CHARACTERIZATION OF A METHYLOTROPHIC BACTERIAL CONSORTIUM AND ITS POTENTIAL IN TREATMENT OF INDUSTRIAL EFFLUENTS

A THESIS SUBMITTED TO

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

(MICROBIOLOGY)

GUIDED BY

Dr. ANURADHA S. NERURKAR

SUBMITTED BY

KRUSHI HINGURAO

DEPARTMENT OF MICROBIOLOGY AND BIOTECHNOLOGY CENTRE,
FACULTY OF SCIENCE,
THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA,
VADODARA-390 002, GUJARAT, INDIA

OCTOBER 2013

DECLARATION

Statement under O. Ph. D. 8/(iii) of The M. S. University of Baroda, Vadodara, India

The work presented in this thesis has been carried out by me under the guidance of Dr. Anuradha S. Nerurkar, Department of Microbiology and Biotechnology Centre, Faculty of Science, The M. S. University of Baroda, Vadodara, Gujarat, India. The data reported herein is original and has been derived from studies undertaken by me.

Place: Vadodara **Krushi Hingurao**

Date: Candidate

This is to certify that the above declaration is true.

Place: Vadodara **Dr. Anuradha S. Nerurkar**

Date: Research Guide

Dedicated to

..... My parents

Acknowledgements

After a long span of five years of my Ph. D., the time has come to thank all those who have helped me in any which way possible during my Ph. D. tenure.

Words are not enough to thank my guide, Dr. Anuradha Nerurkar. It was she who saw the potential in me to pursue Ph. D. Since my first day in the department, my respect and admiration for her has increased by leaps and bounds. She was always there to guide me, no matter what kind of problems I had. Our discussions were more informal, than being formal, and she always tried to understand my difficulties. She gave me a free working environment. She has been the best Ph. D. guide I would have ever asked for.

I would like to thank Prof. T. Bagchi, Head, Department of Microbiology and Biotechnology Centre, M. S. University of Baroda, for providing the various research facilities required for the study. I would also like to thank Prof. Anjana Desai, former Head, Department of Microbiology and Biotechnology Centre, M. S. University of Baroda, for giving me an opportunity to do Ph. D., and Prof. H. S. Chhatpar, former Head, Department of Microbiology and Biotechnology Centre, M. S. University of Baroda, for giving productive suggestions as and when needed.

I express my heartfelt thanks to Prof. G. Archana, Prof. Sanjay Ingle, Dr. Pranav Vyas and Dr. Nandita Baxi for readily extending help as and when required.

I would like to acknowledge Dr. Smita Trivedi, Institute of Management Studies, M. S. University of Baroda, for generously sharing her time and knowledge in statistical evaluation of the media optimization studies.

I am obliged to Dr. Jayalaxmi Iyer, Mr. V. B. Patel and Dr. Sujal Joshi, Gujarat Narmada Valley Fertilizers Company Limited (GNFC), Bharuch, Gujarat, and Choksi Laboratories, Vadodara, Gujarat, for carrying out GC analysis.

Thanks are due to Ms. Reeti Jaiswal, United Phosphorus Limited, Vadodara, Gujarat, for kindly providing the biofilm carriers used for the MBBR studies, and Daystar Laboratories, Vadodara, Gujarat, for helping me in the construction of a bench-scale reactor used for the biotreatment studies.

I am indebted to the University Grant Commision (UGC), New Delhi, for providing the UGC-RFSMS fellowship during the course of my Ph. D.

I take this opportunity to thank Dr. Kunal Shah, Central Salt and Marine Chemicals Research Institute (CSMCRI), Bhavnagar, Gujarat, and Jigar Soni,

Department of Chemistry, M. S. University of Baroda, the two persons whom I could ask for any kind of help at any given time, and they would be more than happy to help me as best they could. I would also like to acknowledge Sonal Bhokarkar, Department of Environmental Sciences, M. S. University of Baroda, for providing all the necessary material for the wastewater treatment studies.

During the five years of my Ph. D., I was fortunate to interact with a large number of Ph. D. students working at our department. I would like to appreciate all my colleagues, especially my seniors, Dr. Anil Kumar Singh, Dr. Aparna Akolkar, Dr. Ketan Patel, Dr. Priyadarshan Keluskar, Dr. Radhika Yadav, Dr. Srinandan Chakraborty and Dr. Vimal Maisuria, and my juniors, Akhilesh Dhanani, Anoop Markande, Jagat Rathod, Sanjukta Samurailaptam, Shweta Talreja, Sneha Garge and Dr. G. Subramanyam.

My colleagues at Research Laboratory – 2 (RL-2), were like an extended family. Dr. Murali Sharaff was always there for me whenever I had any difficulties in my Ph. D. work. Dr. Ruchi Chaturvedi and Dr. Priya Pillai were always eager to help and my SOS whose suggestions were always fruitful whenever I started with a new experiment during my Ph. D. work. Vihang Thite was my helping hand with all the new technologies that I was unaware of, I could rightly ask for his help and he was always ready to do so. Kinnary Iyer has always been my listening ear and I could go on talking to her about any subject possible.

I am fortunate to have such good dissertation students as Manushi Naik, Sangeeta Rathwa, Sangita Yadav, Kintu Vankar, Shivangi Dhebar, Shruti Shah and Payal Shah and other M. Sc. students.

I owe my gratefulness to the non-teaching staff of Department of Microbiology and Biotechnology Centre, M. S. University of Baroda, viz. Shirish Nagarkar, Nirmal Mandwekar, Aparana Shirsat, H. S. Khatri, A. Thomas, Bharat Talati, Pravin Vankar, Seema Patel, Baburao Shinde and others.

I would like to appreciate the staff of workshop of the M. S. University of Baroda, for helping me in construction of the microscopic glass slides used in the biofilm studies.

My family has been my greatest support during the entire tenure of my Ph. D. It was my parents' firm determination that led to the successful completion of my Ph. D. They were always patient with me and encouraged me to carry on with my Ph. D. I hope I have fulfilled my parents' dreams. My sister, Pooja, her husband, Aakash

Mehta, and the youngest family member, Yashvi, were my source of entertainment during the otherwise routine life of Ph. D.

Without the blessings of my in-laws, it would not have been possible for me to complete my Ph. D. It was their patience and the freedom they granted to me which has been one of the strong forces for me to finally finish my Ph. D. I would also like to acknowledge Prof. Deepak Kannal, former Dean, Faculty of Fine Arts, M. S. University of Baroda, for supporting me to pursue Ph. D.

I would not be able to express my luck in words for having such a loving and caring husband, Dr. Harish Suthar. He has been my anchor throughout the tenure of my Ph. D. He was always there for me, being my constant support. It would have been impossible for me to do Ph. D. without his continuous efforts. He has always critically acclaimed my work and made me improvise which has led me to a better understanding of my research topic. It is due to his efforts, more than mine that I have come to the conclusion of my Ph. D. He is the best gift that the department has given to me. Finally, I would like to specially thank the coming member of our family for being so patient and bearing with me while I was busy with my thesis.

Lastly, I would like to thank the Almighty for giving me the strength, patience and commitment to perceive the work for my Ph. D.

Krushi Hingurao

Table of Contents

Acknowledgements i

Table of Contents iv

List of Abbreviations xv

List of Figures xviii

List of Tables xxiii

Abstract xxiv

1. Review of Literature 1

1.1. Methylotrophs 2

1.1.1. Type I methylotrophs 4

1.1.2. Type II methylotrophs 5

1.1.3. Auto/pseudo methylotrophs 6

1.1.4. Methanotrophs 6

1.1.5. Diversity of methylotrophs 8

1.1.6. Methanol dehydrogenase 11

1.1.7. Biotechnological potential of methylotrophs 12

1.2. Xenobiotics 13

1.2.1. Recalcitrance of xenobiotics 15

1.2.2. Adverse effects of xenobiotics 15

1.2.3. Microbial degradation of xenobiotics 17

1.2.4. Xenobiotic degradation by methylotrophs 19

1.2.5. Methyl *tert*-Butyl Ether (MTBE) biodegradation 22

1.2.6. 1,2-Dichloroethane (DCE) biodegradation 25

1.3. Wastewater treatment 26

1.3.1. Activated sludge process 27

1.3.2. Biofilm mediated wastewater treatment 32

1.3.2.1. Biofilms 32

1.3.2.2. Moving bed biofilm reactor (MBBR).....34

1.4. Aim and scope of the present investigation 37

1.5. Objectives of the present investigation 38

1.6. References 39

2. Construction of a bacterial consortium for COD reduction of industrial effluents and characterization of its individual isolates 53

2.1. Introduction 54

2.2. Materials and Methods 55

2.2.1. Isolation 55

2.2.2. Screening 56

2.2.2.1. Screening of isolates on the basis of substrate utilization 56

2.2.2.2. Screening of the selected isolates on the basis of growth in DNR effluent 56

2.2.3. Screening of consortia and sludges for biotreatment potential 57

2.2.4. Growth profile 57

2.2.5. Confirmation of methylotrophy 58

2.2.5.1. Methanol utilization 58

2.2.5.2. *mxaF* gene detection 58

2.2.6. Polyphasic identification 58

2.2.6.1. Morphological and biochemical characterization 58

2.2.6.2. 16S rRNA gene sequence analysis 59

2.2.7. Phylogenetic analysis 59

2.2.8. Carbon substrate utilization 59

2.2.8.1. Single and multi carbon substrate utilization 59

2.2.8.2. Alcohol utilization 60

2.2.9. Methanol dehydrogenase 60

2.2.9.1. MDH isolation 60

2.2.9.2. MDH assay 60

2.2.10. Methanol tolerance....61

2.2.10.1. Exposure to different concentrations of vapor phase methanol on solid media....61

2.2.10.2. Exposure to pre-solubilized shock concentrations of methanol in liquid media....61

2.2.11. Nucleotide sequence accession numbers....61

2.3. Results and Discussion....61

2.3.1. Isolation of potential methylotrophic bacteria....62

2.3.2. Screening of isolates....63

2.3.2.1. Screening of isolates on the basis of substrate utilization....63

2.3.2.2. Screening of the selected isolates on the basis of growth in DNR effluent....64

2.3.3. Screening of consortia and sludges for biotreatment potential....64

2.3.4. Growth profile of the isolates of AC consortium....68

2.3.5. Confirmation of methylotrophy of the isolates of AC consortium....68

2.3.5.1. Methanol utilization by the AC consortium and its individual isolates.68

2.3.5.2. Utilization of methanol present in DNR effluent by the individual isolates of AC consortium....69

2.3.5.3. *mxaF* gene detection in the isolates of AC consortium....71

2.3.6. Polyphasic identification....71

2.3.6.1. Biochemical characterization of the isolates of AC consortium....71

2.3.6.2. Identification of the isolates of AC consortium by 16S rRNA gene sequencing....71

2.3.7. Phylogenetic analysis....75

2.3.8. Carbon substrate utilization....76

2.3.8.1. Differential carbon substrate utilization pattern among the isolates of AC consortium....76

2.3.8.2. Alcohol utilization pattern of AC consortium and its individual isolates 78

2.3.8.3. Biodegradation of different carbon substrates and xenobiotics by AC consortium....79

2.3.9. MDH estimation in the isolates of AC consortium....80

2.3.10. Methanol tolerance of the isolates of AC consortium....80

2.3.10.1. Exposure to vapor phase methanol on solid media....80

2.3.10.2. Exposure to pre-solubilized concentrations of methanol in liquid media....81

2.4. References....82

3. Biodegradation of xenobiotics by the AC consortium....87

3.1. Introduction....88

3A, Methyl *tert*-Butyl Ether biodegradation by the AC consortium....89

3A.1. Introduction....90

3A.2. Materials and Methods....91

3A.2.1. Soluble xenobiotic biodegradation....91

3A.2.2. MTBE biodegradation....91

3A.2.2.1. MTBE biodegradation by AC consortium and its individual isolates 91

3A.2.2.2. Effect of cations on growth of AC consortium and its individual isolates on MTBE....92

3A.2.2.3. Effect of magnesium concentration on the growth of AC consortium and its individual isolates on MTBE....92

3A.2.2.4. Influence of increasing concentration of MTBE on the growth of AC consortium and its individual isolates....92

3A.2.2.5. Effect of different combinations of the isolates of AC consortium on growth on MTBE....92

3A.2.2.6. MTBE utilization by the AC consortium and its individual isolates in optimized medium....93

3A.2.3. TBA biodegradation....93

3A.2.3.1. TBA biodegradation by AC consortium and its individual isolates....93

3A.2.3.2. Effect of different cations on growth of AC consortium and its individual isolates on TBA 93

3A.2.3.3. Effect of cations concentration on growth of AC consortium and its individual isolates on TBA 94

3A.2.3.4. Influence of increasing concentration of TBA on growth of AC consortium and its individual isolates 94

3A.2.3.5. TBA utilization by the AC consortium and its individual isolates in optimized medium 94

3A.2.4. GC-MS analysis of MTBE biodegradation by AC consortium 95

3A.2.5. Reactor studies 95

3A.2.5.1. Treatability studies 95

3A.2.5.2. Batch reactor studies 95

3A.2.5.3. Continuous reactor studies 96

3A.2.6. Statistical analysis 96

3A.3. Results and Discussion 97

3A.3.1. Soluble xenobiotic utilization by AC consortium and its individual isolates 97

3A.3.2. MTBE biodegradation 98

3A.3.2.1. MTBE biodegradation by AC consortium and its individual isolates 99

3A.3.2.2. Effect of cations on growth of AC consortium and its individual isolates on MTBE 99

3A.3.2.3. Effect of magnesium concentration on growth of AC consortium and its individual isolates on MTBE 101

3A.3.2.4. Influence of increasing concentration of MTBE on the growth of AC consortium and its individual isolates 102

3A.3.2.5. Effect of different combinations of the isolates of AC consortium on growth on MTBE 104

3A.3.2.6. MTBE utilization by the AC consortium and its individual isolates in optimized medium 105

3A.3.3. TBA biodegradation.....107

3A.3.3.1. TBA biodegradation by the AC consortium and its individual isolates 108

3A.3.3.2. Effect of different cations on the growth of AC consortium and its individual isolates on TBA 109

3A.3.3.3. Effect of different concentrations of all cations on the growth of AC consortium and its individual isolates on TBA 110

3A.3.3.4. Influence of increasing concentration of TBA on the growth of AC consortium and its individual isolates 110

3A.3.3.5. TBA utilization by the AC consortium and its individual isolates in optimized medium.....112

3A.3.4. Intermediate products of MTBE on biodegradation by the AC consortium 114

3A.3.5. Reactor studies 116

3A.3.5.1. COD reduction of MTBE containing synthetic effluent by the AC consortium at flask level 116

3A.3.5.2. COD reduction of MTBE containing synthetic effluent by the AC consortium at reactor level.....117

3B. 1,2-Dichloroethane biodegradation by the AC consortium 119

3B.1. Introduction 120

3B.2. Materials and Methods 121

3B.2.1. Insoluble xenobiotic biodegradation.....121

3B.2.2. DCE biodegradation.....121

3B.2.2.1. DCE biodegradation by the AC consortium and its individual isolates 121

3B.2.2.2. Effect of different cations on the growth of AC consortium and its individual isolates on DCE 122

3B.2.2.3. Effect of magnesium concentration on the growth of AC consortium and its individual isolates on DCE 122

3B.2.2.4. Influence of increasing concentration of DCE on the growth of AC consortium and its individual isolates 122

3B.2.2.5. Effect of different combinations of the isolates of AC consortium on growth on DCE.....123

3B.2.2.6. DCE utilization by the AC consortium and its individual isolates in optimized medium.....123

3B.2.3. CE biodegradation.....123

3B.2.3.1. CE biodegradation by AC consortium and its individual isolates 123

3B.2.3.2. Effect of different cations on the growth of AC consortium and its individual isolates on CE 124

3B.2.3.3. Effect of cations concentration on the growth of AC consortium and its individual isolates on CE 124

3B.2.3.4. Influence of increasing concentration of CE on the growth of AC consortium and its individual isolates 124

3B.2.3.5. CE utilization by AC consortium and its individual isolates in optimized medium.....124

3B.2.4. GC-MS analysis of DCE biodegradation by the AC consortium 125

3B.2.5. Statistical analysis 125

3B.3. Results and Discussion 125

3B.3.1. Insoluble xenobiotic utilization by the AC consortium and its individual isolates 125

3B.3.2. DCE biodegradation.....126

3B.3.2.1. DCE biodegradation by the AC consortium and its individual isolates 127

3B.3.2.2. Effect of different cations on the growth of AC consortium and its individual isolates on DCE 128

3B.3.2.3. Effect of magnesium concentration on the growth of AC consortium and its individual isolates on DCE 128

3B.3.2.4. Influence of increasing concentration of DCE on the growth of AC consortium and its individual isolates 129

3B.3.2.5. Effect of different combinations of the isolates of AC consortium on growth on DCE 131

3B.3.2.6. DCE utilization by the AC consortium and its individual isolates in optimized medium 131

3B.3.3. CE biodegradation 134

3B.3.3.1. CE biodegradation by the AC consortium and its individual isolates 135

3B.3.3.2. Effect of different cations on the growth of AC consortium and its individual isolates on CE 136

3B.3.3.3. Effect of cations concentration on the growth of AC consortium and its individual isolates on CE 137

3B.3.3.4. Influence of increasing concentration of CE on the growth of AC consortium and its individual isolates 138

3B.3.3.5. CE utilization by the AC consortium and its individual isolates in optimized medium 139

3B.3.4. Intermediate products of DCE on biodegradation by the AC consortium 141

3.2. References 143

4. Biotreatment of different industrial effluents using AC consortium 150

4.1. Introduction 151

4.2. Materials and Methods 153

4.2.1. Wastewater composition 153

4.2.2. Treatability studies 153

4.2.2.1. COD estimation by dichromate reflux method 154

4.2.3. Batch reactor studies 154

4.2.3.1. Estimation of different environmental parameters 155

4.2.3.1.1. BOD estimation by dissolved oxygen dilution method 156

4.2.3.1.2. MLSS estimation 156

4.2.3.1.3. MLVSS estimation 157

4.2.3.1.4. SVI estimation 157

4.2.3.1.5. F/M Ratio calculation 157

4.2.4. Continuous reactor studies 158

4.2.5. Pilot-scale reactor studies 158

4.2.6. Statistical analysis 158

4.3. Results and Discussion 159

4.3.1. Treatability studies 160

4.3.2. Reactor studies 164

4.3.2.1. Batch reactor studies 165

4.3.2.2. Continuous reactor studies 169

4.3.3. Pilot-scale reactor studies 176

4.4. References 178

5. Development of a bench scale Moving Bed Biofilm Reactor (MBBR) for COD reduction of industrial effluents by AC consortium 181

5.1. Introduction 182

5.2. Materials and Methods 184

5.2.1. Biofilm development 184

5.2.1.1. Biofilm formation by the isolates of AC consortium on a microscopic glass slide 184

5.2.1.2. Biofilm forming ability of the isolates of AC consortium using microtiter plate biofilm assay 184

5.2.1.3. Biofilm forming ability of the isolates of AC consortium on biofilm carriers 184

5.2.2. Effect of different parameters on biofilm formation 185

5.2.2.1. Influence of methanol concentration on biofilm formation 185

5.2.2.2. Effect of different carbon, nitrogen and phosphorus sources on the biofilm forming ability of the AC consortium and its individual isolates 186

5.2.2.3. Effect of different concentrations of the selected carbon, nitrogen and phosphorus sources on the biofilm forming ability of the AC consortium and its individual isolates 186

5.2.2.4. Effect of different concentrations of macronutrients on the biofilm forming ability of the AC consortium and its individual isolates 186

5.2.2.5. Effect of physiological parameters on the biofilm forming ability of the AC consortium and its individual isolates 186

5.2.3. Biofilm forming ability of the AC consortium and its members in optimized AMS medium 187

5.2.4. Biofilm forming ability of the AC consortium and its individual isolates in industrial effluent 187

5.2.5. MBBR studies 187

5.2.5.1. Treatability studies 187

5.2.5.2. Biotreatment of DNR effluent by the AC consortium using MBBR 188

5.2.5.3. Biotreatment of different industrial effluents using MBBR 189

5.2.6. Statistical analysis 189

5.3. Results and Discussion 190

5.3.1. Biofilm development 190

5.3.1.1. Biofilm formation by the isolates of AC consortium on a microscopic glass slide 190

5.3.1.2. Biofilm forming ability of the isolates of AC consortium using microtiter plate biofilm assay 192

5.3.1.3. Biofilm forming ability of the isolates of AC consortium on biofilm carriers 192

5.3.2. Effect of different parameters on biofilm formation 193

5.3.2.1. Effect of methanol concentration on biofilm formation by the AC consortium and its individual isolates 193

5.3.2.2. Effect of different carbon, nitrogen and phosphorus sources on biofilm forming ability of the AC consortium and its individual isolates 194

5.3.2.3. Effect of different concentrations of the selected carbon, nitrogen and phosphorus sources on biofilm forming ability of the AC consortium and its individual isolates 197

5.3.2.4. Effect of different concentrations of macronutrients on biofilm forming ability of the AC consortium and its individual isolates 199

5.3.2.5. Effect of physiological parameters on biofilm forming ability of the AC consortium and its individual isolates 202

5.3.3. Biofilm forming ability of AC consortium and its members in optimized AMS medium 204

5.3.4. Biofilm forming ability of the AC consortium and its individual isolates in industrial effluent 205

5.3.5. MBBR studies 206

5.3.5.1. Treatability studies 207

5.3.5.2. Biotreatment of DNR effluent by the AC consortium using MBBR in batch mode 207

5.3.5.3. Biotreatment of different industrial effluents by the AC consortium using MBBR in continuous mode 210

5.4. References 214

6. Summary 216

7. Conclusion 227

8. Publications and presentations 234

List of Abbreviations

ADP – Adenosine diphosphate

AFDW – Ash Free Dry Weight

AMS – Ammonium Mineral Salts

ANOVA – Analysis Of Variance

ATP – Adenosine triphosphate

BLAST – Basic Local Alignment Search Tool

BOD – Biological Oxygen Demand

BTEX – Benzene Toluene Ethylbenzene Xylene

C:N – Carbon:Nitrogen

C:P – Carbon:Phosphorus

CBB – Calvin Benson Bassham

CE – 2-Chloroethanol

CETP – Common Effluent Treatment Plant

cfu – colony forming unit

cfu/ml – colony forming unit per millilitre

COD – Chemical Oxygen Demand

D/W – Distilled Water

DCB – Dichlorobenzene

DCE – 1,2-Dichloroethane

DCM – Dichloromethane

DDW – Double Distilled Water

DNA – Deoxyribonucleic acid

dNTP – Deoxynucleotide triphosphate

DO – Dissolved Oxygen

DW – Dry Weight

EPA – Environmental Protection Agency

ESEM – Environmental Scanning Electron Microscopy

eV – electron Volt

F/M ratio – Food-to-Microorganisms ratio

GC – Gas Chromatography

GC-MS – Gas Chromatography-Mass Spectrometry

HIBA – 2-Hydroxyisobutyric acid

HRT – Hydraulic Retention Time

ICM – Intracytoplasmic membrane

kDa – kilo Dalton

kg/m^3 – kilogram per cubic meter

LB – Luria Bertani

m^3/d – cubic meter per day

MBBR – Moving Bed Biofilm Reactor

MDH – Methanol Dehydrogenase

MLSS – Mixed Liquor Suspended Solids

MLVSS – Mixed Liquor Volatile Suspended Solids

MM2 – Minimal Medium 2

MMO – Methane monooxygenase

MTBE – Methyl *tert*-Butyl Ether

NAD – Nicotinamide Adenine Dinucleotide

ND – Not Determined

ng/l – nanogram per liter

OD_{595} – Optical Density at 595 nm

OD_{600} – Optical Density at 600 nm

PAH – Polycyclic aromatic hydrocarbon

PCR – Polymerase Chain Reaction

PHA – Polyhydroxyalkanoate

PNB – Peptone Nitrate Beef extract

ppb – parts per billion

ppm – parts per million

PQQ – Pyrrolloquinoline quinone

p-value – probability value

rpm – revolutions per minute

rRNA – Ribosomal ribonucleic acid

RubisCO – Ribulose bisphosphate carboxylase

RuBP – Ribulose Bisphosphate Pathway

RuMP – Ribulose monophosphate

SRT – Solids Residence Time

SS- Suspended Solids

STP – Sewage Treatment Plant

SVI – Sludge Volume Index

TAA – *tert*-Amyl Alcohol

TAME – *tert*-Amyl Methyl Ether

TBA – t*ert*-Butyl Alcohol

TBF – tert-Butyl Formate

TCE – Trichloroethylene

TMAH – Trimethylamine hydrochloride

v/v – volume by volume

VSS – Volatile Suspended Solids

w/v – weight by volume

WWTP – Wastewater treatment plant

List of Figures

Figure 1.1. Metabolism of one carbon compounds in aerobic methylotrophs.....3
Figure 1.2. RuMP pathway for formaldehyde assimilation in Type I methylotrophs. .4
Figure 1.3. Serine pathway of formaldehyde assimilation in Type II methylotrophs ..5
Figure 1.4. RuBP pathway for carbon assimilation in auto/pseudo methylotrophs.....6
Figure 1.5. Pathways for the oxidation of methane and assimilation of formaldehyde in methanotrophs.....8
Figure 1.6. Different pathways followed by microorganisms during growth on MTBE 24
Figure 1.7. The catabolic pathway of DCE in *Xanthobacter autotrophicus*.....25
Figure 1.8. Schematic diagram of a conventional activated sludge system.....29
Figure 1.9. Biofilm growth cycle.....33
Figure 1.10. Schematic diagram of a MBBR.....35
Figure 2.1. Fusel oil utilization ability of (a) DAS, (b) DBS, (c) DOE, (d) FTE, (e) WAS and (f) AC isolates on the basis of their growth at 37 ^{o}C under aerobic conditions after 24 h.....65
Figure 2.2. Screening of isolates on the basis of their growth in DNR effluent. 67
Figure 2.3. Biotreatment potential of different consortia and sludge samples based on the COD reduction of DNR effluent at flask level 67
Figure 2.4. Growth profiles of the individual isolates of AC consortium in LB medium. 68
Figure 2.5. Methanol utilization ability of the AC consortium and its individual isolates.....69
Figure 2.6. Gas chromatograms depicting utilization of methanol present in DNR effluent by the isolates of AC consortium. 70
Figure 2.7. PCR amplification of *mxaF* gene in the isolates of AC consortium. 71
Figure 2.8. Phylogenetic analysis of the isolates of AC consortium. 75
Figure 2.9. (a) Single and (b) multi carbon substrate utilization pattern of the isolates of AC consortium.....77
Figure 2.10. Alcohol utilization by the AC consortium and its individual isolates. ...78
Figure 2.11. Carbon substrate/xenobiotic biodegradation ability of the AC consortium in terms of COD reduction.....79

