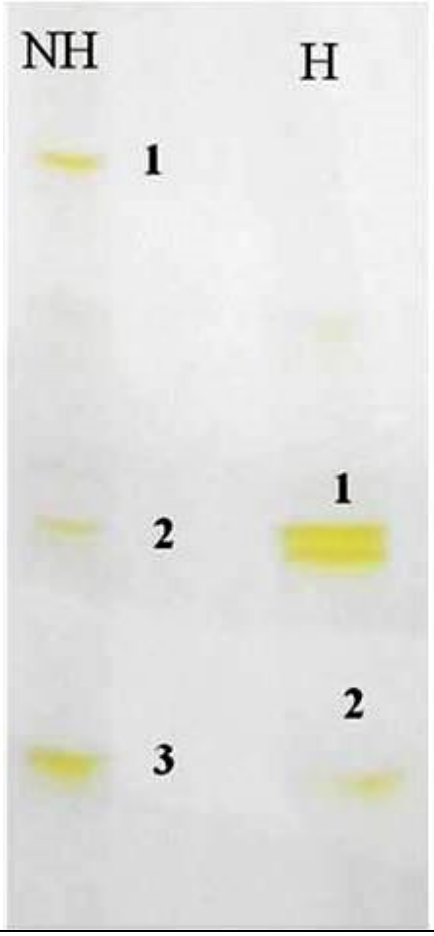

Fig 3.5: DGGE gel showing different bacteria present in Non halo(NH) and Halo (H) consortiums. Lane 1 shows 3 different bands (NH), Lane 2 shows 2 different bands (H).

3.3.4 Biodegradation Studies

3.3.4.1 Effect of hydroxy CB[6] on individual hydrocarbons as sole carbon source

Tetradecane

The percentage of residual tetradecane leftover in the biotic control inoculated with halo and nonhalo consortia at 24 hrs were found to be 74.1% ± 3.5, 76.11% ± 2.1, where as in samples supplied with hydroxyl CB[6] were found to be 75.6% ± 6.7, 77.11% ± 6.7 respectively. At 24 hrs all the samples and their biotic controls have same amount of residual tetradecane. The percentage of residual tetradecane present at 120 hrs i.e., at the end of the experiment in biotic controls of samples inoculated with halo, nonhalo consortia was found to be 43% ± 7.4, 25.56%, whereas in microcosm supplied with hydroxyl CB[6] was found to be 29.3% ± 2.8, 26.73% ± 1.1 respectively. Even though at 120 hrs the residual hexadecane present in nonhalo sample and its biotic control was same, it was found that by 96 hrs in biotic control the residual hydrocarbon was 59 % ± 0.3 and in sample supplied with CB[6] it was 29.85% ± 0.7 (Fig. 3.6).

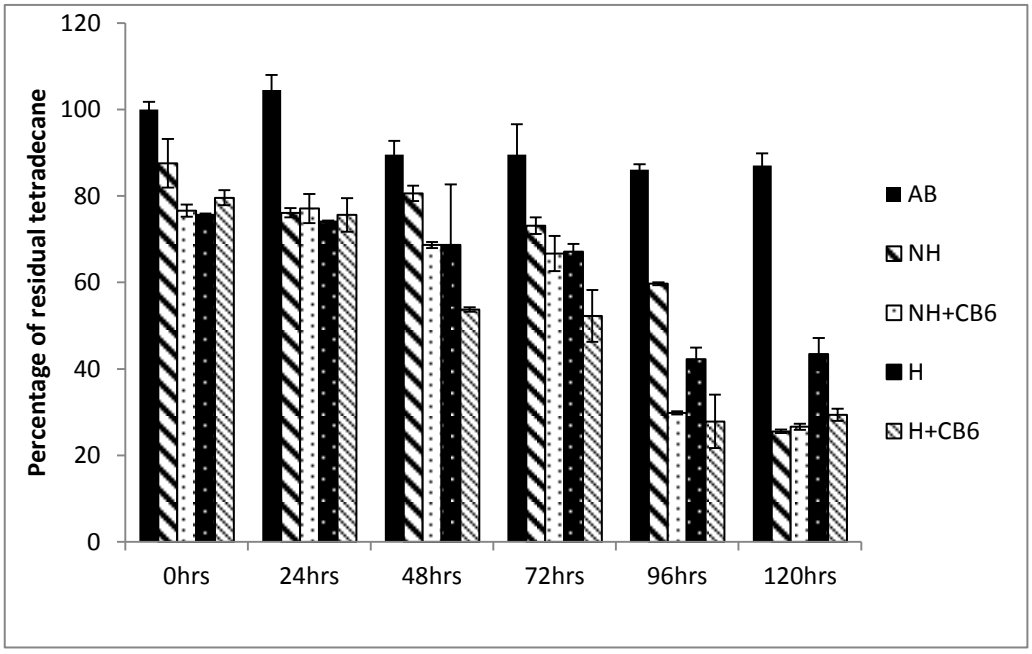

Fig 3.6: Graph representing the degradation of tetradecane as sole carbon source. AB indicates abiotic samples. NH, H, NH+CB6, H+CB6 indicates the samples inoculated with nonhalo and halo consortia and their respective samples supplied with hydroxyl CB[6].

Hexadecane

The percentage of residual hexadecane present in biotic controls inoculated with halo and nonhalo consortia at 24 hrs were found to be 71.09% ± 0.87, 70.04% ± 2.23, where as in samples supplied with hydroxyl CB[6] were found to be 83.03% ± 3.02, 79.87% ± 3.43. At the end of the study i.e., at 120 hrs the residual hexadecane present in biotic samples inoculated with halo and nonhalo consortia were found to be 53.33% ± 7.96, 37.09% ± 1.46 respectively. However in samples supplied with hydroxy CB[6] it was found to be 54.53% ± 0.91, 28.82% ± 0.92 respectively. The percentage of residual hexadecane present in sample inoculated with halo consortium at 96 hrs i.e., 51.7% ± 5.34which was much less to its biotic control i.e., 61.9% ± 2.29 (Fig. 3.7).

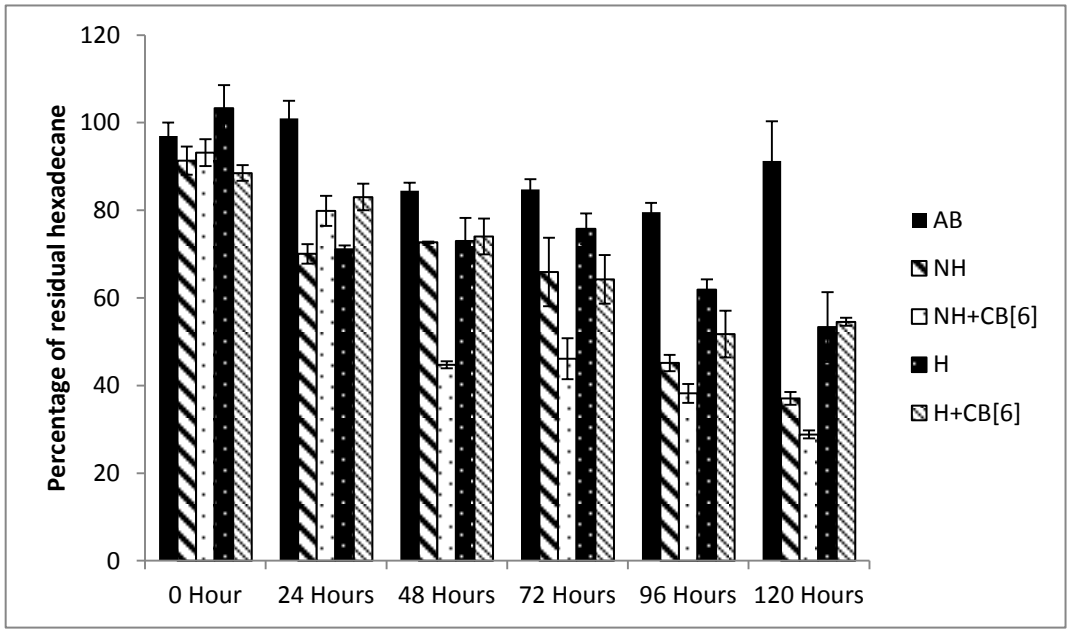

Fig 3.7: Graph representing the degradation of tetradecane as sole carbon source. AB indicates abiotic samples. NH, H, NH+CB[6], H+CB[6] indicates the samples inoculated with nonhalo and halo consortia and their respective samples supplied with hydroxyl CB[6].

Octadecane

The percentage of residual octadecane present in biotic controls inoculated with halo, nonhalo consortia at 24 hrs were found to be 63.74% ± 0.13, 72.44% ± 4.7. Whereas in samples supplied with hydroxy CB[6] were found to be 74.20% ± 3.34, 69.42% ± 1.49 respectively. At 120 hrs the percentage of residual octadecane in sample inoculated with halo and nonhalo consortia were found to be 45.35% ± 0.48, 48.51% ± 2.67, where as in samples inoculated with CB[6] were found to be 50% ± 2.07, 46.25% ± 1.85 respectively. The percentage of degradation was found to be same in all the samples throughout the experiment (Fig. 3.8). So no positive effect was shown by hydroxy CB[6] to towards increasing the bioavailability of octadecane.

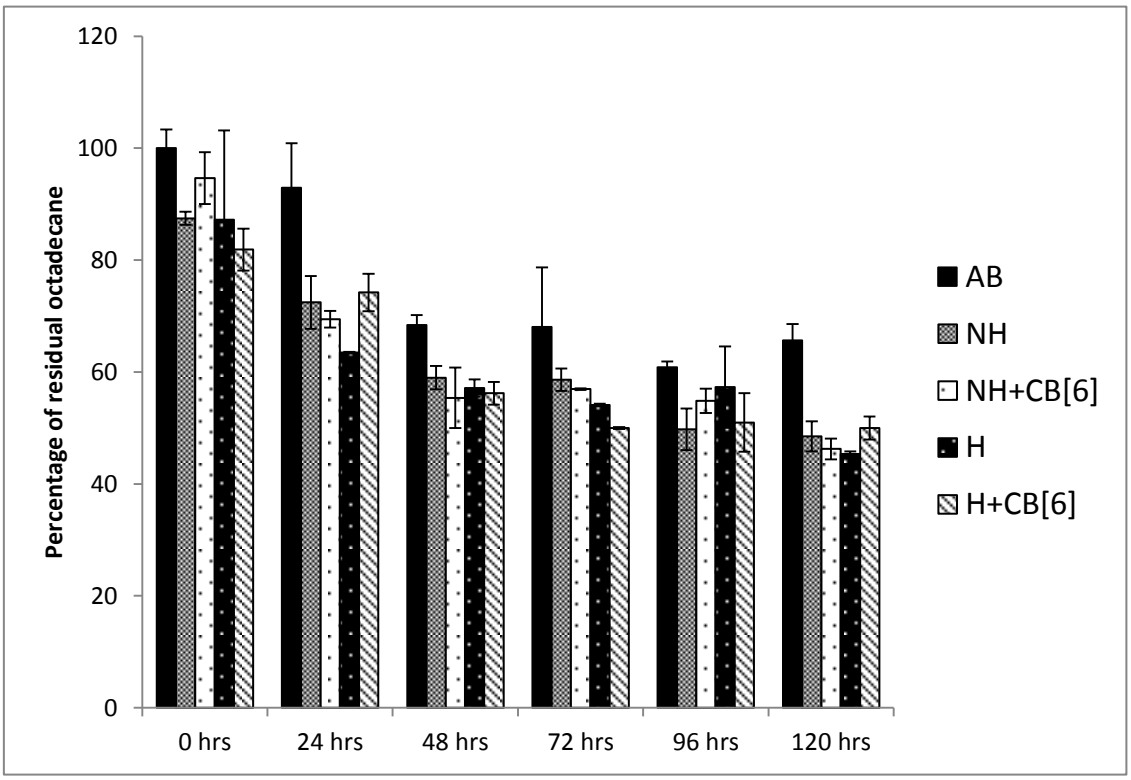

Fig 3.8: Graph representing the degradation of octadecane as sole carbon source. AB indicates abiotic samples. NH, H, NH+CB[6], H+CB[6] indicates the samples inoculated with nonhalo and halo consortia and their respective samples supplied with hydroxyl CB[6].

3.3.4.2 Effect of hydroxy CB[6] on mixture of hydrocarbons

There was no significant effect of hydroxy CB[6] on biodegradation of tetradecane, hexadecane, octadecane when used as mixture with Nonhalo consortium (Fig. 3.9, 3.10, 3.11) whereas all the three hydrocarbon's biodegradation was increased by CB_6 using halo culture. Hydroxy CB[6] has increased the bioavailability of all three hydrocarbons when used as mixture in microcosms (Fig: 3.9, 3.10, 3.11) with only halo culture. The residual hydrocarbons present in microcosm with hydroxy CB[6] were 13% (tetradecane), 15% (hexadecane), 8% (octadecane) less compared to their biotic controls. The degradation of hydrocarbons was found to be less compared to their degradation when used individually as sole carbon source for a very simple reason that when they were used as mixture the need of carbon source by the culture would have been accomplished by all three hydrocarbons than would be by the individual hydrocarbons when supplied as sole carbon source.

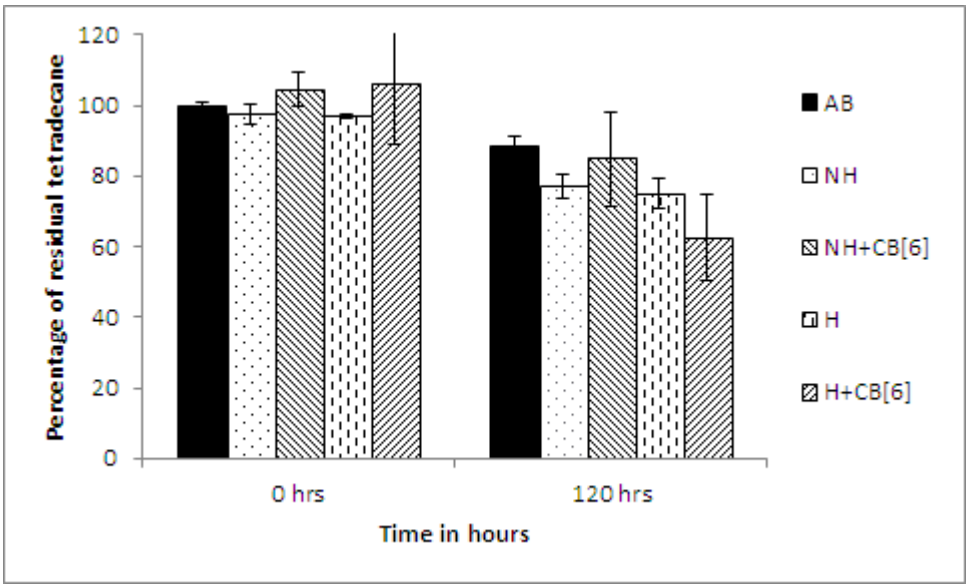

Fig 3.9: Graph representing the degradation of tetradecane. AB indicates abiotic samples. NH, H, NH+CB6, H+CB6 indicates the samples inoculated with nonhalo and halo consortia and their respective samples supplied with hydroxy CB[6].

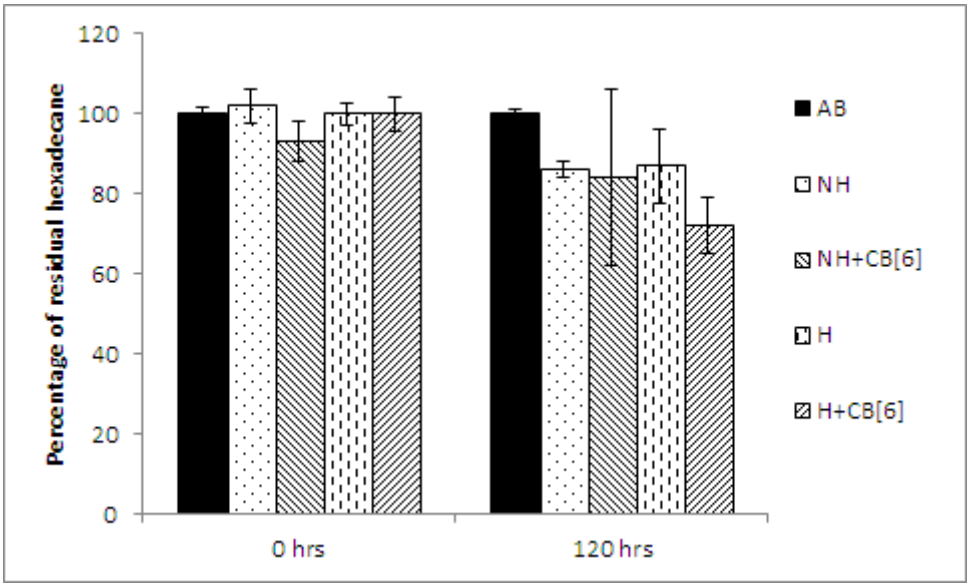

Fig 3.10: Graph representing the degradation of hexadecane. AB indicates abiotic samples. NH, H, NH+CB6, H+CB6 indicates the samples inoculated with nonhalo and halo consortia and their respective samples supplied with hydroxy CB[6].

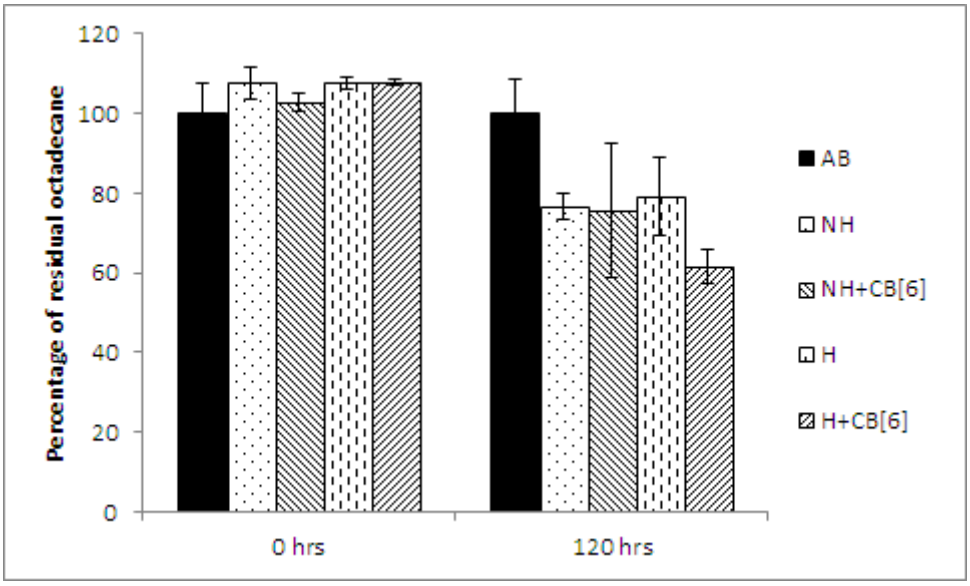

Fig 3.11: Graph representing the degradation of octadecane. AB indicates abiotic samples. NH, H, NH+CB6, H+CB6 indicates the samples inoculated with nonhalo and halo consortia and their respective samples supplied with hydroxy CB[6].

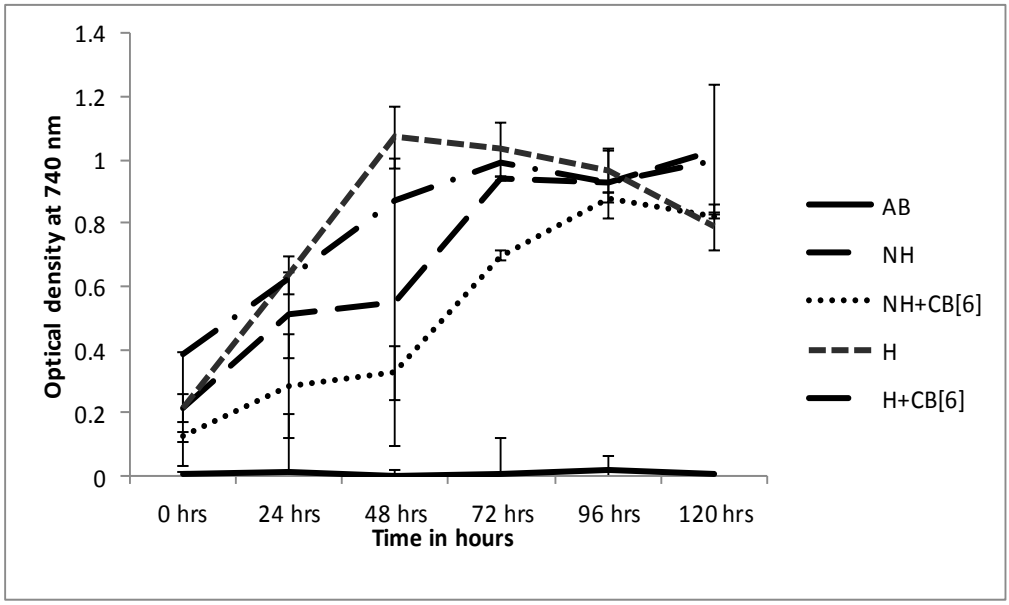

Fig 3.12: Graph representing the protein biomass by Lowry method. AB indicates abiotic samples. NH, H, NH+CB[6], H+CB[6] indicates the samples inoculated with nonhalo and halo consortia and their respective samples supplied with hydroxy CB[6].

3.4 Discussion

Solubility of cucurbiturils has been the issue after the synthesis and characterization of different homologues. It has been reported that cucurbiturils having odd number of glycouril units like CB[5] and CB[7] are more soluble compared to ones having even number of glycourils like CB[6] and CB[8] (Bardeling et al., 2011). Due to its easy synthesis, smooth isolation and high yield, hydroxy CB[6] was preferred to be used in this study. The concentration used in this study was 2.5 mM based on previous reports that solubility range of polyhydroxy CB[6] ($CB[6](OH)_{12}$) was 2 to 3 mM (Lewin et al., 2013). It has been observed that hydroxy CB[6] has shown a positive effect on bioavailability of mixture of hydrocarbons with halophilic culture compared to nonhalo culture which may be due to the effect of 3% NaCl present in the Halo medium. This can be justified by the study stating that the solubility of CB[6] and hydroxy CB[6] can be increased in aqueous solution by addition of alkali metal ions by favorable interaction of sodium ions with the portal of CB[6] (Khan et al., 2009). It is likely that the addition of sodium ions may compete with the organic guest to bind with CB[6] but it has been observed that the degradation of used aliphatic hydrocarbons

were more facile in halophilic medium (3%NaCl) compared to non-halophilic medium. This may be justified considering the fact that hydroxyl CB[6] is more soluble in the presence of 3% NaCl and perform its role more efficiently. At the same time, greater binding affinity of aliphatic hydrocarbons with the cavity of CB[6] helps to dislodge sodium ion and take its place.

3.5. Conclusion

The Halophilic culture can be used for bioremediation of oil spills in marine environment and the organism present in the consortium were already reported for their potential to degrade aliphatic hydrocarbons present in crude oil (Pasumarthi et al., 2013). As hydroxy CB[6] has shown a significant effect on bioavailability of hydrocarbons using halophilic consortium growing in medium containing 3% NaCl, addition of hydroxy CB[6] to the marine oil spills can be a solution to speed up the process.

CHAPTER 4

EFFECT OF RHAMNOLIPID ON BIODEGRADATION OF NON AQUEOUS PHASE LIQUIDS

Publication: Effect of Rhamnolipids on biodegradation of Non aqueous phase liquid. (Communicated to Journal of Bioremediation).

4. Introduction

Hydrocarbon contamination is a serious threat to the environment as they are carcinogenic and mutagenic in nature. Owing to their low aqueous solubility, they exist as non-aqueous phase liquid (NAPL) and bioavailability of these NAPL is of important concern for better biodegradation (Barin et al. 2014). Bioavailability of NAPL can be increased by different ways such as by increasing the solubility of NAPL through micellar solubilization process (Mohanty et al. 2013), by emulsification of NAPL and thereby increasing the mass transfer of NAPL droplets into the cells (Mohanty and Mukherji 2007), by altering the cell surface hydrophobicity and thereby increasing the cell adherence to the hydrocarbons (Chakraborty et al. 2010). Increasing the aqueous solubility of NAPL could be achieved by the addition of surfactants. The role of both chemical and biosurfactants have been studied in the biodegradation of NAPLs (Vanne Hamme and Ward 2001). Bruheim et al., (1997) demonstrated that chemical surfactants like Tween 85, Tergitol 15 could enhance the biodegradation of crude oil by *Rhodococcus* sp. However biosurfactants have many advantages over synthetic surfactants as they are biocompatible, eco-friendly and generally less toxic (Mulligan et al. 2001).

Cell surface hydrophobicity has been reported as an important factor that influences the cell adhesion (Chakraborty et al. 2010). The cell wall composition like lipopolysaccharides, extracellular polymeric substances regulates the cell surface hydrophobicity (Zita and Hermansson 1997). However the types of growth substrate, growth phase, metabolites like biosurfactant, and presence of chemical surfactants may also influence the bacterial cell surface hydrophobicity (Zhang and Miller 1994). Al-Tahhan et al., (2000) demonstrated that increase in cell surface hydrophobicity of *P. aeruginosa* could be achieved by the supplementation of a low concentration of rhamnolipid biosurfactant. Kaczorek et al., (2010) reported that *Aeromonas hydrophilia* cell adherence to hydrocarbons was found to be varying with respect to time and was also affected by saponin, Triton-X 100, and also rhamnolipids. Such changes in adherence of hydrocarbon degrading cultures to NAPLs may enhance the biodegradation of hydrocarbons through direct interfacial uptake. The dispersion of hydrocarbons by solubilization also plays an important role in bioavailability of

hydrocarbons. Several synthetic and biosurfactants were reported for their ability to increase the solubility of hydrocarbons (Desai and Banat 1997; Grimberg et al. 1995).

Rosenberg et al., (1980) reported that the different strains of *Staphylocci, Streptococci and Serratia* were unable to degrade hydrocarbons even though they adhered to hydrocarbons due to their high cell surface hydrophobicity (CSH). Increased solubility does not always result in increased biodegradation (Zhang and Miller 1995). Therefore, it is important that the microorganisms should possess the necessary catabolic machinery to utilize the NAPL. A wide range of organisms are involved as a consortium in biodegradation of NAPL. It is difficult to find single organism to degrade all fractions of petroleum contaminants and hence a consortium of microbes is preferred very often (Obayori et al. 2009). The advantage of using mixed cultures rather than pure cultures is attributed to the synergistic interactions among these associations (Mukred et al. 2008). Cavalca et al., (2008) reported that phenanthrene uptake and assimilation by two mixed cultures and their respective isolates could be attributed to the changes in the CSH.

In the present study, the role of rhamnolipid on biodegradation of NAPL by consortium of marine isolates (*E. fergusonii* AEBBITS2 and *P. aeruginosa* AEBBITS1) was studied. There are limited reports available on the biodegradation of NAPL by *E. fergusonii.* The effect of co-culturing the hydrocarbon degrading consortium (AEBBITS1 & AEBBITS2) with rhamnolipid producing organism i.e., *P. aeruginosa* AMB AS7 was also studied. The effect of changes in CSH on degradation of NAPL was demonstrated elucidating the possible mechanism of NAPL uptake by bacteria in presence of rhamnolipid.