Figure 2.12. Growth of the isolates of AC consortium after 120 h exposure to step concentrations of pre-solubilized methanol in liquid medium 81
Figure 3A.1. A 5 l batch reactor with a settling tank separated by a baffle.....96
Figure 3A.2. Growth of the AC consortium and its individual isolates on soluble xenobiotics. 98
Figure 3A.3. MTBE biodegradation by AC consortium and its individual isolates. 100
Figure 3A.4. Effect of cations on the growth of AC consortium and its individual isolates on MTBE 101
Figure 3A.5. Effect of Mg^{2+} concentration on the growth of AC consortium and its individual isolates on MTBE 102
Figure 3A.6. Effect of MTBE concentration on the growth of AC consortium and its individual isolates 103
Figure 3A.7. Effect of different combinations of AC consortium on growth on MTBE. 105
Figure 3A.8. MTBE utilization by the AC consortium and its individual isolates in optimized medium. 106
Figure 3A.9. Gas chromatograms depicting MTBE utilization by the AC consortium and its individual isolates. 107
Figure 3A.10. TBA biodegradation by the AC consortium and its individual isolates. 108
Figure 3A.11. Effect of different cations on growth of the AC consortium and its individual isolates on TBA 109
Figure 3A.12. Effect of cations concentration on the growth of AC consortium and its individual isolates on TBA 111
Figure 3A.13. Effect of TBA concentration on the growth of AC consortium and its individual isolates 112
Figure 3A.14. TBA utilization by the AC consortium and its individual isolates in optimized medium. 113
Figure 3A.15. Gas chromatograms depicting TBA utilization by the AC consortium and its individual isolates. 114
Figure 3A.16. GC-MS of MTBE biodegradation by the AC consortium. 115
Figure 3A.17. COD reduction of MTBE containing synthetic effluent by the AC consortium at flask level. 116
Figure 3A.18. COD reduction of MTBE containing synthetic effluent by the AC consortium in (a) batch and (b) continuous reactor. 117

Figure 3B.1. Experimental set-up to study growth of the isolates of AC consortium on insoluble xenobiotics 121
Figure 3B.2. Growth of the AC consortium and its individual isolates on insoluble xenobiotics. 126
Figure 3B.3. DCE biodegradation by the AC consortium and its individual isolates. 127
Figure 3B.4. Effect of different cations on the growth of AC consortium and its individual isolates on DCE 130
Figure 3B.5. Effect of Mg^{2+} concentration on the growth of AC consortium and its individual isolates on DCE 130
Figure 3B.6. Effect of DCE concentration on the growth of AC consortium and its individual isolates 131
Figure 3B.7. Effect of different combinations of AC consortium on growth on DCE. .132
Figure 3B.8. DCE utilization by the AC consortium and its individual isolates in optimized medium. 133
Figure 3B.9. Gas chromatograms depicting DCE utilization by the AC consortium and its individual isolates. 134
Figure 3B.10. CE biodegradation by the AC consortium and its individual isolates. ...135
Figure 3B.11. Effect of different cations on the growth of AC consortium and its individual isolates on CE 136
Figure 3B.12. Effect of cations concentration on the growth of AC consortium and its individual isolates on CE 137
Figure 3B.13. Effect of CE concentration on growth of AC consortium and its individual isolates 138
Figure 3B.14. CE utilization by the AC consortium and its individual isolates in optimized medium. 139
Figure 3B.15. Gas chromatograms depicting CE utilization by the AC consortium and its individual isolates. 140
Figure 3B.16. GC-MS of DCE biodegradation by the AC consortium. 142
Figure 4.1. Schematic diagram of a suspended reactor constituting of an aeration zone, a clarified zone and an aeration source 155
Figure 4.2. Treatability studies for COD reduction of DNR, ECO, COR and UPL effluents by the AC consortium at flask level.....161
Figure 4.3. A 6 l bench scale reactor consisting an aeration tank, a setting tank, a baffle and an aeration source 166

Figure 4.4. COD reduction of DNR, ECO, COR and UPL effluents by the AC consortium in batch reactor.....166
Figure 4.5. A 6 l bench scale continuous reactor consisting of an aeration tank, an influent tank, an effluent tank and an aeration source 169
Figure 4.6. COD reduction of DNR, ECO, COR and UPL effluents by the AC consortium in continuous reactor.....170
Figure 4.7. Biotreatment of DNR effluent by the AC consortium in a pilot-scale reactor 177
Figure 5.1. (a) Schematic diagram of a MBBR constituting of an aeration zone, a clarified zone, an aeration source and biofilm carriers (b) Kaldnes type K1 biofilm carriers.....188
Figure 5.2. Biofilm formation by (a) *B. petrii* AC1, (b) *B. licheniformis* AC4, (c) *S. subterranea* AC5, (d) *P. stutzeri* AC8 and (e) AC consortium on a microscopic glass slide observed under a brightfield microscope at 100 X magnification 191
Figure 5.3. Biofilm forming ability of the isolates of AC consortium in AMS medium using microtiter plate biofilm assay. 192
Figure 5.4. (a) Kaldnes type K1 biofilm carrier and (b) Biofilm development by AC consortium on Kaldnes type K1 biofilm carrier 193
Figure 5.5. Comparison of biofilm formation by the AC consortium and its individual isolates at 0.5 % methanol concentration.....194
Figure 5.6. Effect of (a) carbon, (b) nitrogen and (c) phosphorus sources on the biofilm forming ability of the AC consortium.....196
Figure 5.7. Influence of different concentrations of (a) potassium acetate, (b) NH_4Cl and (c) KH_2PO_4 + Na_2HPO_4 on the biofilm forming ability of the isolates of AC consortium.....199
Figure 5.8. Influence of different concentrations of (a) $CaCl_2$, (b) $MgSO_4$ and (c) $FeCl_3$ on biofilm forming ability of the AC consortium and its individual isolates..201
Figure 5.9. Influence of (a) inoculum size, (b) inoculum age and (c) incubation period on biofilm forming ability of the AC consortium and its individual isolates 204
Figure 5.10. Biofilm forming ability of the AC consortium and its individual isolates in optimized AMS medium using microtiter plate biofilm assay. 205
Figure 5.11. Biofilm forming ability of the AC consortium and its individual isolates in DNR effluent.....206

Figure 5.12. Biotreatment of DNR effluent by the AC consortium grown on biofilm carriers at flask level. 207
Figure 5.13. Biotreatment of DNR effluent by the AC consortium using MBBR with (a) 60 % (v/v) and (b) 30 % (v/v) biofilm carriers.....209
Figure 5.14. A MBBR developed for biotreatment of different industrial effluents by the AC consortium 211
Figure 5.15. COD reduction of DNR, ECO, COR and UPL effluents by the AC consortium using continuous MBBR.....211
Figure 5.16. ESEM analysis of the Kaldnes type K1 biofilm carrier showing a mature biofilm formation.....212

List of Tables

Table 1.1. Carbon substrates used by methylotrophic bacteria for growth.....3
Table 1.2. Characteristics of aerobic methylotrophs.....9
Table 1.3. Distribution of methylotrophs among various habitats.....11
Table 1.4. Industrial applications of xenobiotics.....14
Table 1.5. Factors controlling the fate of xenobiotics in the environment.....16
Table 1.6. Microorganisms utilizing xenobiotic compounds for their growth.....18
Table 1.7. MTBE degrading microorganisms.....23
Table 1.8. DCE degrading microorganisms.....26
Table 1.9. Major biological processes used for wastewater treatment.....28
Table 2.1. Distribution of the isolates among various habitats.....63
Table 2.2. Cell morphology of the 24 selected isolates.....66
Table 2.3. Biochemical characteristics of the isolates of AC consortium.....73
Table 2.4. MDH activity of the isolates of AC consortium.....80
Table 4.2(a). Comparison of antibiotic sensitivity of the isolates of AC consortium and the predominant isolates obtained in the treatability studies of the DNR effluent.....163
Table 4.2(b). Growth of the isolates of AC consortium and the predominant isolates obtained in the treatability studies of the DNR effluent on selective agar plates.....163
Table 4.3. Environmental parameters of DNR, ECO, COR and UPL effluents in batch reactor.....168
Table 4.4. Environmental parameters of DNR effluent in continuous reactor.....172
Table 4.5. Environmental parameters of ECO effluent in continuous reactor.....172
Table 4.6. Environmental parameters of COR effluent in continuous reactor.....173
Table 4.7. Environmental parameters of UPL effluent in continuous reactor.....173
Table 4.8. Summary of the COD reduction obtained in flask, batch and continuous reactor studies with DNR, ECO, COR and UPL effluents.....175
Table 4.9. One-Way ANOVA for comparison of difference in % COD reduction obtained in control and AC consortium seeded flask, batch and continuous reactors .175
Table 5.1. Typical characteristics of Kaldnes type K1 biofilm carriers.....185
Table 5.2. COD reduction performance of AC consortium in suspended reactor and MBBR.....213

Abstract

Industrial wastewater treatment is a necessity before introducing the effluents into the ecosystem. Various kinds of chemical, fertilizer and pesticide industries are prevalent in Gujarat, India, which release a large number of hazardous pollutants in their effluents. The indigenous microflora of the industrial effluents is often not capable of treating the effluents completely and, therefore, the industries encounter difficulties to meet the stipulated norms of the regulatory authorities. Hence, development of a special microbial seed is important for the biotreatment of such industrial effluents. Methylotrophic bacteria have been studied extensively with respect to biodegradation and biotreatment, and are known to be versatile in their degradative abilities. Moreover, it has been previously reported that a mixed bacterial consortium is more effective in wastewater treatment as compared to individual microorganisms. Therefore, methylotrophs were the bacteria of choice for the development of a microbial seed for efficient treatment of diverse kinds of industrial effluents. A methylotrophic bacterial consortium comprising specially selected isolates was formulated after screening 118 bacteria isolated from various sludge and effluent samples obtained from chemical and fertilizer industries, and domestic sewage treatment plant on the basis of growth on methanol-rich fusel oil (byproduct of a fertilizer industry).

The AC consortium consisted of 4 isolates identified, on the basis of their phenotypic characters and 16S rRNA gene sequencing, as *Bordetella petrii* AC1, *Bacillus licheniformis* AC4, *Salmonella subterranea* AC5 and *Pseudomonas stutzeri* AC8, out of which *Bordetella petrii* AC1 and *Salmonella subterranea* AC5 are the methylotrophic isolates of the species first reported here. Single carbon substrate utilization, methanol utilization by gas chromatographic analysis and *mxaF* gene amplification confirmed the methylotrophic nature of the 4 isolates. They could tolerate high concentrations of methanol due to their high methanol dehydrogenase activity. The isolates of AC consortium showed a broad metabolic diversity which included many xenobiotics and industrially important alcohols, implying their facultative methylotrophic nature.

The AC consortium could utilize toxic xenobiotics like methyl *tert*-butyl ether (MTBE), *tert*-butyl alcohol (TBA), 1,2-dichloroethane (DCE) and 2-chloroethanol (CE) as sole sources of carbon among other xenobiotics. It showed 98 % MTBE, 94

% TBA, 60 % DCE and 96 % CE degradation. MTBE and DCE were completely degraded by the AC consortium as no intermediates of their metabolism were detected in the GC-MS analysis. 5 l reactor studies with MTBE revealed that 100 % MTBE degradation was achieved by the AC consortium. Considering the spectrum of xenobiotic degradation of the AC consortium, it offers promising opportunities for treatment of effluents generated from various chemical industries, implying that it is a novel consortium possessing a broad range of xenobiotic degradation ability.

The AC consortium showed efficient biotreatment of 4 industrial effluents procured from fertilizer, chemical and pesticide industries and common effluent treatment plant by lowering their COD of 950 – 2000 mg/l to below detection limit in 60 – 96 h in 6 l batch reactor and 9 – 15 d in 6 l continuous reactor. The AC consortium also played an efficient role in maintaining the operating variables of wastewater treatment, viz. COD, BOD, pH, MLSS, MLVSS, SVI and F/M ratio of these effluents, in the permissible range in both batch and continuous reactors. Therefore, formation of the AC consortium has led to the development of an efficient microbial seed capable of treating a variety of industrial effluents containing pollutants generated from their respective industries.

After analyzing the potential of the AC consortium for wastewater treatment in an activated sludge process, its biotreatment potential was studied using moving bed biofilm reactor (MBBR). The isolates of AC consortium showed a complete biofilm formation when present as a consortium. Their biofilm forming ability was enhanced by statistical media optimization using microtiter plate biofilm assay, which was subsequently used to develop the biofilm of AC consortium on commercially available Kaldnes type K1 biofilm carriers to be used for upgradation of the 6 l suspended growth reactor into MBBR. The biofilm carriers coated with the biofilm formed by the AC consortium, as observed in under scanning electron microscope, were recycled with 4 different kinds of industrial effluents. The AC consortium, immobilized in biofilm mode on the carriers, showed efficient biotreatment of the 4 effluents using MBBR by reducing their COD of 1100 – 1700 mg/l to below permissible limit which was maintained there for 120 d of the reactor run. Hence, the studies conducted concluded that the AC consortium has immense potential as a microbial seed for treatment of diverse industrial effluents with the suspended reactor process being amenable to upgradation to MBBR.

Chapter 1

Review of Literature

1.1. Methylotrophs

Bacteria that can use reduced one carbon or multi-carbon compounds with no carbon-carbon bond (C_1 substrates), are called methylotrophs. Methylotrophs were first reported in 1906 (Sohngen, 1906), but it was not until 1970 that their widespread distribution, taxonomic diversity and physiological diversity were described (Colby and Dalton, 1979; Hanson, 1980; Quayle and Ferenci, 1978). Table 1.1 describes those carbon substrates known to support the growth of methylotrophs. Methylotrophs are mainly of 2 types: (1) Obligate methylotrophs, include microorganisms able to grow only on C_1 substrates and (2) Facultative methylotrophs, include microorganisms able to grow on C_1 substrates as well as a variety of multicarbon compounds like dimethyl ether, dimethylamine, trimethylamine, tetramethylammonium, trimethylamine N-oxide, trimethylsulphonium, etc. and other organic carbon compounds like acetate, lactate, pyruvate, hydroxybutyrate, carboxylic acids, etc. (Balachandar *et al*., 2008). The facultative methylotrophs are further divided into many types: (1) The pink facultative methylotrophs (e.g. *Methylobacterium extorquens*), (2) The non-pigmented "pseudomonads" (e.g. *Pseudomonas aminovorans*), (3) Gram negative (or variable), non-motile rods and coccal rods (e.g. *Arthrobacter rufescens*), (4) Gram-positive facultative methylotrophs (e.g. *Bacillus cereus*), (5) Facultative autotrophs or phototrophs growing on methanol or formic acid (e.g. *Rhodopseudomonas palustris*), (6) The Hyphomicrobia (e.g. *Hyphomicrobium* sp.) and (7) Marine bacteria able to grow on methanol and methylated amines with the help of methanol dehydrogenase enzyme and few more unique enzymes (e.g. *Methylomonas thalassica*) (Anthony, 1982).

In all methylotrophic bacteria, C_1 compounds are first oxidized to formaldehyde. Formaldehyde is situated at the branch point between further oxidation to CO_2 for energy formation and assimilation for biosynthesis. Therefore, the fate of formaldehyde is central to understanding methylotrophic metabolism. Figure 1.1 gives an outline of methylotrophic metabolism, showing how different methylotrophic substrates are fed into central metabolic pathways. In most methylotrophs, the pool of formaldehyde generated from methylotrophic substrates is split, with part being oxidised to CO_2 for energy and part being assimilated into the cell carbon via 1 of the 2 unique pathways, the ribulose monophosphate (RuMP) or the serine pathway. Based

on the presence of one of these pathways, methylotrophs are categorized as Type I methylotrophs and Type II methylotrophs (Lidstrom, 2006).

Table 1.1. Carbon substrates used by methylotrophic bacteria for growth (Anthony, 1982)

Compounds containing one carbon atom	**Compounds containing more than one carbon atom**
Methane	Dimethyl ether
Methanol	Dimethylamine
Methylamine	Trimethylamine
Formaldehyde	Tetramethylammonium
Formate	Trimethylamine N-oxide
Formamide	Trimethylsulphonium
Carbon monoxide	

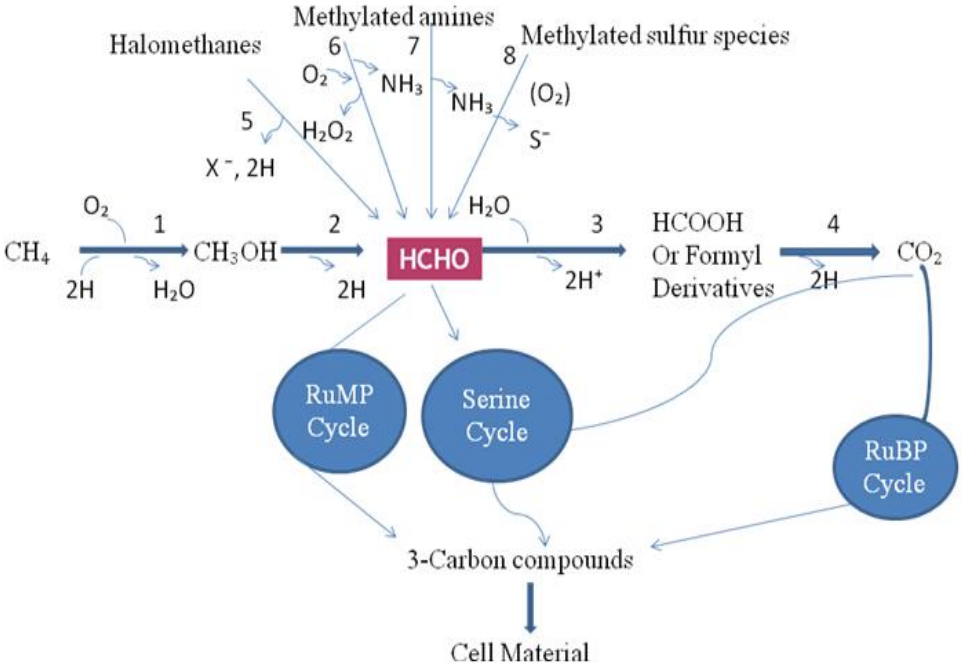


Figure 1.1. Metabolism of one carbon compounds in aerobic methylotrophs. 1 - Methane monooxygenase, 2 - Methanol dehydrogenase, 3 - Formaldehyde dehydrogenase, 4 - Formate dehydrogenase, 5 - Dehalogenase, 6 - Methylated amine oxidase, 7 - Methylamine dehydrogenase, 8 - Methylated sulphur dehydrogenase (Lidstrom, 2006)

1.1.1. Type I methylotrophs

These bacteria use the highly efficient RuMP pathway for formaldehyde assimilation. The RuMP pathway functions as a highly efficient system for trapping free formaldehyde at relatively low concentrations (Dijkhuizen *et al*., 1992). The pathway performs the synthesis of a C_3 compound (pyruvate) from 3 moles of formaldehyde (Figure 1.2). Formaldehyde is fixed with ribulose 5-phosphate to form D-*arabino*-3-hexulose 6-phosphate, which is then isomerised to fructose 6-phosphate. These 2 reactions are catalyzed by 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase respectively. The fructose 6-phosphate formed is then converted to fructose 1,6-bisphosphate, which is subsequently metabolized into pyruvate via the glycolytic pathway (cleavage stage). Ribulose 5-phosphate, the acceptor of formaldehyde, is regenerated from fructose 6-phosphate and glyceraldehyde 3-phosphate through a combination of transketolase, transaldolase and several reactions for pentose phosphate isomerizations (regeneration stage). There are 2 alternative stages for the cleavage of fructose 6-phosphate to C_3 compounds and 2 stages for the regeneration of ribulose 5-phosphate. The combination of 2 cleavage and 2 regeneration stages, thus, leads to a total of 4 possible variants of the RuMP pathway (Kato *et al*., 2006).

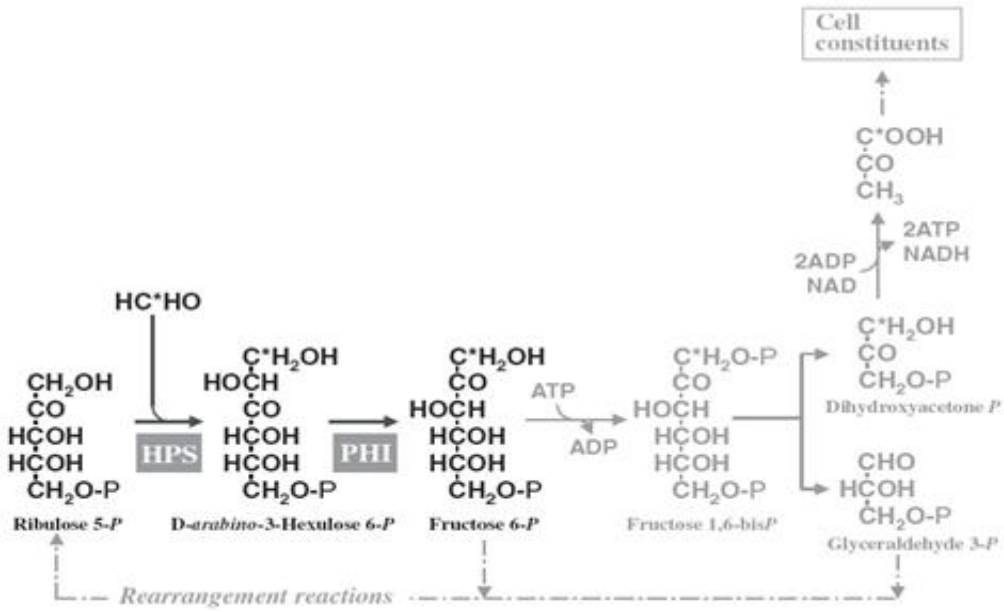


Figure 1.2. RuMP pathway for formaldehyde assimilation in Type I methylotrophs. HPS - 3-hexulose-6-phosphate synthase and PHI - 6-phospho-3-hexuloisomerase (Kato *et al*., 2006)

1.1.2. Type II methylotrophs

These bacteria use the less efficient serine pathway for formaldehyde assimilation. The serine cycle for formaldehyde assimilation is shown in Figure 1.3. This pathway initiates with the condensation of methylene tetrahydrofolate and glycine to form serine. This 3-carbon compound then undergoes a series of transformations to phosphoenolpyruvate, which is carboxylated to form malate. The malate is cleaved into two 2-carbon compounds, which are then converted back into glycine, thus completing the cycle. In most organisms that have the serine cycle, it is not clear how acetyl CoA is converted to glyoxylate. The usual route for this conversion involves isocitrate lyase, but this enzyme is only present in a few strains (Chistoserdova and Lidstrom, 1996). The enzymes specific to the serine cycle are noted in Figure 1.3 and most of the genes encoding these enzymes have been cloned and sequenced from *Methylobacterium extorquens* AM1 (Chistoserdova and Lidstrom, 1997) or *Hyphomicrobium methylovorum* GM2 (Tanaka *et al*., 1997). For each C_3 compound that is generated by the serine cycle, 2 carbons are derived from formaldehyde and 1 from CO_2 (Lidstrom, 2006).