4.1 Materials

4.1.1 Chemicals and Microorganisms

n-Tetradecane (n-C14), n-Hexadecane (n-C16), n-Octadecane (n-C18), Napthalene (NAP) and Phenantherene (PHE) were obtained from Hi-media, India. Organic solvents like ethyl acetate and n-hexane were obtained from S.D. fine chemicals, India. Commercially available Bushnell Hass (BH) medium was obtained from Hi-media, India. Na_2CO_3, NaOH, $CuSO_4$ and $KNaC_4H_4O_6{\cdot}4H_2O$ were obtained from Merck, India. Folin Ciocalteau's reagent was obtained from Fisher Scientific, India. All the chemicals and reagents used were of analytical grade. *Pseudomonas aeruginosa* AMB AS7 (KC822322), Halo consortium (***Escherichia fergusonii*** **AEBBITS2** (AB793686) and *Pseudomonas aeruginosa* **AEBBITS1** (AB793685.1)) was used in this study. *P. aeruginosa* AMB AS7 is a biosurfactant producer isolated from hydrocarbon contaminated soil sample in Tamil Nadu, India and designated as AS7. The consortium of *E. fergusonii* **AEBBITS2** and *P. aeruginosa* **AEBBITS1** is hydrocarbon degraders isolated from crude oil contaminated site, Velsao beach, Goa, India and designated as Halo.

4.2 Methodology

4.2.1. Biosurfactant production

The production of biosurfactant by *P. aeruginosa* AMB AS7 was carried out in BH medium supplemented with 1 % (w/v) of glucose as carbon source. Two percent (v/v) cell suspension of 0.8 OD at 600nm, corresponding to mid log phase of the culture, was inoculated into 500 mL flask containing 100 mL of BH medium and incubated at 37°C and 100 rpm for 48 h. After incubation, biosurfactant was recovered from the cell free supernatant

4.2.2 Extraction of biosurfactant

The biosurfactant extraction from culture broth was carried out as described by Nitschke and Pastore (2006). Briefly, the bacterial cells were harvested by centrifugation at 13000 × g for 2 0 min at 30°C. The cell free supernatant was acidified to pH 2.0 ± 0.1 with 6N HCl, and the a cidified supernatant was left overnight at 4°C for complete precipitation of the biosurfactant. The precipitate was collected by centrifugation at 13000 × g for 20 min at 30°C and dissolve

d in water of pH 8.0 ± 0.1 followed by extraction with a solvent mixture of chloroform-to-m ethanol (2:1) at 30°C. The organic phase was pooled and evaporated, yielded a viscous honey -colored biosurfactant product which was later filtered by 0.22µ filter.

4.2.3 Estimation of biosurfactant

Biosurfactants were measured in the cell-free culture medium using the phenol-sulphuric acid (DuBois et al. 1956). In brief, 0.5 mL of 80% phenol and 2.5 ml of concentrated sulphuric aci d was added to 1 mL of cell-free culture broth. After 10 min of incubation at room temperatur e 30°C, the optical density was measured at 481 nm and the biosurfactant concentration was c alculated using a standard curve prepared using different concentrations of L- rhamnose.

4.2.4 Preparation of model NAPL

The model NAPL was prepared as described by Mukherji et al., (1997). The mole fraction of each solid phase pure component was maintained at less than its fugacity ratio at 30°C. The c omposition of model NAPL was (mole fraction): n-C14 (0.25), n-C16 (0.45), n- C18 (0.11), NAP (0.15), PHE (0.04). The stock solution was prepared by dissolving the required amount of each component in n- Hexane. The aliphatic to aromatic hydrocarbon ratio was maintained at 3:1 to represent the typical composition of diesel.

4.2.5 Microcosm studies

The degradation of hydrocarbons was performed in 100 ml serum vials (microcosm setup). 2 0 mL of sterile BH medium was taken into each vial and 100µl of hexadecane or 100µl NAP L was added to each vial and left for 1 h in laminar air flow chamber to evaporate n-hexane. The following three different experimental setups were used in this study. The serum vials w ere inoculated with 1. Halo, 2. Halo supplemented with rhamnolipid (100 mg/L) and 3. Co-cu lture (mixture of AS7 and Halo). Abiotic samples were maintained which act as control for th e experiments. The vials were incubated in shaker at 30°C and 110 rpm. The residual hydroca rbons were extracted by addition of equal volume of ethyl acetate for hexadecane, n-hexane f or NAPL and estimated by gas chromatography for every 24 h till 120 h as per the protocol d escribed by Pasumarthi et al. (2013). 0.1 mL of 1% naphthalene and 1% fluorine were added as internal standard to the extracted hexadecane and NAPL samples, respectively. All experi ments were carried out in duplicates and the inoculums optical density was adjusted to 1.0 at 600nm.

4.2.6 Cell surface hydrophobicity test (BATH assay)

The bacterial adhesion to hydrocarbon (BATH) assay was carried out as described by Rosenberg et al. (1980). Bacterial cells were separated from culture broth by centrifugation at 13000 × g for 15 min at 30°C and washed thrice with distilled water. The cells were then suspended in distilled water and the initial absorbance at 400 nm was adjusted to 1 and designated as A_0. Hexadecane (0.2 mL) and cell suspension (2.0 mL) were mixed in a test tube for 2 min and left undisturbed for 30 min. The absorbance of the bottom aqueous phase was read at 400 nm (A_1). The cell surface hydrophobicity was calculated using the following formula:

$$Cell\ \ surface\ hydrophobicity\,(\%) = \left(1 - \frac{A_1}{A_0}\right) \times 100$$

4.2.7 Quantification of Microbial biomass

The amount of biomass in the medium was measured by estimation of the protein content usi ng the Lowry method (Lowry et al. 1951). The cells were centrifuged at 13000 x g for 10 min at 30°C and were re-suspended in 1 mL distilled water and lysed by adding 2 mL of 2.5% (w/ v) sodium dodecyl sulphate in 0.2N sodium hydroxide to release the proteins. The samples w ere vortexed and incubated in a boiling water bath for 10 min. The amount of protein was det ermined by Lowry method at 740 nm using a UV-visible spectrophotometer (Shimadzu UV 2 450, Japan). The protein biomass was estimated for all experiment setups.

4.2.8 16S rDNA amplification and Denaturing Gradient Gel Electrophoresis (DGGE)

During the estimation of protein biomass, 1 mL of culture was taken for DNA extraction whi ch was used for PCR and DGGE experiments. Genomic DNA was extracted from every sam ple as per the protocol mentioned by Rochelle (2001). 16SrDNA was amplified by thermal cy cler (Eppendorf master cycler gradient) and DGGE was performed as per the protocol describ ed in chapter 2, section 2.3. The gel was stained by silver staining method.

4.3 Results

4.3.1 Biodegradation of hexadecane in presence of Rhamnolipid

Hexadecane was used as model hydrocarbon to test the effect of rhamnolipid on biodegradation of aliphatic hydrocarbons. The percentage of hexadecane degradation present in samples inoculated with Halo, Halo enriched with rhamnolipid and co-culture, is shown in Fig 4.1. The results reveal that Halo enriched with rhamnolipid degraded more hexadecane (61.9%) followed by co-culture (45.72%) and Halo (30.6%). The percentage of hexadecane degradation in samples of Halo enriched with rhamnolipid was found to be high compared to the abiotic controls indicating increased bioavailability and hence biodegradation.

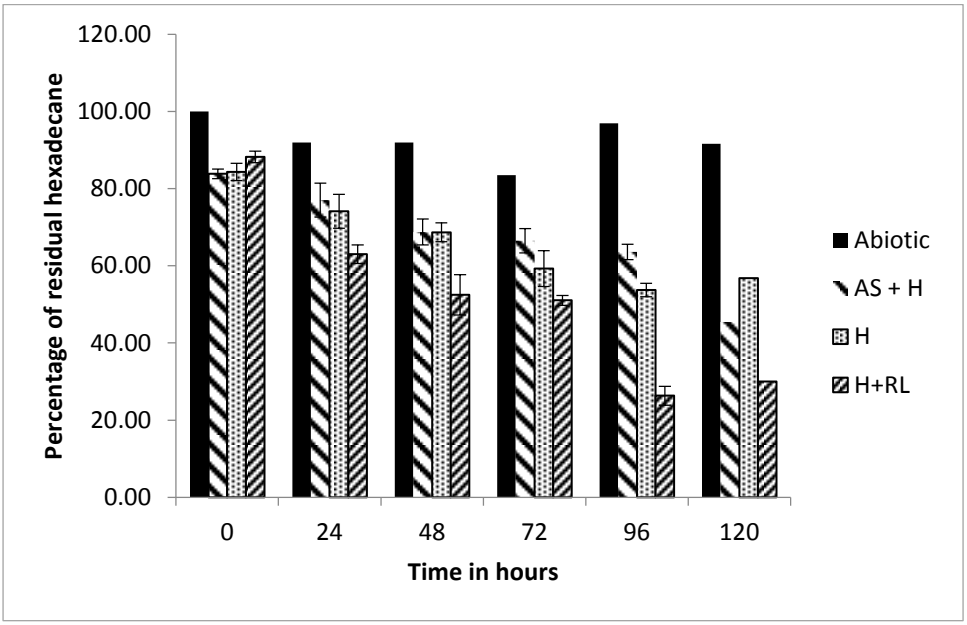

Fig 4.1: Represents degradation of hexadecane as sole carbon source. Abiotic is samples without bacteria, AS represents rhamnolipid producing bacteria, H represents hydrocarbon degrading bacteria, RL represents rhamnolipid (100 ppm)

4.3.2 Biodegradation of NAPL in presence of rhamnolipid

The biodegradation of NAPL (naphthalene, phenantherene, tetradecane, hexadecane and octadecane) was carried out using Halo, Halo enriched with rhamnolipid and co-culture. The percentage of degradation of NAPL as shown in Fig. 4.2 reveal that higher degradation of naphthalene was observed in the experimental setup containing rhamnolipid enriched Halo culture (50%) compared to Halo (40.23%) and co-culture (33.8%). However in case of phenantherene degradation, both rhamnolipid containing Halo culture and co-culture showed relatively higher degradation compared to other setups. The phenantherene degradation was found to be 38% with co-culture followed by rhamnolipid enriched Halo (34.17%) and Halo (22.4%) (Fig.4.3). In the case of degradation of aliphatic hydrocarbons, the order of tetradecane degradation was found to be rhamnolipid enriched Halo (52.45%) > co-culture (16.5%) > Halo (13.8%) as illustrated in Fig. 4.4. About 52.3% of hexadecane was degraded by rhamnolipid containing Halo culture; co-culture and Halo were able to degrade 22.8% and 23.1% of hexadecane, respectively (Fig. 4.5). The degradation result of octadecane reveals that, co-culture showed slightly higher degradation effect than rhamnolipid enriched Halo. The octadecane degradation was found to be 47.8% by co-culture and 46.5 % by rhamnolipid enriched Halo. However Halo showed 25.2% of octadecane degradation as illustrated in Fig. 4.6. In all the experimental setups, rhamnolipid enriched Halo culture showed greater NAPL degradation efficiency except in case of phenantherene and octadecane.

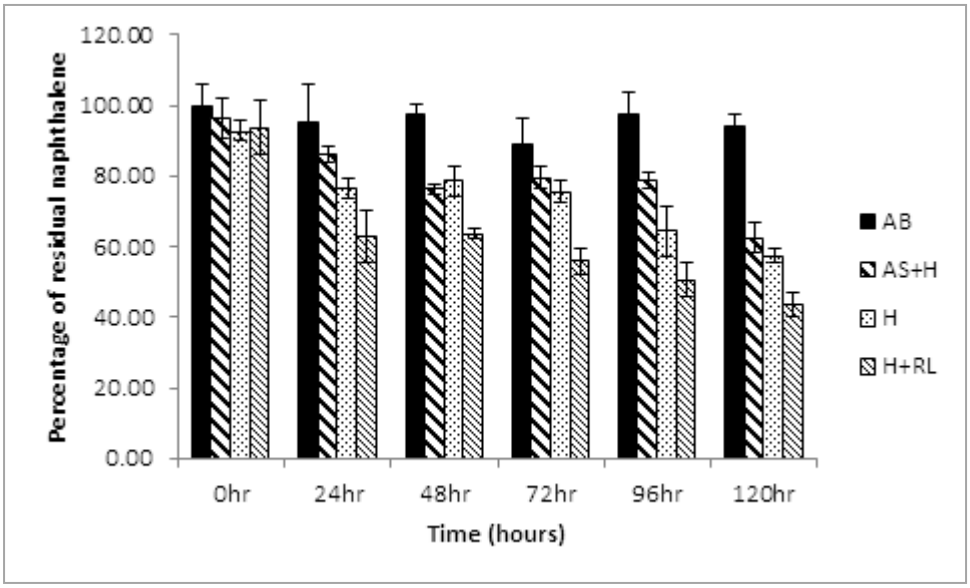

Fig 4.2: Represents degradation of naphthalene present in NAPL. Abiotic is samples without bacteria, AS represents rhamnolipid producing bacteria, H represents hydrocarbon degrading bacteria, RL represents rhamnolipid (100 ppm)

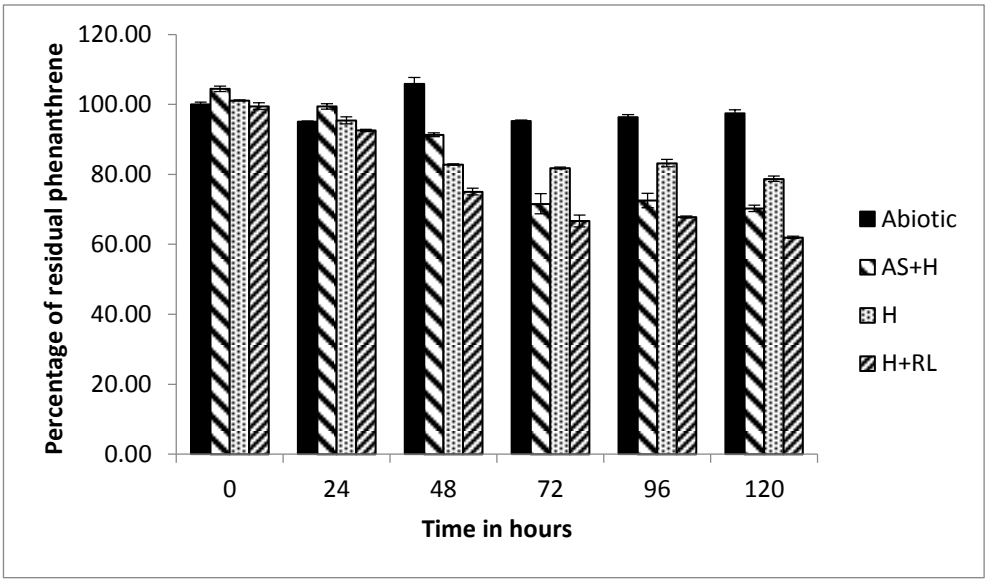

Fig 4.3: Represents degradation of phenanthrene present in NAPL. Abiotic is samples without bacteria, AS represents rhamnolipid producing bacteria, H represents hydrocarbon degrading bacteria, RL represents rhamnolipid (100 ppm)

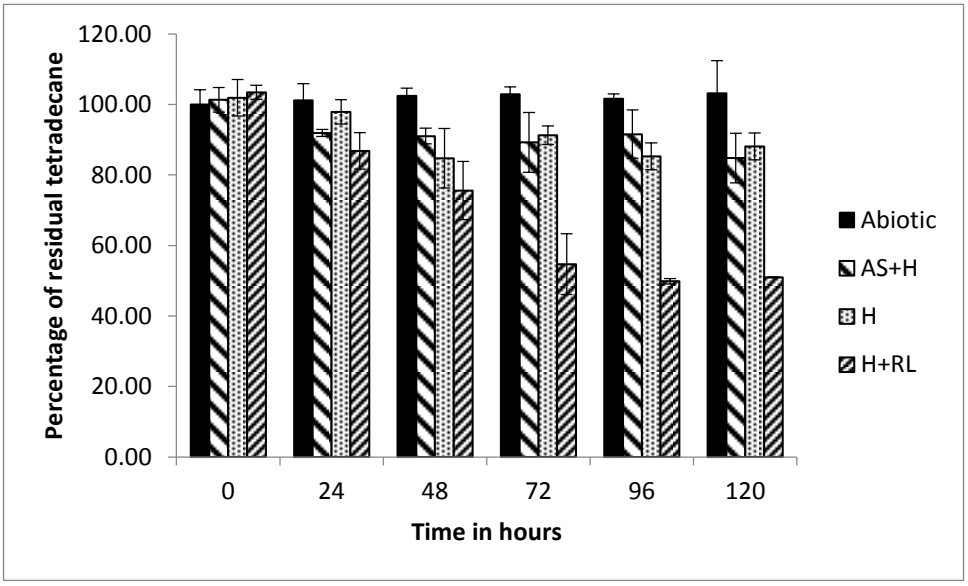

Fig 4.4: Represents degradation of tetradecane present in NAPL. Abiotic is samples without bacteria, AS represents rhamnolipid producing bacteria, H represents hydrocarbon degrading bacteria, RL represents rhamnolipid (100 ppm)

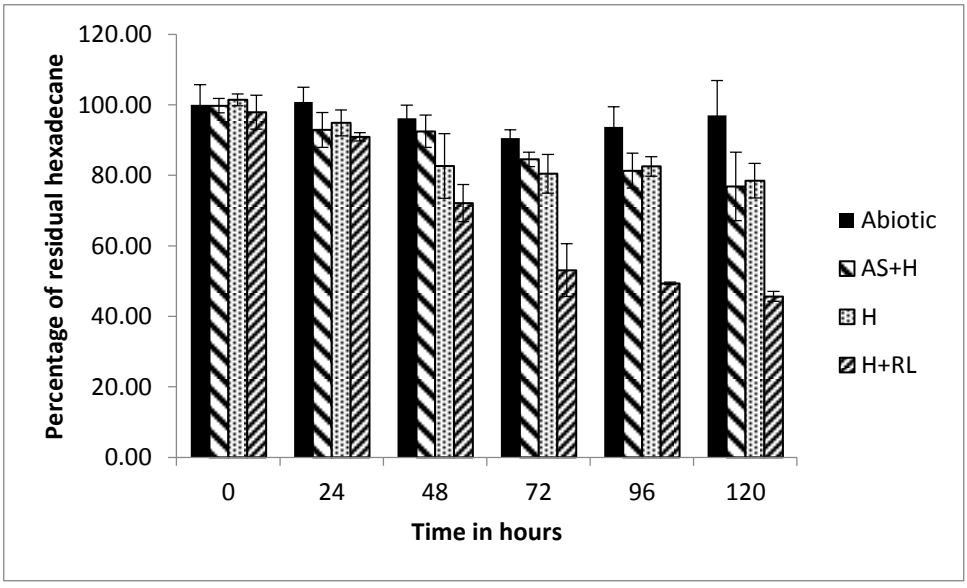

Fig 4.5: Represents degradation of hexadecane present in NAPL. Abiotic is samples without bacteria, AS represents rhamnolipid producing bacteria, H represents hydrocarbon degrading bacteria, RL represents rhamnolipid (100 ppm)

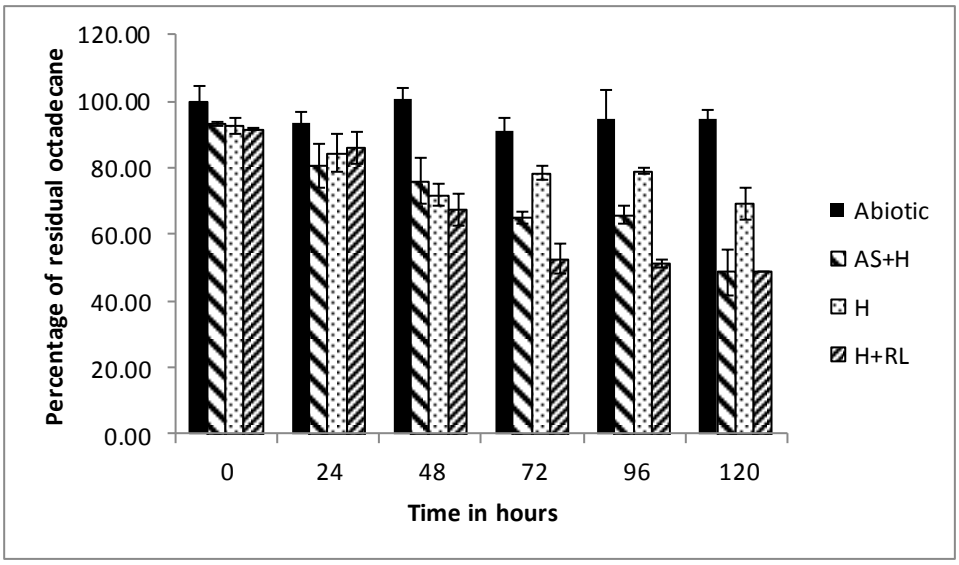

Fig 4.6: represents degradation of octadecane present in NAPL. Abiotic is samples without bacteria, AS represents rhamnolipid producing bacteria, H represents hydrocarbon degrading bacteria, RL represents rhamnolipid (100 ppm)

4.3.3 Rhamnolipid estimation

The pattern of rhamnolipid production was found to be same for both hexadecane and NAPL degradation experimental setups. There were no traces of rhamnolipid in abiotic samples. Halo culture was found to produce in both hexadecane and NAPL degradation setups i.e., 73.94 mg/L and 73.5 mg/L respectively. In the setups with co-culture (Halo+AS7) about 131.42 mg/L and 142.24 mg/L of rhamnolipid has been produced by 96 hrs in hexadecane and NAPL degradation setups respectively. The slight increase in rhamnolipid concentration in experimental setup of Halo culture supplemented with rhamnolipid was observed (Fig. 4.7, 4.8). The amount of hexadecane in co-culture and rhamnolipid enriched setups was found to be same.

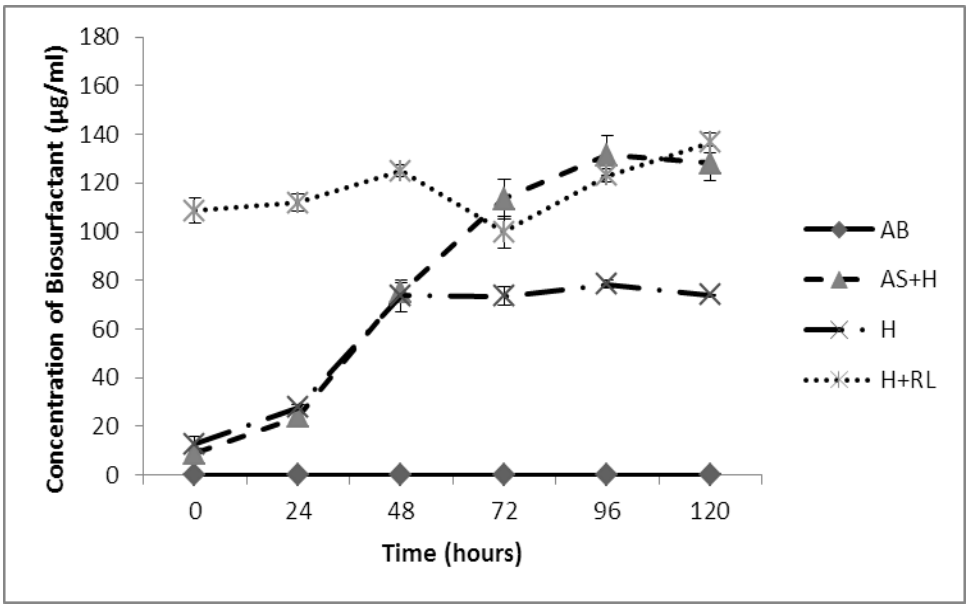

Fig 4.7: Estimation of rhamnolipid during hexadecane degradation as sole carbon source. AS represents rhamnolipid producing bacteria, H represents hydrocarbon degrading bacteria, RL represents rhamnolipid (100 ppm)

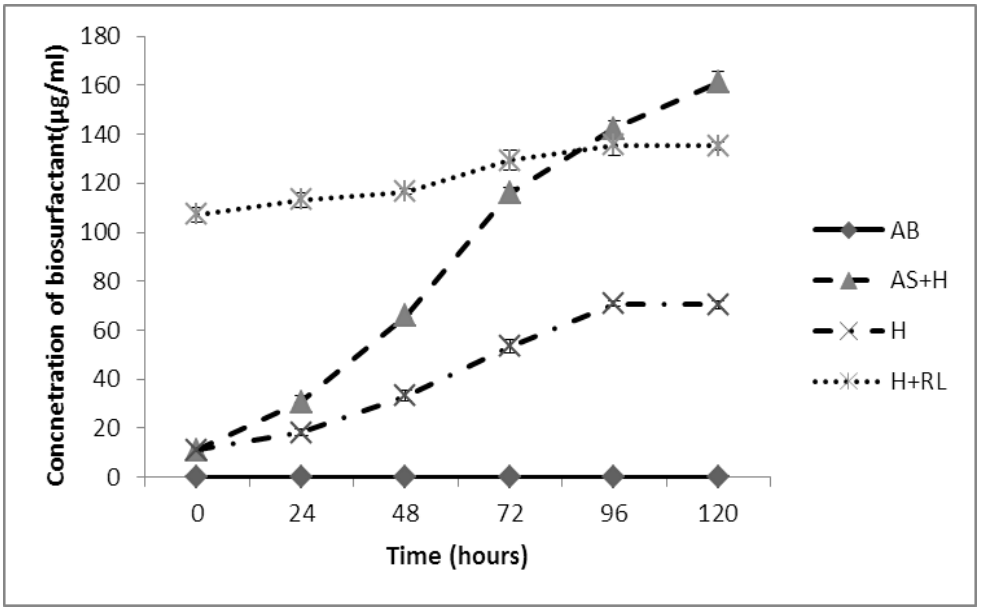

Fig 4.8: Estimation of rhamnolipid during NAPL degradation. AS represents rhamnolipid producing bacteria, H represents hydrocarbon degrading bacteria, RL represents rhamnolipid (100 ppm)

4.3.4 Cell surface hydrophobicity (CSH)

Cell surface hydrophobicity (CSH) of the microbes can be correlated with the hydrocarbon degradation. During hexadecane degradation there was a tremendous increase in CSH of Halo culture from 7.2 % to 69.0% in presence of supplemented rhamnolipid whereas in the absence of rhamnolipid, CSH of Halo culture was found to be a maximum of only 23% by 96 hrs. The increase in CSH in the experimental setup of co-culture i.e., from 17.8% to 43.4%, can be attributed to the AS7 cells present in mixed inoculum (Fig. 4.9). The same pattern of CSH was observed in NAPL degradation setup. CSH of Halo culture increased from 9.1% to 69.5% in presence of rhamnolipid and in absence of rhamnolipid, it was 23.8% by 96 h. whereas 31.9% of CSH was observed in co-culture by 96 hrs (Fig. 4.10).

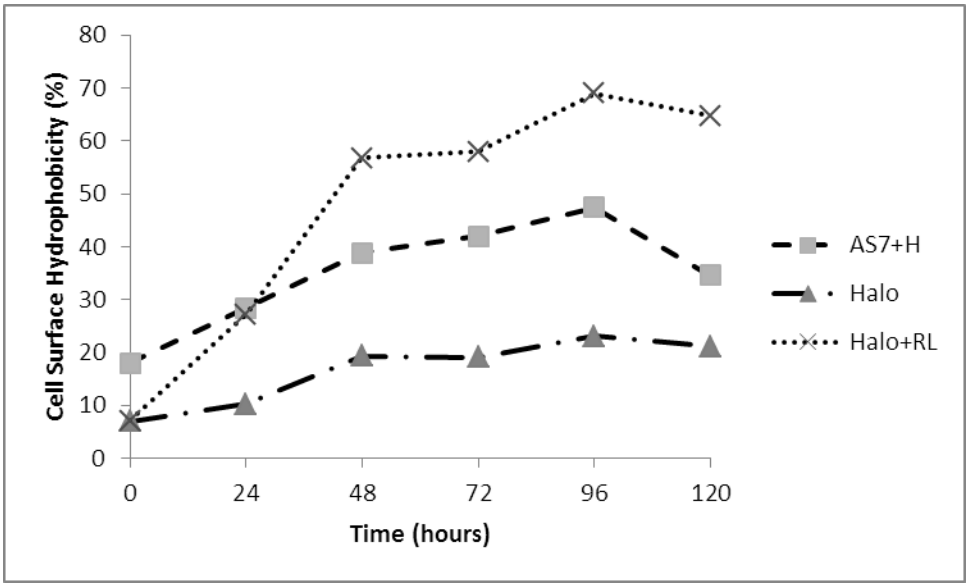

Fig 4.9: Cell surface hydrophobicity during hexadecane degradation as sole carbon source. AS represents rhamnolipid producing bacteria, H represents hydrocarbon degrading bacteria, RL represents rhamnolipid (100 ppm)

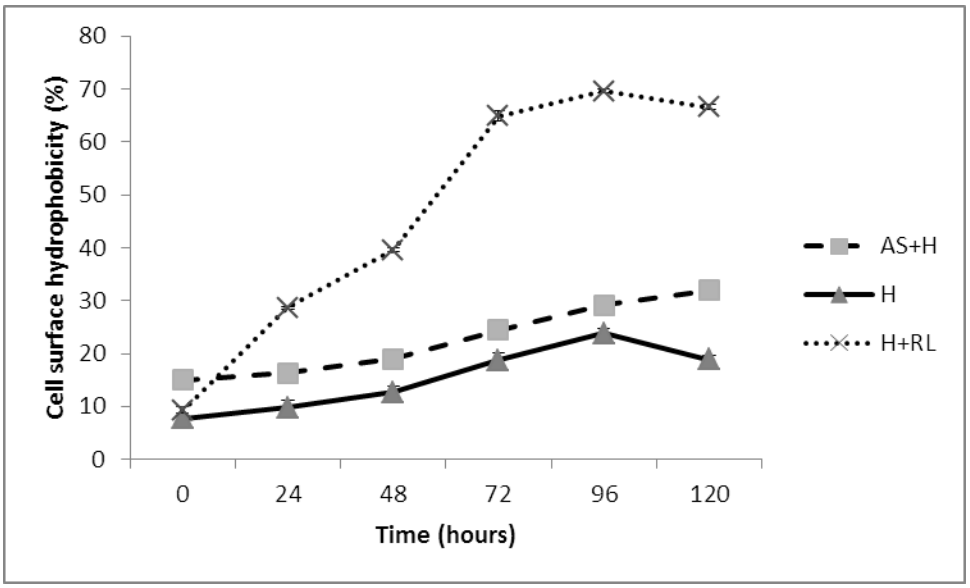

Fig 4.10: Cell surface hydrophobicity during NAPL degradation. AS represents rhamnolipid producing bacteria, H represents hydrocarbon degrading bacteria, RL represents rhamnolipid (100 ppm)

4.3.5 Protein biomass

The growth of microorganisms was estimated by protein biomass and the results are shown in Fig. 9. It was found that the Halo culture in presence of rhamnolipid showed maximum growth in BH medium provided with both hexadecane and NAPL as sole carbon source. This could be correlated with the high degradation rate of hexadecane as well as NAPL by Halo culture in presence of rhamnolipid. In case of medium containing hexadecane, similar growth pattern was observed for Halo and co-culture (Fig. 4.11). However in case of medium containing NAPL, Halo supplemented with rhamnolipid showed high protein biomass followed by co-culture and Halo culture (Fig. 4.12).

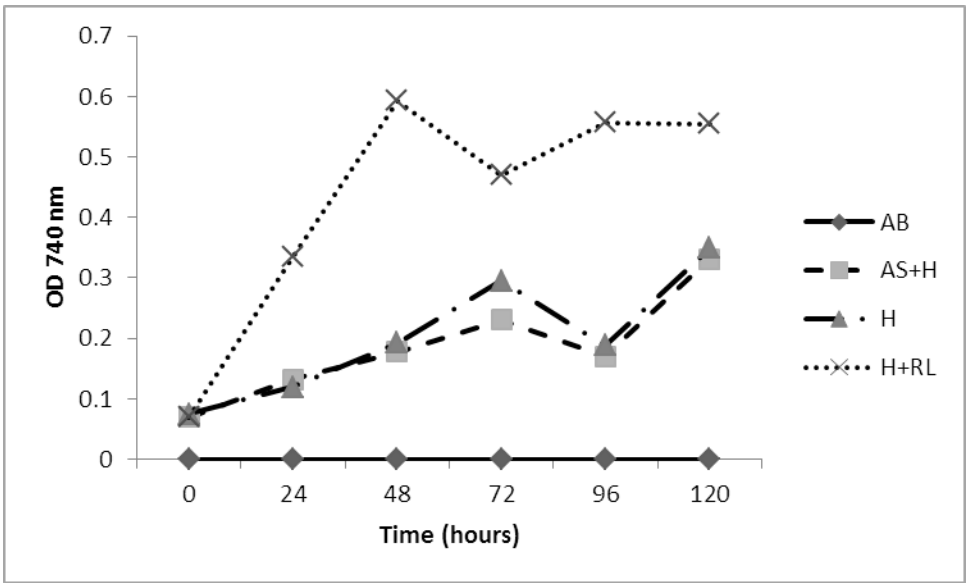

Fig 4.11: Represents total protein biomass during hexadecane degradation. AS represents rhamnolipid producing bacteria, H represents hydrocarbon degrading bacteria, RL represents rhamnolipid (100 ppm)

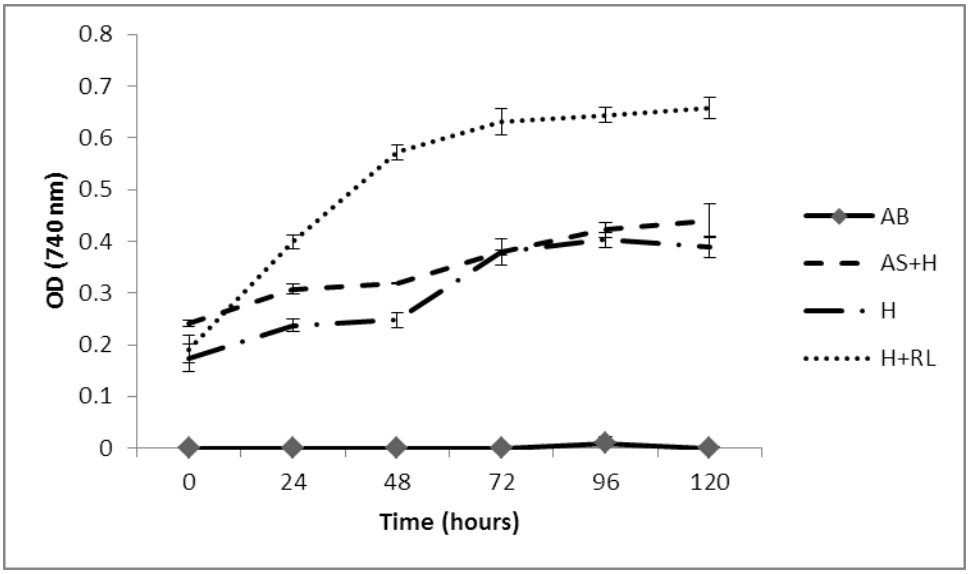

Fig 4.12: Represents total protein biomass during NAPL degradation. AS represents rhamnolipid producing bacteria, H represents hydrocarbon degrading bacteria, RL represents rhamnolipid (100 ppm)

4.3.6 Denaturing Gradient Gel Electrophoresis (DGGE)

The DGGE gel was shown in Fig. 4.13 and the result reveals that both *P. aeruginosa* **AEBBITS1** and *E. fergusonii* survived till last day (120 h) in microcosm setups inoculated with Halo culture. *E. fergusonii* did not survive in the experimental setups inoculated with co-culture (AS7 + Halo) from 48 h as the band indicating *E. fergusonii* has disappeared in the lanes of 72, 96,120 h and also the band indicating *P. aeruginosa* has become faint indicating the culture's decline in growth. This might not be because of rhamnolipid because the amount of rhamnolipid produced in co-culture experimental setup was almost equal to the amount of rhamnolipid added to the Halo i.e., 100 mg/L. However the addition of rhamnolipid producing culture (AS7) might have disturbed the consortial environment and AS7 would have taken over the system.

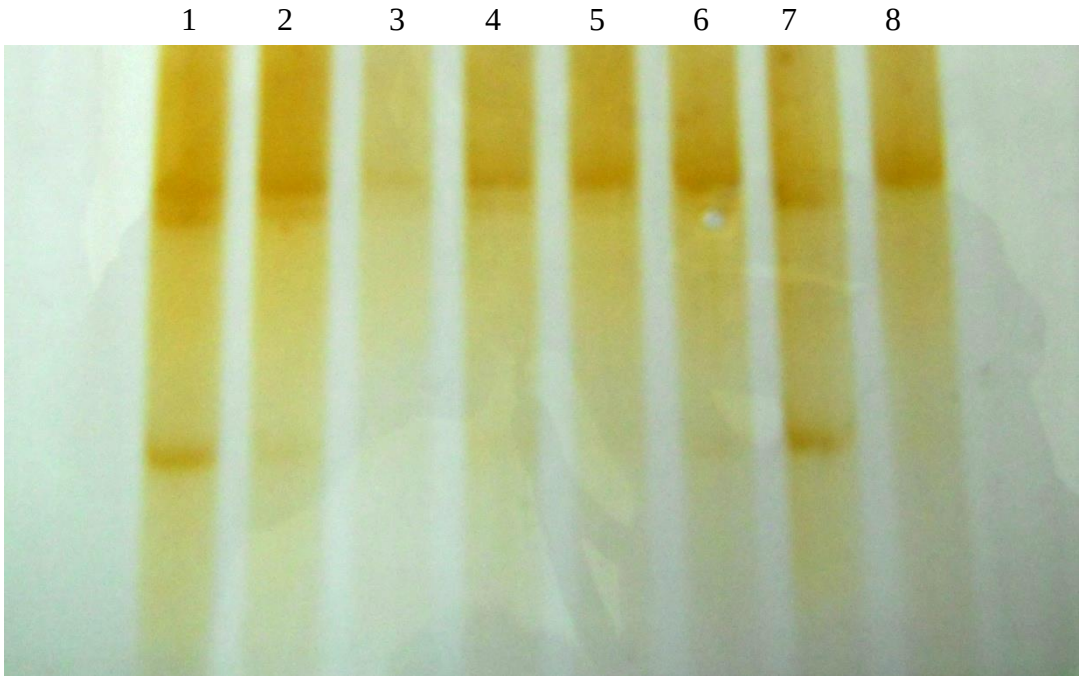

Fig. 4.13: DGGE of the co-culture during degradation of hexadecane. Lane 1 – 6 indicates the co-culture system (AS7+ Halo). Lane 7, 8 indicates Halo and *P. aeruginosa* AMB AS7, respectively. In all lanes, top band corresponds to *P. aeruginosa* and bottom indicates *E. fergusonii*

4.4 Discussion

Biodegradation is a better alternative by which hydrocarbon pollutants can be removed from the environment (Lal and Khanna 1996). This work was initiated to study the influence of rhamnolipid on biodegradation of NAPL by microbial consortia. The hydrocarbon degradation is affected by the limitation in its bioavailability. The addition of rhamnolipid alters the cell surface hydrophobicity which directly influence cell adherence to hydrocarbons and thereby their degradations (Zhao et al. 2011) or may increase the mass transfer of hydrocarbons into aqueous phase (Moscoso et al. 2014). Our findings revealed that both rhamnolipid enriched microbial system as well as mixed culture system showed greater efficiency in NAPL degradation.

In the mixed culture study (co culture), among three strains used, AS7 has the inherent ability to produce rhamnolipid and to grow on both aliphatic and aromatic hydrocarbons. However the other two hydrocarbonoclastic bacterial strains *(E. fergusonii* AEBBITS2 and *P. aeruginosa* AEBBITS1) are marine isolates and showed greater variation in cell surface hydrophobicity during the course of experiment which in turn had greater effect on biodegradation of NAPL. The results proved that rhamnolipid supplemented consortium Halo degraded comparatively more aliphatic model hydrocarbon hexadecane as well as mixed NAPL in all the experiments. The dynamic changes in the cell surface hydrophobicity (CSH) can be correlated with the degradation efficiency. The rhamnolipid addition in Halo culture brought a greater alteration in the CSH (69 %) at 96 h of cultivation which could result in higher degradation of NAPL. The other possibility for the better hydrocarbon degradation might be due transfer hydrocarbons into aqueous phase by rhamnolipids as they have both hydrophobic and hydrophilic domains therefore can interact with both hydrocarbon and aqueous solution. Micelle formation can increase the hydrocarbon solubility which would help for the enhanced biodegradation (Amiriyan et al. 2004). Many researchers have reported that addition of surfactant could alter the CSH drastically during petroleum hydrocarbon degradation (Zhao et al. 2011). Owsianiak et al. (2009), Hua et al. (2003) and AL-Tahhan et al. (2000) demonstrated that the rhamnolipids interaction with the *Pseudomonas* cell surface could result in loss of lipopolysaccharide (LPS). It is well known fact that, LPS is an important hydrophilic component of the cell surface. Therefore, the loss of the LPS could

result in an increase of cell surface hydrophobicity and as a consequence enhanced degradation rate of hydrophobic substrates. Zhang and Miller (1994) observed that the addition of rhamnolipid enhanced the degradation of octadecane by two strains of *P. aeruginosa*. They reported that the increase in CSH of the slow degraders resulted in greater hydrocarbon degradation. Mohanty et al. (2013) reported that the mode of action of both Triton X-100 and rhamnolipid JBR-515 was different. They enhanced the degradation of the model NAPL-A1. A similar mechanism of action of Triton X-100 was also reported for diesel degradation by *B. cepacia* and *Exiguobacterium aurantiacum*. However, depending on the type of NAPL present in the environment and culture used, the same surfactant can exhibit different modes of action. Kaczorek et al. (2010) proved that the presence of Triton X-100 did not exhibit greater diesel degradation by *P. stutzeri* whereas rhamnolipid could show the higher degradation. They also reported that the CSH of *P. stutzeri* was higher in the presence of rhamnolipid as compared to Triton X-100. It has been reported that the effect of rhamnolipid on solubility of hydrocarbons and the dispersion ability depends on the capacity to reduce interfacial tension (Zhang and Miller 1995). In the present study, the rhamnolipid enriched microbial system showed greater degradation than co-culture system. However in case of degradation of phenantherene and octadecane, similar pattern was observed in both rhamnolipid enriched Halo culture as well as co-culture (AS7+Halo). However, rhamnolipid enriched Halo culture was able to degrade comparatively more hydrocarbons in all the cases of NAPL taken, which has been illustrated in Fig. 4.2, 4.3, 4.4, 4.5, 4.6. This proves that not only the increase in CSH or micellar solubility is necessary for the degradation of NAPL, but also the necessary catabolic machinery should be present in the micro-organisms. The low yield of rhamnolipid in co-culture could be due to the presence of low cell number of rhamnolipid producer, *P. aeruginosa* AMB AS7 in co-culture. As shown in Fig.4.7 and 4.8, the dynamic changes in the CSH of microbial system revealed that direct contact between the cells and the hydrocarbons could facilitate the hexadecane and NAPL degradation. In addition to the direct contact, micellar solubilization could happen at low concentrations of rhamnolipid. Therefore, the present study revealed that the possible mechanism of NAPL uptake by rhamnolipid enriched Halo culture as well as co-culture system may be micellar solubilization followed by direct contact between micro-organisms and hydrocarbons (NAPL). In this study the co-culture microcosmic system have one

rhamnolipid producing bacterial isolate *P. aeruginosa* AMB AS7 and two marine halophilic bacterial isolates (*E. fergusonii* AEBBITS2 and *P. aeruginosa* AEBBITS1) which showed synergetic interaction among them. The DGGE result (Fig.4. 13) showed that in co-culture one of the member of Halo culture i.e., *E. fergusonii* AEBBITS2 could not survive beyond 48 h. This may not be attributed to the production of rhamnolipid by *P. aeruginosa* AMB AS7 because only 142.24 mg/L was produced, which was close to the concentration of rhamnolipid present in enriched system (100 mg/L). **I**n NAPL, the degradation of phenanthrene (33%) and octadecane (48%) was found to be more by co-culture system compared to other biotic systems. Similar reports have been documented by others as well. Kim et al. (2009) found a low phenanthrene degradation ability by pure bacterial cultures of *Acinetobacter baumannii, Klebsiella oxytoca*, and *Stenotrophomonas maltophilia*, which was notably increased (>80%) when such bacteria were grown in mixed cultures. Shankar et al. (2014) demonstrated that almost 100% of oil contaminants were removed in 30 days by the isolated microbial consortia. The synergistic interactions involved during hydrocarbon uptake by mixed cultures may be complex and could imply the successive removal of toxic metabolites of the species preceding (Ghazali et al. 2004).

4.5 Conclusion

This study revealed the influence of rhamnolipid on biodegradation of hydrocarbons by microorganisms. The alteration in the cell surface hydrophobicit**y** helped the microorganism to degrade hydrocarbons efficiently. The enhancement of model NAPL degradation also could have happened through micellar solubilization. Rhamnolipid in combination with halophilic consortium showed positive effect on degradation of whole NAPL. However the reason for the death of *E. fergusonii* is not clear in co-culture system, Rhamnolipid can be used to enhance bioavailabilty of hydrocarbons which would result in higher biodegradation.

CHAPTER 5

ROLE OF HORIZONTAL GENE TRANFER IN BIOREMEDIATION

5. Introduction

A bioremediation strategy for any contaminated site requires a prior understanding of the prevailing conditions at the site and the capabilities of the bacteria to be used. It has been observed in many experiments that the viability of the augmented bacteria is a big challenge as different environmental and biotic factors play a role. The indigenous bacteria at the site can also do the degradation but they should be competent enough and should have necessary catabolic genes to degrade the hydrocarbons. To overcome this situation the bacteria used in augmentation process should be supplied with extra nutrients for their survival. Horizontal gene transfer of catabolic genes to the indigenous bacteria from introduced bacteria could also aid in improved bioremediation. The aim of these experiments is to study the possibility of HGT in marine environment.

Horizontal gene transfer (HGT) of hydrocarbanoclastic catabolic genes

There are several retrospective and mechanistic studies proving horizontal gene transfer of catabolic genes as a possible strategy for successful bioremediation. These studies have always been focused on the genes encoding the first enzyme in the degradation pathway which is critical for hydrocarbon degradation. The enzymes like alkane hydroxylase, naphthalene dioxygenase, PAH dioxygenase incorporates oxygen into the hydrocarbon in a process to degrade alkanes, naphthalene, poly aromatic hydrocarbons respectively. Among the above mentioned catabolic genes naphthalene dioxygenase was reported for horizontal gene transfer in many studies. In the retrospective approach, metagenomics studies using TRFLP, DGGE, Sothern hybridization etc., are used as tools to detect the possibility of horizontal gene transfer at a particular site. In mechanistic approach a donor and recipients were selected and grown under controlled conditions to observe the process of HGT. *nahAC* allele is the region of NDO gene responsible for oxidation of naphthalene whereas *nahAA* and *nahAB* are involved in electron transfer to *nahAC*. Herrick et al., (1997) have found an evidence for natural horizontal gene transfer by studying the *nahAC* allele and its distribution in different bacteria which are phylogenetically diverse. They also have witnessed the transfer of *nahAC* gene by confirming that phylogenetically diverse bacteria are sharing the identical allele of *nahAC*. Keil et al., (1998) have used TRFLP and southern hydridization to study the similarity between different size plasmids isolated from a coal tar contaminated site

and found that these naphthalene catabolic plasmids are closely related to each other and pDTG1 Plasmid from *Pseudomonas putida* NCIB 9816. They also have reported the self-transmissible capability of the plasmid by filter mating between donors and their cured progeny. *Marinobacter* strain NCE 312 was isolated by Hedlund et al., (2001) from a chemostat and identified the bacteria by 16srDNA sequencing. They have amplified and sequenced larger subunit of naphthalene 1,2 dioxygenase. The similarity of this gene with that of *Pseudomonas* and *Burkholderia* leads to speculation that horizontal gene transfer might have occurred between these bacteria. Alkane hydroxylases are well characterized structurally and functionally in different bacteria but horizontal gene transfer of the genes coding these enzymes was poorly understood. There were no studies based on mechanistic approach but a very few retrospective experiments reveals that HGT of *alk*B family of alkane hydroxylase might have occurred between bacteria. For instance the T-RFLP studies conducted by Glebler et al., (2013) on 400 alkane degrading soil isolates revealed the possibility of high genetic mobility of *alk*B gene. It was very clear that there were less mechanistic studies on the catabolic genes and the retrospective approach also gives an indirect evidence for HGT. The combination of retrospective and mechanistic methods to monitor the HGT in the contaminated seawater gives a clear idea about the reliability of HGT during bioaugmentation as an alternative. So in the present study DGGE was used to study the changes in the bacterial community patterns in presence of donor and absence of donor (donor is the bacteria used for bioaugmentation). Three microcosms with normal sea water, seawater with diesel oil, sweater supplied with diesel oil and hydrocarbon degrading bacteria were designed to represent three different conditions like normal seawater, diesel oil contaminated seawater and bioaugmentation respectively. The appearance of new bands in the third microcosm ie., bioaugmentation will be due to HGT of catabolic genes from augmented bacteria to the indigenous bacteria. The same DGGE pattern in microcosms of diesel contaminated seawater and bioaugmentation will prove no HGT. The experiment was done in a stipulated time of three weeks to study the reliability of HGT in case of failed bioaugmentaion. The study would provide an insight into strategy that to be designed for oil contamination in Goan seawater.

5.1 Material and methods

5.1.1 Catabolic gene profile of consortium

Genomic DNA was extracted as per the protocol (chapter2, section 2.3) and catabolic genes for degradation of alkanes, naphthalene, pyrene, phenanthrene, poly aromatic hydrocarbons and glutathione s transferase were checked for their presence using Eppendorf flexi lid PCR. The PCR programs for different genes were mentioned in the table 5a. PCR was run for 30 cycles for all the genes mentioned.

Table 5a: PCR program for different catabolic genes

Catabolic gene	**Initial denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final extension**	**Reference**
Naphthalene dioxygenase (NDO)	96°C for 2 minutes	94°C for 1 minute	55°C for 1 minute	72°C for 1 minute	72°C for 7 minutes	Kurkela et al., 1988.
Alkane hydroxylase (*alk*B)	94°C for 3 minutes	94°C for 1 minute	61°C for 1 minute	72°C for 1 minute	72°C for 10 minutes	Wasmund et al., 2009
Pyrene degradation	94°C for 5 minutes	94°C for 1 minute	60°C for 1 minute	72°C for 1 minute	72°C for 3 minutes	Brezna et al., 2003
PAH dioxygenase (pdo)	94°C for 5 minutes	94°C for 30 seconds	62°C for 30 seconds	72°C for 1 minute	72°C for 6 minutes	Krivobok et al., 2003
Glutathione S transferase	94°C for 5 minutes	94°C for 2 minutes	50°C for 1 minute	72°C for 1 minute	72°C for 10 minutes	Lloyd-Jones et al., 1997

5.1.2 Collection of sea water and selection of culture

Sea water was collected from Velsao beach, Goa and was preserved at 4°C until use. The pure *Pseudomonas aeruginosa* (AEBBITS 1) and *Escherichia fergusonii* (AEBBITS 2) have been obtained by spread plate method. The pure colonies were inoculated into 100 ml BH medium with 0.5% diesel oil as sole carbon source and their growth observed at 600nm. Genomic DNA was extracted from both the cultures (Tsai and Rochelle) and checked for the presence of catabolic genes like *alk*B (alkane hydroxylase) and NDO (naphthalene dioxygenase) as per the protocol mentioned in table 5a. The amplified PCR products were confirmed on Gel electrophoresis.

5.1.3 Microcosm studies

For designing the microcosm, three five liter sterile containers were filled with four liters of sea water. Every container has an inlet, outlet and a provision for aeration. Sterile air was supplied using 0.22 micron filters. First microcosm contains only sea water and the other two microcosms were supplied with 0.5% diesel oil as carbon source and also supplied with 3.27g/L of Bushnell Hass medium to support microbial growth. One of the setups having diesel oil was inoculated with *Pseudomonas aeruginosa* (AEBBITS1) to monitor the process of HGT while other two setups act as controls. The change in microbial diversity by addition of diesel oil and *Pseudomonas aeruginosa* (AEBBITS1) was studied using DGGE (fig: 5.1).

Fig 5.1: The microcosm setup for diesel degradation and HGT. The first container contains seawater, second one contains seawater + diesel, third one contains seawater + diesel + *P.aeruginosa* (AEBBITS 1).

5.1.4 Gas chromatography for residual diesel

Diesel oil was extracted from 20 ml of representative volume from seawater microcosm contaminated with diesel and seawater inoculated with *Psedomonas aeruginosa* (AEBBITS 1). The extraction was done by addition of equal volume of ethylacetate to 20 ml of sample and vigorous mixing on a magnetic stirrer for 15 minutes. The upper organic layer was collected and ethylacetate was evaporated at 50°C. The residual diesel was resuspended in 1 ml of ethylacetate and Gas Chromatography was performed.

5.1.5 DNA extraction and DGGE

Genomic DNA was extracted from all the experiment setups as per the protocol suggested by Tsai and Rochelle (2001) but with minor changes (chapter 2, section 2.3). The obtained DNA was confirmed with gel electrophoresis. Nested PCR was applied to amplify 16SrDNA by Eppendorf flexi lid thermal cycler. The primers used are 27F, 1492R for first PCR and 968FGC, 1492R for GC clamping in second PCR. The PCR program mentioned in our earlier studied was used (Nikolausz et al., 2008) (chapter 2, section 2.3.2). DGGE was performed to understand the role and survivability of different bacteria from sea water, survivability of bacteria used in augmentation and as well as the difference between bio-stimulation, bio-augmentation and combination of biostimulation and bioaugmentation. 7.5% acrylamide gel was used with denaturant gradient ranging from 30% to 60% and 10 microliter of sample was loaded and run for 14 hours at 70 volts.

5.1.6 Different Parameters (pH, temperature, optical density, nitrogen, phosphorus)

Nitrogen by total kjeldahl (TKN) and Phosphorus by Vanadomolybdophosphoric acid were estimated as per the standard methods of APHA (2005). The samples were centrifuged to get rid of cell biomass before estimating nitrogen and phosphorus. Temperature was measured using thermometer and salinity was measured by Baume hydrometer (LEMICO). pH was measured by Equiptronics pH meter.

5.2 Results

5.2.1 Catabolic gene profile of consortium

Catabolic genes for degradation of alkane hydroxylase (*alk*B), naphthalene dioxygenase (ndo), pyrene degradation were found to be present (table 5b). The absence of genes for polyaromatic hydrocarbon degradation and phenanthrene leads to confusion as it is contradictory to the degradation results obtained during crude oil degradation (chapter 1). It has been observed that this bacterial consortium could degrade several poly aromatic hydrocarbons like phenanthrene, anthracene, dibenzothipene etc. This could be justified by the study done by Parales et al., (2000) on naphthalene dioxygenase stating that it could degrade several other poly aromatic hydrocarbons based on the amino acids present in the active site making it liberal in substrate selectivity.