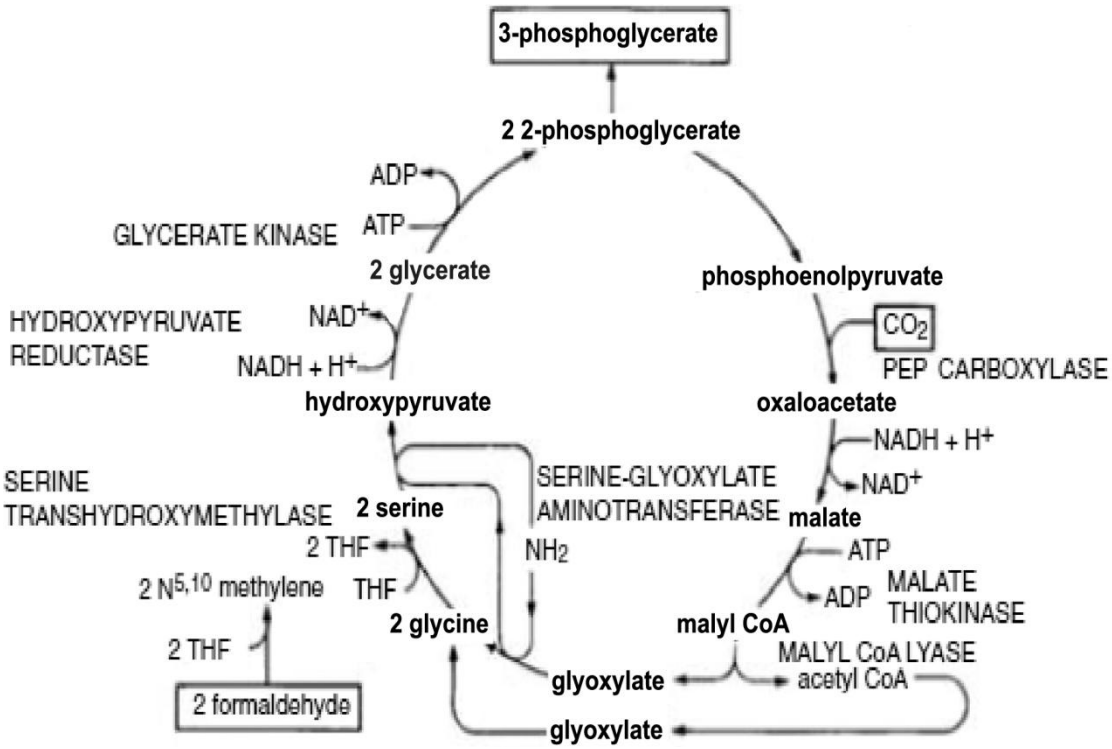


Figure 1.3. Serine pathway of formaldehyde assimilation in Type II methylotrophs (Lidstrom, 2006)

1.1.3. Auto/pseudo methylotrophs

Majority of the methylotrophs use either RuMP pathway or serine pathway for carbon assimilation, but some methylotrophs resemble autotrophic bacteria and photosynthetic organisms which generate precursors for biosynthesis by fixation of CO_2 using Calvin-Benson-Bassham (CBB) cycle or ribulose bisphosphate (RuBP) pathway. Therefore, they are called autotrophic or pseudo methylotrophs. As shown in Figure 1.4, only 2 reactions are unique to this cycle (the others are same as the oxidative pentose phosphate pathway): Phosphoribulokinase, which converts ribulose 5-phosphate to ribulose 1,5-bisphosphate, and Ribulose bisphosphate carboxylase (RubisCO), which converts ribulose 1,5-bisphosphate to 3-phosphoglycerate. This cycle constitutes the dark reaction of photosynthesis. 6 turns of the cycle result in the synthesis of 1 molecule of hexose (Fructose-6-phoshpate) (Madigan and Martinko, 2006).

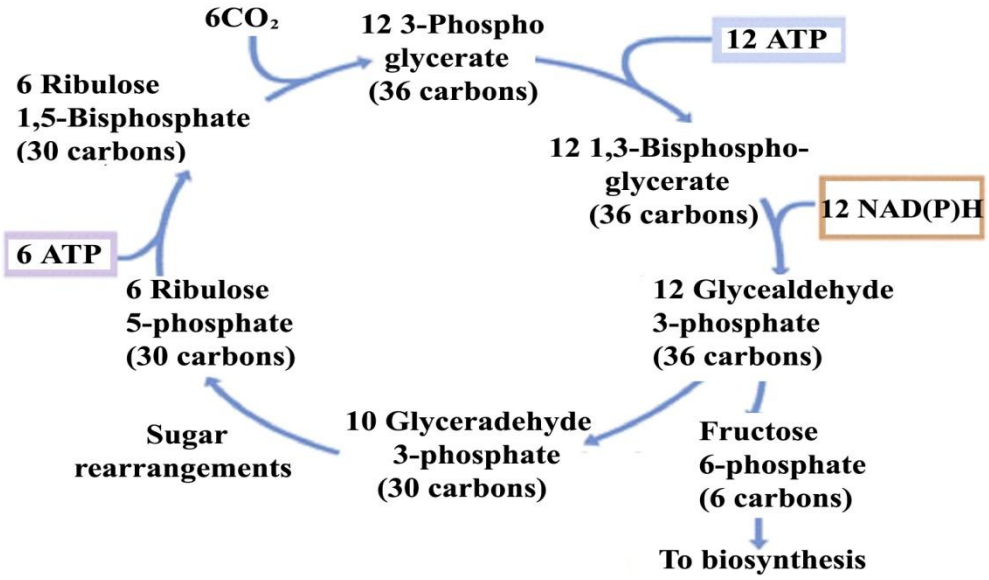


Figure 1.4. RuBP pathway for carbon assimilation in auto/pseudo methylotrophs (Madigan and Martinko, 2006)

1.1.4. Methanotrophs

Methylotrophs may be distinguished into 2 functional groups: those capable of growth on methane, called methanotrophs and those capable of growth on methanol and/or other methylated compounds but not on methane, called non-methane-utilizing methylotrophs (Lidstrom, 2006).

Methanotrophs, a subgroup of the methylotrophs, are capable of utilizing methane and some other C_1-compounds as sole sources of carbon and energy (Whittenbury *et al*., 1970). Methanotrophs have been extensively studied among methylotrophic bacteria (Hanson and Hanson, 1996). A defining characteristic of these organisms is the use of methane monooxygenase (MMO) enzymes to catalyze the oxidation of methane to methanol (Davey *et al*., 1972; Davies and Whittenbury, 1970). For a long time, all methanotrophs were considered to be obligatory methylotrophic, that is, unable to grow on compounds containing C-C bonds. But, recently, some genera of methanotrophic bacteria were reported to be facultative methanotrophs, i.e., capable of growing on methane as well as on some multicarbon substrates (Dedysh *et al*., 2005). Methanotrophs are distinguished from other methylotrophs by the presence of an intracytoplasmic membrane (ICM); non-methane-utilizing methylotrophs do not form an ICM (Lidstrom, 2006). The formation of an ICM in methanotrophs is dependent on growth conditions (Fassel *et al*., 1992). The ICM structure and biochemical characteristics form the basis for classification of methanotrophs. It exists in 2 types of arrangements: vesicular stacks of membranes located throughout the cell and pairs of peripheral membranes located parallel to the cell envelope. The former are characteristic of methanotrophs of type I and type X, and the latter are characteristic of type II methanotrophs. Type X methanotrophs were distinguished from Type I methanotrophs because they also possessed low levels of enzymes of the serine pathway and ribulose bisphosphate carboxylase, an enzyme present in the CBB cycle (Whittenbury and Krieg, 1984). They grew at higher temperatures than type I and type II methanotrophs and possessed DNA with a higher G + C content than that of most type I methanotrophs (Hanson and Hanson, 1996).

All methanotrophs oxidize methane by first initiating reduction of an oxygen atom to H_2O_2 and transformation of methane to methanol using MMOs. Furthermore, 2 types of MMOs have been isolated from methanotrophs: soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO). Cells containing pMMO have demonstrated higher growth capabilities and higher affinity for methane than sMMO containing cells. It is suspected that copper ions may play a key role in both pMMO regulation and the enzyme catalysis, thus limiting pMMO producing cells to more copper-rich environments than sMMO producing cells (Lieberman and Rosenzweig, 2004). Figure 1.5 illustrates the metabolism of

substrates by methanotrophs, the common features of their metabolism including the central role of formaldehyde as an intermediate in catabolism and anabolism, and the unique pathways employed for the synthesis of intermediates of central metabolic routes. As methylotrophs able to grow on methanol but not methane do not possess ICM, then it seems reasonable to conclude that they perform some special function in the initial step in methane hydroxylation. Higgins (1979) proposed that in methanotrophs, membranes may act by anchoring the oxygenase components or they may be involved as a solvent for methane or oxygen.

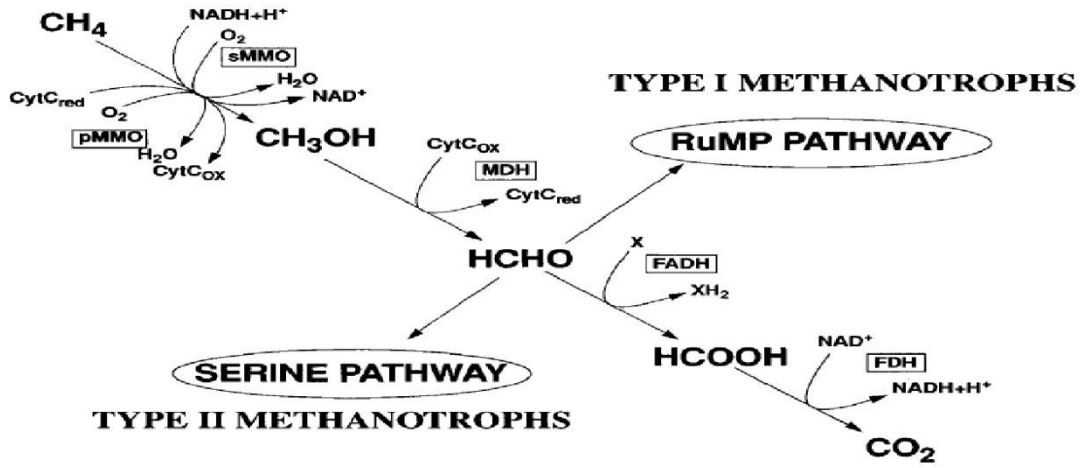


Figure 1.5. Pathways for the oxidation of methane and assimilation of formaldehyde in methanotrophs. CytC - cytochrome C, MDH – methanol dehydrogenase, FADH - formaldehyde dehydrogenase, FDH - formate dehydrogenase (Hanson and Hanson, 1996)

1.1.5. Diversity of methylotrophs

The most prominent organisms that shaped the major concepts and assumptions within the methylotrophy field include *Methylomonas methanica*, *Methylosinus trichosporium* and *Methylococcus capsulatus*, representing the 3 recognized classes (I, II and X) of typical obligate methanotrophs; *Methylobacterium extorquens* and *Paracoccus denitrificans*, representing typical facultative and autotrophic methylotrophs respectively; and a few species within the family *Methylophilaceae*, representing obligate or restricted facultative non-methane-utilizing methylotrophs. The knowledge gained from studying these model organisms has been used to classify methylotrophs into both functional and phylogenetic groups, such as facultative versus obligate methylotrophs, Type I versus Type II

methylotrophs, and autotrophic versus heterotrophic methylotrophs (Trotsenko and Murrell, 2008). Most of these have been placed into a small number of genera within α-, β- and γ-proteobacteria, and actinobacteria. Thus, the focus on easily cultivated strains resulted in a restricted diversity of recognized methylotrophs. Table 1.2 lists the major groups of methylotrophs with examples of the genera that have been described to contain aerobic methylotrophs (Lidstrom, 2006).

Table 1.2. Characteristics of aerobic methylotrophs (Lidstrom, 2006)

Group	Major assimilation pathway	Phylogenetic position
Obligate methylotrophs		
Type I methanotrophs		
Methylomonas	RuMP	γ-Proteobacteria
Methylobacter	RuMP	γ-Proteobacteria
Methylococcus	RuMP	γ-Proteobacteria
Methylomicrobium	RuMP	γ-Proteobacteria
Methylosphaera	RuMP	γ-Proteobacteria
Methylocaldum	RuMP	γ-Proteobacteria
Type II methanotrophs		
Methylosinus	Serine	α-Proteobacteria
Methylocystis	Serine	α-Proteobacteria
Methylocella	Serine	α-Proteobacteria
Restricted facultative methylotrophs		
Methanol utilizers		
Hyphomicrobium	Serine	α-Proteobacteria
Methylophilus	RuMP	β-Proteobacteria
Methylobacillus	RuMP	β-Proteobacteria
Methylophaga	RuMP	γ-Proteobacteria
Facultative methylotrophs		
Methylobacterium	Serine	α-Proteobacteria
Aminobacter	Serine	α-Proteobacteria
Methylorhabdus	Serine	α-Proteobacteria
Methylopila	Serine	α-Proteobacteria
Methylosulfomonas	Serine	α-Proteobacteria
Marinosulfomonas	Serine	α-Proteobacteria
Paracoccus	CBB	α-Proteobacteria
Xanthobacter	CBB	α-Proteobacteria
Ancylobacter	CBB	α-Proteobacteria
Thiobacillus	CBB	α-Proteobacteria
Rhodopseudomonas	CBB	α-Proteobacteria
Rhodobacter	CBB	α-Proteobacteria
Acetobacter	RuMP	γ-Proteobacteria
Bacillus	RuMP	Gram positive
Mycobacterium	RuMP	Gram positive
Arthrobacter	RuMP	Gram positive
Nocardia	RuMP	Gram positive

More recent efforts in methylotroph cultivation, many of which employ conditions mimicking *in situ* conditions rather than standard laboratory conditions, have resulted in a dramatic expansion of the view of phylogenetic distribution and environmental occurrence of methylotrophs. The notion of defined functional groups has also been changing. The terms themselves, obligate versus facultative, appear of questionable validity when it comes to methylotrophs, as traditionally only a standard set of substrates, many of which are sugars or amino acids, are tested when characterizing the new species. Considering environmental distribution, even for the known guilds of methylotrophs (oligotrophic marine waters, lake sediments, soils, plant roots and phyllospheres), these substrates appear to have little classifying power. The notion of methylotrophy as an attribute of specialized bacterial groups is also morphing, because with more sampling, more bacterial groups are identified as capable of methylotrophy (Boden *et al*., 2008). Some of the major groups in which methylotrophy has been discovered to occur include *Verrucomicrobia*, *Burkholderiales*, filamentous bacteria and denitrifying bacteria (Chistoserdova *et al*., 2009).

Methylotrophs are ubiquitous in nature. They are found in a variety of environments including air, tissues of higher organisms, soils, sediments, and freshwater and marine systems (McDonald *et al*., 2008). They are also found in plants, degraded fish and plant compounds and marine algae (Crowther, 2004). They are often associated with the leaf surfaces of plants where methanol is released when the stomata open in the morning (Huve *et al*., 2007). Methylotrophic bacteria have been found in paddy soil in northern Italy (Eller *et al*., 2005). Kalyuzhnaya *et al*. (2006) have isolated type I and type II methylotrophs from Lake Washington sediment. Waturangi *et al*. (2011) have isolated methylotrophic bacteria from human mouth. The pink-pigmented facultative methylotrophs were isolated from the phyllosphere of cotton, maize and sunflower plants exclusively raised in the experimental plots of Tamil Nadu Agricultural University, Coimbatore, India (Balachandar *et al*., 2008). Horz *et al*. (2001) assessed the diversity of methylotrophic bacteria associated with roots of submerged rice plants. Table 1.3 lists the different environmental sources from which methylotrophs have been isolated.

Table 1.3. Distribution of methylotrophs among various habitats

Methylotrophs	Source	Reference
Methylobacterium sp. strain BIP	Waste-treatment bioreactor	Chongcharoen *et al*., 2005
Methylophaga alcalica sp. nov.	Saline soda lake	Doronina *et al*., 2003
Pseudonocardia sp. strain 381	Compost	Eshraghi, 2007
Gordonia, *Leifsonia* and *Microbacterium*	Human oral cavity	Hung *et al*., 2011
Burkholderiales strains RZ18-153 and FAM1	Lake sediment	Kalyuzhnaya *et al*., 2008
Methylobacterium sp. MF1	Soil	Mitsui *et al*., 2005
Bacillus, *Brevibacterium casei*, *Hyphomicrobium sulfonivorans*, *Methylobacterium*, *Micrococcus luteus* and *Variovorax paradoxus*	Tongue, and supra- and subgingival plaque	Anesti *et al*., 2005
Methylophaga spp. and *Gammaproteobacteria* spp.	Coastal marine environment	Neufeld *et al*., 2007
Acidomonas methanolica	Non-sterilized methanol fermentation	Yamashita *et al*., 2004
Methylobacterium nodulans	*Crotalaria* spp. legumes	Sy *et al*., 2001

1.1.6. Methanol dehydrogenase

The activity of methanol dehydrogenase (MDH), first reported in *Methylobacterium extorquens*, has been considered an important attribute of methylotrophic growth, and this enzyme has remained a hallmark of methylotrophy for decades. The genes encoding the MDH subunits were originally identified in *M. extorquens* and later detected in most of the model methylotrophs, along with the accessory genes encoding functions required for generating an active MDH, genes for biosynthesis of the coenzyme pyrroloquinoline quinone (PQQ) and regulatory genes. Studies on the genetics of methanol oxidation in methylotrophic bacteria have revealed that at least 17 genes play a role in this process (Goodwin and Anthony, 1995; Lidstrom, 1992; Lidstrom *et al*., 1994; McDonald and Murrell, 1997). *mxaF* and *mxaI* genes encode the large (α) and small (β) subunits of MDH respectively, and *mxaG* encodes cytochrome C, the primary electron acceptor for MDH. The *mxaF* gene is approximately 1.8 kb in size and encodes a 66-kDa polypeptide, while the

mxaI gene is 290 bp in size and encodes an 8.5-kDa polypeptide. Because the *mxaF* gene is highly conserved among the well-characterized methylotrophs, it has been employed as a tool for environmental detection of methylotrophy. However, some of the newly identified methylotrophs, such as methylotrophic *Burkholderiales*, appeared to lack the *mxaFI* genes, and enzymes responsible for MDH activity were somewhat different from those exhibited by typical *mxaFI*-encoded enzymes. These encoded polypeptides (named Mdh2) had low similarity to MxaF polypeptides (< 35 % amino acid identity), but were highly similar (up to 80 % amino acid identity) to a different class of PQQ-linked dehydrogenases, the well-characterized alcohol dehydrogenases, which have low affinity for methanol (Chistoserdova *et al*., 2009). In methylotrophs capable of growing on methanol, methanol oxidation reaction does not directly involve molecular oxygen but uses an alternative electron acceptor, which is oxidised cytochrome C. The reduced cytochrome C is reoxidised, to enable it to be re-used in the reaction, by a complex chain of electron carriers with oxygen as the final oxidant in the reaction producing water. The metabolism of methanol to formaldehyde by this reaction provides sufficient energy to enable 1 mole of ATP to be synthesised from 1 mole of ADP and 1 mole of inorganic phosphate for each mole of methanol which is so oxidised.

1.1.7. Biotechnological potential of methylotrophs

Methanol as a feedstock has often been cited as providing methylotrophic organisms with economic and quality advantages over other more traditional carbohydrate raw materials. Methylotrophs are most likely to be of commercial utility when they can either synthesize a novel product with desirable properties (e.g. polysaccharides) or provide unique catalytic potential, e.g. MMO.

Methylotrophs have been shown to accumulate a wide variety of potential products (Lidstrom and Stirling, 1990). These range from commodity chemicals (e.g. ethanol, acetone and glycerol) and fine chemicals (e.g. nicotinamide adenine dinucleotide, adenosine triphosphate, vitamin B_{12}) to specialty chemicals (e.g. biopolymers such as polysaccharides and poly-β-hydroxybutyric acid). The types of protein products of potential commercial value derived from methylotrophs include single cell protein (SCP), specific methylotrophic enzymes (e.g. alcohol oxidase) and heterologous gene products (e.g. hepatitis B surface antigen). In cases not involving

unique products, processes using methylotrophs must compete with other organism systems simply on an economic basis. However, lower raw material costs may be an advantage for the commodity-type chemicals.

The biosynthesis of compounds labeled with the stable isotope ^{13}C is an example in which methylotrophic metabolism proved to be superior to that of heterotrophic organisms. ^{13}C -labeled CO can be easily converted by methylotrophs to ^{13}C -labeled products, e.g. ^{13}C sugars, amino acids, etc. These materials are of clinical value in magnetic resonance spectroscopy and ^{13}C carbon dioxide-based diagnostic breath tests. Genetic manipulation capabilities in facultative methylotrophic bacteria have advanced significantly in the past few years (Lidstrom and Stirling, 1990).

Trotsenko *et al*. (2005) reviewed the implementation of the biotechnological potential of aerobic methylotrophs and methanotrophs for obtaining forage proteins, biopolymers (polybutyrate and polysaccharides), enzymes (oxidoreductases) and bioprotectors (ectoine), as well as for degrading toxic C_1 and C_n compounds. In conclusion, the truly revolutionary achievements of genomics and proteomics, which have important implications for basic and applied aspects of methylotrophs, are worth noting.

1.2. Xenobiotics

Anthropogenic activities have generated a number of novel chemicals that are either very rare to exist in nature or have not been reported existing till date. If they have any natural counterpart in existence then it may be degraded, but for others it is obvious that pathways for degradation may not have evolved, as a result, they are either left non-degraded or partially degraded and persist in nature (i.e. show recalcitrance). Being 'foreign' to the nature, they are called xenobiotics. Most of the xenobiotics are highly substituted high molecular weight aliphatic or aromatic compounds. These compounds are generally resistant or recalcitrant to biodegradation. They include both synthetic chemicals such as the halogenated organic compounds (halogenated hydrocarbons, halogenated aromatics, pesticides, etc.) and naturally occurring organic chemicals such as polyaromatic hydrocarbons (PAHs) and some fractions of crude oil and coal. Table 1.4 lists the common xenobiotics present in the environment and their potential industrial applications. Industrial wastewaters contain relatively high concentrations of recalcitrant xenobiotic compounds that may be toxic, mutagenic, carcinogenic, and may be bioaccumulated or biomagnified by the biota (Bitton, 2005).

Table 1.4. Industrial applications of xenobiotics

Xenobiotic	Industrial applications	References
tert-amyl alcohol	Petroleum	Piveteau *et al*., 2001
Methylamine	Pharmaceuticals, pesticides, explosives, surfactants, accelarators and dyeing industries	Doronina *et al*., 1997
2-chloroethanol	Dyes, pharmaceuticals, crop protection chemicals and plasticizers	Strotmann *et al*., 1990
tert-amyl methyl ether	Petroleum	Kharoune *et al*., 2001
Methyl *tert*-butyl ether	Petroleum	Barbera *et al*., 2011
Methacrylic acid	Surface coatings, leather and textile industries, flocculants, ion exchangers	Stellman, 1998
Epichlorohydrin	Textiles, papers, inks, dyes, ion exchange resins, insecticides, bactericides and fungicides	van den Wijngaard *et al*., 1989
Trimethylamine hydrochloride	Ion exchange resins and pesticides	Kim *et al*., 2001
Allyl chloride	Pharmaceutical industries	Yan *et al*., 2009
3-chloroaniline and 4-chloroaniline	Bactericide, biocide agent, agro chemicals, azo dyes and pigments and pharmaceuticals	Radianingtyas *et al*., 2003
1,2-dibromoethane	Grain fumigant, moth control agent in bee hives, wood preservative in timber industry, vinyl bromide, plastic and latex, in the activation of Grignard reagents and in the formulation of flame retardants, polyester dyes, resins and waxes	Poelarends *et al*., 1999
1,2-dichloroethane	Good aprotic solvent, degreaser and paint remover	Olaniran *et al*., 2009
Trichloroethylene	Solvent, food industry, dry cleaning, degreaser	Little *et al*., 1988
1,4-dichlorobenzene	Space deodorants, fumigants, production of various dyes, pharmaceuticals, and resin-bonded abrasives; an agent to control mold and mildew growth on tobacco seeds, leather, and some fabrics; and an extreme pressure lubricant	Schraa *et al*., 1986; Spain and Nishino, 1987

1.2.1. Recalcitrance of xenobiotics

Resistance to xenobiotic biodegradation can result from the following reasons: (1) Molecular structure (e.g. substitutions with chlorine and other halogens), (2) Failure of compound to enter the cell due to absence of suitable permeases, (3) Unavailability of the compound as a result of insolubility or adsorption making it inaccessible for microbial action, (4) Unavailability of the proper electron acceptor, (5) Unfavourable environmental factors such as temperature, light, pH, O_2, moisture or redox potential, (6) Unavailability of other nutrients (e.g. N, P) and growth factors necessary for microorganisms, (7) Compound toxicity affecting the biodegradation potential by microorganisms, which have numerous ways to detoxify chemicals (e.g. catabolic plasmids), (8) Low substrate concentration also affecting biodegradation by microorganisms. Organisms growing at very low substrate concentration have a high affinity for substrates (i.e. very low half-saturation constant K_s). Some microorganisms in the environment may not be able to assimilate and grow on organic substrates below a threshold concentration. Most biodegradation studies have been carried out using relatively high substrate concentrations. This is not a realistic approach as environmental concentrations are much lower (ppm or ppb level) than those that are sometimes used under laboratory conditions (Alexander, 1985). Table 1.5 shows some of the biological, chemical and environmental factors and their consequences which resist the degradation of xenobiotic compounds.

1.2.2. Adverse effects of xenobiotics

The fate of xenobiotic organic compounds (XOC) and other anthropogenic chemicals in the environment is an important domain of research. Information about their biodegradability, metabolic pathways, conjugation and de-conjugation, sorption, transport in the environment, and persistence of these xenobiotic chemicals is needed to understand their fate and impacts, and to help in the formulation of policies to protect humans and the ecosystems from their effects. The fates of xenobiotics in environment include: (1) complete mineralization or (2) stabilization of the parent compound or some metabolite of the compound in environment. If the xenobiotics are toxic and the rate of degradation is very slow, adverse effects on human and ecological health are possible (Ankumah *et al*., 1995).

Table 1.5. Factors controlling the fate of xenobiotics in the environment (Bitton, 2005)

Factors	Consequences
Chemical factors:	
Molecular weight or	Limited active transport
Polymeric nature	Extracellular metabolism required
Aromaticity	Oxygen requiring enzymes (in aerobic environment)
Halogen substitution	Lack of dehalogenating enzymes
Solubility	Competitive partitioning
Toxicity	Enzyme inhibition and cell damage
Environmental factors:	
Dissolved oxygen	O_2 sensitive and O_2 requiring enzymes
Temperature	Mesophilic temperature optimum
pH	Narrow pH optimum
Dissolved carbon	Concentration dependence or organic/pollutant complexes for growth
Particulates, surfaces	Sorptive competition for substrates
Light	Photochemical enhancement
Nutrients and trace elements	Limitations on growth and enzyme synthesis
Biological factors:	
Enzyme ubiquity	Low frequency of degradative species
Enzyme specificity	Analogous substrates not metabolized
Plasmid encoded enzymes	Low frequency of degradative species
Enzyme regulation	Repression of catabolic enzyme synthesis, required acclimation or induction
Competition	Extinction of low density populations
Habitat selection	Lack of establishment of degradative populations

The persistence of xenobiotics in soil causes different types of problems. If the pesticide remains in soil for a long period of time it may (1) be assimilated by plants and accumulate in edible portions, (2) be transported with eroding soil particles or with surface and subsurface waters to locations where its presence may cause harm or (3) may accumulate in the animal food chain and change the ecological balance of nature. Compounds that are short-lived in the environment are less likely to achieve these fates (Skladany and Metting, 1992).

Sewage treatment plants (STPs) are the usual receptors of xenobiotic compounds that have to be co-treated with municipal wastewaters before being discharged to water bodies. The presence of trace metals, metalloids and synthetic organic chemicals, such as surfactants, PAHs, phthalates, pesticides, pharmaceuticals and their primary degradation products in the influents of STPs may irreversibly inhibit sensitive biological processes, such as nitrification. It is known that the first step of nitrification, i.e. the oxidation of ammonium to nitrite, is particularly sensitive to the presence of certain chemicals. Inhibition of this step under uncontrolled conditions may completely inhibit biological nitrogen production (Dokianakis *et al*., 2006).

Xenobiotics can exert adverse effects on human health by disrupting or interacting with multiple cellular communication pathways that direct growth, development and normal physiological function. These interactions or disturbances can result in heritable and non-heritable changes in gene expression and function. Origin of xenobiotic compounds affecting humans can be exogenous or endogenous. Exogenous means the foreign molecules are not normally ingested or utilized by the organism, but they gain entry through dietary food stuffs or in the form of certain drugs used for therapeutic cause or are inhaled through environment (e.g. drugs, food additives, pollutants, insecticides, chemical carcinogens, etc.). Endogenous xenobiotics are not foreign substances but they are synthesized in the body or produced as metabolites of various processes in the body (e.g. bilirubin, bile acids, steroids, eicosanoids and certain fatty acids). Xenobiotics produce a variety of biological effects including pharmacological responses, toxicity, immunological responses and cancer.

1.2.3. Microbial degradation of xenobiotics

Biological processes play a major role in the removal of contaminants and take advantage of the astonishing catabolic versatility of microorganisms to degrade such compounds. New methodological breakthroughs in sequencing, genomics, proteomics, bioinformatics and imaging are producing vast amounts of information. In the field of environmental microbiology, genome-based global studies open a new era providing unprecedented *in silico* views of metabolic and regulatory networks, as well as clues to the evolution of degradation pathways and the molecular adaptation strategies to changing environmental conditions. Functional genomic and metagenomic approaches

are increasing our understanding of the relative importance of different pathways and regulatory networks to carbon flux in particular environments and for particular compounds and they will certainly accelerate the development of bioremediation technologies and biotransformation processes.

Microbes found in natural waters and soils have a broad ability to utilize (catabolize) many xenobiotic compounds as their sources of carbon and energy, thus recycling the fixed organic carbon back into harmless biomass and CO_2 (Table 1.6). Identification of such microorganisms and development of a suitable consortium system with favourable biological, chemical and environmental parameters, can effectively biodegrade or biotransform the toxic pollutants generally found in wastewater plants.

Table 1.6. Microorganisms utilizing xenobiotic compounds for their growth

Xenobiotics	Degrading microorganisms	References
tert-amyl alcohol	*Burkholderia cepacia* CIP I-2052	Piveteau *et al*., 2001
Methylamine	*Pseudomonas* sp. strain MA	Bellion *et al*., 1981
2- chloroethanol	*Mycobacterium* sp. strain GP1	Poelarends *et al*.,1999
	Pseudomonas stutzeri	Dijk *et al*., 2004
Methyl *tert*-butyl ether	*Methylobacterium, Rhodococcus* and *Arthrobacter*	Mo *et al*., 1997
Epichlorohydrin	*Pseudomonas, Arthrobacter*	van den Wijngaard *et al*., 1989
	Xanthobacter strain Py2	Small *et al*., 1995
Allyl chloride	*Chloroflexi* strain BL-DC8 and BL-DC9	Yan *et al*., 2009
3-chloroaniline and 4-chloroaniline	*Acinetobacter baumanii* CA2	Vangnai and Petchkroh, 2007
	Pseudomonas putida CA16	
1,2-dibromoethane	*Mycobacterium* sp. strain GP1	Poelarends *et al*., 1999
1,2-dichloroethane	*Ancylobacter aquaticus*	van den Wijngaard *et al*., 1992
	Xanthobacter autotrophicus G10	Baptista *et al*., 2006
Trichloroethylene	Methanotroph strain 46-1	Little *et al*., 1988
1,4-dichlorobenzene	*Xanthobacter flavus* 14p1	Spiess *et al*., 1995
	Alcaligenes sp. strain A175	Schraa *et al*., 1986
	Pseudomonas sp.	Spain and Nishino, 1987

In case of microbial degradation, the xenobiotic may be consumed as a source of carbon and/or nitrogen and/or energy. For example, *Desulfomonile tiedjei*, a bacterium derives energy from dechlorination of chlorobenzoates (Mohn and Tiedje, 1991). Alternatively it may be transformed/degraded as a part of co-metabolic activity where a non-growth substrate is utilized in the obligate presence of another growth substrate. For example, lipid enrichment is required to degrade hydrocarbons such as hexane. Co-metabolism does not lead to energetic or nutritional gain. Co-metabolic degradation may be gratuitous (unprovoked or unjustifiable) or fortuitous (accidental or chance) as the degradation takes place due to similarity of the xenobiotic to a natural substrate or as a result of non-specificity of the enzyme. Here, it may be difficult to induce the enzyme in absence of a natural substrate. Co-metabolic degradation can still be adaptive, as it may reduce local toxicity. Methanotrophs express an enzyme called MMO, which is quite unspecific. It is used by microorganisms to degrade a number of hydrocarbons (Little *et al*., 1988). In general, xenobiotics cannot be degraded to CO_2 and H_2O by 1 type of organism alone and, thus, it requires cooperation of many other organisms.

Transformation does not always yield a simpler molecule. Sometimes an intermediate product might be more toxic than the original xenobiotic, e.g. Trichloroethylene (TCE) > Polychloroethylene > Vinyl chloride. A xenobiotic can be biodegraded aerobically as well as anaerobically (Yan *et al*., 2009). Hydrocarbons (petroleum) are quite stable under anaerobic conditions. The more chlorinated an organic molecule is the more readily it will be degraded anaerobically. The rate slows down with the loss of chlorine (Dijk *et al*., 2004). Hence, use of methylotrophs can be prompted in order to degrade and further minimize wastes, especially hazardous wastes. This combination is fully eco-friendly and increasingly of interest as part of a long-term waste minimization strategy for industry. Therefore, the concept that the methylotrophic bacteria could serve as non-polluting multistage catalysts using the wastes as feedstock is a highly effective one.

1.2.4. Xenobiotic degradation by methylotrophs

A large number of methylotrophs capable of degrading a variety of xenobiotics have been reported in literature. Evidence for the occurrence of the non-

enzymatic route involving an initial oxygen incorporation step with the formation of trimethylamine N-oxide in trimethylamine-grown *Pseudomonas aminovorans* and *Hyphomicrobium vulgare* NQ has been reported (Large *et al*., 1972). Microbial enzymes catalyzing the demethylation of trimethylamine N-oxide yielding dimethylamine and formaldehyde have been observed in *Bacillus* PM6 (Myers and Zatman, 1971) and *P. aminovorans* (Large, 1971). 12 methylotrophic bacterial isolates that grow on methylamine, dimethylamine and trimethylamine as sole source of carbon and energy were obtained by Colby and Zatman (1973). The obligate methylotrophs, bacteria 4B6 and C2A1, and the facultative methylotrophs, bacterium 5B1 and *Pseudomonas* 3A2, all grew on trimethylamine and oxidized trimethylamine, dimethylamine, formaldehyde and formate; only bacterium 5B1 and *Pseudomonas* 3A2 oxidized trimethylamine N-oxide (Colby and Zatman, 1973).

Bellion *et al*. (1980) described an uptake system for methylamine in a methylotrophic bacterium *Pseudomonas* sp. strain MA. The organism had been grown on methylamine as the sole source of carbon and nitrogen for this study. The uptake of methylamine as the sole nitrogen, but not carbon, source by *Pseudomonas* sp. strain MA was investigated by Bellion and Wayland (1982). Methylotrophs of the genera *Hyphomicrobium* and *Methylobacterium* dehalogenate dichloromethane (DCM) to formaldehyde by a glutathione transferase reaction catalyzed by the DCM dehalogenase (Kohler-Staub and Leisinger, 1985).

Fogel *et al*. (1986) and Janssen *et al*. (1987) have shown that mixed cultures enriched by growth on methane degraded several chlorinated derivatives of methane, ethane and ethylene. Vinyl chloride was degraded more rapidly than dichloroethylene and TCE, whereas DCM was oxidized faster than chloroform (McCarty, 1988). Little *et al*. (1988) reported the oxidation of TCE by a pure culture of a methane-utilizing bacterium. *Methylosinus trichosporium* oxidized dichloroethylene, dichloroethanes and vinyl chloride (Fox *et al*., 1990). Hanson and Hanson (1996) observed that 4 species of type II methanotrophs classified in the genus *Methylosinus* and the type X methanotroph *Methylococcus capsulatus* (Bath) were able to degrade TCE. *Methylosinus trichosporium* OB3b was shown to oxidize TCE much more rapidly than did the nitrifying bacteria that possess ammonia monooxygenase (Arciero *et al*., 1989), *Pseudomonas cepacia* that possesses toluene 2-monooxygenase (Folsom *et al*., 1990), *Pseudomonas mendocina* containing toluene 4-monooxygenase (Winter *et al*., 1989), *Pseudomonas putida* F1 containing toluene dioxygenase and *Mycobacterium*

sp. with propane monooxygenase (Wackett and Gibson, 1988). Alvarez-Cohen (1993) has studied the degradation of TCE by a mixed culture of bacteria enriched with methane and oxygen from aquifer material from Moffett Field Naval Air Station, Mountain View, California. TCE is completely degraded by mixed cultures of bacteria that grow with methane as the sole carbon and energy source (Chang and Alvarez-Cohen, 1995).

Methylotrophic DCM-degrading strains express a glutathione-dependent DCM dehalogenase that is encoded by the gene *dcmA* (Bader and Leisinger, 1994) and is one of the bacterial glutathione *S*-transferases whose function is known (Vuilleumier, 1997). The thiolytic substitutive dehalogenation process has been extensively studied in methylotrophic bacteria. Dechlorination of DCM by facultative methylotrophic bacteria is catalyzed by inducible glutathione *S*-transferases. DCM is converted to formaldehyde and inorganic chloride with *S*-chloromethylgutathione as intermediate and the formaldehyde so formed is a central metabolite of methylotrophic growth (Fetzner, 1998). *Pseudomonas* strains, *Hyphomicrobium* strains and several *Methylobacterium* sp. strains have been shown to contain these enzymes (Bhatt *et al*., 2007). *Methylobacterium* sp. strain DM4 and *Methylophilus* sp. strain DM11 can grow with DCM as the sole source of carbon and energy by virtue of homologous glutathione-dependent DCM dehalogenases with markedly different kinetic properties (Gisi *et al*., 1998). 3 aerobic DCM-utilizers have been fully characterized and described as representatives of the new taxa, *Methylorhabdus multivorans DM13* (Doronina *et al*., 1995), *Methylophilus leisingerii* DM11 (Doronina and Trotsenko, 1994) and *Paracoccus methylutens* DM12 (Doronina *et al*., 1998).

Methylopila helvetica sp. nov. strain DM9 and *Methylobacterium dichloromethanicum* sp. nov. strain DM4 utilized DCM, methanol and methylamine as well as a variety of polycarbon compounds as the carbon and energy source (Doronina *et al*., 2000). *Burkholderia cepacia* CIP I-2052, a methylotroph isolated from an activated sludge sample, was able to use *tert*-butyl alcohol (TBA), a product of methyl *tert*-butyl ether and ethyl *tert*-butyl ether degradation, as well as *tert*-amyl alcohol as its sole carbon and energy source (Piveteau *et al*., 2001).

Methanotrophs have been studied for their potential to be used directly in bioremediation due to the MMO enzyme(s) they possess. These oxygenases have broad substrate specificity and have been shown to co-oxidize pollutants such as aromatics (Jechorek *et al*., 2003) and TCE (Bowman *et al*., 1993). 4 novel

methylotrophic bacterial strains, isolated from a range of soil and sediment sources (both pristine and polluted) under different enrichment regimes, can be considered for their ability to withstand extremely high concentrations of a variety of pollutants (de Marco *et al*., 2004).

1.2.5. Methyl *tert*-Butyl Ether (MTBE) biodegradation

Fuel oxygenates, mainly MTBE, are added to gasoline in replacement of lead tetraethyl to enhance its octane index. Its addition also improves the combustion efficiency and, therefore, decreases the emission of pollutants (CO and hydrocarbons). On the other hand, MTBE, being highly soluble in water (water solubility = 48 g/l) (Kharoune *et al*., 2001) and recalcitrant to biodegradation, is a major pollutant of water in aquifers contaminated by MTBE-supplemented gasoline during accidental release. When an MTBE-supplemented gasoline is spilled, the plumes generated by MTBE are indeed much larger than those generated by benzene (water solubility = 1.75 g/l) (Lopes Ferreira *et al*., 2006).

The retardation factor (ratio of water speed to pollutant speed) was 1.8 for benzene and 1.1 for MTBE, which means that MTBE migrates practically as rapidly as the aquifer. This is the consequence of (1) its low adsorption onto organic matter, (2) its high solubility and (3) its recalcitrance to biodegradation. Thus, the half-life of MTBE in groundwater is estimated as 2-3 years, whereas that of benzene is 2-3 months under similar conditions. MTBE was shown to be degraded either by co-metabolism or by being used as a sole carbon and energy source by a number of microorganisms (Fayolle and Monot, 2005). Table 1.7 lists the microorganisms able to biodegrade MTBE.

The different MTBE degradation pathways proposed are summarized in Figure 1.6. Hanson *et al*. (1999) isolated a facultative methylotroph, *Methylibium petroleiphilum* PM1^{T} gen. nov., sp. nov., capable of degrading MTBE. No TBA accumulated during growth on MTBE, even when it was provided at a concentration of 500 mg/l. The strain was also able to grow on the metabolic intermediates of MTBE, viz. *tert*-butyl formate (TBF), TBA and hydroxyisobutyric acid (HIBA). Steffan *et al*. (2000) isolated *Hydrogenophaga flava* ENV735 for its capacity to grow on MTBE. TBA and HIBA were detected as degradation intermediates during growth on MTBE, whereas no TBF could be detected, which would suggest that *tert*-butoxy

methanol undergoes a direct dismutation to TBA and formaldehyde (Figure 1.6). *Mycobacterium austroafricanum* IFP 2012 was isolated from an activated sludge for its capacity to grow on TBA and was shown to grow on MTBE by Francois *et al*. (2002), with the detection of several intermediates of MTBE degradation, viz., TBF, TBA, 2-methyl 1,2-propanediol (2-M1,2-PD), HIBA and acetone (Lopes Ferreira *et al*., 2006).

Table 1.7. MTBE degrading microorganisms

MTBE degrading microorganisms	**References**
Rhodococcus aetherivorans IFP 2017 *Rhodococcus wratislaviensis* IFP 2016	Auffret *et al*., 2009
Achromobacter xylosoxidans MCM1/1 *Enterobacter cloacae* MCM2/1 *Ochrobactrum anthropi* MCM5/1 *Exophiala dermatitidis* MCM3/4	Barbera *et al*., 2011
Methylibium petroleiphilum PM1	Chen *et al*., 2007; Kane *et al*., 2007
Mycobacterium austroafricanum IFP 2012	Fayolle *et al*., 2003; Francois *et al*., 2002; Lopes Ferreira *et al*., 2006
Gordonia terrae strain IFP 2001	Hernandez-Perez *et al*., 2001
Ochrobactrum cytisi	Lin *et al*., 2007
Hydrogenophaga flava ENV735	Steffan *et al*., 2000
Mycobacterium vaccae JOB5	Johnson *et al*., 2004; Smith *et al*., 2003
Methylobacterium mesophilicum *Rhodococcus* sp. *Arthrobacter ilicis*	Mo *et al*., 1997
Pseudomonas putida CAM	Steffan *et al*., 1997

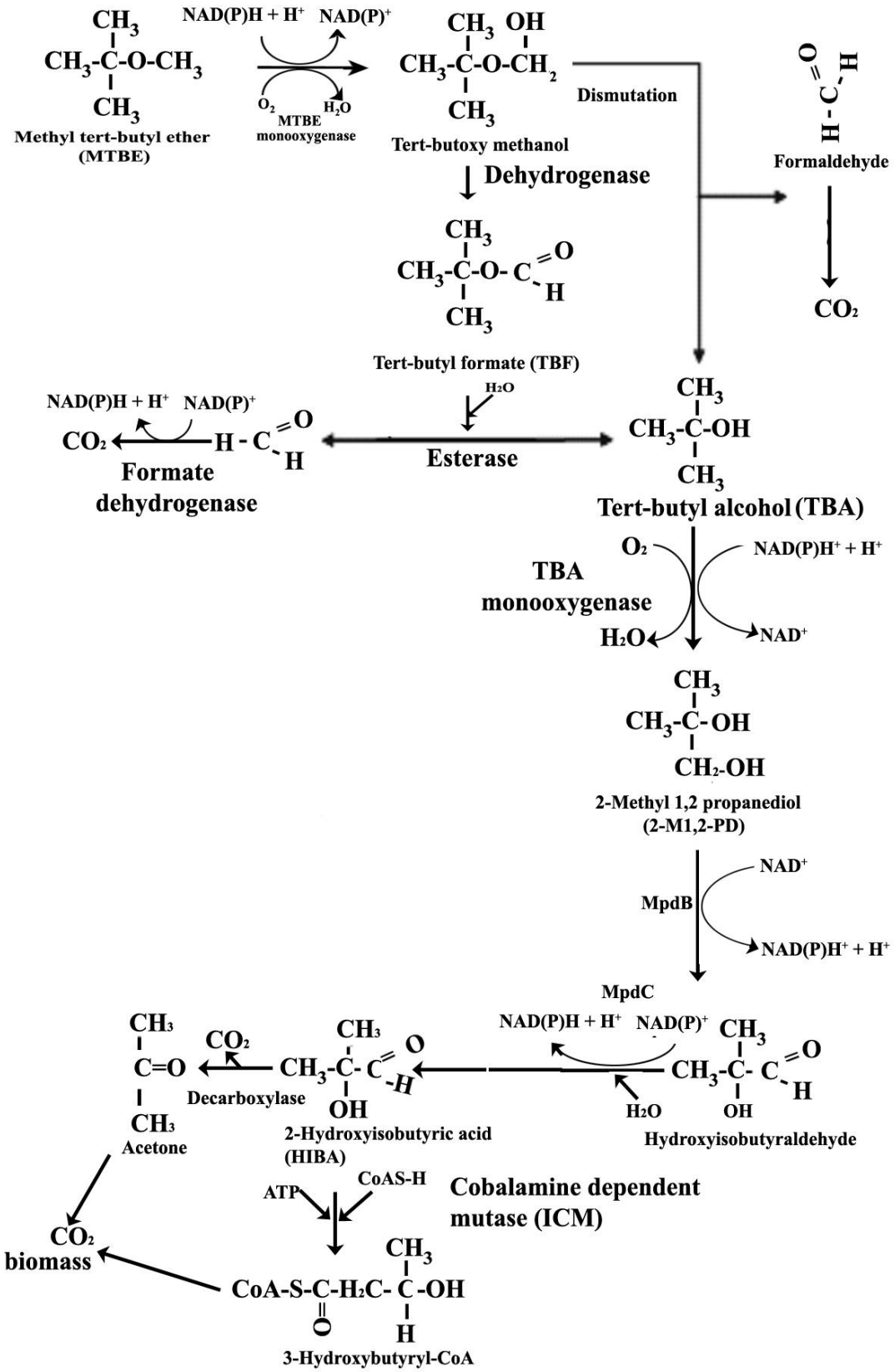


Figure 1.6. Different pathways followed by microorganisms during growth on MTBE (Lopes Ferreira *et al*., 2006)

1.2.6. 1,2-Dichloroethane (DCE) biodegradation

The bacterial degradation of chlorinated synthetic compounds is dependent on enzymes that recognize molecules that do not occur at significant concentrations in nature. The chlorinated compound with the largest production volume is DCE. Its industrial synthesis started in 1923 (Janssen *et al*., 1994). DCE is mainly used for the synthesis of vinyl chloride for polyvinyl chloride (PVC) production, the synthesis of amines and other derivatives, and as a solvent in pharmaceutical synthesis. Environmental contamination of DCE is usually caused by accidents, such as leaking tanks or pipelines and accidental spillage. The catabolic pathway for DCE has been resolved in *Xanthobactor autotrophicus* GJ10 and strains of *Ancylobacter aquaticus* (Janssen *et al*., 1985; van den Wijngaard *et al*., 1992). The first step is the hydrolytic conversion of DCE to 2-chloroethanol (CE). This intermediate is oxidised to chloroacetaldehyde by a periplasmic alcohol dehydrogenase. Further oxidation by an NAD-dependent aldehyde dehydrogenase yields chloroacetate, which is cleaved by a second hydrolytic dehalogenase to glycolate. The glycolate formed further enters the central metabolic pathway (Figure 1.7). DCE can also be biodegraded by reductive dehalogenation which involves the removal of a halogen substituent from a molecule with concurrent addition of electrons to the molecule (Maymo-Gatell *et al*., 1999). *Dehalococcoides* spp. and *Dehalobacter* spp. are some of the examples of bacteria degrading DCE by reductive dehalogenation (van der Zaan *et al*., 2009). Table 1.8 lists the microorganisms able to degrade DCE.