Table 5b: amplification of different catabolic genes of the consortium

Gene	**Function**	**Size (base pairs)**	**Result**
NAH	Naphthalene degradation	377	Positive
Pdo	PAH dioxygenase	372	Negative
P3-24	Glutathione S-transferase	480	Negative
NidA	Pyrene degradation	1400	Positive
Ndo	Naphthalene dioxygenase	1041	Positive
phnAC	Phenanthrene degradation	994	Negative
*alk*B	Alakne hydroxylase	500	Positive

5.2.2 Collection of sea water and selection of bacteria

Sea water was collected from Velsao beach. The selected bacteria were isolated from oil contaminated sand sample and it has been proven to degrade aliphatic hydrocarbons extensively compared to aromatic hydrocarbons as per the results in our previous studies (chapter 1).

As per results obtained from growth curve experiment (fig: 5.2), it has been observed that *Pseudomonas aeruginosa* (AEBBITS1) showed a log phase till 2 hours and reached stationary phase with 6 hours and remained in stationary phase till 30 hrs. There was no increase in optical density in the flask inoculated with *Escherichia fergusonii* (AEBBITS 2). The reason was understood well by the amplification of two catabolic genes, which are the initial catabolic attackers of hydrocarbon. The amplification of *alk*B and NDO genes from the bacteria showed that *Pseudomonas aeruginosa* has both the genes but *Escherichia fergusonii* doesn't have either of the genes. The amplified product was confirmed based on size (fig: 5.3). Based on the growth curve results and catabolic gene detection *Pseudomonas aeruginosa* (AEBBITS1) has been selected for further studies.

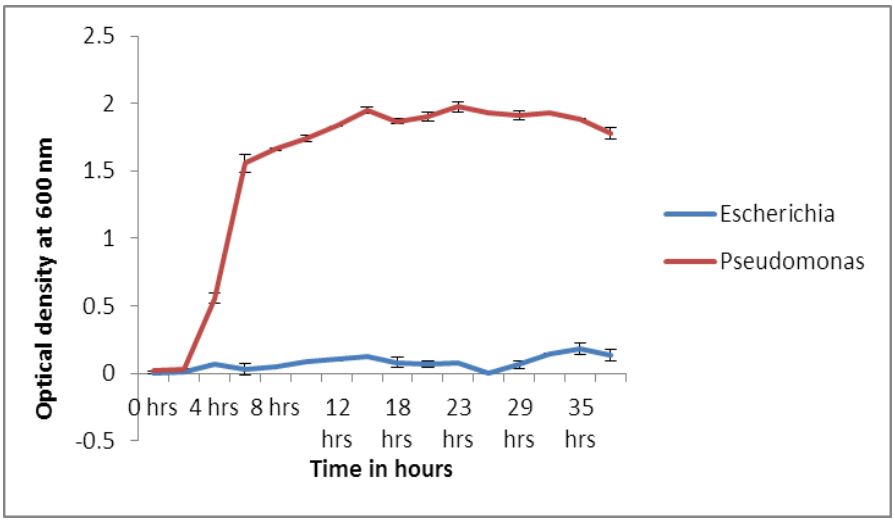

Fig 5.2: Growth of AEBBITS1 and AEBBITS2 using diesel oil as sole carbon source

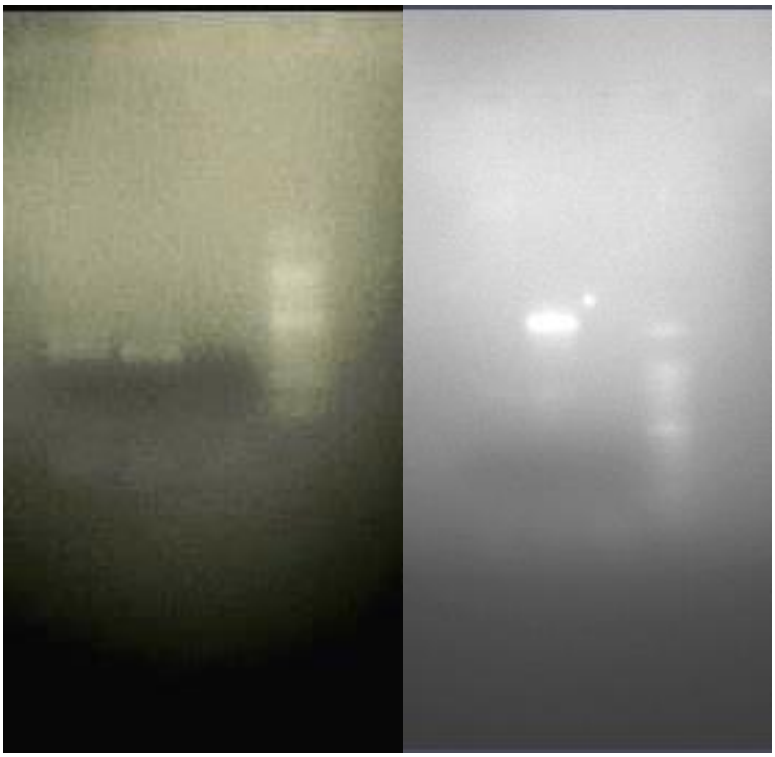

Fig 5.3: *alk*B (alkane hydroxylase) and NDO (Naphthalene dioxygenase) genes on gel electrophoresis after PCR. Lanes 1, 2, 4 represents *alk*B, NDO genes respectively. Lanes 3, 5 represents 100 base pair ladder

5.2.3 Biodegradation of Diesel

In the microcosm setup having diesel and the microcosm setup inoculated with AEBBITS1 there were about 10 different fractions of diesel observed. Gas chromatography was performed on every seventh day followed by addition of 0.5% diesel. The Gas chromatography results showed that 0% of residual diesel was present in both the microcosms (fig: 5.4, 5.5). There was no difference in degradation between the microcosm just with diesel and the one inoculated with *Pseudomonas aeruginosa* (AEBBITS1).

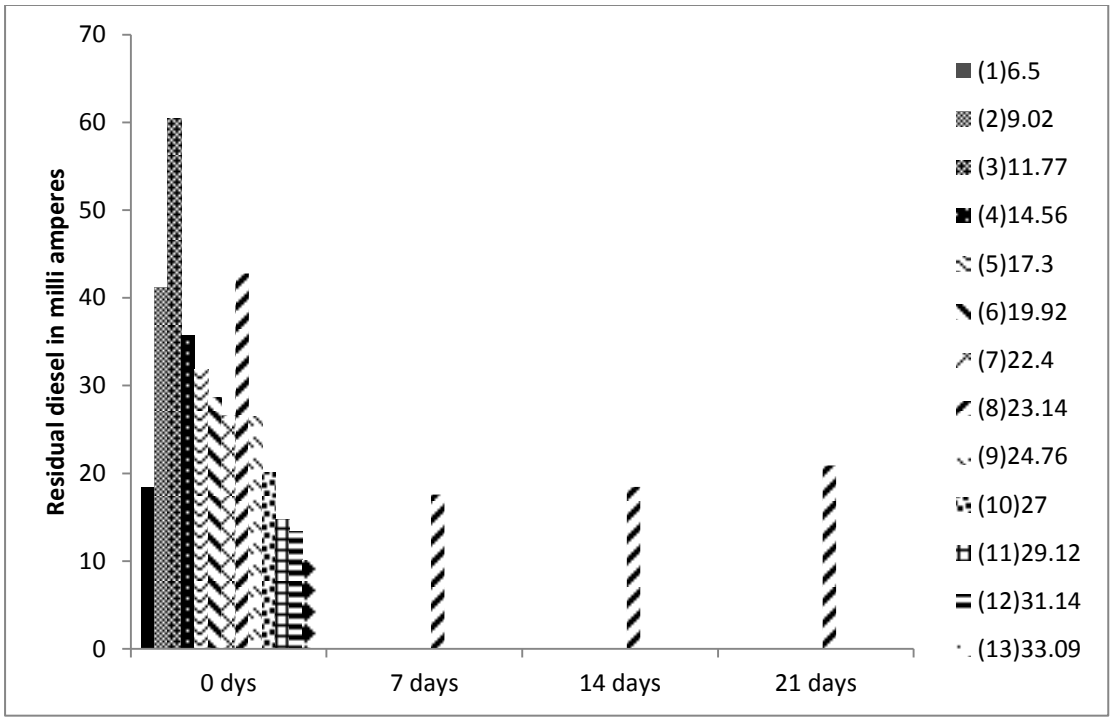

Fig 5.4: Degradation of diesel in microcosm (seawater +diesel) study for HGT. The numbers 1-10 represents different fractions of diesel and their retention time. The peak on7, 14, 21 days is of internal standard. The arrow mark indicates addition of seawater and diesel.

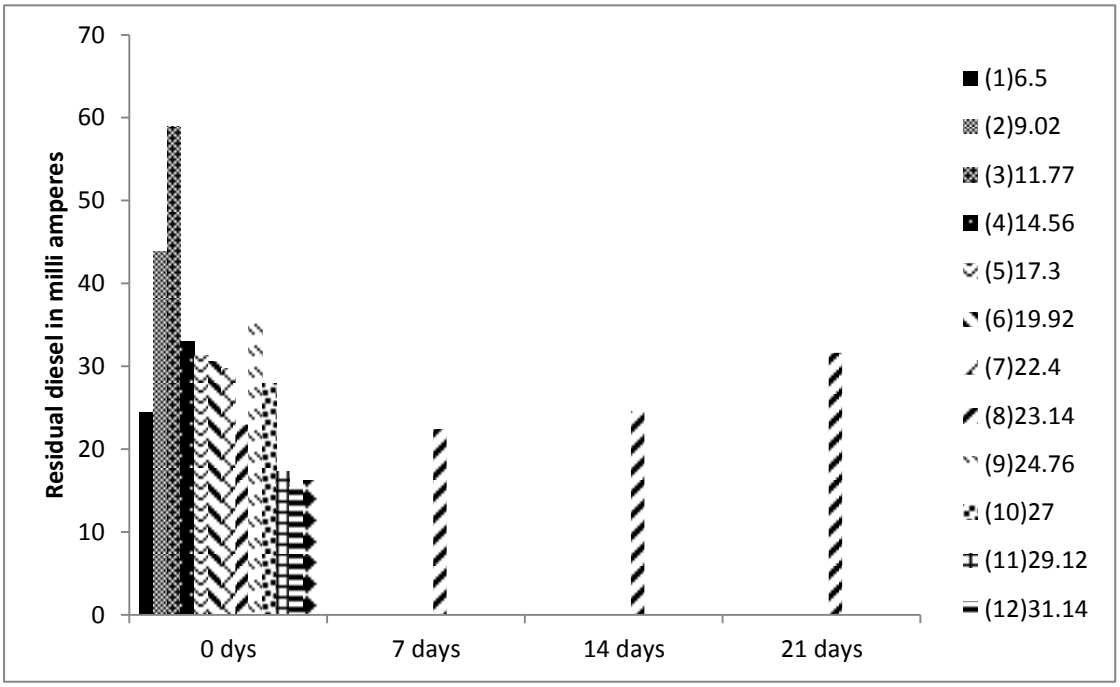

Fig 5.5: Degradation of diesel in microcosm (seawater+diesel+AEBBITS1) study for HGT. The numbers 1-10 represents different fractions of diesel and their retention time. The peak on7, 14, 21 days is of internal standard. The arrow mark indicates addition of seawater and diesel.

5.2.4 Different parameters

Different parameters like pH, temperature, salinity, nitrogen, phosphorus were monitored throughout the experiment. It has been observed that pH has dropped from 7 to 5.5 or 5.6 may be due to production of acids as secondary metabolites during growth of bacteria. There was no much change in other parameters. Salinity remained 4% in all the samples throughout the experiment. Nitrogen, phosphorus, temperature was found to be almost same throughout the experiment. The results are detailed in table 5c. The elevated level of nitrogen and phosphorus was due to addition of BH medium for the support of bacterial growth.

Table 5c: Different parameters on day 7, 14, 21 of the experiment

Parameter	Seawater (day 0&21)		Seawater + diesel (contaminated seawater)			Seawater+diesel+AEBBITS1 (bioaugmentation)		
pH	7	7	5.63	5.58	5.55	5.57	5.63	5.66
Salinity (%)	4	4	4	4	4	4	4	3.5
Nitrogen(ppm)	315	315	315	-	280	315	315	315
Phosphorus(ppm)	282	291	289	279	293	318	285	302
Temperature	is around 29 to 30°C throughout the experiment							

5.2.5 Denaturing gradient gel electrophoresis during diesel degradation

The DNA extraction of seawater samples was done using 0.22 micron vacuumed filtration. For microcosm supplied with diesel and *Pseudomonas aeruginosa* (AEBBITS 1), DNA extraction was done by centrifuging the enriched samples followed by Tsai and Rochelle protocol for environmental samples. So there is difference in DGGE patterns between only seawater and seawater with diesel. The sea water has shown same community pattern from first to last day of the experiment showing 7-8 prominent bands representing different bacteria. There were less number of bands observed in lanes representing seawater + diesel and seawater + diesel + AEBBITS 1. These bands represent the bacteria that were enriched by utilizing diesel as sole carbon source. However there was no change in enriched community by addition of AEBBITS 1 as the DGGE pattern remains same in both microcosms i.e., seawater + diesel and seawater + diesel + AEBBITS 1. The appearance of no extra bands in the DGGE in presence of AEBBITS 1 leads to a possible conclusion of no horizontal gene transfer based on the retrospective approach. It was also observed that *Pseudomonas* (AEBBITS1) has grown in seawater when supplied with diesel (fig: 5.6).

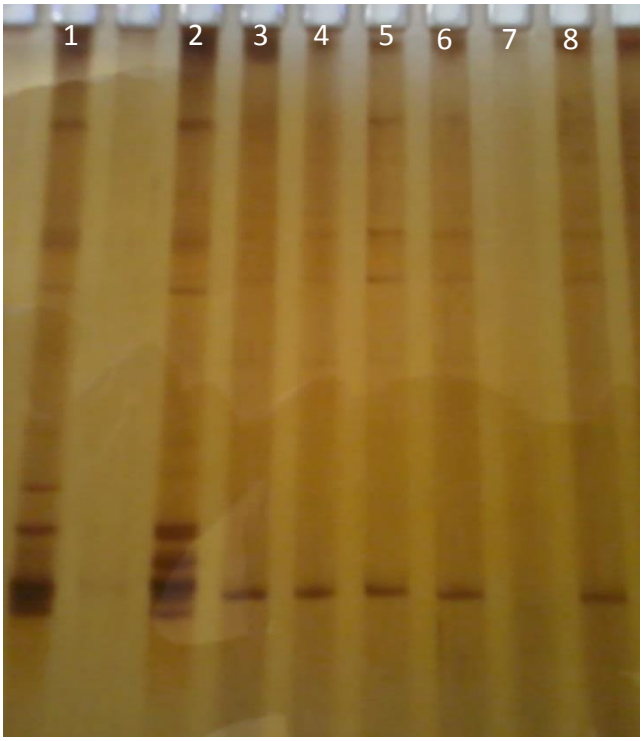

Fig 5.6: DGGE during biodegradation of diesel. First two lanes (1, 2) represent seawater on first and last day of experiment. The remaining lanes represent seawater + diesel (3, 5, 7), seawater + diesel + AEBBITS1 (4, 6, 8) on 7^{th}, 14^{th}, 21^{st} days respectively.

5.3 Discussion

It has been proved by many researchers that horizontal gene transfer is a method adapted by different bacteria to survive in polluted environments. As per the studies done with phylogenetic analysis comparing 16srDNA and catabolic gene distribution, different scientific groups have proposed that there might be HGT happening between different distantly related bacterial species in presence of pollutants. For instance Herrick et al., (1997) has proposed HGT of *nahAC* allele of naphthalene dioxygenase in a retrospective way as it is present in plasmid hosted by phylogenetically diverse bacteria. It also has been proposed that HGT can be a reliable technique in case of failed bioaugmentation, if the catabolic genes could be transferred to the native bacteria (Leahy et al., 1990). It was identified that alkane hydroxylase (alkB) and naphthalene dioxygenase (ndo) are present in *Pseudomonas aeruginosa* (AEBBITS 1). The position of catabolic genes i.e., on genomic DNA or plasmid plays a critical role as the fragment of gene undergoing HGT should have all necessary elements required for its expression in the recipient bacteria. Catabolic plasmids like pDTG1 harboring complete NDO gene has been reported for HGT and degradation of naphthalene by recipient bacteria (Herrick et al., 1997). For instance alkane hydroxylase system have three gene (*alk*B, rebredoxin, rubredoxin reductase) required for hydroxylating alkanes but in many bacteria the rubredoxin reductase is located away from alkB and rebredoxin (van Beilen et al., 2003). This type of organization of genes may not play a key role in horizontal gene transfer as alkane hydroxylase will not function without the support of rubredoxin and rubredoxin reductase. DGGE is a molecular finger printing technique used to study the diversity of microorganisms in different ecosystem, later it has been adapted to study the diversity of catabolic genes also. DGGE is used to study the difference of microbial community between pristine and contaminated environments and also the community changes during bioremediation process (Ferguson et al., 2007, Vinas et al., 2005). The diversity and distribution of alkane hydroxylase and naphthalene dioxygenase have been investigated from contaminated sites using DGGE as potential tool. It has been proposed that the quantifying the catabolic genes from a contaminated site can be an indicator for bioremediation process (Fuentes et al., 2014). So, in the present study DGGE has been used to study the change in diversity of enriched hydrocarbon degrading bacteria to study the possibility of horizontal gene transfer. DGGE was performed on samples from seawater on

first and last day of the experiment using metagenomics DNA. There was no difference in the DGGE pattern of seawater. The DGGE patterns of microcoms representing contaminated seawater and augmented seawater is same but different from normal seawater. This might be due to difference in DNA extraction procedure but it very clearly shows the enrichment of hydrocarbon degradation bacteria. As there was no difference of DGGE pattern between diesel oil contaminated seawater and augmented seawater, it was clear that HGT did not take place. If HGT is not a reliable technique for immediate remedy, the diesel degradation results arises a question if bioaugmentation is necessary. If bioaugmentation is necessary, what is the role of nutrients in bioremediation or rate of degradation?

5.4 Conclusion

In this study the similar patterns of DGGE in microcosms representing diesel oil contaminated seawater and bioaugmented seawater shows there was no HGT happened during bioaugmentation. A similar band as that of *Pseudomonas aeruginosa* (AEBBITS 1) used for bioaugmentation appeared in all the lanes representing its presence in seawater. Unreliability of HGT, successful diesel degradation and existence of donor (AEBBITS 1) in seawater leads to a question if bioaugmentation is required. So, further studies were carried out to evaluate the effect of nutrient availability on bioremediation.

CHAPTER 6

BIOSTIMULATION VERSUS BIOAUGMENTATION

6. Introduction

Oil pollution is one of the major concerns of Goa tourism. Goa is located on the Arabian stretch of Indian peninsular region (15°29'N, 73°48'E) and its shoreline provides site for public recreational activities. Marine accidental oil spills or intentional disposal of oil is a major concern as the spilled oil floods the beaches with tarballs. It has been stated by National Institute of oceanography (NIO, Goa) that the occurrence of tarballs or mats of oil on beaches is a seasonal process. The trajectory of tarballs or oil slicks has been studied according to the wind currents and action of waves and found the chances of oil and tarballs flooding Goa's coast is very high (Vethamony et al., 2007). Though it has been proposed that bioremediation is a solution for the oil spills and tarballs of Goan beaches (Rodrigues et al., 2011, Sivaraman et al., 2011, Pasumarthi et al., 2013), bioaugmentation versus biostimulation has been never investigated. Bioaugmentation is addition of hydrocarbon degrading bacteria to the contaminated site whereas biostimulation is addition of nutrients to enhance degradation by the indigenous bacteria. There were bioaugmentation studies conducted in lab scale and *Pseudomonas aeruginosa*, *Pseudomonas mendocina* were proposed to be used for bioremediation via augmentation (Pasumarthi et al., 2013, Sivaraman et al., 2010, Prakash et al., 2008). It has been reported that the indigenous bacteria play a role in degradation but a consortium is required as a single bacteria cannot degrade all the fractions of oil (Shankar et al., 2014). Existence of specific groups of bacteria in marine ecosystem specialized in hydrocarbon degradation has been reported (Yakimov et al., 2007). Apart from microorganisms, biodegradation of hydrocarbons depends on availability of oxygen, water, nitrogen and phosphorus (Rosenberg et al., 1992). In the process of bioremediation a balanced nutrient availability is essential as the limitation or a change in nutrients sometimes inhibits microbial activity (Braddack et al., 1997). In the incident of oil spill even though high content of oil is available, the rate of degradation will be limited due to low availability of nitrogen and phosphorus (Atlas et al., 1972). Gertler et al., (2012) has reported the increase in number of hydrocarbon degrading bacteria by addition of nitrogen and phosphorus. Availability of these nutrients results in eutrophication with higher concentration and suboptimal degradation with low concentration. The selection of technique depends on the circumstances that prevail at the contaminated site. The specialization of augmented microorganisms and the diversity of indigenous hydrocarbon degrading bacteria play an

important role in selection of natural attenuation, biostimulation and bioaugmentation. In the present study *Pseudomonas aeruginosa* (AEBBITS1) has been used and it has been reported in our earlier study for its capacity to degrade crude oil sediment (Pasumarthi et al., 2013). This study will provide an insight into conditions that play a role in bioremediation strategies for marine oil pollution of Goa.

6.1 Materials and Methods

6.1.1 Collection of sea water and selection of culture

Sea water was collected from Velsao beach, Goa and was preserved at 4^0C until use. *Pseudomonas aeruginosa* (AEBBITS 1) has been chosen for its capability to degrade hydrocarbons as per the results obtained in our previous study. The culture was isolated from oil contaminated soil of Velsao beach using enrichment culture technique (Pasumarthi et al., 2013, 2014).

6.1.2 NAPL design

The model non aqueous phase liquid (NAPL) was prepared as described by Mukherji et al., (1997). The NAPL was prepared by maintaining the mole fraction of each component less than its fugacity ratio. The mole fraction of each hydrocarbon used is as follows: n-Tetradecane (0.25), n-Hexadecane (0.45), n-Octadecane (0.11), n-Naphthalene (0.15), and n-Phenanthrene (0.04). As NAPL should represent diesel, hexadecane to Naphthalene ratio has been maintained 3:1.

6.1.3 Microcosm studies

Microcosm setup has been designed in 100 ml serum vials so that a comparison will be possible between natural attenuation, Biostimulation and Bioaugmentation (Sivaraman et al., 2010). In each microcosm 20 ml of sea water was taken and supplied with 100 µl of NAPL. Abiotic setup acts as a control and it was taken care that sterile sea water was used. In the biostimulated microcosm setup, 0.077 g/L of KH_2PO_4, 0.2 g/L of NH_4Cl, 0.1 g/L of $NaNO_3$ were added as additional nutrients (Hassanshahian et.al., 2014). In setup of biostimulated bioaugmentation the above mentioned additional nutrients were added to sea water and also augmented with *Psedomonas aeruginosa* (AEBBITS 1). In bioaugmented setup, sea water was enriched with NAPL and inoculated with *Pseudomonas aeruginosa* (AEBBITS 1).

6.1.4 Gas chromatography

For every 24 hours the NAPL was extracted from duplicates of microcosm representing Abiotic, natural attenuation, bio-stimulation, bio-augmentation, bio-stimulated bio-augmentation. NAPL was extracted by adding equal volume of ethyl acetate and stirring on magnetic stirrer for 15 minutes at room temperature. 1 ml of the upper organic layer was pipetted into clean 5ml culture vial, sealed and preserved at 4^0C until Gas chromatography. Two microliter was used as injection volume into CHEMITO (GC 7610) Gas chromatograph equipped with BPX5 capillary column. The injection port and Flame ionization detector were maintained at 250^0C and the temperature of oven was increased at the rate of 5^0C per minute. Hundred microliter of 1% fluorine was added to each sample as an internal standard.

6.1.5 DNA extraction and DGGE

Genomic DNA was extracted from all the experiment setups as per the protocol suggested by Tsai and Rochelle (2001) but with minor changes (Pasumarthi et al., 2013). The obtained DNA was confirmed with gel electrophoresis. Nested PCR was applied to amplify 16SrDNA by Eppendorf flexi lid thermal cycler. The primers used are 27F, 1492R for first PCR and 968FGC, 1492R for GC clamping in second PCR. The PCR program mentioned in our earlier studied was used (Nikolausz et al., 2008). DGGE was performed to understand the role and survivability of different bacteria from sea water, survivability of bacteria used in augmentation and as well as the difference between bio-stimulation, bio-augmentation and combination of bio-stimulation and bio-augmentation. 7.5% acrylamide gel was used with denaturant gradient ranging from 30% to 60% and 10 microliter of sample was loaded and run for 14 hours at 70 volts. The gel was stained by silver staining (Pasumarthi et al., 2013). DNA from bands was eluted and re-amplified with primers 968FGC and 1492R and subjected for sequencing. nd phylogenetic analysis was carried out using MEGA software.

6.1.6 Total Protein, Nitrogen and Phosphorus Estimation

All the samples of microcosms were centrifuged at 10,000 rpm for 10 minutes in Remi C-24 centrifuge and the obtained cell pellet was suspended in 1 milliliter of distilled water. The cells were lysed and total protein was estimated by Lowry's method as mentioned in the earlier chapter (chpter2, section 2.5). Optical density was measured at 740 nm. Nitrogen by

total kjeldahl and Phosphorus by Vanadomolybdophosphoric acid were estimated for first and last day samples as per the standard methods of APHA (2005).

6.2 Results

6.2.1 Collection of sea water and selection of bacteria

Sea water was collected from Velsao beach. The selected bacteria were isolated from oil contaminated sand sample and it has been proven to degrade aliphatic hydrocarbons extensively compared to aromatic hydrocarbons as per the results obtained in the previous studies (chapter 2). The nitrogen and phosphorus were found to be more in the bio-stimulated setups compared to the control microcosm setup (table 6a).

	Nitrogen		**Phosphorus**	
	0 hrs	**96 hrs**	**0 hrs**	**96 hrs**
Abiotic	20.3 ± 0.808	20.06 ± 3.63	0.303 ± 0.05	0.2 ± 0.02
Natural attenuation	20.3 ± 0.808	19.95 ± 3.6	0.303 ± 0.05	0.61 ± 0.06
Biostimulation	29.75 ± 6.06	18 ± 2.82	11.89 ± 0.13	10.13 ± 0.01
Bioaugmentation	20.3 ± 0.808	10.1 ± 0.404	0.303 ± 0.05	1.66 ± 0.3
Biostimulation & Bioaugmentation	29.75 ± 6.06	14.7 ± 0.8	11.89 ± 0.01	9.22 ± 0.01

Table 6a: Represents the amount of nitrogen and phosphorus present in different microcosm setups on first and last day of experiment

6.2.2 Biodegradation of NAPL

Aliphatic hydrocarbons

Tetradecane, hexadecane, octadecane are the aliphatic hydrocarbons used as components of NAPL mixture. All three were mixed as per their fugacity ratio. Fugacity ratio is the ratio of the solid to liquid state fugacities. Degradation of all three has started around 72 hours but considerable degradation was found by 96 hrs. This might be due to the availability of other carbon source in seawater or the bacteria might have taken some time to acclimatize to the microcosm environment. All three aliphatic hydrocarbons were found to be degraded more by *Pseudomonas aeruginosa* in the presence of bio-stimulants (biostimulated bioaugmnetation) by 96 hrs. The percentage of residual tetradecane, hexadecane, octadecane present in microcosm inoculated with *Pseudomonas aeruginosa* and supplemented with bio-stimulants was 39.3%, 42.3%, 63.2% respectively (Fig. 6.1, 6.2, 6.3:) which was very less compared to other microcosm setups. There was no considerable degradation in the setups of abiotic, natural attenuation, bio-stimulation and bio-augmentation till last day of the experiment. Residual tetradecane found to be present in abiotic, natural attenuation, bio-stimulation, bioaugmentation by 96 hours was 100.9%, 97.4%, 102.5%, 94.5% respectively (Fig. 6.1). Percentage of residual hexadecane present in microcosms of abiotic, natural attenuation, biostimulation, bioaugmentation was found to be 102.1%, 101.2%, 103.2%, 93.5% by 96 hours (Fig. 6.2) and for octadecane it was about 95.2%, 95.7%, 101.3%, 98.4% respectively (Fig. 6.3).

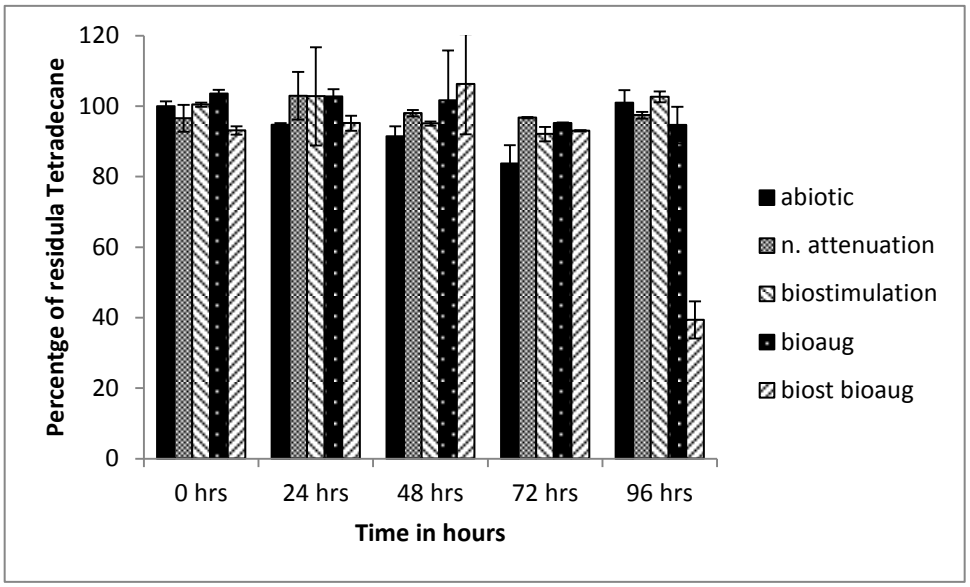

Fig 6.1: Represents degradation of tetradecane in different microcosms

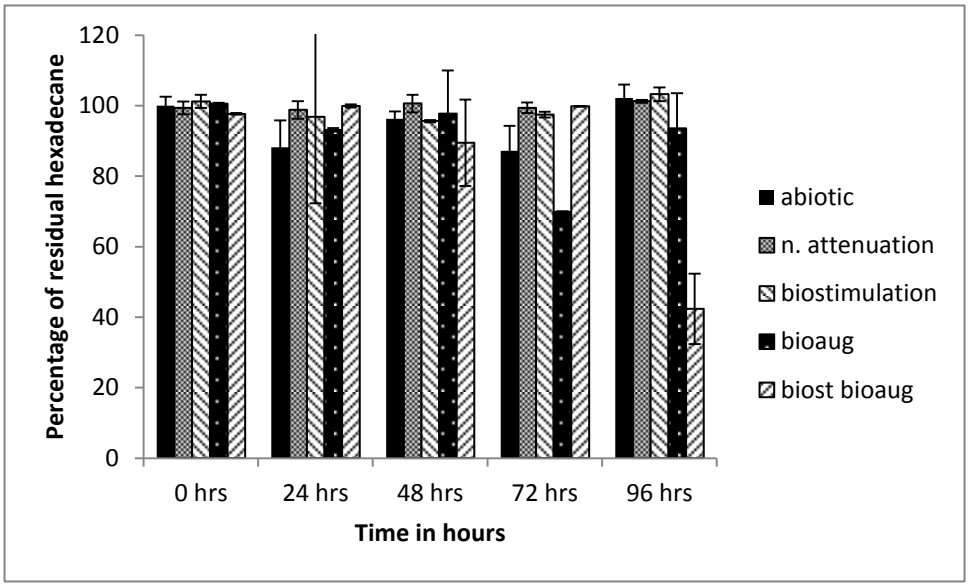

Fig 6.2: Represents degradation of hexadecane in different microcosms

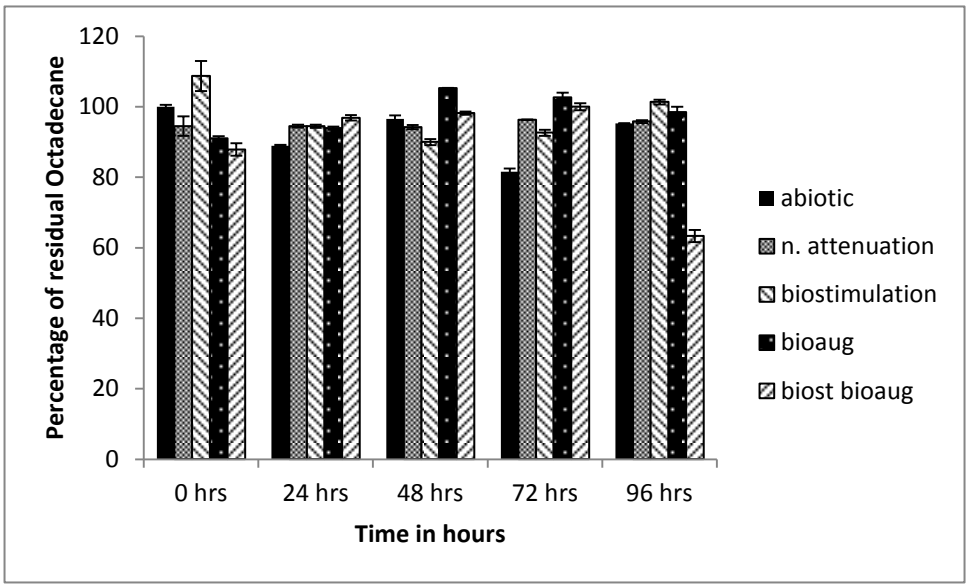

Fig 6.3: Represents degradation of octadecane in different microcosms

Aromatic hydrocarbons

Naphthalene and Phenanthrene are the two aromatic hydrocarbons used as components in the NAPL mixture. As per the results obtained from Gas Chromatograph, degradation of both the hydrocarbons was found to be more in microcosm inoculated with *P.aeruginosa* in presence of bio-stimulants (biostimulated bioaugmentation). The percentage of residual naphthalene present in microcosm inoculated with *P.aeruginosa* (AEBBITS 1) and supplemented with bio-stimulants was 26.3% by 96 hours and the amount present in abiotic samples, natural attenuation, biostimulation, bioaugmentation was 70.7%, 70.8%, 76.7%, 66.7% respectively by 96 hours (Fig. 6.4). The amount of residual phenanthrene in bioaugmentation setup supplemented with bio-stimulants was 28.4% where as in abiotic, natural attenuation, bio-stimulation, bioaugmentation was 71.4%, 79.9%, 86.2%, 57.3% respectively (Fig. 6.5). The reduction in residual aromatic hydrocarbons in abiotic samples might be due to evaporation.

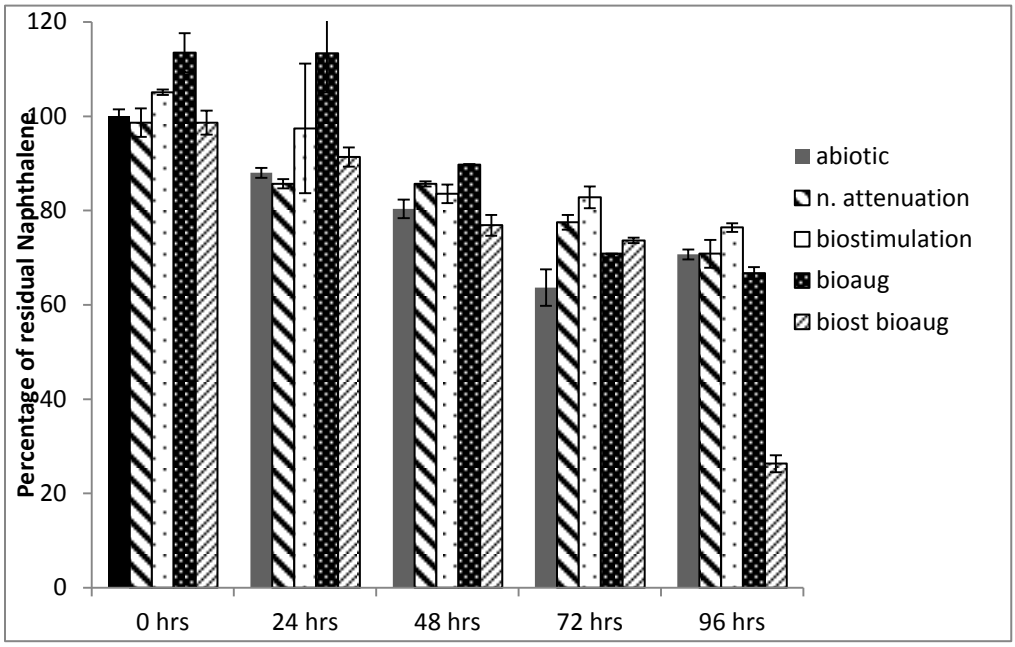

Fig 6.4: Represents degradation of naphthalene in different microcosms

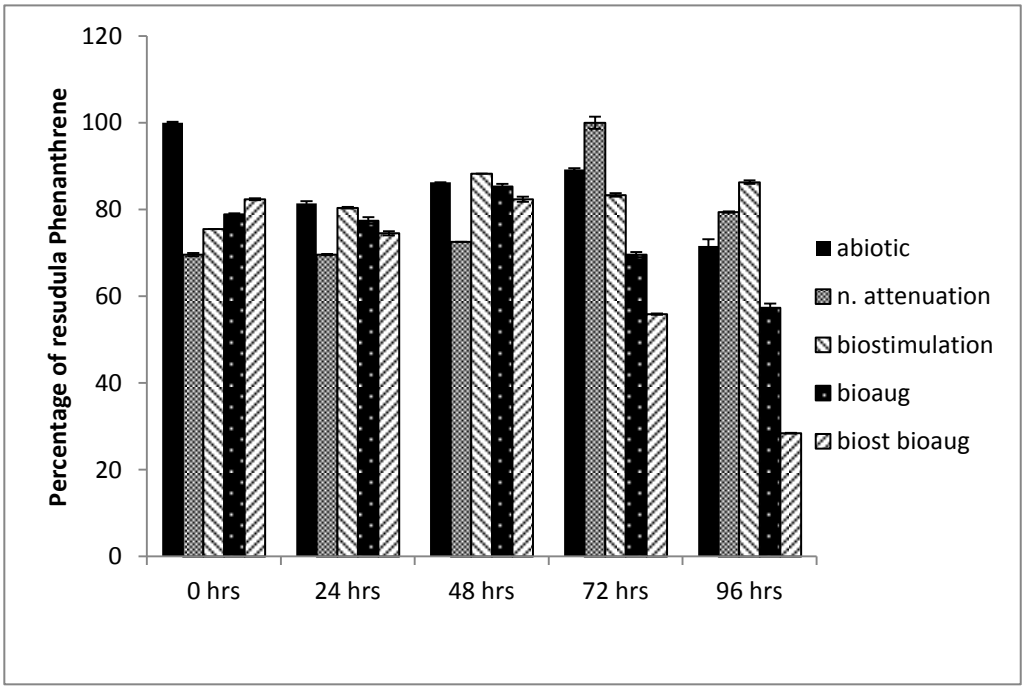

Fig 6.5: Represents degradation of phenanthrene in different microcosms

6.2.3 Total protein estimation

Total estimation of protein of the samples representing different microcosm setups at different time intervals gives a picture of bacterial growth in the experimental setup. As per the spectrophotometric readings obtained it has been observed there was growth in microcosms inoculated with *P.aeruginosa* (bio-augmentation) and also in biostimulated bioaugmentation. There was mild growth observed in bio-stimulated setup but no degradation was observed in this setup. There was no increase in protein biomass i.e., no growth in abiotic and natural attenuation setup clearly indicating the role of bio-stimulants for bacterial growth in sea water. Maximum growth was observed with bio-stimulated bio-augmentation by 48 hours and entered decline phase by 96 hours. In bio-augmentation setup maximum growth was obtained by 96 hours. The increase protein biomass also supports the high degradation in microcosm of biostimulated bioaugmentation (Fig. 6.6).

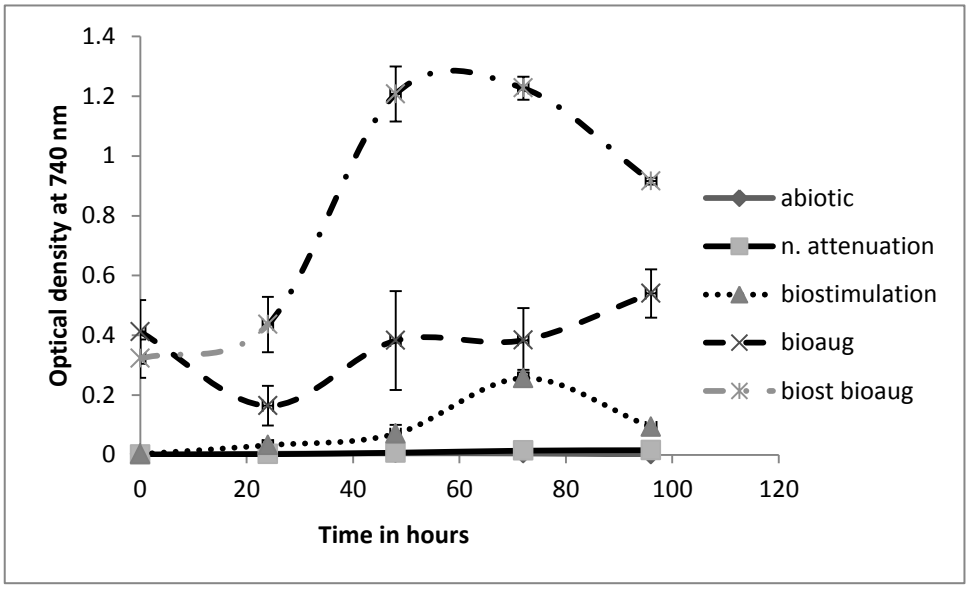

Fig 6.6: Represents estimation of protein by Lowry's method

6.2.4 Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed only to the enriched cultures. The abiotic and natural attenuation microcosms were excluded from DGGE as there was no growth observed. It has been observed that NAPL enriched diversity is same in biostimulated setup, bioaugmented setup, biostimulated bioaugmentation setup. All these lanes have six prominent bands representing six different bacteria. By 72 & 96 hours only four of them remained prominent in bioaugmented setup and biostimulated bioaugmentation (Fig 6.7). This indicates the difference in the diversity of NAPL enriched hydrocarbon degrading bacteria and non dergrading bacteria. After sequencing, the sequences were analyzed by NCBI BLAST and found that bands 1, 2, 3, 4 are *Sphingomonas* and 5, 6 are AEBBITS1, *Pseudomonas* sp. respectively.

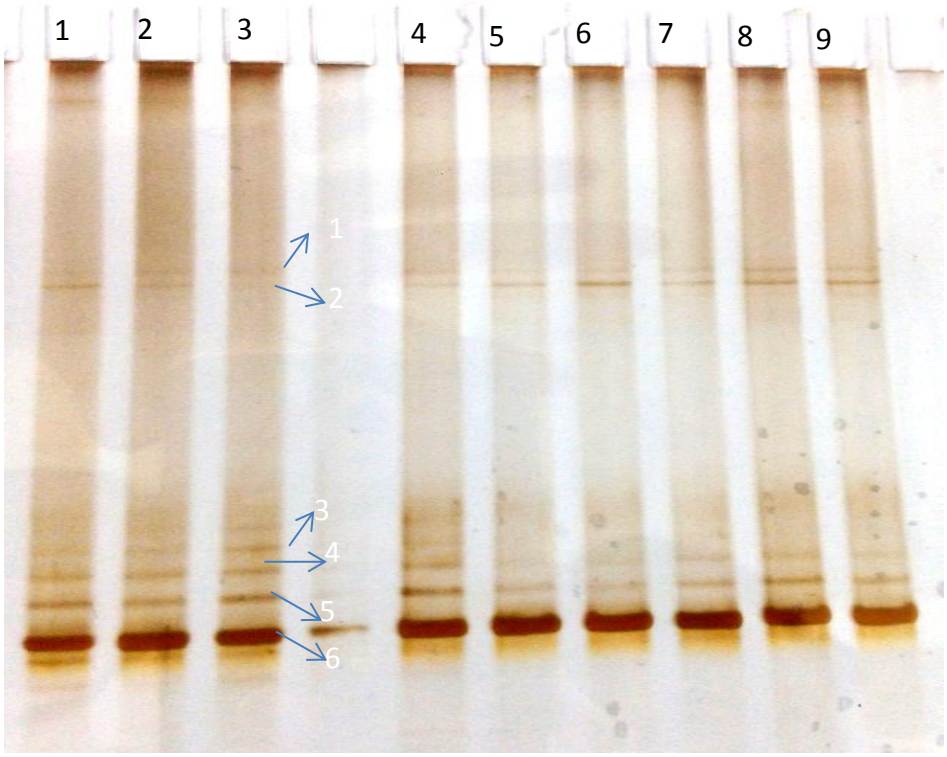

Fig 6.7: DGGE gel represents diversity of NAPL enriched bacteria in different microcosms. 1, 2, 3 lanes represent biostimulation, bioaugmentation, biostimulated bioaugmentation on 24 hrs respectively. Lanes 4, 5, 6 represents 72 hrs, 7, 8, 9 lanes represent 96 hrs. The band no.5 represents AEBBITS1

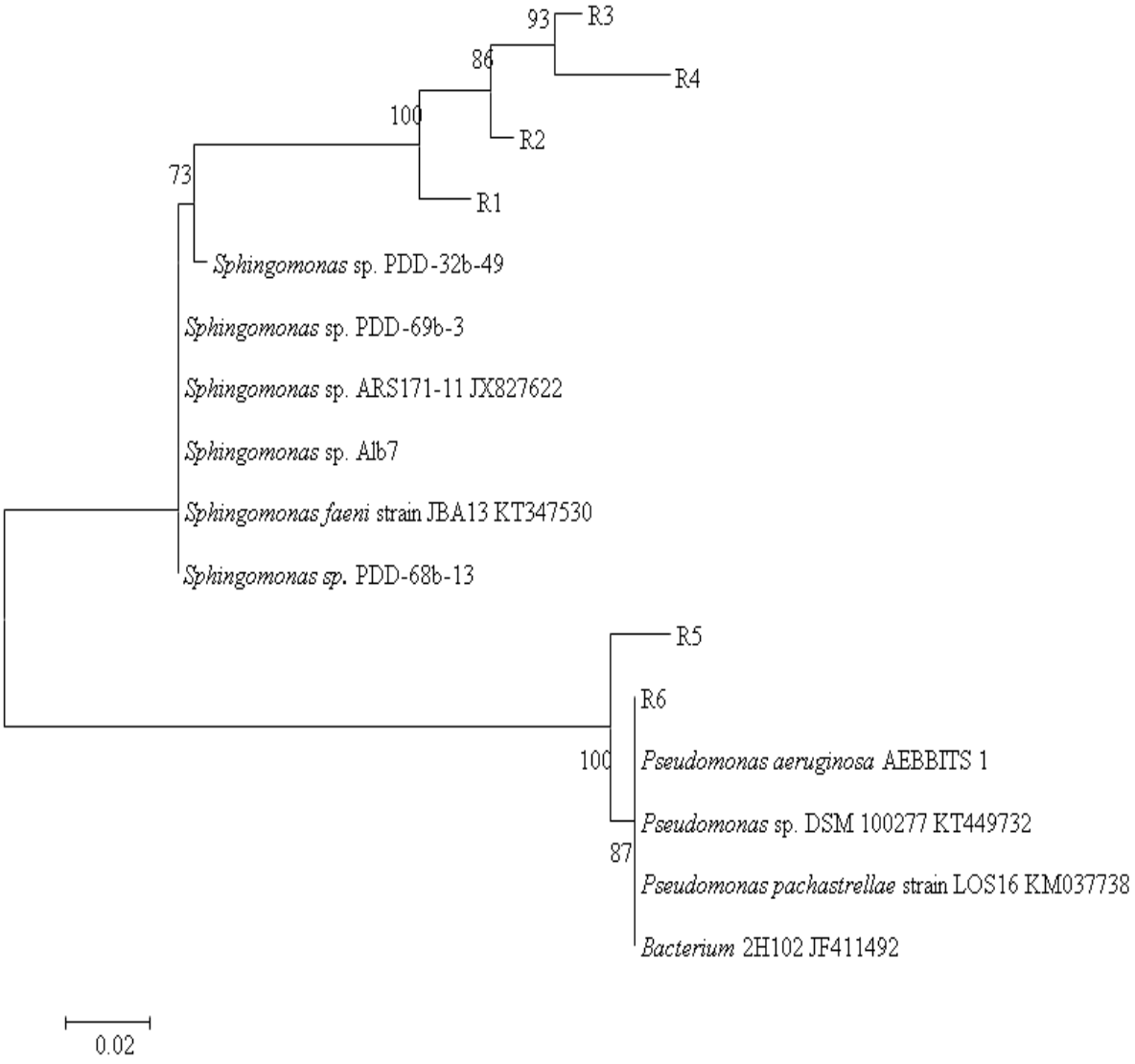

Fig 6.8: Phylogenetic analysis of different bacteria from the DGGE gel. R5 1492 is AEBBITS1

6.3 Discussion

As per the results obtained it has been observed that degradation of hydrocarbon by *P.aeruginosa* was more in presence of Na_2HPO_4, $NaNO_3$, and NH_4Cl (biostimulated bioaugmentation) compared to natural attenuation, biostimulation and bioaugmentation experiments. The degradation data has been supported by the total protein analysis stating that maximum growth was observed in microcosm inoculated with *P.aeruginosa* in the presence of biostimulants. There was some increase in protein biomass in biostimulated setup which might be due to utilization of other carbon sources present in seawater by the indigenous bacteria. The importance of nitrogen and phosphorous sources in hydrocarbon degradation has been discussed many times before and it has been reported that low availability of nitrogen and phosphorus limits the rate of degradation (Prince et al., 1997, Lee et al., 1999). In marine waters low amounts of nitrogen and phosphorus is one of the important reasons of nutrient limitations (Das et al., 2006). It has been estimated that 20.3±0.8 ppm of nitrogen and 0.303±0.05 ppm of phosphorus was present in seawater collected from Velsao beach, Goa and the amount increased after addition of biostimulants to 29.75±6 and 11.89±0.13 ppm respectively. Both elements were decreased by 96 hrs in all the microcosm setups except in natural attenuation and abiotic samples which was understandable as there was no growth observed in these two microcosm setups. It has been an unanswered debate whether bioaugmentation or biostimulation is a better choice for bioremediation as there are different studies leading to different conclusions. In some cases it has been proved that selection of right microorganism capable of dealing the contaminated site defines successful bioremediation where as in other studies it is opposite proving the importance of biostimulation (Hamdi et al., 2007, Bento et al., 2005). The studies conducted by Swannell et al., 1996, Roling et al., 2002 proved that the growth and degradation of oil by bacteria can be enhanced by addition of inorganic nitrogen and phosphorus. In the present study both bio-augmentation and biostimulation have proved to be slow compared to combination of both as it was proved by Hamdi et al., (2007) with successful bioremediation of PAH contaminated soil using both bioaugmentation and biostimulation. In a mecocosm study conducted by Darmayati et al., (2015) in Indonesian sandy beaches, it has been proved that combination of biostimulation and bioaugmentation degraded more oil compared to biostimulation. How ever there was no degradation, growth and change in nutrients with

natural attenuation proving that natural acclimatization by indigenous microbial population takes a longer time due to extended log phase in natural environmental circumstances resulting in very slow bioremediation as described by Lendvay et al., (2003). DGGE has been used for studying the diversity of hydrocarbonoclastic bacteria in many instances. Yakimov et al., (2004) has reported crude oil cultural shift of bacteria in Ross Sea. The change in DGGE pattern indicates the enrichment of hydrocarbon degrading bacteria and their survivability. The changes in marine microbial diversity by addition of inorganic nitrogen and phosphorus has been reported before in a seawater mecocosm study (Jimenez et al., 2007, Evans et al., 2004) and it also has been stated that addition of nitrogen and phosphorus has enhanced the growth of hydrocarbon degrading bacteria (Capello et al., 2007). In this study the change in the NAPL enriched diversity is might be due to nitrogen and phosphorus or by addition or enrichment of *Pseudomonas* (AEBBITS 1) resulting in changes in synergistic relation among the existing bacteria in seawater.

6.4 Conclusion:

From the present study it can be concluded that combination of bioaugmentation and biostimulation with addition of Na_2HPO_4, $NaNO_3$, and NH_4Cl has led to higher degradation of hydrocarbons present in the designed NAPL. The failure of indigenous bacteria was clearly observed in natural attenuation and biostimulation even though the presence of hydrocarbon degrading bacteria in seawater was reported before. This work will be continued at field level to evaluate the importance of other environmental factors apart from nutrient availability.

CHAPTER 7

MOLECULAR INTERACTIONS OF HYDROCARBONS TO OUTER MEMBRANE PROTEIN (TodX) OF *Pseudomonas*

7. Introduction

There are three major steps involved in hydrocarbon degradation 1) adsorbing substrate onto the cell membrane 2) transport of substrate into the cell 3) enzymatic digestion of substrate. As the enzymes for hydrocarbon degradation are located intracellularly, it is important that hydrocarbons should be transported into cell (Hau et al., 2013). From the above mentioned three steps, contact of hydrocarbon to bacterial cell and enzymatic action on hydrocarbon are well understood but the critical step of transport was not studied extensively. Hydrocarbon transport can be broadly classified into passive diffusion and active transport. Many studies have been conducted to understand whether hydrocarbon uptake by bacterial cells is energy dependent or independent. However it was not concluded as some studies proved that the transport of hydrocarbons is energy dependent and some studies proves the opposite (Miyata et al., 2004, Verdin et al., 2005, Kallimanis et al., 2007). The transport mechanism depends on the type of bacteria and type of hydrocarbon. Recently crystal structures of two outer membrane proteins from *Pseudomonas putida* and *Rostelinia picketti* were reported which play a role in aromatic hydrocarbon transport (Hearn et al., 2008). It was evident from the study that these two protein structures are similar to FadL transporter protein from *E.coli*. They are monomeric beta barrel proteins with 14 strands having an internal hatch domain. The authors have reported that the N-terminus of the hatch domain is flexible and undergoes conformational change during hydrocarbon transport. So, the proposed transport model is that the hydrophobic cleft formed by the extracellular loops traps the hydrocarbons, moves through the hydrophobic barrel and the hatch domain is flexible enough to allow the hydrocarbon into the cytoplasm. Another FadL protein from *Pseudomonas aeruginosa* has been reported by Hearn et al., (2009). The protein structure is similar to that of TodX and TbuX having a lateral opening created by a kink in beta sheet S3 of the barrel. In this study the authors have observed the presence of a detergent molecule at the S3 kink which leads to idea of transport of hydrocarbons through lateral opening. The mutated protein having no lateral opening was not capable of transporting hydrocarbons. So, it was proposed that the hydrocarbon enters the interior of the beta barrel protein followed by movement into the cell membrane through lateral opening and then diffuses into the cytoplasm. The studies involving the crystal structures was more focused on the interaction of detergent molecules to the proteins but no study was done whether hydrocarbons are interacting with outer

membrane proteins. So, the present study focuses on interaction of hydrocarbon molecules to TodX protein from *P. putida* and investigates if S3 kink is playing a role in hydrocarbon transport.