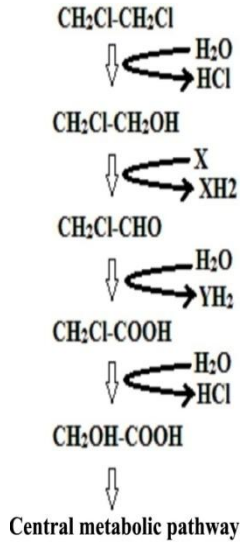


Figure 1.7. The catabolic pathway of DCE in *Xanthobacter autotrophicus* (Janssen *et al*., 1985)

Table 1.8. DCE degrading microorganisms

DCE degrading microorganisms	References
Xanthobacter autotrophicus GJ10	Baptista *et al*., 2006; Janssen *et al*., 1985
Pseudomonas sp. strain DCA1	Hage and Hartmans, 1999
Pseudomonas sp. DM1 *Hyphomicrobium* sp. DM2 *Pseudomonas* sp. DM4 *Hyphomicrobium* sp. GJ21	Kohler-Staub *et al*., 1986
Ancylobacter aquaticus AD20, AD25, AD27	van den Wijngaard *et al*., 1992
Klebsiella oxytoca VA 8391	Mileva *et al*., 2008
Dehalococcoides spp. *Dehalobacter* spp. *Desulfitobacterium* spp. *Sulfurospirillum* spp.	van der Zaan *et al*., 2009
Dehalococcoides ethenogenes 195	Maymo-Gatell *et al*., 1999
Desulfitobacterium dichloroeliminans strain DCA1	Marzorati *et al*., 2007

1.3. Wastewater treatment

Raw wastewaters are since long being discharged directly to receiving waters hoping their self-purification would take care of the waste. Unfortunately, most receiving bodies failed to do so as they were overcharged with organic and nitrogen pollution resulting in a chronic state of degradation. In most cases, wastewater treatment is, therefore, needed before effluent discharge. One of the most popular types of treatment is the biological one which more or less mimics some of the natural processes found in a self-purifying receiving body, mainly organic degradation and nitrogen conversion through bacterial action. The overall objectives of the biological treatment of domestic wastewater are to: (1) transform (i.e. oxidise) dissolved and particulate biodegradable constituents into accepted end products, (2) capture and incorporate suspended and non-settleable colloidal solids into a biological floc or biofilm, (3) transform and reuse waste as nutrients and (4) in some cases, remove specific trace organic constituents and compounds. For industrial wastewater, the objective is to remove or reduce the concentrations of organic and inorganic

compounds. Because some of the constituents and compounds present in industrial wastewater are toxic to microorganisms, pre-treatment may be required before the industrial wastewater can be discharged to a municipal collection system. For agricultural irrigation return wastewater, the objective is to remove nutrients, specifically nitrogen and phosphorus, which are capable of stimulating the growth of aquatic plants (Tchobanoglous *et al*., 2003).

The principal biological processes for wastewater treatment can be divided into 2 main categories: suspended growth and attached growth (or biofilm) processes (Table 1.9). The successful design and operation of the processes require an understanding of the types of microorganisms involved, the specific reaction that they perform, the environmental factors that affect their performance, their nutritional needs and their reaction kinetics (Tchobanoglous *et al*., 2003).

1.3.1. Activated sludge process

The activated sludge process is a widely used aerobic suspension type of liquid waste treatment system (Figure 1.8). After primary settling, the sewage containing dissolved organic compounds is introduced into an aeration tank. Air injection and mechanical stirring provide the aeration. The rapid development of microorganisms is also stimulated by the reintroduction of most of the settled sludge from a previous run. Thus, the process derives its name from this inoculation with activated sludge (Atlas, 1997).

During the holding period in the aeration tank, the heterogeneous nature of organic substrates in sewage allows the vigorous development of diverse heterotrophic bacterial populations, including Gram-negative rods, predominantly *Escherichia*, *Enterobacter*, *Pseudomonas*, *Achromobacter*, *Flavobacterium* and *Zooglea* sp.; other bacteria, including *Micrococcus*, *Arthrobacter*, *Bdellovibrio*, various coryneforms and mycobacteria, *Sphaerotilus*, *Nocardia*; the 2 most common nitrifying bacteria, *Nitrosomonas* and *Nitrobacter*, and other large filamentous bacteria; and low numbers of filamentous fungi, yeasts and protozoa, mainly ciliates. The protozoa and rotifers are important predators of bacteria; they control the growth of bacterial population. The bacteria in the activated sludge tank occur in free suspensions and as aggregates and flocs.

Table 1.9. Major biological processes used for wastewater treatment (Tchobanoglous *et al*., 2003)

Type	Common name	Use
Aerobic processes		
Suspended growth	Activated sludge process	Carbonaceous BOD removal, nitrification
	Aerated lagoons	Carbonaceous BOD removal, nitrification
	Aerobic digestion	Stabilization, carbonaceous BOD removal
Attached growth	Trickling filters	Carbonaceous BOD removal, nitrification
	Roughing filters	Carbonaceous BOD Removal
	Rotating biological contactors	Carbonaceous BOD removal, nitrification
	Packed-bed reactors	Carbonaceous BOD removal, nitrification
Hybrid (combined) suspended and attached	Trickling filter/activated sludge	Carbonaceous BOD removal, nitrification
Anoxic processes		
Suspended growth	Suspended-growth denitrification	Denitrification
Attached growth	Attached-growth denitrification	Denitrification
Anaerobic processes		
Suspended growth	Anaerobic contact processes	Carbonaceous BOD removal
	Anaerobic digestion	Stabilization, solids destruction, pathogen kill
Attached growth	Anaerobic packed and fluidized bed	Carbonaceous BOD removal, waste stabilization, denitrification
Sludge blanket	Upflow anaerobic sludge blanket	Carbonaceous BOD removal, especially high-strength wastes
Hybrid	Upflow sludge blanket/attached growth	Carbonaceous BOD removal
Combined aerobic, anoxic and anaerobic processes		
Suspended growth	Single or multi stage processes, various proprietary processes	Carbonaceous BOD removal, nitrification, denitrification and phosphorus removal
Hybrid	Single or multi stage processes with packing for attached growth	Carbonaceous BOD removal, nitrification, denitrification and phosphorus removal
Lagoon processes		
Aerobic lagoons	Aerobic lagoons	Carbonaceous BOD removal
Maturation (tertiary) lagoons	Maturation (tertiary) lagoons	Carbonaceous BOD removal, nitrification
Facultative lagoons	Facultative lagoons	Carbonaceous BOD removal
Anaerobic lagoons	Anaerobic lagoons	Carbonaceous BOD removal, waste stabilization

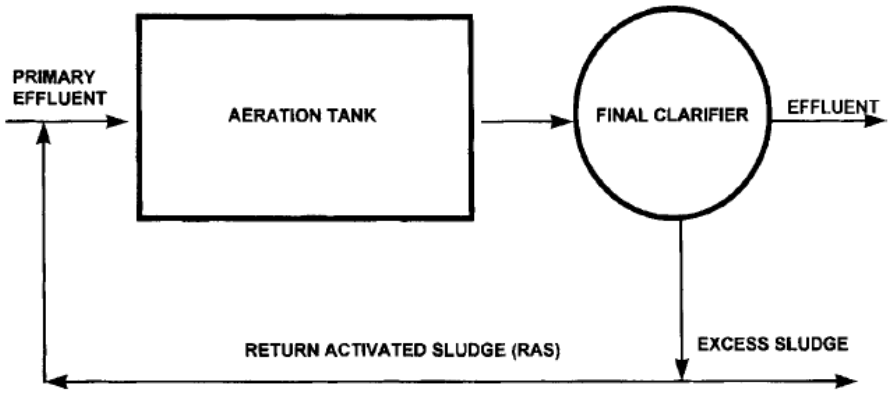


Figure 1.8. Schematic diagram of a conventional activated sludge system (Bitton, 2005).

The flocs comprise microbial biomass held together by bacterial slimes. Most of the ciliate protozoa, like *Vorticella*, are of the attached filter-feeding type. They adhere to the flocs, while feeding predominantly on the suspended bacteria. The floc is too large to be ingested by the ciliates and rotifers and, hence, acts as a defence mechanism against predation. Suspended bacteria predominate in the raw sewage, but during the holding period in the aeration tank their number decreases, while the bacteria associated with flocs greatly increase in number (Tchobanoglous *et al*., 2003).

A significant portion of the dissolved organic substrates is mineralized and another portion is converted to microbial biomass as a result of the microbial metabolism of organic compounds in the sewage. In the later stages of aeration, most of the microbial biomass becomes associated with flocs that can be removed from the suspension by settling. The settling characteristic of the sewage sludge flocs is critical for their efficient removal. Poor settling produces 'bulking' of sewage sludge, caused by proliferation of filamentous bacteria such as *Sphaerotilus*, *Beggiatoa*, *Thiothrix* and *Bacillus* and filamentous fungi such as *Geotrichum*, *Cephalosporium*, *Cladosporium* and *Penicillium*. Bulking is frequently associated with high C:N (Carbon:Nitrogen) and C:P (Carbon:Phosphorus) ratios and/or low dissolved oxygen concentrations. A portion of the settled sewage sludge is recycled into the aeration tank for use as an inoculum for the incoming raw sewage. The remaining portion of the sludge requires additional treatment by composting or anaerobic digestion.

The activated sludge process along with primary settling reduces the Biological Oxygen Demand (BOD) of the effluent to 10-15 % of that of the raw

sewage. The treatment also reduces the number of intestinal pathogens in the sewage. This reduction is due to the combined effects of competition, adsorption, predation and settling. Ciliates, rotifers and *Bdellovibrio* are predators of pathogens and non-pathogenic heterotrophs. Non-pathogenic heterotrophs proliferate vigorously. Hence, the non-pathogenic heterotrophs reproduce to compensate for their removal by predation, whereas the pathogens continuously decrease in number. Settling of the flocs also removes additional pathogens.

In activated sludge process, a variety of terms are used which require precise definition. The most important ones are listed below (Verstraete and van Vaerenbergh, 1986).

The amount of oxygen required to oxidize by chemical means organic carbon compounds completely to CO_2 and H_2O is referred to as Chemical Oxygen Demand (COD). In practice, organic matter in water is oxidized by $K_2Cr_2O_7$ under rather stringent conditions (concentrated H_2SO_4 medium, 160 ^oC). The amount of dichromate oxygen used is determined and expressed as COD. As a thumb rule, 1 g carbohydrate or 1 g protein equals about 1 g COD. The COD does not comprise the oxygen needed to convert the reduced nitrogen to nitrate. Reduced sulphur (R-SH, S^{2-}), is oxidized to sulphate (SO_4^{2-}) by the chemical reagents and, therefore, included in the COD-value. An important advantage of the method is that it quantifies both dissolved and particulate organic matter. In view of the fact that wastewater treatment deals with the removal of both types of organic matter, the COD measurement is widely used as a quantitative parameter.

BOD is defined as the amount of oxygen (mg/l or mg/kg) used by the non-photosynthetic microorganisms at 20 ^oC to metabolize biologically degradable organic compounds. The oxygen consumed is measured over a period of 5 d (normally noted as BOD_5) to obtain a stable reading. During the first 2 d, microbial cells rapidly metabolize the available organics. From day 2 onwards, the substrate becomes limited and the cells shift to endogenous metabolism. Such kinetics are obtained by providing appropriate environmental conditions for the test such as: (1) neutral pH, (2) presence of sufficiently large acclimatized microbial inoculum, (3) presence of adequate amounts of necessary mineral nutrients for microbial growth (N, P, Ca, Mg, Fe and S) and (4) incubation in the dark.

When microorganisms aerobically metabolize 1 g COD, they immobilize about half of that organic matter in the form of biomass and consume oxygen to

oxidize the other half. This removal can be either immobilization into cell biomass or oxidation to CO_2 and H_2O. Upon prolonged incubation, the cells will completely mineralize and the so-called ultimate BOD (BOD_∞) approaches the initial COD value. In order to design an activated sludge treatment plant, it is necessary to know how much biodegradable organic matter is present in the wastewater. It is, therefore, needed to determine the BOD_5. However, it is advisable to measure in parallel the COD value. Furthermore, the BOD_5/COD ratio can be used to monitor and operate the treatment plant.

Volatile Suspended Solids (VSS) actually represents the amount of organic matter, present in particulate form in the water. It is often used to indicate the approximate amount of activated sludge present in the aeration tank. Ash Free Dry Weight (AFDW) is a crude measure of the total amount of organic matter (soluble and suspended) present in the water.

The influent is often subjected to pre-treatment before entering the biological treatment. Typical pre-treatments are: bar screen or rack, grit remover, fat trap and primary settler to remove coarse solids which decant readily. The biological treatment process comprises both the removal and breakdown of dissolved particulate organic matter.

The effluent flowing over the final clarifier should be free of dissolved and suspended organic matter and contain a minimum of mineral components such as NH_4^+, NO_3^- and PO_4^{3-} because the latter could subsequently induce eutrophication. Typical criteria for discharge on inland surface waters are: COD - 100 mg/l, BOD_5 - 20 mg/l and SS - 30 mg/l.

The mixture of sludge and water in the aeration tank is called mixed liquor. The mixed liquor suspended solids (MLSS) represents the total amount of sludge present in the mixed liquor. The term which more closely reflects the active biomass is the mixed liquor volatile suspended solids (MLVSS). Only part of the organic fraction of the sludge is truly viable microbial biomass. The efficiency of the activated sludge treatment depends both on the degradation activity of the biomass in the aeration tank and on the separation of the total amount of suspended solids from the effluent in the final clarifier. Hence, it is normally preferred to model and design on the basis of the VSS as a reasonable approximation of both active biomass and total sludge.

The volume (in ml) occupied by 1 g of sludge (MLSS) after 30 min of sedimentation is called the sludge volume index (SVI). Good settling sludge has an SVI of 40-60 ml/g DW; for bulking sludge the SVI is 200 or more. This parameter is of major importance in an activated sludge operation. In case the sludge does not flocculate or sediment, no clearing is obtained.

The amount of substrate added daily for bacterial metabolism is called the sludge loading rate (B_x). The substrate is usually expressed as COD or BOD. The average time of residence of the sludge in the aerated system (Solids or Cell Residence Time, SRT, θ_x) is mainly controlled by the rate of sludge withdrawal from the reactor. The sludge wastage is normally operated so that the concentration of sludge remains in the appropriate range. The imposed loading rate should give sludge growth values for θ_x in the range of 10-20 d.

The amount of biomass formed per kg substrate removed varies according to the type of waste treated and the treatment parameters imposed (temperature, B_x, pH, etc.). 1 kg of substrate removed means 1 kg of oxygen demand removed in the context of wastewater treatment. The oxygen required can comprise only the BOD load or the BOD and the NOD (Nitrification Oxygen Demand) loads together, depending on the type of treatment. Hence, the oxygenation capacity (OC) load ratio should be in the range of 1.5-2.5.

1.3.2. Biofilm mediated wastewater treatment

1.3.2.1. Biofilms

Biofilms can be defined as communities of bacteria attached to a surface. It is the evolutionary strategy used by microorganisms to enhance the ability of a community to adapt to quick changes in environmental selective pressures. Bacterial extracellular polymers are the construction materials of the biofilm. They form a 3-dimensional, gel-like, highly hydrated and often charged biofilm matrix, in which the microorganisms are embedded (Sutherland, 1977). This matrix interacts with the environment; e.g. by attaching biofilms to surfaces and through its sorption properties, which allow for sequestering of dissolved and particulate substances from the environment, providing nutrients for biofilm organisms. The matrix also facilitates the retention of exo-enzymes, cellular debris and genetic material; it can be considered as a microbial recycling yard (Flemming *et al*., 2007). In the biofilm, microorganisms

can establish stable arrangements and function multicellularly as synergistic microbial consortia. By pooling their biochemical resources, several species of bacteria, each armed with different enzymes, can break down food supplies that no single species could digest alone. Compared with their planktonic counterparts, the compact microbial consortia present in biofilms show extraordinary resistance to conventional biocides and antimicrobial treatments.

Development of biofilm is initiated by the attachment of bacteria to a solid surface (Figure 1.9). Permanent attachment of bacteria requires 2 stages: (1) reversible attachment, which is a transitory physicochemical attraction and (2) irreversible attachment, which is a biologically mediated stabilization reaction. The initial reversible attachment of organisms to surfaces can become permanent, with time. Irreversible attachment is initiated by the excretion of extracellular polymers by the reversibly attached bacteria. The extracellular polymers create a matrix that surrounds the cell and forms a strong chemical bridge to the solid surface. The transition from initial microbial attachment to the development of a mature biofilm is due to both the proliferation of the attached cells and the deposition of cells from the bulk fluid (Maier *et al*., 2000). The properties of a solid surface may be modified by the presence of an adherent conditioning film of organic compounds and bacterial attachment can be promoted by coating of dissolved organic matter of limited water solubility on the surface of solid substrates.

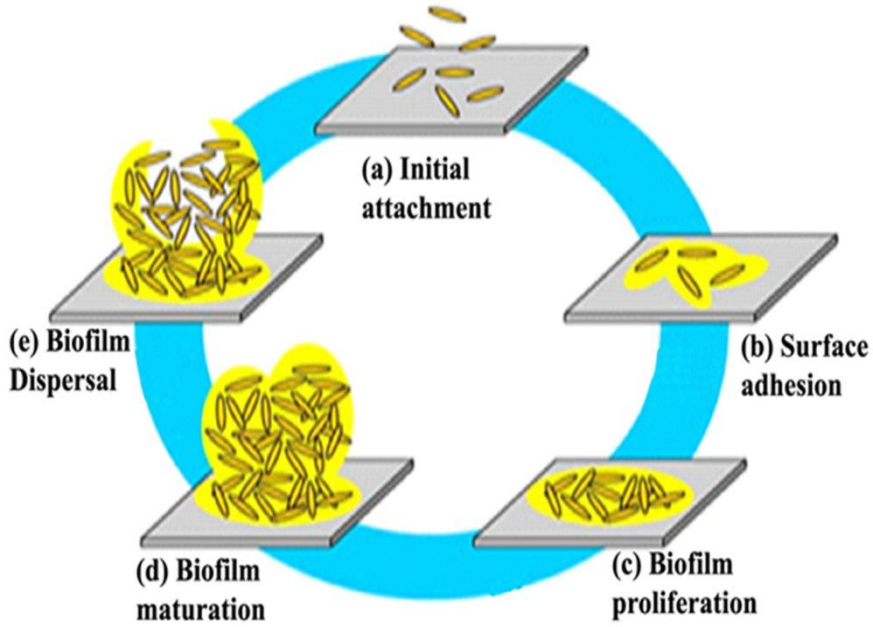


Figure 1.9. Biofilm growth cycle (Rosche *et al*., 2009)

1.3.2.2. Moving bed biofilm reactor (MBBR)

The MBBR has been successfully used for biological treatment of industrial wastewater (Rusten *et al*., 2006). Figure 1.10 depicts an ideal set-up of MBBR. The MBBR has many salient features: (1) Robust – (a) Stable under load variations, (b) Insensitive to temporary limitation, (c) Consistent treatment results; (2) Efficient – (a) Low solids generation, (b) Low or no polymer required for liquid/solids separation; (3) Compact – (a) Small footprint, (b) Low capital cost; (4) Flexible – (a) Customizable reactor shapes, (b) Utilization of existing tanks, (c) Upgradation of existing plants; (5) Trouble-free – (a) Easy to operate, (b) No media clogging, (c) No sludge return.

The MBBR was developed in Norway in the late 1980s and early 1990s. It is covered by several patents and has been a huge success world-wide for treatment of municipal and industrial wastewaters (Rusten *et al*., 2006). Contrary to most biofilm reactors, the MBBR utilizes the whole tank volume for biomass growth. It also has a very low head-loss. Contrary to the activated sludge reactor, it does not need any sludge recycle. This is achieved by having the biomass grow on carriers that move freely in the water volume of the reactor and are kept within the reactor volume by a sieve arrangement at the reactor outlet. The reactor may be used for aerobic, anoxic or anaerobic processes. In aerobic processes, the biofilm carrier movement is caused by the agitation set up by the air, while in anoxic and anaerobic processes a mixer (normally a horizontal shaft mounted banana mixer) keeps the carriers moving. Proper design of aeration grids and sieves is very important for optimum performance of the MBBR process. An important advantage of the MBBR is that the filling fraction of biofilm carriers in the reactor may be subject to preferences. In order to be able to move the carrier suspension freely, it is recommended that filling fractions should be below 70 %. As in every biofilm process, diffusion of compounds in and out of the biofilm plays a key role. Because of the importance of diffusion, the thickness of the effective biofilm (depth of the biofilm to which the substrates have penetrated) is important. Since this depth of full substrate penetration is normally less than 100 µm, the ideal biofilm in the moving bed process is thin and evenly distributed over the surface of the carrier. In order to obtain this, the turbulence in the reactor is of importance, both in order to transport the substrates to the biofilm and to maintain a low thickness of the biofilm by shearing forces (Rusten *et al*., 2006).

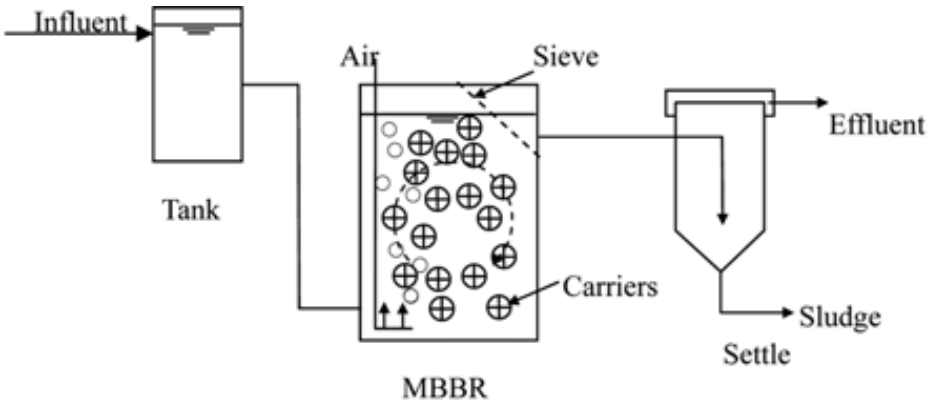


Figure 1.10. Schematic diagram of a MBBR (Jing *et al*., 2009)

The biofilm carrier elements are made of high density polyethylene and are shaped like small cylinder (10 mm x 7 mm) with a cross inside the cylinder and longitudinal fins on the outside. Depending on the treatment required and strength of a given wastewater, the filling of carrier elements in a reactor can be decided for each case giving considerable flexibility in the specific biofilm surface area. The reactor volume is totally mixed and consequently there is no dead or unused space in the reactor. Different reactor shapes can be used and the MBBR process is ideal for upgrading of overloaded treatment plants or converting unused tanks into biofilm reactors (Johnson *et al*., 2000). Two Kaldnes MBBR™ full scale treatment plants were commissioned by Johnson *et al*. (2000) in 1999, following successful pilot tests which generated the design data. The MBBR plants were incorporated into each facility as roughing reactors ahead of existing activated sludge processes.