7.1 Methodology

7.1.1 Multiple sequence alignment

The MSA sequence alignment data from study of TodX and TbuX crystal structures, TmoX of *P. mendocina* and xylN from *P.putida* (Hearn et., 2008) was taken into consideration. As it has been already reported by the authors that some sites in hatch domain are conserved, the present study also focuses to observe conserved amino acids and their role in interaction to hydrocarbon molecules.

7.1.2 Molecular docking

Molecular docking has been carried out by AUTODOCK4.2 software (Morris et al., 2009). Autodock was developed by Garrett Morris in Olson group at Scripps Research Institute La Jolla. Autodock 4.2 is faster than earlier versions; it allows flexible docking of macromolecule. It has free energy scoring function that is based on a linear regression analysis, the AMBER force field. Autodock combines both the rapid grid-based energy evaluation and efficient search of torsional freedom. Autodock calculations require at least four input files:

- A PDBQT file for the ligand;
- A PDBQT file for the receptor;
- A Grid Parameter File (GPF) for the Auto Grid calculation;
- A Docking Parameter File (DPF) for Auto Dock calculation.

Auto Dock calculations were performed in several steps:

- ✓ Coordinate file preparation using Auto Dock Tools
- ✓ Atomic Affinities pre calculation using Auto Grid
- ✓ Docking of ligands using Auto Dock
- ✓ Analysis of results using Auto Dock Tools

Step 1 Coordinate File Preparation

The PDBQT files of different hydrocarbons and TodX protein were prepared. PDBQT coordinate file includes atomic partial charges and atom types. PDBQT files also include information on torsional degrees of freedom.

Step 2 Auto Grid Calculations

Pre-calculating the atomic affinity potentials for each atom type for the evaluation of energy. In Auto Grid procedure, the protein was embedded in a three dimensional grid and a probe atom is placed at each grid point. The energy of interaction was assigned to grid point. Auto Grid affinity grid was calculated for each type of atoms in the ligand, typically carbon, oxygen and hydrogen, as well as grids of electrostatic and desolvation potentials. The energy of a particular ligand configuration is evaluated using the values from the grids.

Step 3 Docking using Auto Dock

There were several search methods used in docking. The most efficient method was a Lamarckian Genetic Algorithm (LGA), Auto Dock was run several times to give several docked conformations and energy was analyzed to identify the best solution.

Step 4 Analysis using Auto Dock Tools

It includes several methods for analyzing the results of docking simulations, including tools for clustering results by conformational similarity, visualizing interactions between ligands and proteins and visualizing the affinity potentials created by Auto Grid.

Every hydrocarbon was docked individually to see the interaction sites and the conformations near to interior hatch domain and S3 kink are taken into consideration. The binding energy and ligand efficiency was noted down.

7.2 Results

7.2.1. Molecular interactions

Interaction of aliphatic hydrocarbons with TodX

TodX is the outer membrane protein involved in the transport of hydrocarbon in *P. putida*. Aliphatic hydrocarbons like Butane, Hexane, Heptane, Tetradecane, Hexadecane, Octadecane are tested for their interaction with TodX. They are found to have interaction with amino acids near hatch domain and S3 kink (Fig 7.1, Fig 7.2). It has been observed that all the hydrocarbons have interacted with TodX near the Hatch domain and S3 kink. The amino acids involved in interaction with different hydrocarbon are given in the figures 7.1 and 7.2. Some of the hydrocarbons did not interact at interior of the barrel in the first conformation but they showed interaction near the hatch domain (interior of the barrel) in other conformations. The interactions interior to the beta barrel are taken into consideration neglecting the conformations having interaction with outer surface of barrel. None of the hydrocarbons are found to interact with the extracellular loops of the protein. The binding energy of Butane, Hexane, Heptane, Decane, Tetradecane, Octadecane, Benzene, Toluene to amino acids near to hatch domain are or interior of the barrel are -2.61, -3.23, -3.09, -3.69 -3.65, -3.91, -3.56, -3.58, -3.69 respectively. The binding energy of Hexane, Tetradecane, Hexadecane, Octadecane, Benzene and Toluene near S3 kink is -3.26, -3.91, -3.39, -4.2, -3.41 and -3.91 respectively.

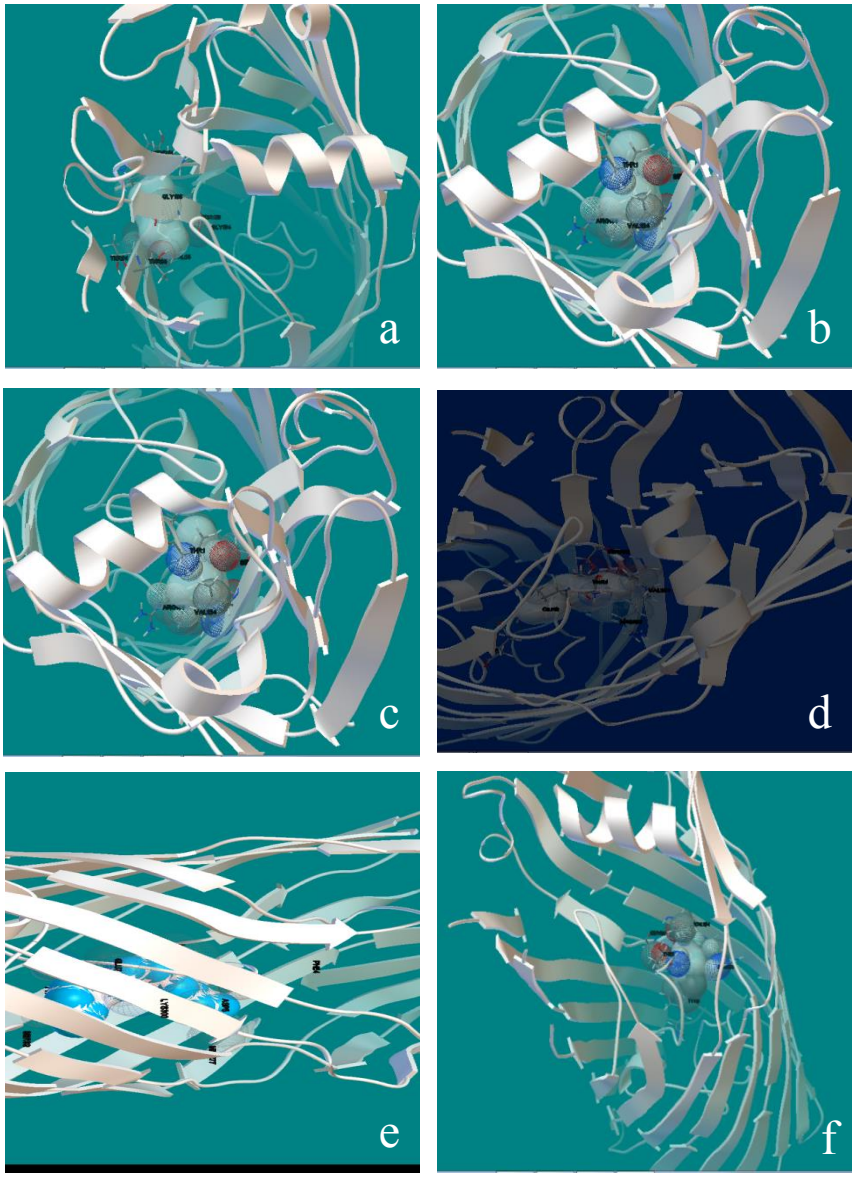

Fig 7.1: represents interaction of aliphatic hydrocarbons to TodX and different amino acids involved a) Butane (Tyrosine-9, Threonine-1, Isoleucine-131, Phenylalanine-100, Serine-102, Arginine-136, Valine 134) b) Hexane c) Heptane (Tyrosine-9, Threonine-1, Phenylalanine-100, Serine-102, Arg-136, Valine-134) d) Decane (Tyrosine-9, Glutamine-7, Glutamic acid- 2, Threonine-1, Phenylalanine-100, Serine-102, Arginine-136, Valine-134) e) Tetradecane f) Octadecane (Glycine-8, Tyr-9, Glutamine7, Aspartic acid-5, Glutamine-2, Threonine-1, Isoleucine-31, Arginine-14, Serine-104, Arginine-136, Methionine-277, Lysine-300) .

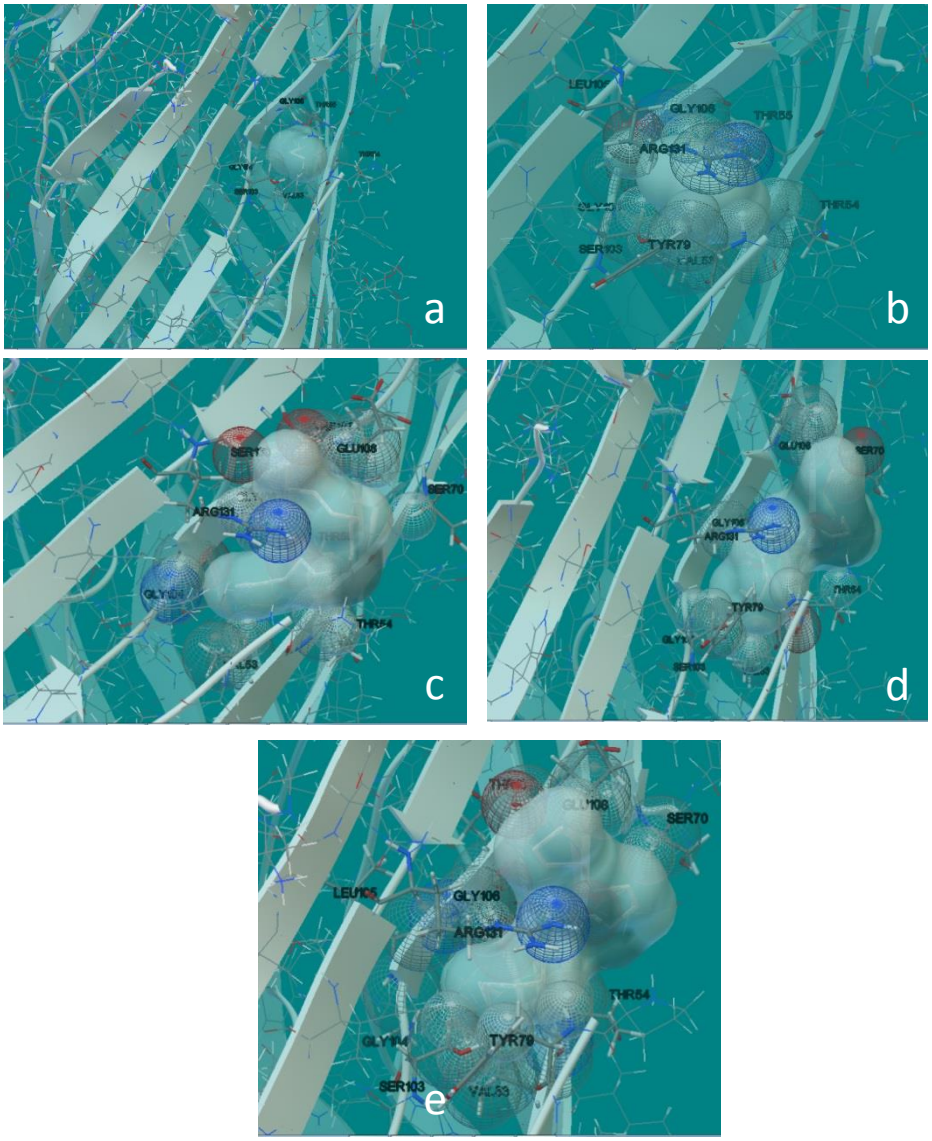

Fig 7.2: Represents the interaction of hydrocarbons to S3 kink (lateral opening) and the amino acids involved. a) Butane (Glycine-104, Glycine-106, Serine-103, Valine-53, Threonine-54, 55). b) Hexane (Leucine-105, Glycine-104, 106, Threonine-54, 55, Tyrosine-79, Valine-53, Serine-103, Arginine-131). c) Tetradecane (Glycine-104, 106, Threonine-107, Glutamic acid- 108, Valine-53, Threonine- 54, 55, Arginine-131, Serine-130, Serine-70). d) Hexadecane (Glycine-104, 106, Glutamic acid- 108, Valine-53, Threonine-54, 55, Serine-103, Serine-70, Tyrosine-79, Arginine-131). e) Octadecane (Leucine-105, Glycine-104, 106, Serine-103, Glutamic acid-108, Threonine-107, Valine-53, 54, Tyrosine-79, Threonine-55, Arginine-131)

Interaction of aromatic hydrocarbons with TodX:

Toluene and Benzene are the aromatic hydrocarbons used to study the interactions with TodX. TodX was reported to transport these Benzene and Toluene based on the crystal structure of the protein by Hearn et al., (2008). The authors have stated that the narrowest interior diameter of the barrel of the protein is about 4.5–5-Å and these two aromatic hydrocarbons can pass through the barrel. The amino acids present near to the narrowest channel are Gln-83, Phe-100, and Tyr-9. Benzene and Toluene found to interact near hatch domain as well as near S3kink. The amino acids interacting with Benzene and Toluene are listed in the figures 7.3 and 7.4.

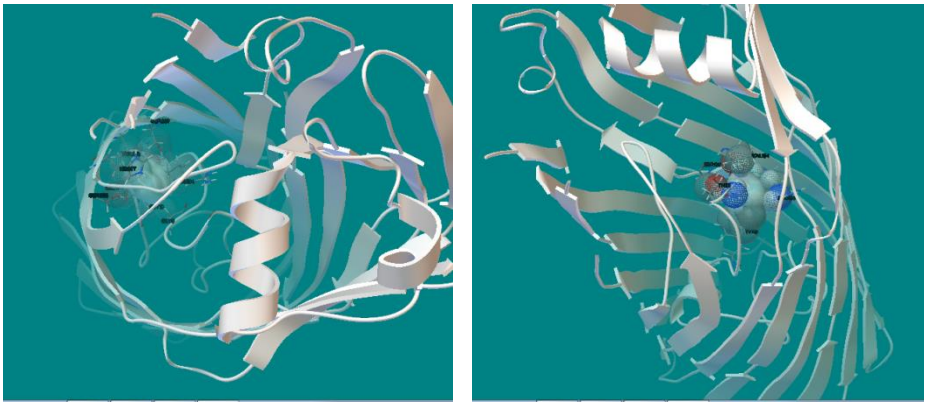

Fig 7.3: TodX interaction with Benzene and Toluene near hatch domainBenzene (Glycine-8, 17, 18, Arginine-14, Serine-355, Histidine-377,Threonine-379, Serine-397). Toluene (Threonine-1, Tyrosine-9, Phenyalanine-100, Serine-102, Valine-134, Arginine-136

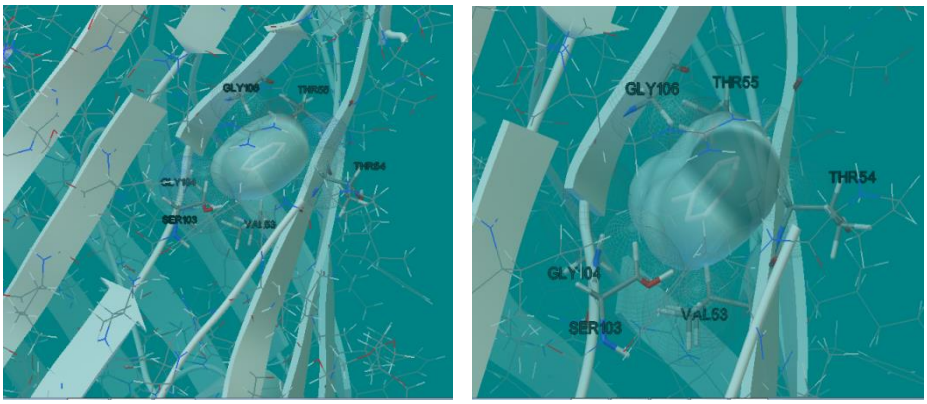

Fig 7.4: TodX interaction with Benzene and Toluene near S3 kink. Benzene(Valine-53, Threonine-53, 54, S Glycine-104, 106, Serine-103). Toluene(Valine-53, Threonine-54, 55, Serine-103, Glycine-104, 106)

Table 7a: Interaction of amino acids with different hydrocarbons near hatch domain and their presence in different hydrocarbon transport proteins (Multiple sequence alignment)								
Gly 8	benzene					Tetradecane	Octadecane	-
Tyr 9	-	Toluene	Butane	Heptane	Decane	Tetradecane	Octadecane	Conserved
Glutm 7	-	-	-	-	Decane	Tetradecane	Octadecane	Conserved
Glut 2	-	-	-	-	Decane	Tetradecane	Octadecane	Conserved
Thr 1	-	Toluene	Butane	Heptane	Decane	Tetradecane	Octadecane	Conserved
Arg 14	Benzene	-	-	-	-	-	Octadecane	Conserved
Phe 100	-	Toluene	Butane	Heptane	Decane	-	Octadecane	Conserved
Ser 102	-	Toluene	Butane	Heptane	Decane	-	Octadecane	todx, tbux
Arg 136	-	Toluene	Butane	Heptane	Decane	-	Octadecane	Conserved
Val 134	-	Toluene	Butane	Heptane	Decane	-	-	Conserved

Table 7b: Interaction of amino acids with different hydrocarbons near S3 Kink and their presence in different hydrocarbon transport proteins (Multiple sequence alignment -MSA)

Position in protein (strand)	Butane	Hexane	Tetradecane	Hexadecane	Octadecane	Toluene	MSA
S3	Glycine 106	Gly106	Gly106	Gly106	Gly106	Gly106	Conserved
S1	Threonine 54	Thr54	Thr54	Thr54	Thr54	Thr54	No
S1	Threonine 55	-	Thr55	Thr55	Thr55	Th55	Conserved (not in FadL)
S3	Glycine 104	Gly104	Gly104	Gly104	Gly104	Gly104	Conserved
S3	Serrine103	Ser103	Ser103	Ser103	Ser103	Ser103	Not
S3	Valine 53	Val 53	Val 53	Val 53	Val 53	Val 53	Conserved
S3		Leucine 105	-	-	Leu105		Conserved
S3		Glutamic Acid 108	Glu A 108	Glu A 108	Glu A 108		Conserved
S3		Threonine 107	Thr107	-	Thr107		Not
S1		Serine70	Ser70	Ser70	Ser70		Conserved
S1		Tyrosine 79	-	Tyr79	Tyr79		Conserved (not in FadL)
S4		Arginine 131	Arg131	Arg131	Arg131		-

7.3 Discussion

It was found from the results that hydrocarbon can interact with outer membrane protein TodX. The amino acids involved in the interactions are conserved in most of the outer membrane proteins reported for hydrocarbon transport (Table 7a, 7b). It was evident from the study of Hearn et al. (2008) that Glycine-83, Phenyalanine-100, and Tyrosine-9 are close to the narrowest point of the channel. From the data obtained from interaction of different hydrocarbon to interior of protein, the hydrocarbon bind near to hatch domain and Tyrosine-9 is one of the amino acids involved. The position of Tyrosine-9 is in N-terminus of the hatch domain and it was reported by Hearn et al. that 10–12 residue of N-terminus in the TodX and TbuX have higher B-factor relative to rest of the hatch domain indicating its flexibility. The amino acids involved in the interactions near to hatch domain are Glycine-8, Glutamine-7, Glutamicacid-2, Threonine-1, Arginine-14, Phenylalanine-100, Serine-102, Arginine-136, and Valine-134. Among these amino acids Glycine-8, Phenyalanine-100, Valine-134 are hydrophobic in nature. Glutamine-7, Threonine-1, Serine-102 are polar in nature and the rest are charged. The presence of combination of both hydrophobic and hydrophilic amino acids in the channel is required for movement of the hydrophobic substrate. If the passage is of only hydrophobic amino acids the hydrocarbon might get struck due to high binding energy. In some of the conformations both aliphatic and aromatic hydrocarbons are found to bind to S3 kink supporting the information given by Hearn et al., (2009) that the hydrocarbon may pass into membrane through lateral opening made by S3 kink. Tyrosine-9, Threonine-54, 55, Serine-70 from sheet 1 (S1) and Valine-53, Serine-103, Glycine-104, Leucine-105, Glycine-106, Threonine-107, Glutamic acid-108 from sheet 3 (S3) are found to interact to hydrocarbons. These groups of amino acids are also combination of hydrophobic and hydrophilic amino acids providing better substrate movement.

Though molecular interactions don't give complete information of the movement of substrate, it helps in understanding the substrate interaction with the transport protein. As the earlier studies concluded the transport models based on the binding of detergent molecules in the crystal structure of protein. The sites of interaction of hydrocarbon and the reported detergent molecules are different, but the position of S3 kink binding site is same. It was evident from the substrate transport assay performed by Hear et al., (2009) that the mutated

transport protein from *P.aeruginosa* having no lateral opening failed to transport the substrate.

It has been proposed the hydrophobic cleft present in the extracellular loop L3 will first bind to substrate based on the detergent C_8E_4 position in the crystal structure (Hearn et al., 2008). The amino acids from loop L3 which are in close proximity to C8E4 were also been reported (Serine-171, Valine-173, Threonine-177, Leucine-182, Valine-187, Alanine-191, Valine-194, Alanine-199). But any of these amino acids did not interact with the hydrocarbons using Autodock4, this might due to the difference in molecular characteristics of hydrocarbons and detergent C_8E_4. By this information and the molecular interactions studies, it was clear that the hydrocarbon will come into hydrophobic protein barrel and further move into the membrane through lateral opening and diffuse into the cytoplasm.

7.4 Conclusion

The molecular interaction studies using AUTODOCK gives insight into the binding sites of hydrocarbons to Outer membrane protein TodX. The data supports the transport model proposed by Hearn et al., (2008). So, the hydrocarbons might go into the interior of the barrel and there might be a conformational change in hatch domain allowing the hydrocarbon to pass through lateral opening and finally into the cytoplasm.

Conclusion and summary

Hydrocarbon degrading bacteria have been successfully isolated from crude oil sediment of Velsao beach, Goa. It was found that *Pseudomonas aeruginosa* and *Escherichia fergusonii* are the two bacteria present in the enriched consortium and was able to degrade alkanes extensively compared to aromatic hydrocarbons. It was found that the consortium could degrade alkanes ranging from C_{12} to C_{42} by 98 to 60% respectively in 45 days. No considerable degradation of alkylated aromatic hydrocarbons has been observed while unalkylated were degraded. The degradation of aromatic hydrocarbon was decreased with increase in ring number. As bioavailability is one of the major constraint of bioremediation of hydrocarbons, Hydroxy Cucurbit[6]uril and Rhmanolipid were used to increase the bioavailability. Hydroxy Cucurbit[6]uril was used to increase the degradation of aliphatic hydrocarbons but not aromatic hydrocarbons as the internal cavity volume might not accommodate aromatic ring. It was found that degradation of tetradecane (14%), hexadecane (10%) are increased in presence of Hydroxy Cucurbit[6]uril when used as sole carbon sources individually. When used as a mixture tetradecane, hexadecane and octadecane were degraded more (13%, 15%, 8% respectively) in presence of Hydroxy Cucurbit[6]uril in halophilic medium (3% NaCl). In the medium without NaCl Hydroxy Cucurbit[6]uril did not show any effect on hydrocarbon degradation. This might be because the solubility of hydroxy Cucurbit[6]uril is more in presence of NaCl. It was found that Rhamnolipid can increase the degradation of both aliphatic and aromatic hydrocarbons (Non aqueous phase liquid). The percentage of degradation was also found to be more compared to Hydroxy Cucurbit[6]uril but it found was that *Escherichia fergusonii* (AEBBITS 2) was unable to survive in presence of rhamnolipid producing bacteria. Alkane hydroxylase and Naphthalene dioxygenase are found to be present in AEBBITS 1 but not in AEBBITS 2. So AEBBITS 1 was used as donor in horizontal gene transfer experiments but found to have no effect on the indigenous bacteria of sea when contaminated with diesel oil. The seawater from Velsao beach was found to have very less amount of nitrogen and phosphorus. It was also found that rather than relying on horizontal gene transfer of catabolic genes biostimulation is a better option to support AEBBITS 1 during bioaugmentation in sea water. Finally outer membrane protein TodX of *P. putida* found to interact with both aliphatic hydrocarbons and aromatic hydrocarbon supporting the studies done by Hearn et al., (2008, 2009). The hydrocarbons may enter the

channel of beta barrel protein and pass into the cell memebrane through lateral opening. The movement through lateral opening will be facilitated by conformational changes in hath domain present inside the channel of the protein. By understanding that the isolated bacteria could degrade hydrocarbon and role of Hydroxy CB[6], Rhmnolipid, Nitrogen and Phosphorus in biodegradation of hydrocarbons. The combination of these different elements could provide a better solution for oil contamination in Goan seawater.

Future Prospects

- Field scale application of *Pseudomonas aeruginosa* (AEBBITS 1)

- Detailed study of hydrocarbon transport by OMP by Molecular Dynamic simulation

References:

Pashin, YV., Bakhitora, LM., 1979. Mutagenic and carcinogenic properties of polycyclic aromatic hydrocarbons. Environ. Health Perspect. 30, 185-189.

Masiol, M., Hofer, A., Squizzato, S., Piazza, R., Rampazza, G., Pavoni, B., 2012. Carcinogenic and mutagenic risk associated to air born particle-phase polycyclic aromatic hydrocarbons: A source apportionment. Atmospheric Environment. 60, 375-382.

Dasgupta, D., Ghosh, R., Sengupta, T.K., 2013. Biofilm-Mediated Enhanced Crude Oil Degradation by Newly Isolated Pseudomonas Species. ISRN Biotechnology 2013, 13.

D'Adamo, R., Pelosi, S., Trotta, P., Sansone, G., 1997. Bioaccumilation and biomagnification of polyaromatic hydrocarbons in aquatic organisms. Marine Chemistry. 56, 45-49.

Dhargalkar, VK., Kureishy, TW., Bhandare, MV., 1997. Deposition of tarballs (oil residues) on beaches along the west coast of India. Bulletin of the National Institute of Oceanography, Mahasagar, 103-108.

Salako, A., Sholeye, O., Ayankoya, S., 2012. Oil spills and community health: Implications for resource limited settings. J. Toxicol. Environ. Health. Sci. 4, 145-150.

Valdi, M., Bioremediation. An overview, 2001. Pure. Appl. Chem. 73, 1163-1172.

Saadoun, I., 2002. Isolation and characterization of bacteria from crude petroleum oil contaminated soil and their potential to degrade diesel fuel. J. Basic. Microbiol. 42, 422- 430.

Head, IM., Jones, DM., Roling, WFM., 2006. Marine microorganisms make a meal of oil. Nature Reviews Microbiology. 4, 173-182.

Halvorson, HO., Pramer, D., Rogul, M., 1985. Engineered organisms in the environment: Scientific issues. American Society of Microbiology, Washington DC, 239.

Maier, RM., 2000. Bioavailability and Its Importance to Bioremediation. Bioremediation. Springer Netherlands, 59-78.

Peters, KE., Moldowan, JM., 1993. The Biomarker Guide: Interpreting Molecular Fossils in Petroleum and Ancient Sediments. Prentice Hall, Englewood Cliffs, NJ, 363.

Harayama, S., Krishna, H., Kasai, Y., Shutsubo, K., 1999. Petroleum Biodegradation in Marine Environment. J. Mol. Microbiol. Biotechnol. 1, 63-70.

Kvenvolden, KA., Cooper, CK., 2003. Natural seepage of crude oil into the marine environment. Geo-Marine Letters. 23, 140-146.

Aguilera, F., Mendez, J., Pasaro, E., Laffon, B., 2010. Review on the effects of exposure to spilled oils on human health. J. Appl. Toxicol. 30, 291-301.

Ektin, DS., 1999. Estimating Cleanup Costs for Oil Spills. International Oil Spill Conference Proceedings, 1, 35-39.

Das, N., Chandram, P., 2011. Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview. Biotechnol. Res. Int. 1-13.

Kontovas, CA., Psaraftis, HN., Ventikos, NP., 2010. An empirical analysis of IOPCF oil spill cost data. Mar. Pol. Bul. 60, 1455-1466.

Teal, JM., Howarth, RW., 1984. Oil spill studies: A review of ecological effects. Environmental Management. 8, 27-43.

Piatt, JF., Lensink, CJ., Butler, W., Kendziorek, M., Nysewander, DR., 1990. Immediate Impact of the ‘Exxon Valdez’ Oil Spill on Marine Birds. The Auk. 107, 387-397.

Onwurah, INE., Ogugua, VN., Onyike, NB., Ochonogor, AE., Otitoju, OF., 2007. Crude Oil Spill in the Environment, Effects and Some Innovative Clean-up Biotechnologies. Int. J. Environ. Res. 1, 307-320.

Bamforth, SM., Singleton, L., 2005. Bioremediation of polycyclic aromatic hydroocarbons: current knowledge and future directions. 80, 723-736.

Harvey, RG., 1996. Mechanisms of carcinogenesis of polycyclic aromatic hydrocarbons . Polycyclic Aromatic Compounds. 9, 1-25.

Juhasz, AL., Naidu, R., 2000. Bioremediation of high molecular weight poly aromatic hydrocarbons: a review of the microbial degradation of Benzo[a]Pyrene. Int. Bioterior. Biodegrad. 45, 57-88.

Fingas, M., Fieldhouse, B., 2011. Review of solidifiers. Oil spill Sci. Technol. 713-733.

Vergetis, E., 2002. Oil pollution in Greek sea and spill confrontation means-methods, National Technical University of Athens, Greece.

Larson, H., 2010. Responding to oil spill disasters, the regulations that govern their response.

Hammoud, AH., 2001. Enhanced oil spill recovery rate using the weir skimmer.

Jensen, H., McClimans, TA., Johannessen, BO., 1995. Evaluation of weir skimmers without testing, Eighteenth AMOP, Technical Seminar proceedings, Edmonton, Alberta, Canada, 689-704.

Ventikos, NP., Vergetis, E., Psaraftis, HN., Triantafyllou, G., 2004. A high level synthesis of oil spill response equipment and countermeasures. J. Har. Mat. 107, 1-58.

Adebajo, MO., Frost, RL., Kloprogge, JT., Casmody, O., Kokot, S., 2003. Porous Materials for oil spill cleanup: A review of synthesis and absorbing properties. J. Porous. Mater. 10, 159-170.

Karakasi, OK., Moutsatsou, A., 2010. Surface modification of high calcium fly ash for its application in oil spill cleanup. Fuel. 89, 3966-3970.

Holakoo, L., 2001. On the capability of Rhamnolipids for oil spill control of surface water.

NRC., 1989. Using oil spill dispersants on the sea. National Research Council, National Academy Press. Washington, DC. ISBN-13, 978-0-309-09045-2.

Fingas, MF., Kyle, DA., Larouche, N., Filedhouse, B., Sregy, G., Stoodley, G., 1995. Effectiveness Testing of Oil spill-treating Agents, ASTM Special Technical Publications. 1252, 286-298.

Dave, D., Ghaly, AE., 2011. Remediation Technologies for Marine Oil Spills: A Critical Review and Comparative Analysis. American Journal of Environmental Sciences. 7, 423-440.

Hassanshahian, M., Capello, S., 2013. Crude Oil Biodegradation in the Marine Environments. Biodegradation Engineering and Technology. 101-127.

Kostka, JE., Prakash, O., Overholt, WA., Green, SJ., Freyer, G., Canion, A., Delgardio, J., Norton, N., Hazen, TC., Huettel, M., 2011. Hydrocarbon-Degrading Bacteria and the Bacterial Community Response in Gulf of Mexico Beach Sands Impacted by the Deepwater Horizon Oil Spill. Appl. Environ. Microb. 77, 7962-7974.

Zhang, Z., Hou, Z., Yang, C., Ma, C., Tao, F., Xu, P., 2011. Degradation of n-alkanes and polycyclic aromatic hydrocarbons in petroleum by a newly isolated *Pseudomonas aeruginosa* DQ8. Bioresource Technol. 102, 4111-4116.

Rojo, F., 2009. Degradation of alkanes by bacteria. Environmental Microbiology. 11, 2477-2490.

Fritsche, W., Hofrichter, M., 2000. Aerobic degradation by microorganisms. In: Klein J, editor. Environmental Processes- Soil Decontamination. Weinhein, Germany: Wiley- VCH, 146- 155.

Mrozik, A., Piotrowska-Seget, Z., Labuzek, S., 2003. Bacterial Degradation and Bioremediation of Polycyclic Aromatic Hydrocarbons. Pol. J. Environ. Stud. 12, 15-25.

Seo, JS., Keom, YS., Li, QX., 2009. Bacterial degradation of aromatic compounds. Int. J. Environ. Res. Public Health. 6, 278-309.

Sugiura, K., Ishihara, M., Shimauchi, T., Harayama, S., 1997. Physiochemical Properties and Biodegradability of Crude Oil. Environ. Sci. Technol. 31, 45-51.

Mukred, AM., Hamid, AA., Hamzad, A., Yusoff, WMW., 2008. Development of three bacteria consortium for the bioremediation of crude oil petroleum oil in contaminated water. J. Bio. Sci. 8, 73-79.

Atlas, RM., 1981. Microbial Degradation of Petroleum Hydrocarbons: an Environmental Perspective. 45, 180-209.

Atlas, RM., Bartha, R., 1973. Abundance, distribution and oil biodegradation potential of microorganisms in Rartian Bay. Environ. Pollut. 4, 291-300.

Jannasch, HW., Wirsen, CO., 1984. Variability of pressure adaptation in deep sea bacteria. Arch. Microbiol. 139, 281-288.

Lauro, FM., McDougald, D., Thomas, T., Williams, TJ., Egan, S., Rice, S., DeMaere, MZ., Ting, L., Ertan, H., Johnson, J., Ferriera, S., Lapidus, A., Anderson, I., Kyrpides, N., Munk,

AC., Detter, C., Hang, CS., Brown, MV., Robb, FT., Kjelleberga, S., Cavicchiol, R., 2009. The genomic basis of trophic strategy in marine bacteria. PNAS. 106, 15527– 15533.

Rahman, KSM., Rahman, JT., Lakshmanaperumalsamy, P., Banat, IM., 2002. Towards efficient crude oil degradation by a mixed bacterial consortium. Bioresource Technol. 85, 257-261.

Rainbow, PS., 1995. Bio monitoring of heavy metal availability in the marine environment. Mar. Poll. Bull. 31, 183–192.

Maneerat, S., Phetrong, K., 2007. Isolation of biosurfactant-producing marine bacteria and characteristics of selected biosurfactant. Songklanakarin J. Sci. Technol. 29,781–791.

Sakalle, K., Rajkumar S., 2009. Isolation of crude oil degrading marine bacteria and assessment for biosurfactant production. The Internet J. Microbiol. 7(2). doi:10.5580/1d0e.

Piskorska, M., Smith, G., Weil, E., 2007. Bacteria associated with the coral Echinopora lamellosa (Esper 1795) in the Indian Ocean—Zanzibar Region. Afr. J. Environ. Sci. Technol. 1, 93–98.

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

Brusseau, ML., 1998. The impact of physical, chemical and biological factors on biodegradation: implications for *in situ* bioremediation. In: *Biotechnology for soil remediation. Scientific bases and practical applications.* R. Serra. C.I.P.A. S.r.l. Milan, Italy, 81-98.

Tiwari, G., Tiwari, R., Rai, AK., 2010. Cyclodextrins in delivery systems: Applications. J. Pharm. Bioallied. Sci. 2, 72-79.

Valle, MD., 2004. Cyclodextrins and their uses: a review. Process Biochem. 39, 1033-1046.

Freeman, WA., Mock, WL., 1981. Cucurbituril. J. Am. Chem. Soc. 103, 7367-7368.

Kim, J., Jung, IS., Kim, SY., Lee, E., Kang, JK., Sakamoto, S., Yamaguchi, K., Kim, K., 2000. New cucurbituril homologs: Synthesis, isolation, characterization, and X-ray crystal structures of cucurbit[n]uril (n=5,7 and 8). J. Am. Chem. Soc. 122, 540-541.

Marquez, C., Hudgins, RR., Nau, WM., 2004. Mechanism of Host-Guest Complexation of Cucurbituril. J. Am. Chem. Soc. 126, 5806-5816.

McInnes, FJ., Anthony, NG., Kennedy, AR., Wheate, NJ., 2010. Solid state stabilization of the orally delivered drugs atenolol, glibenclamide, memantine and paracetmol through their complexation with cucubit[7]uril. Org. Biomol. Chem. 8, 765-773.

Wheate, NJ., Buck, DP., Day, AI., Collis, JG., 2006. Cucurbit[n]uril binding of platinum anticancer complexes. Dalton Trans. 451-458.

Wang, R., Bardelang, D., Waite, M., Udachin, KA., Leek, DM., Yu, K., Ratcliffe, CI., Ripmeester, JA., 2009. Inclusion complexes of coumarin in cucurbiturils. Org. Biomol. Chem. 7, 2435-2439.

Parkinson, M., 1985. Biosurfactants. Biotechnol. Adv. 3, 65-83.

Ron, EZ., Rosenberg, E., 2002. Biosurfactants and oil bioremediation. Curr. Opin. Biotechnol. 13, 249-252.

Taylor, WH., Juni, E., 1961. Pathways for biosynthesis of a bacterial capsular polysaccharide. I. Characterization of the organism and polysaccharide. J. Bacteriol. 81, 688-693.

Novan, VS., Zosim, Z., Gottlieb, A., Legmann, R., Carmeli, S., Ron, EZ., Rosenberg, E., 1995. Alasan, a new bioemulsifier from *Acinetobacter radioresistens*. Appl. Environ. Microbiol. 61, 3240-3244.

Manresa, MA., Bastida, J., Macrada, ME., Robert, M., De Andres, C., Guinera, J., 1991. Kinetic studies on surfactant production by *Pseudomonas aeruginosa.* J. Appl. Microbiol. 8, 133.

Sekhan Randhawa, KK., Rahman, PKSM., 2014. Rhamnolipid biosurfactants- past, present and future scenario of global market. Frontiers in Microbiology. 1, 1-7.

Toribio, J., Escalante, AE., Soberon-Chavez, G., 2010. Rhamnolipds: Production in bacteria other than Pseudomonas aeruginosa. Eur. J. Lipid. Sci. Technol. 112, 1082-1087.

Roony, AP., Price, NP., Ray, KJ., Kuo, TM., 2009. Isolation and characterization of rhamnolipids-producing bacterial strains from a biodiesel facility. FEMS Microbiol. Lett. 295, 82-87.

Guo-lang, Z., Tye-ting, W., Xin-ping, Q., Qin, M., 2005. Biodegradation of crude oil by Pseudomonas aeruginosa in the presence of rhamnolipids. J. Zheijiang Univ. Sci. 6B(8), 725-730.

Abalos, A., Vinas, M., Sabate, J., Manresa, MA., Solanos, AM., 2004. Enhanced biodegradation of Casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by Pseudomonas aeruginosa AT10. Biodegradation. 15, 249-260.

Zhang, Y., Maier, WJ., Miller, RM., 1997. Effect of Rhamnolipids on the Dissolution, Bioavailability, Biodegradation of Phenanthrene. Environ. Sci. Technol. 31, 2211-2217.

Mata-Sandoval, JC., Karns, J., Torrents, A., 2001. Influence of rhamnolipids and Triton X-100 on the biodegradation of three pesticides in aqueous phase and soil slurries. J. Agric. Food Chem. 49, 3296.

Hassanshahian, M., Cappello, S., 2003. Crude oil Biodegradation in Marine Environment. Biodegradation Engineering-Technology. 101-135(chapter 5).

Tackeuchi, K., Fujioka, Y., Kawasaki, Y., Shirayama, Y., 1997. Impacts of high concentrations of CO_2 ocean sequestration. Energy Convers. Mgmt. 38, S337-S341.

Lebaron, P., Servais, P., Trousellier, M., 1999. Changes in bacterial community structure in seawater mecocosms differing in their nutrient status. Aquatic Microbial Ecology. 19, 255-267.

Leahy, JG., Colwell, RR., 1990. Microbial Degradation of Hydrocarbons in the Environment. Microbiological Reviews. 54, 305-315.

Tschape, H., 1994. The spread of plasmids as a function of bacterial adaptability. FEMS Microbiol. Ecol. 15, 23-32.

Mazel, D., Davies, J., 1999. Antibiotic resistance in microbes. Cell. Mol. Life Sci. 56, 742-754.

Syvanen, M., 1994. HORIZONTAL GENE TRANSFER: Evidence and possible consequences. Annual. Rev. Gennet. 28, 237-261.

Tsuda, M., Tan, HM., Nishi, A., Furukawa, K., 1999. Mobile catabolic genes in bacteria. J. Biosci. Bioeng. 87, 401-410.

Top, EM., Springael, D., Boon, N., 2002. Catabolic mobile genetic elements and their potential use in bioaugmentation of polluted soils and waters. FEMS. Microbiol. Ecol. 42, 199-208.

Van Elsas JDJC, Fry P, Hirsch, Molin. 2000. Ecology of plasmid transfer and spread. In: ThomasCM, eds. The horizontal gene pool; bacterial plasmids and gene spread. Amsterdam, The Netherlands: Harwood Academic Publishers.

Stuart-Keil, KG., Hohnstock, AM., Drees, KP., Herrick, JB., Madsen, EL., 1998. Plasmids Responsible for Horizontal Transfer of Naphthalene Catabolism Genes between Bacteria at a Coal Tar-Contaminated Site Are Homologous to pDTG1 from *Pseudomonas putida* NCIB 9816-4. Appl. Environ. Microbiol. 64, 3633-3640.

Lieberman, RL., and Rosenzweig, AC., 2004. Biological methane oxidation: regulation, biochemistry, and active site structure of particulate methane monooxygenase. *Crit. Rev. Biochem. Mol. Biol.* 39**,** 147–164.

Dubbels, BL., Sayavedra-Soto, LA., and Arp, DJ., 2007. Butane monooxygenase of '*Pseudomonas butanovora*': purification and biochemical characterization of a terminalalkane hydroxylating diiron monooxygenase. *Microbiology* .153, 1808–1816.

Kotani, T., Yamamoto, T., Yurimoto, H., Sakai, Y., and Kato, N., 2003. Propane monooxygenase and NAD+-dependent secondary alcohol dehydrogenase in propane metabolism by *Gordonia* sp. strain TY-5. *J. Bacteriol.* 185, 7120–7128.

Kotani, T., Kawashima, Y., Yurimoto, H., Kato, N., and Sakai, Y., 2006. Gene structure and regulation of alkane monooxygenases in propane-utilizing *Mycobacterium* sp. TY-6 and *Pseudonocardia* sp. TY-7. *J. Biosci. Bioeng.* 102, 184–192.

Kotani, T., Yurimoto, H., Kato, N., and Sakai, Y., 2007. Novel acetone metabolism in a propane-utilizing bacterium, *Gordonia* sp. strain TY-5. *J. Bacteriol.* 189, 886–893.

van Beilen, JB., Smits, TH., Roos, FF., Brunner, T., Balada, SB., Rothlisberger, M., and Witholt, B.. 2005. Identification of an amino acid position that determines the substrate range of integral membrane alkane hydroxylases. *J. Bacteriol.* 187, 85–91.

Maeng, JH., Sakai, Y., Tani, Y., Kato, N., 1996. Isolation and Characterization of a Novel Oxygenase That Catalyzes the First Step of n-Alkane Oxidation in Acinetobacter sp. Strain M-1. J. Bacteriol. 178, 3695-3700.

Van Beilen, JB., Veenhoff, L., Witholt, B., 1998. Alkane hydroxylase systems in Pseudomonas aeruginosa strain able to grow on n-octane. New Frontiers in screening for microbial biocatalysts. 211-215.

Marin, MM., Smits, THM., Van Beilen, JB., Rojo, F., 2001. The Alkane Hydroxylase Gene of *Burkholderia cepia* RR10 is under catabolite repression control. J. Bacteriol. 183, 4202-4209.

Jurelevicius, D., Alvarez, VM., Peixoto, R., Rosado, AS., Seldin, L., 2013. The Use of a Combination of alkB Primers to Better Characterize the Distribution of Alkane-Degrading Bacteria. PLoS ONE. 8, e66565.

Smits, THM., Ro¨thlisberger, M., Witholt, B., van Beilen, J. B.. 1999. Molecular screening for alkane hydroxylase genes in gram–negative and gram–positive strains. Environ. Microbiol. 1, 307–317.

Glebler, J., Wick, LY., Schloter, M., Harms, H., Chatzinotas, A., 2013. Evaluating the Assignment of alkB Terminal Restriction Fragments and Sequence Types to Distinct Bacterial Taxa. Appl. Environ. Microbiol. 79, 3129-3132.

Parales, RE., Lee, K., Resnick, SM., Jiang, H., Lessner, DJ., Gibson, DT., 2000. Substrate specificity of naphthalene dioxygenase: effect of specific aminoacids at the active site of the enzyme. J. Bacteriol. 132, 1641-1649.

Wasmud, K., Burns, KA., Kurtboke, DI., Bourne, DG., 2009. Novel Alkane Hydroxylase Gene (*alk*B) Diversity in sediments Associated with Hydrocarbon Seeps in the Timor Sea, Australia. Appl. Environ. Microbiol. 75, 7391-7398.

Nie. Y., Chi, CQ., Fang, H., Liang, JL., Lu, SL., Lai, GL., 2014. Diverse alkane hydroxylase genes in microorganisms and environments. Sci. Rep. 4, 4968–4978.

Dinamarca, MA., Aranda-Olmedo, I., Puyet, A., Rojo, F., 2003. Expression of the Pseudomonas putida OCT Plasmid Alkane Degradation Pathway Is Modulated by Two Different Global Control Signals: Evidence from Continuous Cultures. 185, 4772-4778.

Gibson, DT., Parales, RE., 2000. Aromatic hydrocarbon dioxygenase in environmental biotechnology. Curr. Opin. Biotechnol. 11, 236-243.

Kauppi, B., Lee, K., Carrdano, E., Parales, RE., Gibson, DT., Eklund, H., Ramaswamy, S., 1998. Structure of an aromatic ring hydroxylating dioxygenase-naphthalene 1,2-dioxygenase. Structure. 6, 571-586.

Coulon, F., McKew, BA., Osborn, AM., McGenity, JJ., Timmis, KN., 2006. Effect of Temperature and Biostimualtion of Oil-Degrading Microbial Communities in Temperate Estuarine Waters. Environ. Microbiol. 9, 177-186.

Hoeppel, RE., Hinchee, RE., 1994. Enhanced biodegradation for on-site remediation of contaminated soils and ground water. Marcel Dekker Inc. New York.

Goswami, P., Singh, HD., 1991. Different modes of hydrocarbon uptake by two *Pseudomonas* species. Biotechnol. Bioeng. 37, 1-11.

Wiener, MC., Horanyi, PS., 2011. How hydrophobic molecules traverse the outer membranes of Gram-negative bacteria. PNAS. 108, 10929-10930.

Whitman, BE., Leuking, DR., Mihelcic, JR., 1998. Naphthalene uptake by a *Pseudomonas fluorescens* isolate. Can. J. Microbiol. 44, 1086-1093.

Kim, IS., Foght, JM., Gray, MR., 2002. Selective transport and accumulation of alkanes by *Rhodococcus erythropolis* S+14He. Biotechnol. Bioeng. 80, 650-659.

Verdin, A., Sahraoui, ALH., 2005. Newsam, R., Robinson, G., Durand, R., 2005. Polycyclic aromatic storage by *Fusarium solani* in intracellular lipid vesicles. Environ. Pollut. 133, 283-291.

Bateman, JN., Speer, B., Feduik, L., Hartline, RA., 1986. Naphthalene association and uptake in *Pseudomonas putida*. J. Bacteriol. 166, 155-161.

Kallimanis, A., Frillingos, S., Drainas, C., Koukou, AL., 2007. Taxonomic identification, phenanthrene uptake activity, and membrane lipid alterations of the PAH degrading Arthrobacter sp. Strain Sphe3. Appl. Microbiol. Biotechnol. 76, 709-717.

Van den Berg, B., Black, PN., Clemons, WM., Rapport TA., 2004. Crystal structure of the long-chain fatty acid transporter FadL. Science. 304, 1506-1509.

Hearn, EM., Patel, DR., van den Berg, B., 2008. Outer-membrane transport of aromatic hydrocarbons as a first step in biodegradation. PNAS. 165, 8601-8606.

Hearn EM., Patel, DR., Lepore, BW., Indic, M., van den Berg, B., 2009. Transmemebrane passage of hydrophobic compounds through a protein channel wall. Nature. 458, 367-370.

Chauhan, A., Fazlurrahman, Oakeshott, J., Jain, R., 2008. Bacterial metabolism of polycyclic aromatic hydrocarbons: strategies for bioremediation. Indian J Microbiol 48, 95-113.

Dasgupta, D., Ghosh, R., Sengupta, T.K., 2013. Biofilm-Mediated Enhanced Crude Oil Degr

adation by Newly Isolated Pseudomonas Species. ISRN Biotechnology 2013, 13.

Dua, M., Singh, A., Sethunathan, N., Johri, A., 2002. Biotechnology and bioremediation: suc cesses and limitations. Appl Microbiol Biotechnol 59, 143-152.

Golovlev, E.V., 2001. Enrichment culture has long been the method of choice for isolating ba cteria expressing specific phenotypes Microbiol 70, 379-383.

Guerlava, P., Izac, V., Tholozan, J.-L., 1998. Comparison of Different Methods of Cell Lysis and Protein Measurements in Clostridium perfringens: Application to the Cell Volume Deter mination. Curr Microbiol 36, 131-135.

Harayama, S., Kasai, Y., Hara, A., 2004. Microbial communities in oil-contaminated seawate r. Current Opinion in Biotechnology 15, 205-214.

Hasanuzzaman, M., Ueno, A., Ito, H., Ito, Y., Yamamoto, Y., Yumoto, I., Okuyama, H., 200 7. Degradation of long-chain n-alkanes (C36 and C40) by Pseudomonas aeruginosa strain Wa tG. International Biodeterioration & Biodegradation 59, 40-43.

Hidayat, A., Tachibana, S., 2012. Biodegradation of aliphatic hydrocarbons in three types of crude oil by Furasium sp.F092 under stress with artificial sea water. Journal of Environmenta l Science and Technology 5, 64-73.

Itah, A.Y., Essien, J.P., 2005. Growth Profile and Hydrocarbonoclastic Potential of Microorg anisms Isolated from Tarballs in the Bight of Bonny, Nigeria. World J Microbiol Biotechnol 21, 1317-1322.

Jimenez, N., Morris, B.E.L., Cai, M., Grundger, F., Yao, J., Richnow, H., H., Kruger, M., 20 12. Evidence for in situ methanogenic oil degradation in the Dagang oil field. Org. Geochem. 52, 44-54.

Kanaly, R., A., Harayama, S., 2000. Biodegradation of High-Molecular-Weight polycyclic ar omatic hydrocarbons by Bacteria. JOURNAL OF BACTERIOLOGY 182, 2059-2067.

Khan, Z., Troquet, J., Vachelard, C., 2005. Sample preparation and analytical techniques for determination of polyaromatic hydrocarbons in soil. Int. J. Environ. Sci. Tech. 2, 275-286.

Mackay, D., Shu, W.-Y., Ma, K.-C., 1992. Illustrated hand book of physical – chemical prop erties and environmental fate for organic chemicals. Boca Raton ; London : CRC Lewis.

Morozik, A., Seget, P., Labuzek, S., 2003. Bacterial degradation and Bioremediation of Polyc ylcic Aromatic Hydrocarbons. Pol.J.Environ.Stud 12, 15-25.

Mukherji, S., Jagadevan, S., Mohapatra, G., Vijay, A., 2004. Biodegradation of diesel oil by an Arabean Sea sediment culture isolated from the vicinity of an oil field. Bioresource Techn ology 95, 281-286.

Murray, A., E., Hollobaugh, J., T., Orrego, C., 1996. Phylogenetic composition of Bacteriopl anktons from two California estuaries compared by Denaturing gradient gel electrophoresis o f 16s rDNA fragments. Applied and Environmental Microbiology 62, 2676-2680.

Nikolausz, M., Kappelmeyer, U., Székely, A., Rusznyák, A., Márialigeti, K., Kästner, M., 20 08. Diurnal redox fluctuation and microbial activity in the rhizosphere of wetland plants. Eur opean Journal of Soil Biology 44, 324-333.

Ojo, O.A., 2006. Petroleum – hydrocarbon utilization by native bacterial population from a w aste water canal Southwest Nigeria. African Journal of Biotechnology 5, 333-337.

Prince, R.C., Elmendorf, D.L., Lute, J.R., Hsu, C.S., Haith, C.E., Senius, J.D., Dechert, G.J., Douglas, G.S., Butler, E.L., 1994. 17.alpha.(H)-21.beta.(H)-hopane as a conserved internal m arker for estimating the biodegradation of crude oil. Environmental Science & Technology 2 8, 142-145.

Rochelle, P., A., 2001. Environmental Molecular Microbiology: Protocols and Applications. Horizon Scientific Press, Wymondham, UK.

Rodrigues, j., Madhukar, A., Sangodkar, U.M.X., 2011. Isolation and characterization of a m arine bacterium belonging to the genus alkaligenes capable of the complete mineralization of the dibenzothiopene. Indian Journal of Marine Science 40, 391-397.

Seidman, C.E., Struhl, K., Sheen, J., Jessen, T., 2001. Introduction of Plasmid DNA into Cell s, Current Protocols in Molecular Biology. John Wiley & Sons, Inc.

Seifert, W.K., Michael Moldowan, J., 1979. The effect of biodegradation on steranes and terp anes in crude oils. Geochimica et Cosmochimica Acta 43, 111-126.

Sivadas, S., George, A., Ingole, B.S., 2008. How vulnerable is Indian coast to oil spills? Impa ct of MV ocean seraya oil spill. Current Science 95, 504-512.

Sivaraman, C., Ganguly, A., Mutnuri, S., 2010. Biodegradation of hydrocarbons in the presen ce of cyclodextrins. World J Microbiol Biotechnol 26, 227-232.

Sivaraman, C., Ganguly, A., Nikolausz, M., Mutnuri, S., 2011. Isolation of hydrocarbonoclas tic bacteria from bilge oil contaminated water. International Journal of Environmental Scienc e and Technology 8, 461-470.