MBBRs have increasing application in domestic and industrial wastewater treatment, making them worthy of intensive study. Attributes which differentiate them from other biological wastewater treatment systems have been identified as: simplicity, low space requirement, and no sludge separation requirement for effective operation (advantages over activated sludge); low head loss, no channelling or requirement for backwashing (advantages over fixed-bed biofilters/trickling filters); large surface area for colonization, and high specific biomass activity; protection of slow growing microorganisms from excessive abrasive removal (advantage over turbulent reactor biofilm systems such as fluidized beds); versatility; ability for retrofitting into existing tank volumes and/or addition to existing treatment systems to improve overall performance (Gapes and Keller, 2009). Biofilm-internal and external mass transfer resistance was investigated by Gapes and Keller (2009) in laboratory-

scale nitrifying MBBR, demonstrating the importance of these factors for MBBR. External mass transfer exerts significant control over the overall reaction rate, thus biofilm models must adequately account for this resistance.

Biofilm processes have proved to be very reliable for tertiary nitrification, because of the higher volumetric loading rates that can be applied and the low solids build-up in the reactor. This allows the use of smaller reactors usually with no need of tertiary settling tanks. 2 bench-scale reactors, fed with the secondary effluent of a municipal wastewater treatment plant (WWTP), were used by Bonomo *et al*. (2000) in order to study tertiary nitrification in pure oxygen moving bed biofilm reactors (PO-MBBRs) with patented KMT® media as biofilm carriers.

MBBR is self-cleaning and has low head loss (Rusten *et al*., 2000). Bioreactors with up to 60 % of the working volume occupied by carriers have been studied and shown to perform effectively (Maurer *et al*., 2001). The MBBR has been successfully used in the biological treatment of different effluents, such as the wastewater from pulp and paper industry, refineries, lactic industry, and in municipal wastewater treatment plants, as well as in different conditions like mesophilic and thermophilic environments (Moreno-Andrade *et al*., 2009).

A mathematical model of nitrification and denitrification in a moving-fixed bed biofilm reactor was developed by Lin (2008) to describe ammonium utilization by nitrifying biomass as well as nitrate and organic carbon utilization by denitrifying biomass, respectively. A pilot-scale moving-fixed bed biofilm reactor including suspended and attached growths of nitrifying and denitrifying biomasses was set up to verify the model system. The removal efficiencies of NH_4^+–N, NO_3^-–N and COD in specific area and volumetric loadings were about 75 %, 92 % and 70 % at a steady-state condition respectively.

The hydrodynamic behaviour and denitrification capacity of downflow self-cleaning MBBR using floating packed-bed carriers were investigated by Dupla *et al*. (2006). Increase in the overall liquid velocity profile led to an increase of up to 30 % in the denitrification rate with a 1-month-old biofilm. The influence of starvation and shock loads on the performance of a moving bed sequencing batch reactor degrading 4-chlorophenol was investigated by Moreno-Andrade *et al*. (2009). The experiments showed that the moving bed showed great robustness against starvation periods and shock loads.

1.4. Aim and scope of the present investigation

Microbially mediated environmental protection and restoration processes involve process cultures comprising multiple microbial consortia and it is the consortium performance, rather than individual strain performance, that is critical as far as both process efficiency and economics are concerned. Moreover, a mixed bacterial consortium comprises of different kinds of bacteria with varied metabolic activities. Hence, a microbial consortium is a better candidate to be studied with respect to biodegradation and biotreatment purposes.

Different industries manufacture different kind of products, thus leading to emittance of a wide variety of pollutants in the environment. Fertilizer, pesticide and chemical industries are prevalent on a large scale in India. These industries discharge their treated effluents directly into the water bodies. Generally, these industries experience difficulties in complying with the Pollution Control Board norms for discharge of industrial effluents into water bodies, as their effluents contain a large number of not easily biodegradable pollutants. These pollutants cannot be treated effectively in the common effluent treatment plants (CETPs) since they require a special bacterial seed having the potential to degrade them. If amenable to biological treatment, it affords a cost effective option of reducing the carbon to below permissible limit.

Industrial effluents having high COD are environmentally hazardous. The biodegradability index (BOD/COD ratio) varies from 0.4 to 0.8 for domestic wastewaters. If BOD/COD ratio is between 0.3 and 0.6, then seeding is required to treat it biologically. This means that the indigenous microflora of the respective industrial effluents is not sufficient for their biotreatment. Hence, a special microbial seed with high biodegradation and biotreatment abilities needs to be developed and incorporated for biotreatment of such industrial effluents.

Methylotrophs have been studied for their xenobiotic biodegradation abilities. Thus, methylotrophic bacteria are an efficient type to be studied with respect to xenobiotic biodegradation. The effective use of methylotrophic bacterial consortium for biodegradation of many xenobiotics has been previously reported. As compared to an individual methylotroph, a methylotrophic consortium would be more effective for xenobiotic biodegradation. Use of a methylotrophic bacterial consortium for

biodegradation studies of some specific xenobiotics has limited mention in literature. Hence, this aspect needs more attention.

Activated sludge process is the most preferred type of biological wastewater treatment process due to its efficiency in carbon removal. A variety of methylotrophs have been effectively used in activated sludge processes for industrial wastewater treatment. MBBR is a highly advanced biological wastewater treatment system. It has many advantages over an activated sludge process like simplicity, low space requirement, and no sludge separation requirement for effective operation; the most important one being no need of sludge recycle. Another notable advantage of MBBR is that an existing activated sludge process can be easily upgraded to a MBBR for more efficient biotreatment of different kinds of industrial effluents.

In this perspective, the work presented in this thesis aims at development of a methylotrophic mixed bacterial consortium and characterization of its individual isolates; biodegradation of selected xenobiotics by this consortium, and biotreatment of different kinds of industrial effluents by the methylotrophic consortium using activated sludge process and MBBR.

1.5. Objectives of the present investigation

1. Construction of a bacterial consortium for reducing the COD of industrial effluent containing methanol as a significant component

2. Characterization of individual isolates of the methylotrophic consortium

3. Development of a bench scale Moving Bed Biofilm Reactor for COD reduction of industrial effluents by the methylotrophic bacterial consortium

1.6. References

Alexander, M. 1985. Biodegradation of organic chemicals. *Environmental Science and Technology*, 19, 106-111.

Alvarez-Cohen, L., 1993. Application of methanotrophic bacteria for the bioremediation of chlorinated organics, in: Murrell, J.C., Kelley H.P. (Eds.), Microbial growth on C1 compounds. Intercept Press Ltd., United Kingdom, pp. 337-350.

Anesti, V., McDonald, I.R., Ramaswamy, M., Wade, W.G., Kelly, D.P., Wood, A.P., 2005. Isolation and molecular detection of methylotrophic bacteria occurring in the human mouth. *Environmental microbiology*, 7, 1227-1238.

Ankumah, R.O., Dick, W.A., McClung, G., 1995. Metabolism of carbamothioate herbicide,EPTC, by *Rhodococcus* strain JE1 isolated from soil. *Soil Science Society of America Journal*, 59, 1071-1077.

Anthony, C., 1982. The biochemistry of methylotrophs, Academic Press, London.

Arciero, D., Vannelli, T., Logan, M., Hopper, A.B., 1989. Degradation of trichloroethylene by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. *Biochemical and biophysical research communications*, 159, 640-643.

Atlas, R.M., 1997. Principles of microbiology, second ed. Von Hoffmann Press, USA.

Auffret, M., Labbe, D., Thouand, G., Greer, C.W., Fayolle-Guichard, F., 2009. Degradation of a mixture of hydrocarbons, gasoline, and diesel oil additives by *Rhodococcus aetherivorans* and *Rhodococcus wratislaviensis*. *Applied and environmental microbiology*, 75, 7774-7782.

Bader, R., Leisinger, T., 1994. Isolation and characterization of the *Methylophilus* sp. strain DM11 gene encoding dichloromethane dehalogenase/glutathione S-transferase. *Journal of bacteriology*, 176, 3466-3473.

Balachandar, D., Raja, P., Sundaram, S.P., 2008. Genetic and metabolic diversity of pink-pigmented facultative methylotrophs in phyllosphere of tropical plants. *Brazilian Journal of Microbiology*, 39, 68-73.

Baptista, I.I.R., Peeva, L.G., Zhou, N.-Y., Leak, D.J., Mantalaris, A., Livingston, A.G., 2006. Stability and performance of *Xanthobacter autotrophicus* GJ10 during 1, 2-dichloroethane biodegradation. *Applied and environmental microbiology*, 72, 4411-4418.

Barbera, M.J., Mateo, E., Monkaityte, R., Constanti, M., 2011. Biodegradation of methyl *tert*-butyl ether by newly identified soil microorganisms in a simple mineral solution. *World Journal of Microbiology and Biotechnology*, 27, 813-821.

Bellion, E., Khan, M.Y., Romano, M.J., 1980. Transport of methylamine by *Pseudomonas* sp. MA. *Journal of bacteriology*, 142, 786-790.

Bellion, E., Bolbot, J.A., Lash, T.D., 1981. Generation of glyoxylate in methylotrophic bacteria. *Current Microbiology*, 6, 367-372.

Bellion, E., Wayland, L., 1982. Methylamine uptake in *Pseudomonas* species strain MA: utilization of methylamine as the sole nitrogen source. *Journal of bacteriology*, 149, 395-398.

Bhatt, P., Kumar, M.S., Mudliar, S., Chakrabarti, T., 2007. Biodegradation of chlorinated compounds-a review. *Critical Reviews in Environmental Science and Technology*, 37, 165-198.

Bitton, G., 2005. Wastewater microbiology, Third ed. Wiley-Liss, New Jersey.

Boden, R., Thomas, E., Savani, P., Kelly, D.P., Wood, A.P., 2008. Novel methylotrophic bacteria isolated from the River Thames (London, UK). *Environmental microbiology*, 10, 3225-3236.

Bonomo, L., Pastorelli, G., Quinto, E., Rinaldi, G., 2000. Tertiary nitrification in pure oxygen moving bed biofilm reactors. *Water science and technology*, 41, 361-368.

Bowman, J.P., Sly, L.I., Nichols, P.D., Hayward, A.C., 1993. Revised taxonomy of the methanotrophs: description of *Methylobacter* gen. nov., emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. *International Journal of Systematic Bacteriology*, 43, 735-753.

Chang, H.L., Alvarez-Cohen, L., 1995. Transformation capacities of chlorinated organics by mixed cultures enriched on methane, propane, toluene, or phenol. *Biotechnology and bioengineering*, 45, 440-449.

Chen, J., Chen, D., Zhong, W., Zhang, J., Chen, X., 2007. Biodegradation of methyl *tert*-butyl ether by *Methylibium petroleiphilum* PM1 in poor nutrition solution. *Journal Of Environmental Science And Health Part A*, 42, 2123-2129.

Chistoserdova, L.V., Lidstrom, M.E., 1996. Molecular characterization of a chromosomal region involved in the oxidation of acetyl-CoA to glyoxylate in the isocitrate-lyase-negative methylotroph *Methylobacterium extorquens* AM1. *Microbiology*, 142, 1459-1468.

Chistoserdova, L., Lidstrom, M.E., 1997. Molecular and mutational analysis of a DNA region separating two methylotrophy gene clusters in *Methylobacterium extorquens* AM1. *Microbiology*, 143, 1729-1736.

Chistoserdova, L., Kalyuzhnaya, M.G., Lidstrom, M.E., 2009. The expanding world of methylotrophic metabolism. *Annual review of microbiology*, 63, 477-499.

Chongcharoen, R., Smith, T.J., Flint, K.P., Dalton, H., 2005. Adaptation and acclimatization to formaldehyde in methylotrophs capable of high-concentration formaldehyde detoxification. *Microbiology*, 151, 2615-2622.

Colby, J., Dalton, H., 1979. Characterization of the second prosthetic group of the flavoenzyme NADH-acceptor reductase (component C) of the methane mono-oxygenase from *Methylococcus capsulatus* (Bath). *Biochemical Journal*, 177, 903-908.

Colby, J., Zatman, L.J., 1973. Trimethylamine metabolism in obligate and facultative methylotrophs. *Biochemical Journal*, 132, 101-112.

Crowther, G., 2004. Alcohol consumption as a way of life: the metabolism of methylotrophic bacteria. Lidstrom Lab, Washington.

Davey, J.F., Whittenbury, R., Wilkinson, J.F., 1972. The distribution in the methylobacteria of some key enzymes concerned with intermediary metabolism. *Archives of Microbiology*, 87, 359-366.

Davies, S.L., Whittenbury, R., 1970. Fine structure of methane and other hydrocarbon-utilizing bacteria. *Journal of general microbiology*, 61, 227-232.

Dedysh, S.N., Knief, C., Dunfield, P.F., 2005. *Methylocella* species are facultatively methanotrophic. *Journal of bacteriology*, 187, 4665-4670.

Dijk, J.A., Gerritse, J., Schraa, G., Stams, A.J.M., 2004. Degradation pathway of 2-chloroethanol in *Pseudomonas stutzeri* strain JJ under denitrifying conditions. *Archives of microbiology*, 182, 514-519.

Dijkhuizen, L., Levering, P.R., De Vries, G.E., 1992. The physiology and biochemistry of aerobic methanol-utilizing gram-negative and gram-positive bacteria, in: Murrell, J.C., Dalton, H. (Eds.), Methane and methanol oxidizers. Plenum Press, New York, pp. 149–181.

Dokianakis, S.N., Kornaros, M., Lyberatos, G., 2006. Impact of five selected xenobiotics on isolated ammonium oxidizers and on nitrifying activated sludge. *Environmental Toxicology*, 21, 310-316.

Doronina, N.V., Trotsenko, Y.A., 1994. *Methylophilus leisingerii*, a new species of restricted facultatively methylotrophic bacteria. *Microbiology*, 63, 298-302.

Doronina, N.V., Braus-Stromeyer, S.A., Leisinger, T., Trotsenko, Y.A., 1995. Isolation and Characterization of a New Facultatively Methylotrophic Bacterium: Description of *Methylorhabdus multivorans*, gen. nov., sp. nov. *Systematic and applied microbiology*, 18, 92-98.

Doronina, N.V., Krauzova, V.I., Trotsenko, Y.A., 1997. *Methylophaga limanica* sp. nov.: A new species of moderately halophilic, aerobic, methylotrophic bacteria. *Microbiology*, 66, 434-439.

Doronina, N.V., Trotsenko, Y.A., Krausova, V.I., Suzina, N.E., 1998. *Paracoccus methylutens* sp. nov. - a new aerobic facultatively methylotrophic bacterium utilizing dichloromethane. *Systematic and applied microbiology*, 21, 230-236.

Doronina, N.V., Trotsenko, Y.A., Tourova, T.P., Kuznetsov, B.B., Leisinger, T., 2000. *Methylopila helvetica* sp. nov. and *Methylobacterium dichloromethanicum* sp. nov. - Novel aerobic facultatively methylotrophic bacteria utilizing dichloromethane. *Systematic and applied microbiology*, 23, 210-218.

Doronina, N.V., Darmaeva, T.D., Trotsenko, Y.A., 2003. *Methylophaga alcalica* sp. nov., a novel alkaliphilic and moderately halophilic, obligately methylotrophic bacterium from an East Mongolian saline soda lake. *International journal of systematic and evolutionary microbiology*, 53, 223-229.

Dupla, M., Comeau, Y., Parent, S., Villemur, R., Jolicoeur, M., 2006. Design optimization of a self-cleaning moving-bed bioreactor for seawater denitrification. *Water research*, 40, 249-258.

Eller, G., Kruger, M., Frenzel, P., 2005. Comparing field and microcosm experiments: a case study on methano- • and methyl-trophic bacteria in paddy soil. *FEMS microbiology ecology*, 51, 279-291.

Eshraghi, S., 2007. NAD^+-dependent methanol dehydrogenase from one-carbon compound utilizer Thermoactinomycete. *Journal of Biological Sciences*, 7, 205-209.

Fassel, T.A., Buchholz, L.A., Collins, M.L., Remsen, C.C., 1992. Localization of methanol dehydrogenase in two strains of methylotrophic bacteria detected by immunogold labeling. *Applied and environmental microbiology*, 58, 2302-2307.

Fayolle, F., Francois, A., Garnier, L., Godefroy, D., Mathis, H., Piveteau, P., Monot, F., 2003. Limitations in MTBE biodegradation. *Oil & gas science and technology*, 58, 497-504.

Fayolle, F., Monot, F., 2005. Biodegradation of fuel ethers, in: Magot, M., Ollivier, B. (Eds.), Petroleum Microbiology. American Society for Microbiology, Washington, DC, pp. 301–316.

Fetzner, S., 1998. Bacterial dehalogenation. *Applied microbiology and biotechnology*, 50, 633-657.

Flemming, H.-C., Neu, T.R., Wozniak, D.J., 2007. The EPS matrix: the "house of biofilm cells". *Journal of bacteriology*, 189, 7945-7947.

Fogel, M.M., Taddeo, A.R., Fogel, S., 1986. Biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. *Applied and environmental microbiology*, 51, 720-724.

Folsom, B.R., Chapman, P.J., Pritchard, P.H., 1990. Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: kinetics and interactions between substrates. *Applied and environmental microbiology*, 56, 1279-1285.

Fox, B.G., Borneman, J.G., Wackett, L.P., Lipscomb, J.D., 1990. Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. *Biochemistry*, 29, 6419-6427.

Francois, A., Mathis, H., Godefroy, D., Piveteau, P., Fayolle, F., Monot, F., 2002. Biodegradation of methyl *tert*-butyl ether and other fuel oxygenates by a new strain, *Mycobacterium austroafricanum* IFP 2012. *Applied and environmental microbiology*, 68, 2754-2762.

Gapes, D.J., Keller, J., 2009. Impact of oxygen mass transfer on nitrification reactions in suspended carrier reactor biofilms. *Process Biochemistry*, 44, 43-53.

Gisi, D., Willi, L., Traber, H., Leisinger, T., Vuilleumier, S., 1998. Effects of bacterial host and dichloromethane dehalogenase on the competitiveness of methylotrophic bacteria growing with dichloromethane. *Applied and environmental microbiology*, 64, 1194-1202.

Goodwin, P.M., Anthony, C., 1995. The biosynthesis of periplasmic electron transport proteins in methylotrophic bacteria. *Microbiology*, 141, 1051-1064.

Hage, J.C., Hartmans, S., 1999. Monooxygenase-mediated 1, 2-dichloroethane degradation by *Pseudomonas* sp. strain DCA1. *Applied and environmental microbiology*, 65, 2466-2470.

Hanson, R.S., 1980. Ecology and diversity of methylotrophic organisms. *Advances in Applied Microbiology*, 26, 3-39.

Hanson, R.S., Hanson, T.E., 1996. Methanotrophic bacteria. *Microbiological reviews*, 60, 439-471.

Hanson, J.R., Ackerman, C.E., Scow, K.M., 1999. Biodegradation of methyl *tert*-butyl ether by a bacterial pure culture. *Applied and environmental microbiology*, 65, 4788-4792.

Hernandez-Perez, G., Fayolle, F., Vandecasteele, J.-P., 2001. Biodegradation of ethyl *t*-butyl ether (ETBE), methyl *t*-butyl ether (MTBE) and *t*-amyl methyl ether (TAME) by *Gordonia terrae*. *Applied microbiology and biotechnology*, 55, 117-121.

Higgins, I.J., 1979. Microbial Biochemistry, in: Quayle, J.R. (Ed.), International Review of Biochemistry, vol. 21. University Park Press, Baltimore, pp. 300-353.

Horz, H.-P., Yimga, M.T., Liesack, W., 2001. Detection of methanotroph diversity on roots of submerged rice plants by molecular retrieval of *pmoA*, *mmoX*, *mxaF*, and 16S rRNA and ribosomal DNA, including *pmoA*-based terminal restriction fragment length polymorphism profiling. *Applied and environmental microbiology*, 67, 4177-4185.

Hung, W.-L., Wade, W.G., Boden, R., Kelly, D.P., Wood, A.P., 2011. Facultative methylotrophs from the human oral cavity and methylotrophy in strains of *Gordonia*, *Leifsonia*, and *Microbacterium*. *Archives of microbiology*, 193, 407-417.

Huve, K., Christ, M.M., Kleist, E., Uerlings, R., Niinemets, U., Walter, A., Wildt, J., 2007. Simultaneous growth and emission measurements demonstrate an interactive control of methanol release by leaf expansion and stomata. *Journal of experimental botany*, 58, 1783-1793.

Janssen, D.B., Scheper, A., Dijkhuizen, L., Witholt, B., 1985. Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. *Applied and environmental microbiology*, 49, 673-677.

Janssen, D.B., Keuning, S., Witholt, B., 1987. Involvement of a quinoprotein alcohol dehydrogenase and an NAD-dependent aldehyde dehydrogenase in 2-chloroethanol metabolism in *Xanthobacter autotrophicus* GJ10. *Journal of general microbiology*, 133, 85-92.

Janssen, D.B., van der Ploeg, J.R., Pries, F., 1994. Genetics and biochemistry of 1, 2-dichloroethane degradation. *Biodegradation*, 5, 249-257.

Jechorek, M., Wendlandt, K.D., Beck, M., 2003. Cometabolic degradation of chlorinated aromatic compounds. *Journal of biotechnology*, 102, 93-98.

Jing, J., Feng, J., Li, W., Xu, Y., 2009. Removal of COD from coking-plant wastewater in the moving-bed biofilm sequencing batch reactor. *Korean Journal of Chemical Engineering*, 26, 564-568.

Johnson, C., Page, M., Blaha, L., 2000. Full scale moving bed biofilm reactor results from refinery and slaughter house treatment facilities. *Water science and technology*, 41, 401-407.

Johnson, E.L., Smith, C.A., O'Reilly, K.T., Hyman, M.R., 2004. Induction of methyl *tertiary* butyl ether (MTBE)-oxidizing activity in *Mycobacterium vaccae* JOB5 by MTBE. *Applied and environmental microbiology*, 70, 1023-1030.

Kalyuzhnaya, M.G., Zabinsky, R., Bowerman, S., Baker, D.R., Lidstrom, M.E., Chistoserdova, L., 2006. Fluorescence in situ hybridization-flow cytometry-cell sorting-based method for separation and enrichment of type I and type II methanotroph populations. *Applied and environmental microbiology*, 72, 4293-4301.

Kalyuzhnaya, M.G., Hristova, K.R., Lidstrom, M.E., Chistoserdova, L., 2008. Characterization of a novel methanol dehydrogenase in representatives of *Burkholderiales*: implications for environmental detection of methylotrophy and evidence for convergent evolution. *Journal of bacteriology*, 190, 3817-3823.

Kane, S.R., Chakicherla, A.Y., Chain, P.S.G., Schmidt, R., Shin, M.W., Legler, T.C., Scow, K.M., Larimer, F.W., Lucas, S.M., Richardson, P.M., 2007. Whole-genome analysis of the methyl *tert*-butyl ether-degrading beta-

proteobacterium *Methylibium petroleiphilum* PM1. *Journal of bacteriology*, 189, 1931-1945.

Kato, N., Yurimoto, H., Thauer, R.K., 2006. The physiological role of the ribulose monophosphate pathway in bacteria and archaea. *Bioscience, biotechnology, and biochemistry*, 70, 10-21.

Kharoune, M., Pauss, A., Lebeault, J.M., 2001. Aerobic biodegradation of an oxygenates mixture: ETBE, MTBE and TAME in an upflow fixed-bed reactor. *Water research*, 35, 1665-1674.

Kim, H.S., Katayama, H., Takizawa, S., Ohgaki, S., 2001. Removal of coliphage Qb and organic matter from synthetic secondary effluent by powdered activated carbon-microfiltration (PAC-MF) process, in: Proceedings of IWA Specialized Conference on Membrane Technology, Israel, pp. 211–219.

Kohler-Staub, D., Leisinger, T., 1985. Dichloromethane dehalogenase of *Hyphomicrobium* sp. strain DM2. *Journal of bacteriology*, 162, 676-681.

Kohler-Staub, D., Hartmans, S., Galli, R., Suter, F., Leisinger, T., 1986. Evidence for identical dichloromethane dehalogenases in different methylotrophic bacteria. *Journal of general microbiology*, 132, 2837-2843.