Sriram, M.I., Gayathiri, S., Gnanaselvi, U., Jenifer, P.S., Mohan Raj, S., Gurunathan, S., 201 1. Novel lipopeptide biosurfactant produced by hydrocarbon degrading and heavy metal toler ant bacterium Escherichia fergusonii KLU01 as a potential tool for bioremediation. Bioresour ce Technology 102, 9291-9295.

Stucki, G., Alexander, M., 1987. Role of dissolution rate and solubility in biodegradation of a romatic compounds. Appl. Envirin. Microbial. 53, 292-297.

Székely, A., Sipos, R., Berta, B., Vajna, B., Hajdú, C., Márialigeti, K., 2009. DGGE and T-R FLP Analysis of Bacterial Succession during Mushroom Compost Production and Sequence-aided T-RFLP Profile of Mature Compost. Microb Ecol 57, 522-533.

Zakaria, M.P., Horinouchi, A., Tsutsumi, S., Takada, H., Tanabe, S., Ismail, A., 2000. Oil Pol lution in the Straits of Malacca, Malaysia: Application of Molecular Markers for Source Iden tification. Environmental Science & Technology 34, 1189-1196.

Bardelang, D., Udachin, K.A., Leek, D.M., Margeson, J.C., Chan, G., Ratcliffe, C.I., Ripmeester, J.A., 2011. Cucurbit[n]urils (n = 5–8): A Comprehensive Solid State Study. Crystal Growth & Design 11, 5598-5614.

Bardi, L., Mattei, A., Steffan, S., 2000. Hydrocarbon degradation by a soil microbial population with β- cyclodextrin as a surfactant to enhance bioavailability. Enzyme Microb. Technol. 7, 709-713.

Day, A., Arnold, A.P., Blanch, R.J., Snushall, B., 2001. Controlling Factors in the Synthesis of Cucurbituril and Its Homologues. The Journal of Organic Chemistry 66, 8094-8100.

Eirc, M., Xioaxi, L., Royman, J., Lawrence, K., Mensah., Xiayong, L., 2012. Cucurbituril chemistry: a tale of supramolecular success. R S C Advances. 2, 1213-1247.

Florea, M., Nau, W.M., 2011. Strong Binding of Hydrocarbons to Cucurbituril Probed by Fluorescent Dye Displacement: A Supramolecular Gas-Sensing Ensemble. Angew. Chem. Int. Ed. 50, 9338-9342.

Golovlev, E.V., 2001. Enrichment culture has long been the method of choice for isolating bacteria expressing specific phenotypes Microbiol 70, 379-383.

Guerlava, P., Izac, V., Tholozan, J.-L., 1998. Comparison of Different Methods of Cell Lysis and Protein Measurements in Clostridium perfringens: Application to the Cell Volume Determination. Curr Microbiol 36, 131-135.

Hidayat, A., Tachibana, S., 2012. Biodegradation of aliphatic hydrocarbons in three types of crude oil by Furasium sp.F092 under stress with artificial sea water. Journal of Environmental Science and Technology 5, 64-73.

Itah, A.Y., Essien, J.P., 2005. Growth Profile and Hydrocarbonoclastic Potential of Microorganisms Isolated from Tarballs in the Bight of Bonny, Nigeria. World J Microbiol Biotechnol 21, 1317-1322.

Jin Jeon, Y., Kim, S.-Y., Ho Ko, Y., Sakamoto, S., Yamaguchi, K., Kim, K., 2005. Novel molecular drug carrier: encapsulation of oxaliplatin in cucurbit[7]uril and its effects on stability and reactivity of the drug. Organic & Biomolecular Chemistry 3, 2122-2125.

Jon, S.Y., Selvapalam, N., Oh, D.H., Kang, J.-K., Kim, S.-Y., Jeon, Y.J., Lee, J.W., Kim, K., 2003. Facile Synthesis of Cucurbit[n]uril Derivatives via Direct Functionalization: Expanding Utilization of Cucurbit[n]uril. J. Am. Chem. Soc. 125, 10186-10187.

Khan, M.S.A., Heger, D., Necas, M., Sindelar, V., 2009. Remarkable salt effect on stability of supramolecular complex between modified Cucurbit[6]uril and Methylviologen in aqueous media. J. Phys. Chem. B. 113, 11054-11057.

Lewin, V., Rivollier, J., Coudert, S., Buisson, D.-A., Baumann, D., Rousseau, B., Legrand, F.-X., Kouřilová, H., Berthault, P., Dognon, J.-P., Heck, M.-P., Huber, G., 2013. Synthesis of Cucurbit[6]uril Derivatives and Insights into Their Solubility in Water. Eur. J. Org. Chem. 2013, 3857-3865.

Mock, W.L., Shih, N.Y., 1986. Structure and selectivity in host-guest complexes of cucurbituril. The Journal of Organic Chemistry 51, 4440-4446.

Mohnaty, S., Mukherji, S., 2011. Alteration in cell surface properties of Burkholderia spp. during surfactant aided biodegradation of petroleum hydrocarbons. Applied Microbial and Cell Physiology, 3703-3707.

Nikolausz, M., Kappelmeyer, U., Székely, A., Rusznyák, A., Márialigeti, K., Kästner, M., 2008. Diurnal redox fluctuation and microbial activity in the rhizosphere of wetland plants. European Journal of Soil Biology 44, 324-333.

Ojo, O.A., 2006. Petroleum – hydrocarbon utilization by native bacterial population from a waste water canal Southwest Nigeria. African Journal of Biotechnology 5, 333-337.

Pasumarthi, R., Chandrasekaran, S., Mutnuri, S., 2013. Biodegradation of crude oil by Pseudomonas aeruginosa and Escherichia fergusonii isolated from the Goan coast. Mar. Pollut. Bull. 76, 276-282.

Rochelle, P., A., 2001. Environmental Molecular Microbiology: Protocols and Applications. Horizon Scientific Press, Wymondham, UK.

Sivaraman, C., Ganguly, A., Mutnuri, S., 2010. Biodegradation of hydrocarbons in the presence of cyclodextrins. World J Microbiol Biotechnol 26, 227-232.

Székely, A., Sipos, R., Berta, B., Vajna, B., Hajdú, C., Márialigeti, K., 2009. DGGE and T-RFLP Analysis of Bacterial Succession during Mushroom Compost Production and Sequence-aided T-RFLP Profile of Mature Compost. Microb Ecol 57, 522-533.

Torsvik, V., Ovreas, L., Thrigstand, T.F., 2002. Procaryotic diversity - magnitude, dynamics and controlling factors. Science 296, 1064-1066.

Wick, L.Y., Munain, A.R., Spingael, D., Harms, H., 2002. Responses of mycrobacterium sp. LB50IT to the low bioavailability of solid anthracene. Appl Microbiol Biotechnol 58, 378-385.

Zhao, Z., Selvam, A., Wong, J., 2011. Effects of rhamnolipids on cell surface hydrophobicity of PAH degrading bacteria and biodegradation of phenanthrene. Bioresource Technol. 102, 3999-4007.

Abbasnezhad, H, Gray, MR., Foght, JM., 2008. Two different mechanisms for adhesion of Gram-negative bacterium, Pseudomonas fluorescens LP6a, to an oil–water interface. Colloids. Surf. B. Biointerfaces. 62, 36-41.

Al-Tahhan R, A., Sandrin T, R., Bodour A, A., Maier R, M., 2000. Rhamnolipid Induced Removal of Lipopolysaccharide from Pseudomonas aeruginosa. Appl. Envirin. Microbiol. 66, 3262-3268.

Amiriyan, A., Mazaheri Assadi, M., Saggadian, V., Noohi, A., 2004. Bioemulsan Production by Iranian Oil Reservoirs Microorganisms. Iranian Journal of Environmental Health Science & Engineering. 1, 28-35.

Bruheim, P., Bredholt, H., Eimhjellen, K., 1997. Bacterial degradation of emulsified crude oil and the effect of various surfactants. Can. J. Microbiol. 43, 17-22.

Cavalca, L., Roa, MA., Bernasconi, S., Colombo, M., Andreoni, V., Gianfreda, L., 2008. Biodegradation of phenanthrene and analysis of degrading cultures in the presence of a model organic mineral matrix and of a simulated NAPL phase. Biodegradation. 19, 1-13.

Chakraborty, S., Mukherji, S., 2010. Surface hydrophobicity of petroleum hydrocarbon degrading Burkholderia strains and their interactions with NAPLs and surfaces. Colloids. Surf. B. Biointerfaces. 78, 101-108.

Desai, JD., Banat, IM., 1997. Microbial production of surfactant and their commercial potential. Microbiol. Mol. Biol. Rev. 61, 47–64.

DuBois, M., Gilles, KA., Hamilton, JK., Rebers, PA, Smith, F., 1956. Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem. 28, 350-356.

Ghazali, FM., Rahman, RNZA., Salleh, AB., Basri, M., 2004. Biodegradation of hydrocarbons in soil by microbial consortium. International Biodeterioration & Biodegradation. 54, 61-67.

Grimberg, SJ., Nagel, J., Aitken, MD., 1995. Kinetics of Phenanthrene Dissolution into Water in the Presence of Nonionic Surfactants. Environmental Science & Technology 29:1480-1487 doi:10.1021.

Hua, Z., Chen, J., Lun, S., Wang, X., 2003. Influence of biosurfactants produced by Candida antarctica on surface properties of microorganism and biodegradation of n-alkanes. Water Res. 37, 4143-4150.

Kaczorek, E., Urbanowicz, M., Olszanowski, A., 2010. The influence of surfactants on cell surface properties of Aeromonas hydrophila during diesel oil biodegradation. Colloids. Surf. B. Biointerfaces. 81, 363-368.

Kim, YM., Ahn, CK., Woo, SH., Jung, GY., Park, JM., 2009. Synergic degradation of phenanthrene by consortia of newly isolated bacterial strains. J. Biotechnol. 144, 293-298.

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

Mohanty, G., Mukherji, S., 2007. Effect of an emulsifying surfactant on diesel degradation by cultures exhibiting inducible cell surface hydrophobicity. J. Chem. Technol. Biotechnol. 82, 1004-1011.

Mohanty, S., Jasmine, J., Mukherji, S., 2013. Practical Considerations and Challenges Involved in Surfactant Enhanced Bioremediation of Oil. BioMed. Research. International. 2013:16.

Mukherji, S., Peters, CA., Weber, WJ., 1997. Mass Transfer of Polynuclear Aromatic Hydrocarbons from Complex DNAPL Mixtures. Environmental Science & Technology. 31, 416-423.

Mukred, AM., Hamid, AA., Yusoff, WMW., 2008. Development of Three Bacteria Consortium for the Bioremediation of Crude Petroleum-oil in Contaminated Water. OnLine Journal of Biological Sciences. 8, 73-79.

Mulligan, CN., Yong, RN., Gibbs, BF., 2001. Heavy metal removal from sediments by biosurfactants. J. Hazard. Mater. 85, 111-125.

Nitschke, M., Pastore, GM., 2006. Production and properties of a surfactant obtained from Bacillus subtilis grown on cassava wastewater. Bioresource Technology. 97, 336-341.

Obayori, O., Ilori, M., Adebusoye, S., Oyetibo, G., Omotayo, A, Amund, O., 2009. Degradation of hydrocarbons and biosurfactant production by Pseudomonas sp. strain LP1. World J. Microbiol. Biotechnol. 25, 1615-1623.

Oberbremer, A., Müller-Hurtig, R., Wagner, F., 1990. Effect of the addition of microbial surfactants on hydrocarbon degradation in a soil population in a stirred reactor. Appl. Microbiol. Biotechnol. 32, 485-489.

Owsianiak, M., Szult, A., Chrzanowski, L., Cyplik, P., Bagacki, M., Olejnik-Schmidt, AK., 2009. Biodegradation and Surfactant mediated biodegradation of diesel fuel bi 218 microbial consortia are not corrected to cell surface hydrophobicity. Appl. Microbiol. Biotechnol. 84, 545-553.

Rosenberg, M., Gutnick, D., Rosenberg, E., .1980. Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. 9, 29-33.

Vanne Hamme, JD., Ward, OP., 2001. Physical and metabolic interactions of Pseudomonas sp. Strain JA5-B45 and Rhodococcus sp. Strain F9-D79 during growth on crude oil and effect of a chemical surfactant on them. Appl. Environ. Microbiol. 67, 4874-4879.

Volkering F., Breure, AM., Rulkens, WH., 1997. Microbiological aspects of surfactant use for biological soil remediation. Biodegradation. 8, 401-417.

Zhang, Y., Miller, RM., 1994. Effect of a Pseudomonas rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. Appl. Envirin. Microbial. 60, 2101-2106.

Zhang, Y., Miller,, RM., 1995. Effect of Rhamnolipid (Biosurfactant) Structure on solubilization and Biodegradation of n-alkanes. Appl. Envirin. Microbiol. 61, 2247-2251.

Zhao, Z., Selvam, A., Wong, JW-C., 2011. Effects of rhamnolipids on cell surface hydrophobicity of PAH degrading bacteria and the biodegradation of phenanthrene. Bioresource Technology. 102, 3999-4007.

Zita, A., Hermansson, M., 1997. Effect of bacterial cell surface structures and hydrophobicity on attachment to activated sludge flocs. Appl. Envirin. Microbial. 63, 1168-1170.

Herrick, JB., Stuart-Keil, KG., Ghiorse, WC., Modsen, EL., 1997. Natural Horizontal Transfer of a Naphthalene Dioxygenase Gene between Bacteria Native to a Coal Tar-Contaminated Field Site. Appl. Environ. Microbiol. 63, 2330-2337.

Stuart-Keil, KG., Hohnstock, AM., Drees, KP., Herrick, JB., Madsen, EL., 1998. Plasmids responsible for Horizontal Transfer of Naphthalene Catabolism Genes between Bacteria at a

Coal Tar-Contaminated Site are Homologous to pDTG1 from Pseudomonas putida NCIB 9816-4. Appl. Environ. Microbiol. 64, 3633-3640.

Hedlund, BP., Geiselbrecht, AD., Staley, JT., 2001. *Marinobacter* strain NCE 312 has a *Pseudomonas*-like naphthalene dioxygenase. FEMS. Microbiol. Letters. 201, 47-51.

Giebler, J., Wick, LY., Schloter, M., Harms, H., Chatzinotas, A., 2013. Evaluating the Assignment of *alk*B Terminal Restriction Fragment and Sequence Types to Distinct Bacterial Taxa. Appl. Environ. Microbiol. 79, 3129-3132.

Kurkela, S., Lehvaslaiho, H., Palva, ET., Teeri, TH., 1988. Cloning, nucleotide sequence and characterization of genes encoding naphthalene dioxygenase of *Pseudomonas putida* strain NCIB 9816. Gene. 73, 355-362.

Brezna, B., Khan, AA., Cerniglia, CE., 2003. Molecular characterization of dioxygenases from polycyclic aromatic hydrocarbon degrading *Mycobacterium* spp. FEMS, microbiology letters. 223, 177-183.

Krivobok, S., Kony, S., Meyer, C., Louwagie, M., Willison, JC., Jouanneau, Y., Identification of Pyrene-Induced Proteins in *Mycobacterium sp.* Strain 6PY1: Evidence for Two Ring-Hydroxylating Dioxygenases. J. Bacteriol. 185, 3828-3841.

Lloyd –Jones, G., Lau, PC., 1997. Glutithione s-transferase encoding gene as a potential probe for environmental bacterial isolates capable of degrading polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. 63, 3286-3290.

Vethamony, P., Sudheesh, K., Babu, MT., Jayakumar, S., Manimurali, R., Saran, AK., Sharma, LH., Rajan, B., Srivastava, M., 2007. Trajectory of an oil spill of Goa, eastern Arabian Sea: field observations and simulations. Environ. Pollut. 148, 438-444.

Rodrigues, J., Madhukar, A., Sangodkar, U.M.X., 2011. Isolation and characterization of a marine bacterium belonging to the genus Alkaligenes capable of the complete mineralization of the dibenzothiopene. Indian J. Mar. Sci. 40, 391–397.

Sivaraman, C., Ganguly, A., Nikolausz, M., Mutnuri, S., 2011. Isolation of hydrocarbanoclastic bacteria from bilge oil contaminated water. Int. J. Environ. Sci. Technol. 8, 461-470.

Pasumarthi, R., Chandrasekaran, S., Mutnuri, S., 2013. Biodegradation of crude oil by *Pseudomonas aeruginosa* and *Escherichia fergusonii* isolated from the Goan coast. Mar. Pollut. Bull. 76, 276-282.

Eaton AD., APHA., AWW., WEF., 2005. Standard Methods for the Examination of water and waste water, 21st ed. American Public Health Association, Washington, D.C.

Sivaraman, C., Ganguly, A., Mutnuri, S., 2010. Biodegradation of hydrocarbons in the presence of cyclodextrins. World J. Microbiol. Biotechnol. 26, 227-232.

Hassanshahian, M., Emitiazi, G., Caruso, G., Cappello, S., 2014. Bioremediation (bioaugmentation/biostimulation) trails of polluted seawater: a mecocosm simulation study. Mar. Environ. Res. 95, 28-38.

Prakash, D., Raushan, RK., Sangodkar, UMX., 2008. Isolation and characterization of meta-toluic acid degrading marine bacterium. Indian J. Mar. Sci. 37, 322-325.

Shankar, S., Kansrajh, C., Dinesh, MG., Satyam, RS., Kiruthika, S., Tharanipriya, A., 2014. Application of indigenous microbial consortia in bioremediation of oil-contaminated soils. Int. J. Environ. Sci. Technol. 11, 367-376.

Yakimov MM., Timmiski N., Golyshin PN., 2007. Obligate oil-degrading marine bacteria. Cuur. Opin. Biotechnol. 18, 257-266.

Rosenberg, E., Legmann, R., Kushmaro, A., Taube, R., Adler, E., 1992. Petroleum bioremediation- a multiphase problem. Biodegradation. 3, 337-350.

Braddock JF., Ruth ML., Catterall PH., Walworth JL., McCarthy CA., 1997. Enhancement and inhibition of microbial activity in hycrocarbon-contaminated Arctic soils: Implications for nutrients amended bioremediation. Environ. Sci. Technol. 31, 2078-2084.

Atlas, RM., Bartha, R., 1972. Degradation and mineralization of petroleum in seawater: limitations by nitrogen and phosphorus. Biotechnol. Bioeng. 14, 309-318.

Gertler, C., Nather, DJ., Capello, S., Gerdts G., Quilliam, RS., Yakimov, MM., Golyshin PN., 2012. Composition and dynamics of biostimulated indigenous oil-degrading microbial consortia from the Irish, North and Mediterranean seas: a mecocosm study. FEMS Microbiol. Ecol. 81(3): 530-536.

Mukherji, S., Peters, CA., Weber, WJ., 1997. Mass transfer of poly nuclear aromatic hydrocarbons from complex DNAPL mixtures. Environmental Science and Technology. 31, 416-423.

Rochelle, P.A., 2001. Environmental Molecular Microbiology: Protocols and Applications. Horizon Scientific Press. Wymondham. UK.

Nikolausz, M., Kappelmeyer, U., Szekely, A., Rusznyak, A., Marialigeti, K., Kastner, M., 2008. Diurnal redox fluctuation and microbial activity in rhizosphere of wetland plants. Eur. J. Soil Biol. 44, 324-333.

Prince, RC., Bragg, JR., 1997. Shoreline bioremediation following the *exxon valdez* oil spill in Alaska. Biormediation J. 97-104.

Lee, k., Merline, FX., 1999 Bioremediation of oil on shoreline environments: development of techniques and guidelines. 71, 161-171.

Das, K., Ashis, K., Mukherjee, F., 2006. Crude petroleum-oil biodegradation efficiency *Bacillus subtilis* and *Pseudomonas aeruginosa* strain isolated from a petroleum-oil contaminated soil from North-East India. Bioresource Technology. 98, 1339-1345.

Bento, FM., Camargo, FAO., Okeke, BC., Frankenberger, WT., 2005. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresour. Technol. 96, 1049-1055.

Swannell, RPJ., Lee, K., McDongh, M., 1996. Field evaluation of marine oil spill bioremediation. Microbiol. Rev. 60, 342-365.

Roling, WFM., Milner, MG., Jones, DM., Lee, K., Daniel, F., Swannell, RJP., Head, IM., 2002. Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient enhanced oil spill bioremediation. Appl. Environ. Microbiol. 68, 5537-5548.

Darmayati, Y., Sanusi, HS., Prartono, T., Santosa, DA., Nuchsin, R., 2015. The Effect of Biostimulation and Biostimulation-Bioaugmentation on Biodegradation of Oil-Pollution on Sandy Beaches Using Mesocosms. International Journal of Marine Science, 5, 1-11.

Yakimov, MM., Gentile, G., Bruni, V., Cappello, S., DAuria, G., Golyshin, PN., Giuliano, L., 2004. Crude oil induced cultural shift of coastal bacterial communities of Rod Bay (Terra Nova Bay, Ross Sea, Antarctica) and characterization of cultured cold- adapted hydrocarbonoclastic bacteria. FEMS Microbiol. Ecol. 49, 419-432.

Capello, S., Denaro, R., Genoress, M., Guiliano, L., Yakimov, MM., 2007. Predominant growth of *Aleanivorax* during experiments on “Oil spill bioremediation” in mecocosms. Microbial Research. 10, 185-190.

Jimenez, N., Vinas, M., Bayona, JM., Albaiges, J., Solanas, AM., 2007. The prestige oil spill: bacterial community dynamics during a field biostimulation assay. Appl. Microbiol. Biotech. 77, 935-945.

Evans, FF., Rosando, AS., Sebastian, GV., Cesella, R., Machando, PLOA., Holmstrom, C., Kjelleberg, S., Elasas, JDV., Seldin, L., 2004. Impact of oil contamination and biostimulation on the diversity of indigenous bacterial communities in soil microcosms. FEMS Microbiol. Ecol.

Morris, GM., Huey, R., Lindstrom, W., Sanner, MF., Belew, RK., Goodshell, DS., Oslon, AJ., 2009. Autodock 4 and AutoDock Tools4: automated docking with selective receptor flexibility. J. Comput. Chem. 30, 2785-2791.

Appendices

BH Medium:

Ingredient	Gms/Liter
Magnesium sulphate	0.2
Calcium chloride	0.02
Monopotassium phosphate	1.0
Dipotassium phosphate	1.0
Ammonium nitrate	1.0
Ferric chloride	0.05
Final pH	7.0 ± 0.2

Primers:

27F: AGA GTT TGA TCM TGG CTC AG

1492R: CGG TTA CCT TGT TAC GAC TT

968FGC: CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT

*alk*BF: AAYACNGCNCAYGARCTNGGNCAYAA

*alk*BR: GCRTGRTGRTCNGARTGNCGYTG

NDOF: CAC TCA TGA TAG CCT TGA TTC CTG CCC CCG GCG

NDOR: CCG TCC CAC AAC ACA CCC ATG CCG CTG CCG

Mixed indicator solution:

a) Dissolve 200 mg of Methyl red indicator in 100 ml 95 % Ethyl alcohol.
b) Dissolve 100 mg Methylene blue in 50 ml 95 % Ethyl alcohol. (mix a and b)

Indicator boric acid solution:

Dissolve 20 gm of H_3BO_3 in water, add 10 ml mixed indicator solution and dilute to 1000 ml.

Catalyst mixture:

Mix sodium/potassium sulphate and copper sulphate in 5:1 ratio (w/w).

TKN Estimation:

- 3gm digestion mixture + 2ml sample + 10ml H_2SO_4- digestion at 400°C for 2 hrs- cool down
- Add 10ml 0f dH_2O- 30-40ml of 40% NaOH- distilled for 6 min
- Collect the condensate into 12.5 ml boric acid indicator flask
- Titrate it with 0.1N HCl

% Nitrogen = [14 X Normality of acid X Titrant value burette reading X 100] / [volume or

weight of sample x 1000]

Phosphorus estimation:

- Stock solution of Phosphorus(0.16g/L)- 179mg/250ml d H_2O
- Reagent A- 25g Ammonium molybdate in 400ml d H_2O
- Reagent B- 1.25g Ammonium vandate in 300ml d H_2O
- Mixed reagent (freshly prepared, 50ml):
  20ml Reagent A + 15ml Reagent B + 12.5ml conc. HNO_3 + 2.5ml d H_2O
- 3.5ml sample + 1ml mixed reagent + 0.5ml d H_2O- O.D. 540nm

Appendix 1:

List of Publications:

i) Pasumarthi, R., Sivaraman, C., Mutnuri, S., Biodegradation of crude oil by *Pseudomonas aeruginosa* and *Escherichia fergusonii* isolated from the Goan coast. Mar. Poll. Bull. 76, 276-282.

ii) Pasumarthi, R., Kumar, V., Chandrasekharan, S., Ganguly, A., Banerjee, M., Mutnuri, S., Biodegradation of aliphatic hydrocarbons in the presence of hydroxy cucurbit[6]uril. Mar. Poll. Bull. 2014, 88, 148-54.

iii) Work titled "Effect of rhamnolipids in biodegradation of non-aqueous phase liquids" has been communicated to journal of Bioremediation.
(authors: Rajesh Pasumarthi, Maria Amal Raj, Srikanth Mutnuri)

Appendix 2:

Conferences attended:

iv) Conference: **Oil Spill India 2014 (conference and exhibition, Goa)**.
Theme: "Practice to Perfection" (September 18, 2014).

Appendix 3: Brief Biography of the Candidate

Personal details

Name	Mr. Rajesh Pasumarthi
Education	M.Sc. Microbiology, Sri. Y.N. College, Andhra University (2010)
	B.Sc. (Microbiology, Biochemistry, Biotechnology), AKRG Degree College, Andhra University (2007)
E-mail	rajesh.pasumarthi9@gmail.com

Work experience:

- Worked in BITS PILANI, Hyderabad campus as project assistant in NPMASS project (Urinary tract infections) under the supervision of Dr.Suman Kapoor. From January 2011 to April 2011.

Research experience:

- Worked as Ph.D. student from August 2011 availing Institute fellowship under the supervision of Dr. Srikanth Mutnuri at BITS PILANI, K K BIRLA GOA CAMPUS.

Number of Publications: 02

Appendix 4: A Brief Biography of the supervisor

Dr. M. Srikanth was a recipient of DAAD-UGC scholarship to complete his Doctoral Research at UFZ – Centre for Environmental Research, Germany and obtained his degree from Anna University Chennai in the year of 2004. He joined BITS Pilani K K Biral Goa Campus as a full time faculty by 2005. He worked as convener for three International Conferences in Environmental Biotechnology held in the year 2009, 2011 and 2014 at BITS Pilani, K K Birla Goa Campus. Dr. M. Srikanth conducted International Workshop on Bioremediation in association with Dr. Haggblom, Rutgers University USA for two weeks from January 4-16, 2010. He was principal investigator for four research projects funded by DST, DBT, UGC and GEDA and currently he has research projects funded by CSIR and DBT BIRAC & Bill and Mellinda Gates foundation. He has published 21 research papers in International Journals and written two Book Chapters. He received Helmholtz association's Junior Scientist Award and FEMS Young Scientist Award to participate in International conference on Environmental Biotechnology, Leipzig, Germany, 2006 and 14th International Biodeterioration and Biodegradation symposium Sicily, Italy, 2008 respectively. He had attended National and International conferences to present his research work as Oral and Poster presentations. He has Research collaborations with scientists from IISc Bangalore, INRA France, UFZ Germany, GTZ-BMU Germany and Rutgers University USA. He is a recipient of American Society for Microbiology & Indo US Science and Technology Forum (ASM IUSSTF) Indo Research Professorship for October 2010.