Large, P.J., 1971. Non-oxidative demethylation of trimethylamine *N*-oxide by *Pseudomonas aminovorans*. *FEBS letters*, 18, 297-300.

Large, P.J., Boulton, C.A., Crabbe, M.J., 1972. The reduced nicotinamide-adenine dinucleotide phosphate-and oxygen-dependent N-oxygenation of trimethylamine by *Pseudomonas aminovorans*. *Biochemical Journal*, 128, 137P-138P.

Lidstrom, M.E., Stirling, D.I., 1990. Methylotrophs: genetics and commercial applications. *Annual Reviews in Microbiology*, 44, 27-58.

Lidstrom, M.E., 1992. The genetics and molecular biology of methanol-utilizing bacteria, in: Murrell, J.C., Dalton, H. (Eds.), Methane and methanol utilizers. Plenum Press, New York, pp. 183–206.

Lidstrom, M.E., Anthony, C., Biville, F., Gasser, F., Goodwin, P., Hanson, R.S., Harms, N., 1994. New unified nomenclature for genes involved in the oxidation of methanol in Gram-negative bacteria. *FEMS microbiology letters*, 117, 103-106.

Lidstrom, M.E., 2006. Aerobic methylotrophic prokaryotes, in: Balows, A., Truper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (Eds.), *The Prokaryotes*. Springer-Verlag, New York, USA, pp. 618–634.

Lieberman, R.L., Rosenzweig, A.C., 2004. Biological methane oxidation: regulation, biochemistry, and active site structure of particulate methane monooxygenase. *Critical reviews in biochemistry and molecular biology*, 39, 147-164.

Lin, C.-W., Cheng, Y.-W., Tsai, S.-L., 2007. Influences of metals on kinetics of methyl *tert*-butyl ether biodegradation by *Ochrobactrum cytisi*. *Chemosphere*, 69, 1485-1491.

Lin, Y.-H., 2008. Kinetics of nitrogen and carbon removal in a moving-fixed bed biofilm reactor. *Applied Mathematical Modelling*, 32, 2360-2377.

Little, C.D., Palumbo, A.V., Herbes, S.E., Lidstrom, M.E., Tyndall, R.L., Gilmer, P.J., 1988. Trichloroethylene biodegradation by a methane-oxidizing bacterium. *Applied and environmental microbiology*, 54, 951-956.

Lopes Ferreira, N., Malandain, C., Fayolle-Guichard, F., 2006. Enzymes and genes involved in the aerobic biodegradation of methyl *tert*-butyl ether (MTBE). *Applied microbiology and biotechnology*, 72, 252-262.

Madigan, M.T., Martinko, J. M., 2006. Brock Biology of Microorganisms, eleventh ed. Prentice Hall, NJ, USA.

Maier, M., Maier, D., Lloyd, B., 2000. The role of biofilms in the mobilisation of polycyclic aromatic hydrocarbons (PAHs) from the coal-tar lining of water pipes. *Water science and technology*, 41, 279-285.

de Marco, P., Pacheco, C.C., Figueiredo, A.R., Moradas-Ferreira, P., 2004. Novel pollutant-resistant methylotrophic bacteria for use in bioremediation. *FEMS microbiology letters*, 234, 75-80.

Marzorati, M., de Ferra, F., Van Raemdonck, H., Borin, S., Allifranchini, E., Carpani, G., Serbolisca, L., Verstraete, W., Boon, N., Daffonchio, D., 2007. A novel reductive dehalogenase, identified in a contaminated groundwater enrichment culture and in *Desulfitobacterium dichloroeliminans* strain DCA1, is linked to dehalogenation of 1, 2-dichloroethane. *Applied and environmental microbiology*, 73, 2990-2999.

Maurer, M., Fux, C., Graff, M., Siegrist, H., 2001. Moving-bed biological treatment (MBBT) of municipal wastewater: denitrification. *Water science and technology*, 43, 337-344.

Maymo-Gatell, X., Anguish, T., Zinder, S.H., 1999. Reductive dechlorination of chlorinated ethenes and 1, 2-dichloroethane by "*Dehalococcoides ethenogenes*" • 195. *Applied and environmental microbiology*, 65, 3108-3113.

McCarty, P.L., 1988. Bioengineering issues related to *in-situ* remediation of contaminated soils and groundwater, in: Omenn, G.S. (Ed.), Environmental biotechnology: reducing risks from environmental chemicals through biotechnology. Plenum Press, New York, pp. 143-162.

McDonald, I.R., Murrell, J.C., 1997. The methanol dehydrogenase structural gene *mxaF* and its use as a functional gene probe for methanotrophs and methylotrophs. *Applied and environmental microbiology*, 63, 3218-3224.

McDonald, I.R., Bodrossy, L., Chen, Y., Murrell, J.C., 2008. Molecular ecology techniques for the study of aerobic methanotrophs. *Applied and environmental microbiology*, 74, 1305-1315.

Mileva, A., Sapundzhiev, T., Beschkov, V., 2008. Modeling 1, 2-dichloroethane biodegradation by *Klebsiella oxytoca* va 8391 immobilized on granulated activated carbon. *Bioprocess and biosystems engineering*, 31, 75-85.

Mitsui, R., Omori, M., Kitazawa, H., Tanaka, M., 2005. Formaldehyde-limited cultivation of a newly isolated methylotrophic bacterium, *Methylobacterium* sp. MF1: enzymatic analysis related to C_1 metabolism. *Journal of bioscience and bioengineering*, 99, 18-22.

Mo, K., Lora, C.O., Wanken, A.E., Javanmardian, M., Yang, X., Kulpa, C.F., 1997. Biodegradation of methyl *t*-butyl ether by pure bacterial cultures. *Applied microbiology and biotechnology*, 47, 69-72.

Mohn, W.W., Tiedje, W.M., 1991. Evidence for chemiosmotic coupling of reductive dechlorination and ATP synthesis in *Desulfomonile tiedjei*. *Archives of Microbiology*, 157, 1-6.

Moreno-Andrade, I., Buitron, G., Vargas, A., 2009. Effect of starvation and shock loads on the biodegradation of 4-chlorophenol in a discontinuous moving bed biofilm reactor. *Applied biochemistry and biotechnology*, 158, 222-230.

Myers, P.A., Zatman, L.J., 1971. The metabolism of trimethylamine N-oxide by *Bacillus* PM6. *Biochemical Journal*, 121, 10P.

Neufeld, J.D., Schafer, H., Cox, M.J., Boden, R., McDonald, I.R., Murrell, J.C., 2007. Stable-isotope probing implicates *Methylophaga* spp and novel

Gammaproteobacteria in marine methanol and methylamine metabolism. *The ISME journal*, 1, 480-491.

Olaniran, A.O., Balgobind, A., Pillay, B., 2009. Impacts of heavy metals on 1, 2-dichloroethane biodegradation in co-contaminated soil. *Journal of Environmental Sciences*, 21, 661-666.

Piveteau, P., Fayolle, F., Vandecasteele, J.P., Monot, F., 2001. Biodegradation of *tert*-butyl alcohol and related xenobiotics by a methylotrophic bacterial isolate. *Applied microbiology and biotechnology*, 55, 369-373.

Poelarends, G.J., van Hylckama Vlieg, J.E.T., Marchesi, J.R., Dos Santos, L.M.F., Janssen, D.B., 1999. Degradation of 1,2-Dibromoethane by *Mycobacterium* sp. Strain GP1. *Journal of bacteriology*, 181, 2050-2058.

Quayle, J.R., Ferenci, T., 1978. Evolutionary aspects of autotrophy. *Microbiological reviews*, 42, 251-273.

Radianingtyas, H., Robinson, G.K., Bull, A.T., 2003. Characterization of a soil-derived bacterial consortium degrading 4-chloroaniline. *Microbiology*, 149, 3279-3287.

Rosche, B., Li, X.Z., Hauer, B., Schmid, A., Buehler, K., 2009. Microbial biofilms: a concept for industrial catalysis? *Trends in biotechnology*, 27, 636-643.

Rusten, B., Hellstrom, B., Hellstrom, F., Sehested, O., Skjelfoss, E., Svendsen, B., 2000. Pilot testing and preliminary design of moving bed biofilm reactors for nitrogen removal at the FREVAR wastewater treatment plant. *Water science and technology*, 41, 13-20.

Rusten, B., Eikebrokk, B., Ulgenes, Y., Lygren, E., 2006. Design and operations of the Kaldnes moving bed biofilm reactors. *Aquacultural engineering*, 34, 322-331.

Schraa, G., Boone, M.L., Jetten, M.S., van Neerven, A.R., Colberg, P.J., Zehnder, A.J., 1986. Degradation of 1,4-dichlorobenzene by *Alcaligenes* sp. strain A175. *Applied and environmental microbiology*, 52, 1374-1381.

Skladany, G.J., Metting, F.B., 1992. Bioremediation of contaminated soil, in: Metting, F.B. (Ed.), Soil Microbial Ecology. Marcel Dekker, New York, pp. 483-513.

Small, F.J., Tilley, J.K., Ensign, S.A., 1995. Characterization of a new pathway for epichlorohydrin degradation by whole cells of *Xanthobacter* strain Py2. *Applied and environmental microbiology*, 61, 1507-1513.

Smith, C.A., O'Reilly, K.T., Hyman, M.R., 2003. Characterization of the initial reactions during the cometabolic oxidation of methyl *tert*-butyl ether by propane-grown *Mycobacterium vaccae* JOB5. *Applied and environmental microbiology*, 69, 796-804.

Sohngen, N. L. 1906. Uber Bakterien, welche Methan als Kohlenstoffnahrung und Energiequelle gebrauchen. *Centr. Bakt. Parasitenk.*, Abt. II, 16, 513-517.

Spain, J.C., Nishino, S.F., 1987. Degradation of 1, 4-dichlorobenzene by a *Pseudomonas* sp. *Applied and environmental microbiology*, 53, 1010-1019.

Spiess, E., Sommer, C., Gorisch, H., 1995. Degradation of 1,4-dichlorobenzene by *Xanthobacter flavus* 14p1. *Applied and environmental microbiology*, 61, 3884-3888.

Steffan, R.J., McClay, K., Vainberg, S., Condee, C.W., Zhang, D., 1997. Biodegradation of the gasoline oxygenates methyl *tert*-butyl ether, ethyl *tert*-butyl ether, and *tert*-amyl methyl ether by propane-oxidizing bacteria. *Applied and environmental microbiology*, 63, 4216-4222.

Steffan, R.J., Vainberg, S., Condee, C., McClay, K., Hatzinger, P., 2000. Biotreatment of MTBE with a new bacterial isolate, in: Wickramanayake, G.B., Gavaskar, A.R., Alleman, B.C., Magar, V.S. (Eds.), Bioremediation and phytoremediation of chlorinated and recalcitrant compounds. Batelle Press, Columbus, Ohio, pp. 165-173.

Stellman, J.M., 1998. Encyclopaedia of occupational health and safety, fourth ed., vol. 3. International Labour Office, Geneva.

Strotmann, U.J., Pentenga, M., Janssen, D.B., 1990. Degradation of 2-chloroethanol by wild type and mutants of *Pseudomonas putida* US2. *Archives of microbiology*, 154, 294-300.

Sutherland, I.W., 1977. Enzyme action on bacterial surface carbohydrates, in: Sutherland, I.W. (Ed.), Surface carbohydrates of the prokaryotic cell. Academic Press, New York, pp. 209-245.

Sy, A., Giraud, E., Jourand, P., Garcia, N., Willems, A., de Lajudie, P., Prin, Y., Neyra, M., Gillis, M., Boivin-Masson, C., 2001. Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. *Journal of bacteriology*, 183, 214-220.

Tanaka, Y., Yoshida, T., Watanabe, K., Izumi, Y., Mitsunaga, T., 1997. Cloning and analysis of methanol oxidation genes in the methylotroph *Hyphomicrobium methylovorum* GM2. *FEMS microbiology letters*, 154, 397-401.

Tchobanoglous, G., Burton, F.L., Metcalf & Eddy, Stensel, H.D., 2003. Suspended growth biological treatment processes, in: Tchobanoglous, G., Burton, F.L., Stensel, H.D. (Eds.), Wastewater Engineering Treatment and Reuse, fourth ed. McGraw-Hill, Inc., New York, USA, pp. 659-886.

Trotsenko, Y.A., Doronina, N.V., Khmelenina, V.N., 2005. Biotechnological potential of aerobic methylotrophic bacteria: a review of current state and future prospects. *Applied Biochemistry and Microbiology*, 41, 433-441.

Trotsenko, Y.A., Murrell, J.C., 2008. Metabolic Aspects of Aerobic Obligate Methanotrophy. *Advances in applied microbiology*, 63, 183-229.

Vangnai, A.S., Petchkroh, W., 2007. Biodegradation of 4-chloroaniline by bacteria enriched from soil. *FEMS microbiology letters*, 268, 209-216.

Verstraete, W., van Vaerenbergh, E., 1986. Aerobic activated sludge, in Rehm, H.J., Reed, G. (Eds.), Biotechnology. VCH, Weinheim, Germany, pp. 43-112.

Vuilleumier, S., 1997. Bacterial glutathione S-transferases: what are they good for? *Journal of bacteriology*, 179, 1431-1441.

Wackett, L.P., Gibson, D.T., 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. *Applied and environmental microbiology*, 54, 1703-1708.

Waturangi, D., Marko, N., Suhartono, M.T., 2011. Purification of methanol dehydrogenase from mouth methylotrophic bacteria of tropical region. *Malaysian Journal of Microbiology*, 7, 226-229.

Whittenbury, R., Phillips, K.C., Wilkinson, J.F., 1970. Enrichment, isolation and some properties of methane-utilizing bacteria. *Journal of general microbiology*, 61, 205-218.

Whittenbury, R., Krieg, N.R., 1984. *Methylococcacea* fam. nov., in: Krieg, N.R., Holt, J.G. (Eds.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore, pp. 256-262.

van den Wijngaard, A.J., Janssen, D.B., Witholt, B., 1989. Degradation of epichlorohydrin and halohydrins by bacterial cultures isolated from freshwater sediment. *Journal of general microbiology*, 135, 2199-2208.

van den Wijngaard, A.J., van der Kamp, K.W., van der Ploeg, J., Pries, F., Kazemier, B., Janssen, D.B., 1992. Degradation of 1,2-dichloroethane by *Ancylobacter aquaticus* and other facultative methylotrophs. *Applied and environmental microbiology*, 58, 976-983.

Winter, R.B., Yen, K.-M., Ensley, B.D., 1989. Efficient degradation of trichloroethylene by a recombinant *Escherichia coli*. *Nature Biotechnology*, 7, 282-285.

Yamashita, S.-i., Uchimura, T., Komagata, K., 2004. Emendation of the genus *Acidomonas* Urakami, Tamaoka, Suzuki and Komagata 1989. *International journal of systematic and evolutionary microbiology*, 54, 865-870.

Yan, J., Rash, B.A., Rainey, F.A., Moe, W.M., 2009. Isolation of novel bacteria within the *Chloroflexi* capable of reductive dechlorination of 1,2,3-trichloropropane. *Environmental microbiology*, 11, 833-843.

van der Zaan, B., de Weert, J., Rijnaarts, H., de Vos, W.M., Smidt, H., Gerritse, J., 2009. Degradation of 1,2-dichloroethane by microbial communities from river sediment at various redox conditions. *Water research*, 43, 3207-3216.

Chapter 2

Construction of a bacterial consortium for COD reduction of industrial effluents and characterization of its individual isolates

2.1. Introduction

Methylotrophs are the bacteria which grow on C_1 compounds with no C-C bonds as sole source of carbon and energy. These compounds include methane, methyl halides, methanol, methylamine, methyl sulfides and formate (Chapter 1, Table 1.1). They can utilize CO_2 as a carbon source but not as an energy source. Almost all methylotrophs are aerobic. They grow on methanol produced by plants, methylated amines from degradation of fish and plant compounds as well as methyl halides and methyl sulfides produced by marine algae. They co-metabolize many toxic compounds. They have the ability to convert C_1 molecules to C_3 by forming serine or ribulose monophosphate via the serine or ribuolse monophosphate (RuMP) pathway respectively (Crowther, 2004). The facultative methylotrophs are also able to additionally degrade multi-carbon compounds including many xenobiotics (Piveteau *et al*., 2001).

Methylotrophs are ubiquitous in nature. They can be found in diverse habitats. Eller *et al*. (2005) isolated methylotrophic bacteria from a rice field in northern Italy. Kalyuzhnaya *et al*. (2006) detected and enriched methylotroph populations from Lake Washington sediment using a fluorescence in situ hybridization-flow cytometry (FISH/FC)-based method. Facultative methylotrophs have been isolated from agricultural soil, woodland leaf litter and coastal seawater, environments where methyl halides are produced (Miller *et al*., 2001).

Methanol dehydrogenase (MDH) is the first enzyme of both serine and RuMP pathways of methylotrophs, which converts methanol to formaldehyde (Anthony, 1982). MDH is a water soluble quinoprotein that is involved in the metabolism of small molecules such as methanol and methane in methylotrophic bacteria (Anthony and Williams, 2003). In facultative methylotrophs, this enzyme is produced at high concentration, up to 10-15 % in bacterial cell. MDH has the ability to metabolize wide range of substrates, such as methanol, methane, formaldehyde and methylamine. Due to the presence of mechanisms to utilize single carbon compounds, MDH can be applied to many industrial and environmental applications (Waturangi *et al*., 2011).

MDH was reported to consist of α subunit (62 kDa) and β subunit (8.5 kDa) and arranged in α2β2 configuration (Nunn *et al*., 1989). *mxaF* and *mxaI* genes encode the α and β subunits of MDH respectively (Zhang and Lidstrom, 2003). Hence, many researchers used these genes as a tool to identify methylotrophic bacteria in various

habitats. Eller *et al*. (2005) used *mxaF* primer sets in a PCR-based approach to identify methylotrophic bacteria present in a rice field in northern Italy. Christoserdova (2011) reported that *mxaF* is one of the highly specialized probes used to assess the methylotrophic populations in different environmental samples.

Methylotrophs are useful in carbon cycle, bioremediation and biocatalysis (Crowther, 2004). A large number of reports are available on methylotrophs with respect to co-metabolism of toxic compounds. *Methylopila helvetica* sp. nov. and *Methylobacterium dichloromethanicum* sp. nov., aerobic facultatively methylotrophic bacteria, utilize dichloromethane, methanol and methylamine as well as a variety of polycarbon compounds as the carbon and energy source (Doronina *et al*., 2000). 4 novel methylotrophic bacterial strains, isolated from a range of soil and sediment sources, possessed the ability to withstand extremely high concentrations of a variety of pollutants (de Marco *et al*., 2004). *Burkholderia cepacia* CIP I-2052 degraded *tert*-butyl alcohol (TBA), *tert*-amyl alcohol (TAA) and methanol (Piveteau *et al*., 2001). Near complete nitrate and nitrite removal was obtained using a methylotrophic enrichment consortium during sequencing batch denitrifying reactor (SBDR) operation (Baytshtok *et al*., 2008). Industrial effluents have high COD, mainly contributed by toxic chemicals. COD enhancement is environmentally hazardous and needs to be addressed by developing a special microbial seed for reducing the COD of industrial effluents to < 100 ppm, which is the stipulated permissible limit. Hence, in order to develop a suitable microbial seed for COD reduction of different industrial effluents, the methylotrophic bacteria were selected in the present study.

This chapter deals with the isolation of methylotrophic bacteria from different sources and their screening on the basis of methanol utilization ability in order to prepare an efficient bacterial consortium. The methylotrophic bacterial consortium constructed in this study was further characterized and utilized for treatment of different industrial effluents at a bench scale reactor level in the subsequent studies.

2.2. Materials and Methods

2.2.1. Isolation

To isolate potential methylotrophic bacteria, samples were collected from the following sources: Denitrifying reactor A, denitrifying reactor B, denitrifying reactor outlet and storage tank of a fertilizer company, Bharuch, Gujarat, India; Domestic

sewage treatment plant, Wadi, Vadodara, Gujarat, India, and Common effluent treatment plant, Ankleshwar, Gujarat, India.

Isolation was carried out by the direct isolation method. The media used for isolation were Luria Bertani (LB) medium containing (per liter): Casein enzymatic hydrolysate, 10.0 g; Yeast extract, 5.0 g; NaCl, 5.0 g, pH 7.0, and Peptone Nitrate Beef extract (PNB) medium containing (per liter): Peptone, 5.0 g; KNO_3, 5.0 g; Beef extract, 3.0 g, pH, 7.2. Direct samples and their serial dilutions were spread on LB and PNB agar plates and incubated at 37 ^oC for 48 h. Isolates obtained were selected on the basis of different colony morphologies.

2.2.2. Screening

2.2.2.1. Screening of isolates on the basis of substrate utilization

Fusel oil utilization ability of the isolates was checked in terms of their growth in MM2 medium containing (per liter): KNO_3, 1.0 g; K_2HPO_4, 1.0 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.02 g; $MnCl_2.4H_2O$, 0.002 g; $NaMoO_4.2H_2O$, 0.001g; $FeSO_4.7H_2O$, 0.05 g; Yeast extract, 0.5 g; Fusel oil, 1 % (v/v), pH, 7.0 (Srinandan *et al*., 2010) with fusel oil as the carbon source. Fusel oil is a byproduct of the fertilizer company and used as a substrate in the denitrifying reactor, since methanol is its major component and an exogenous carbon source for denitrifiers. Fusel oil contains (v/v) water, 64.50 %; methanol, 33.18 %; ethanol, 1.15 %; N-propanol, 0.38 %; 2-butanol, 0.31 %; 1-butanol, 0.18 %; isopropanol, 0.17 %, N-butanol, 0.13 %. A colony of isolates was inoculated in a test tube containing 5 ml PNB medium and incubated at 37 ^oC for 12 h at 180 rpm. It was then centrifuged at 10,000 rpm for 5 min and the cell pellet obtained was suspended in 1 ml MM2 medium which was used as an inoculum. 0.1 ml of the inoculum was inoculated in a test tube containing 4.9 ml MM2 medium and incubated at 37 ^oC for 24 h at 180 rpm. The growth of the isolates was measured in terms of their OD at 600 nm. The isolates showing maximum growth were selected for the further studies.

2.2.2.2. Screening of the selected isolates on the basis of growth in DNR effluent

Growth in DNR effluent was recorded with fusel oil (1 %, v/v) as a carbon source in the DNR effluent. The same experimental procedure, as for MM2 medium (section 2.2.2.1), was followed for the DNR effluent. The isolates showing maximum

growth were chosen for preparation of a mixed bacterial consortium. While making the consortia, colony morphology of the isolates and their Gram nature were also considered. For this purpose, the isolates were streaked on PNB agar plates and incubated at 37 ^{o}C for 48 h. Their colony characters were observed thereafter and Gram staining was done.

2.2.3. Screening of consortia and sludges for biotreatment potential

AC consortium, FW consortium, DNR A sludge, DNR B sludge and Wadi activated sludge were screened for their biotreatment potential on the basis of their ability to reduce the COD of DNR effluent. 2 % of the 12 h old consortium/sludge was inoculated into 100 ml DNR effluent in a 250 ml Erlenmeyer flask and incubated at 37 ^{o}C for 6 h at 180 rpm. 1 ml of the effluent was collected after the incubation period and centrifuged at 10,000 rpm for 5 min. The supernatant obtained was used as a sample for COD estimation. The COD of the DNR effluent was estimated by the dichromate reflux method (Tomar, 1999).

2.2.4. Growth profile

A colony of the isolates was inoculated into a test tube containing 5 ml LB medium and incubated at 37 ^{o}C for 12 h at 180 rpm. 1 ml of the inoculum was then inoculated in an Erlenmeyer flask containing 49 ml LB medium and incubated at 37 ^{o}C for 48 h at 180 rpm. Samples were collected after intervals of 1 h and used for measuring growth (OD_{600}). The specific growth rate of the isolates was calculated using the following formula (Widdel, 2007):

$$\text{Specific growth rate } (\mu) = \frac{2.303(\log OD_2 - \log OD_1)}{T_2 - T_1}$$

Where,

OD_1 = Growth at initial time, OD_{600}

OD_2 = Growth at final time, OD_{600}

T_1 = Initial time, h

T_2 = Final time, h

2.2.5. Confirmation of methylotrophy

2.2.5.1. Methanol utilization

Methanol utilization ability of the isolates of AC consortium was checked in terms of their growth and COD reduction in MM2 medium. A colony of isolates was inoculated into a test tube containing 5 ml PNB medium and incubated at 37 ^{o}C for 12 h at 180 rpm. The culture obtained was then centrifuged at 10,000 rpm for 5 min and the cell pellet obtained was suspended in 1 ml MM2 medium, containing methanol (1 %, v/v) as the sole carbon source, which was used as an inoculum. 1 ml of the inoculum was inoculated in an Erlenmeyer flask containing 49 ml MM2 medium and incubated at 37 ^{o}C for 120 h at 180 rpm. The sample was collected after intervals of 24 h and used for growth (OD_{600}) and COD estimation (Tomar, 1999).

The same experimental procedure, used for MM2 medium, was followed for the DNR effluent. The cell culture obtained after the incubation period of 120 h was distilled and the methanol unutilized was quantified by gas chromatography (GC) (Fayolle *et al*., 1998).

2.2.5.2. *mxaF* gene detection

The *mxaF* gene in the isolates was detected with specific primers mxaf1003 (5'GCG GCA CCA ACT GGG GCT GGT3') and mxar1561 (5'GGG CAG CAT GAA GGG CTC CC3') (McDonald *et al*., 1995). PCR reactions were carried out in an Applied Biosystems thermal cycler in a 30 µl system containing 50 ng genomic DNA, 1 µl; 0.2 mM dNTP mixture, 1 µl; 1 µM primers, 3 µl; 2.5mM $MgCl_2$, 5 µl, and 2 U *Taq* DNA polymerase, 0.5 µl (Bangalore Genei, India) under the following conditions: 95 ºC for 5 min followed by 35 cycles of 94 ºC for 1 min, 56 ºC for 2 min, 72 ºC for 1 min and final extension at 72 ºC for 10 min (Balachandar *et al*., 2008). PCR products were separated by electrophoresis on 1 % agarose gels stained with ethidium bromide and analyzed using Alphaimager HP documentation and analysis system.

2.2.6. Polyphasic identification

2.2.6.1. Morphological and biochemical characterization

Gram staining and endospore staining were used to characterize the morphological features of the 4 isolates of AC consortium. The biochemical tests included: Glucose fermentation, indole test, methyl red test (MR), Voges Proskauer test

(VP), citrate utilization test, triple sugar iron test (TSI), catalase test, oxidase test, growth on Mac Conkey's agar, growth on EMB agar, growth in LB broth with NaCl and β-galactosidase assay (Holt *et al.*, 1994). The media used for biochemical tests were prepared as per MacFaddin's manual (MacFaddin, 1980). For isolate AC5, biochemical tests were carried out according to the standard tests for *Enterobacteriaceae* family (Himedia). These tests included ONPG (*o*-nitrophenyl-β-D-galactopyranoside), lysine utilization, ornithine utilization, urease, phenylalanine deamination, nitrate reduction, H_2S producton, citrate utilization, VP, MR, indole production, malonate utilization, esculin hydrolysis, acid production from arabinose, xylose, adonitol, rhamnose, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose, lactose, and oxidase (Shelobolina *et al*., 2004).

2.2.6.2. 16S rRNA gene sequence analysis

The isolates of AC consortium were further identified on the basis of 16S rRNA gene sequence analysis. Universal eubacterial 16S rRNA primers, 27F (5'GAGAGTTTGATCCTGGCTCAG3') and 1541R (5'AAGGAGGTGATCCAGCCGC3'), were used for amplification of the 16S rRNA gene from the genomic DNA of the isolates (Pillai and Archana, 2008). The 16S rRNA genes of all the 4 isolates were sequenced at Xcelris Labs Ltd., India, and the sequences obtained were matched with the database of National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov).

2.2.7. Phylogenetic analysis

Sequences from *Bordetella*, *Bacillus*, *Salmonella* and *Pseudomonas* species and other genera were obtained from GenBank for phylogenetic analysis. Phylogenetic analysis was performed using the Ribosomal Database Project available at Centre for Microbial Ecology (Michigan State University, East Lansing, MI). Phylogenetic trees were constructed by distance analysis with the Jukes-Cantor correction (Shelobolina *et al*., 2004).

2.2.8. Carbon substrate utilization

2.2.8.1. Single and multi carbon substrate utilization

Carbon substrate utilization profile of the isolates of AC consortium was analyzed in terms of their growth in MM2 medium supplemented with different single and multi carbon substrates at 0.5 % (w/v) (Balachandar *et al*., 2008). The single carbon substrates used included methanol, methyl amine, methyl fluoride, methyl chloride, methyl bromide, methyl iodide, dichloromethane, methyl ammonium, formaldehyde

and formate. The multi carbon substrates used included ethanol, N-propanol, isopropanol, N-butanol, 1-butanol, 2-butanol, fusel oil, glucose, fructose, xylose, citrate, L-glutamate and tartaric acid. The ability of AC consortium to utilize the constituents of fusel oil, viz. methanol, ethanol, propanol and butanol, as well as different xenobiotic compounds as the sole carbon and energy sources was determined in terms of their growth and COD reduction in MM2 medium (Piveteau *et al*., 2001).

2.2.8.2. Alcohol utilization

The ability of AC consortium and its members to utilize various industrially important alcohols was also checked. The alcohols used were methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, *tert*-butanol and isobutyl alcohol (Piveteau *et al*., 2001). The inoculum was prepared as described in section 2.2.2.1. 0.1 ml of the inoculum was inoculated in a test tube containing 4.9 ml MM2 medium supplemented with different alcohols at 0.5 % (v/v) and incubated at 37 ^{o}C for 120 h at 180 rpm. The growth of the isolates was measured in terms of their OD at 600 nm.

2.2.9. Methanol dehydrogenase

2.2.9.1. MDH isolation

Cell paste of the individual isolates of AC consortium (1 g) was suspended in 5 ml D/W and placed in an ice bath to slowly thaw while stirring. The cells were then lysed by ultrasonication (Sonics, Vibra Cell) with 30 repetitive cycles of ultrasonication for 9 s followed by cooling for 9 s in an ice bath. Cell debris was removed by centrifugation at 12,000 rpm for 30 min at 4 °C. The resulting supernatant was mixed with 100 mM Mes, pH 5.5 (v/v = 3:1) at 4 °C to a final buffer concentration of 25 mM (Liu *et al*., 2006).

2.2.9.2. MDH assay

Alcohol-dependent NADH production was measured at 25 ^{o}C, by following the increase in absorbance at 340 nm, in a reaction mixture (1 ml) containing: 50 mM sodium pyrophosphate buffer (pH 8.8), 260 µl; 15 mM β-NAD, 300 µl; extract, 20 µl. The reaction was started with 220 µl of 5 M methanol. 1 unit of MDH activity was defined as the amount of enzyme that converts 1 µmole of methanol to formaldehyde per minute at pH 8.8 at 25 ^{o}C (Arfman *et al*., 1989).

2.2.10. Methanol tolerance

2.2.10.1. Exposure to different concentrations of vapor phase methanol on solid media

The isolates of AC consortium were streaked on MM2 agar plates and placed inside a 6 l desiccator plugged with non-absorbent cotton wool to allow for the diffusion of air. A 10 ml glass beaker was filled with methanol and placed inside the desiccator to expose the plates to vapor phase methanol. Approximately 2 g/l of methanol was maintained at this level until the methanol totally volatilized from the 10 ml beaker. Growth on the plates was monitored daily by visual inspection. Growth of the isolates was measured in terms of cfu after the incubation period of 120 h at 37 ^{o}C (Gutierrez, 1999).

2.2.10.2. Exposure to pre-solubilized shock concentrations of methanol in liquid media

A 5 ml culture of the isolates of AC consortium was grown in LB broth. These cells were then inoculated into 5 Erlenmeyer flasks containing 50 ml MM2 medium supplemented with 0.3, 0.5, 0.8, 1.0 and 1.2 g/l of pre-solubilized methanol. A methanol unexposed control was also included. All the cell-methanol mixtures were incubated at 37 °C at 180 rpm for 120 h. The growth of the isolates was measured in terms of their OD at 600 nm (Gutierrez, 1999).

2.2.11. Nucleotide sequence accession numbers

The 16S rRNA gene sequences of the isolates AC1, AC4, AC5 and AC8 were deposited in GenBank under the accession numbers KF410859, KF410860, KF410861 and KF410862 respectively.

2.3. Results and Discussion

Methylotrophic bacteria are recognized by their ability to grow on carbon compounds more reduced than CO_2, without any C-C bonds. They use methane, methanol, methylamines, halomethanes, or other reduced one carbon compounds as energy sources and assimilate formaldehyde as the major carbon source (Anthony, 1982). Methylotrophic bacteria are found in a variety of ecosystems, such as freshwater lakes, ponds, marshes, marine sediments and soil. Eller *et al*. (2005)

reported that the methylotrophic bacteria from different compartments of a rice field in northern Italy play an important role in reducing the methane emission from these compartments.

Methylotrophs are ubiquitous and belong to a heterogenous group of bacteria, reflecting the physiological diversity of this vast group of microorganisms. The type of methylotrophs and their niche depends on the predominant single carbon substrate and its concentration; the presence or absence of O_2 and alternative terminal electron acceptors (nitrate and sulphate); the available nitrogen source (amino acids, amines, ammonia, nitrate, nitrite and N_2), and the prevailing conditions of temperature and pH. One of the most remarkable features of methylotrophic metabolism is the general use of formaldehyde, a highly reactive molecule and potent poison to living systems (Grafstrom *et al*., 1983). In addition to numerous other applications in biotechnology and bioremediation (Chapter 1, section 1.1.7), methylotrophs may be used for removal of the toxic and reactive pollutant formaldehyde (Chongcharoen *et al*., 2005). Considering the vast metabolic diversity and wide prevalence of methylotrophic bacteria, their isolation was undertaken with an aim to construct a consortium possessing a broad ability of biotreatment of a variety of industrial effluents.

2.3.1. Isolation of potential methylotrophic bacteria

Direct isolation of bacteria was performed from the samples collected from a fertilizer company, domestic sewage treatment plant (STP) and common effluent treatment plant (CETP), since it gives the numerically dominant and actively metabolizing, fast growing bacteria. The effluent from a fertilizer company denitrifying reactor was selected for isolation of methylotrophs since it contains methanol as the main organic carbon. The domestic STPs and CETPs offer a variety of heterotrophic bacteria; considering this, samples from domestic STP and CETP were collected. A total of 118 isolates were obtained from different sources (Table 2.1). These isolates were aerobic, heterotrophic, mesophilic bacteria which were distinguished on the basis of their colony morphology. The denitrifying reactor effluent yielded a maximum of 28 isolates, indicating wide bacterial diversity, while the denitrifying reactor B sludge yielded a minimum of 8 isolates (Table 2.1). Here, the denitrifying reactor being a specialized environment shows less diversity in its sludge sample, whereas the STP sludge sample shows more diversity in the heterotrophic bacteria.

Table 2.1. Distribution of the isolates among various habitats

Source	No. of isolates	Name of isolates
Denitrifying reactor A sludge	13	DAS1-DAS13
Denitrifying reactor B sludge	8	DBS1-DBS8
Denitrifying reactor outlet effluent	28	DOE1-DOE28
Final treated effluent	26	FTE1-FTE26
Wadi activated sludge	27	WAS1-WAS27
Ankleshwar CETP effluent	16	AC1-AC16

2.3.2. Screening of isolates

The isolates were tested for growth on fusel oil and DNR effluent (containing fusel oil) to screen for methylotrophs.

2.3.2.1. Screening of isolates on the basis of substrate utilization

In biotechnology which uses microorganisms as tools, selection of the raw material for the culture substrate is of paramount importance, as is the pinpointing of the screening of microorganisms. C_1 compounds have attracted much attention as the culture substrate for the methylotrophs, and methanol is anticipated to be the most suitable substrate for the selection of methylotrophic bacteria (Tani and Sahin, 2013).

Fusel oil is a byproduct of a fertilizer company and used as a substrate in the denitrifying reactor due to its high proportion of methanol. Hence, the isolates obtained from the specially chosen sources were screened on fusel oil used as the sole carbon source in order to obtain efficient methylotrophs. Fusel oil utilization ability of the 118 isolates was observed in terms of their growth in MM2 medium supplemented with fusel oil as the sole carbon source. Almost all the isolates showed growth at 37 ^{o}C under aerobic conditions after 24 h. The isolates showing growth (OD_{600}) more than 0.5 were selected for the further studies. These 24 selected isolates included DAS9, obtained from denitrifying reactor A sludge (Figure 2.1(a)); DBS4, from denitrifying reactor B sludge (Figure 2.1(b)); DOE11 and DOE13, from denitrifying reactor outlet effluent (Figure 2.1(c)); FTE1, FTE3, FTE5, FTE8, FTE15, FTE19, FTE22 and FTE23 from final treated effluent (Figure 2.1(d)); WAS2, WAS7, WAS10, WAS23, WAS24 and WAS26, from domestic STP activated sludge (Figure 2.1(e)), and AC1, AC2, AC4, AC5, AC8 and AC9, from CETP effluent (Figure 2.1(f)).

2.3.2.2. Screening of the selected isolates on the basis of growth in DNR effluent

24 selected putative methylotrophic isolates were distinguished on the basis of their colony and cell morphologies (Table 2.2). Growth of the putative methylotrophs in DNR effluent indicates their suitability for use for the effluent treatment. Therefore, the ability of these isolates to degrade DNR effluent was observed in terms of their growth in DNR effluent containing fusel oil. Almost all the isolates showed growth at 37 ^{o}C under aerobic conditions (Figure 2.2). The isolates showing higher growth were selected to prepare a mixed bacterial consortium. Based on this criterion, 2 mixed bacterial consortia were constructed. The isolates FTE3, FTE8, FTE15, FTE22 and WAS2 were selected to prepare FW consortium, whereas the isolates AC1, AC4, AC5 and AC8 were selected to prepare AC consortium.

2.3.3. Screening of consortia and sludges for biotreatment potential

The biotreatment potential of AC consortium, FW consortium, DNR A sludge, DNR B sludge and Wadi activated sludge was checked with DNR effluent at flask level. It was found that the AC consortium reduced the initial COD of DNR effluent from 770 mg/l to 60 mg/l, FW consortium reduced its COD to 120 mg/l, DNR A sludge to 310 mg/l, DNR B sludge to 190 mg/l and Wadi activated sludge to 700 mg/l in 6 h (Figure 2.3). Hence, as the AC consortium showed maximum biotreatment potential, it was selected for the further studies.

According to Hamer (1997), microbially mediated environmental protection and restoration processes involve process cultures comprising multiple microbial consortia and it is the consortium performance, rather than the individual strain performance, that is critical as far as both process efficiency and economics are concerned. Biotreatment processes involve multiple substrates (pollutants) and highly complex mixed microbial cultures, irrespective of whether it is wastewater, waste gas or waste slurry stream that is undergoing treatment. Coats *et al*. (2007) reported polyhydroxyalkanoates (PHAs) production using a mixed microbial consortium indigenous to an activated sludge process on carbon present in municipal wastewaters. Hence, a mixed bacterial methylotrophic consortium, rather than the individual isolates, is effective for wastewater treatment.

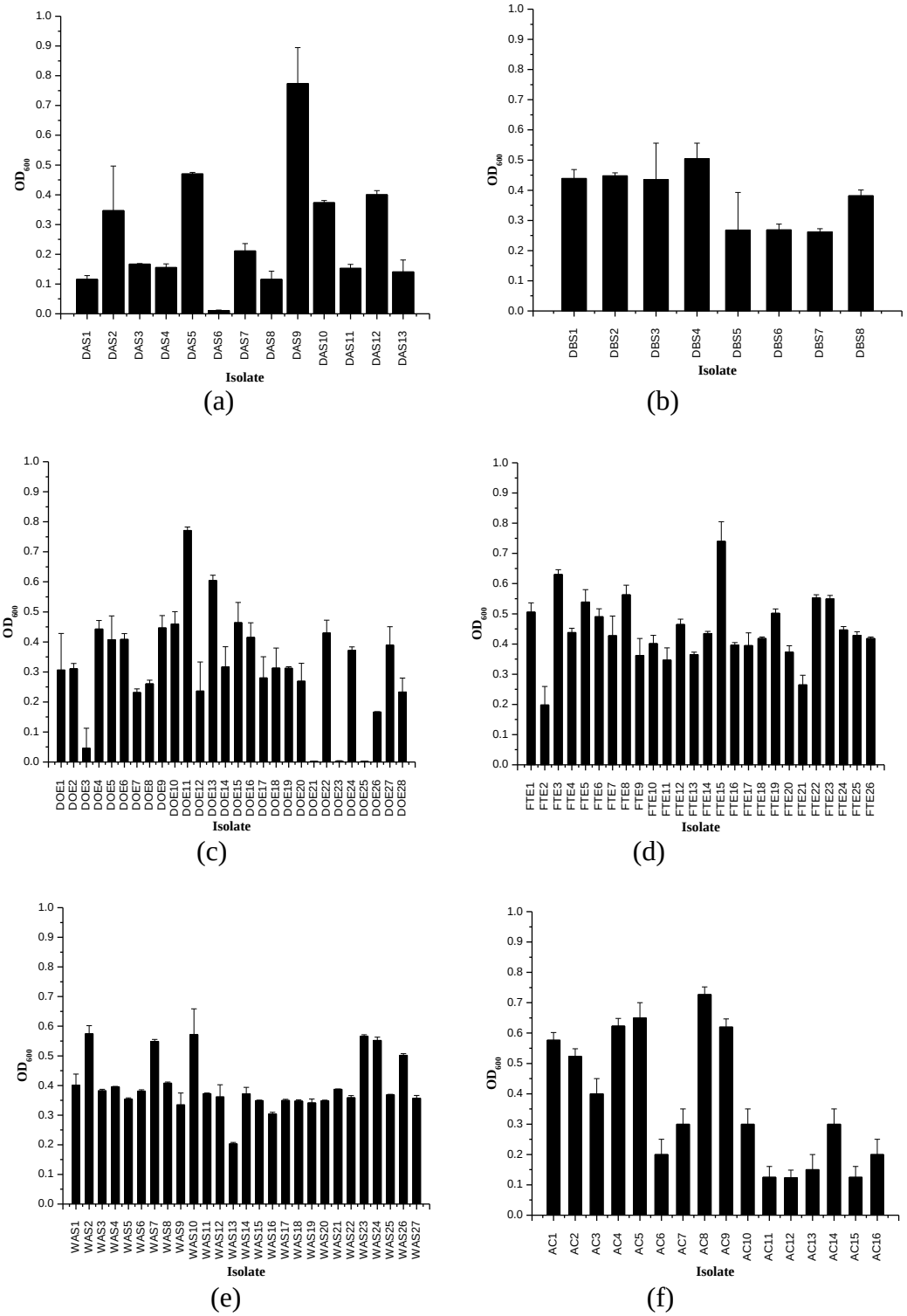


Figure 2.1. Fusel oil utilization ability of (a) DAS, (b) DBS, (c) DOE, (d) FTE, (e) WAS and (f) AC isolates on the basis of their growth at 37 ^{o}C under aerobic conditions after 24 h. Error bars represent standard deviation from the mean, n = 3.

Table 2.2. Cell morphology of the 24 selected isolates

Isolate	Cell morphology	Gram reaction
DAS9	Cocci	Gram negative
DBS4	Thick rods	Gram negative
DOE11	Thick rods	Gram negative
DOE13	Coccobacilli	Gram negative
FTE1	Coccobacilli	Gram negative
FTE3	Cocci	Gram negative
FTE5	Thin rods	Gram positive
FTE8	Cocci	Gram positive
FTE15	Cocci	Gram positive
FTE19	Coccobacilli	Gram negative
FTE22	Cocci	Gram negative
FTE23	Cocci	Gram negative
WAS2	Thin rods	Gram positive
WAS7	Cocci	Gram negative
WAS10	Short rods	Gram positive
WAS23	Thick rods	Gram positive
WAS24	Thin rods	Gram positive
WAS26	Short rods	Gram positive
AC1	Coccobacilli	Gram negative
AC4	Long rods	Gram positive
AC5	Coccobacilli	Gram negative
AC8	Thin rods	Gram negative

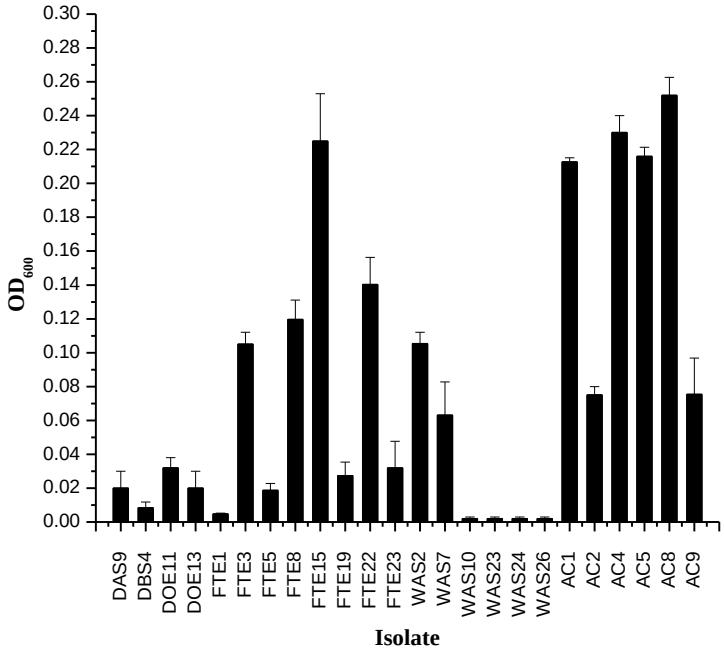


Figure 2.2. Screening of isolates on the basis of their growth in DNR effluent. Error bars represent standard deviation from the mean, n = 3.

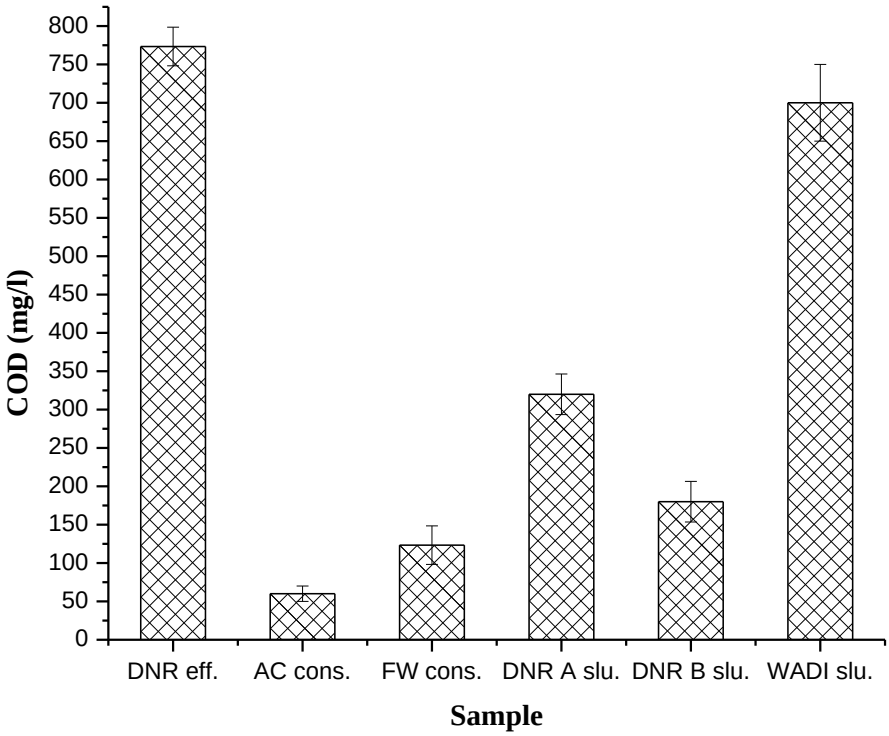


Figure 2.3. Biotreatment potential of different consortia and sludge samples based on the COD reduction of DNR effluent at flask level

2.3.4. Growth profile of the isolates of AC consortium

Growth profiles of the isolates of AC consortium were examined and found comparable. It was observed that the isolates showed a lag phase between 1 - 7 h, followed by a log phase up to 16 - 24 h (Figure 2.4). The specific growth rate of the isolate AC1 was 0.15 h^{-1}, that of isolate AC4 was 0.47 h^{-1}, that of isolate AC5 was 0.38 h^{-1} and that of isolate AC8 was 0.33 h^{-1}. Thus, it can be concluded that the growth curves of all the 4 isolates of AC consortium were similar, which is an important attribute of individual isolates comprising a consortium.

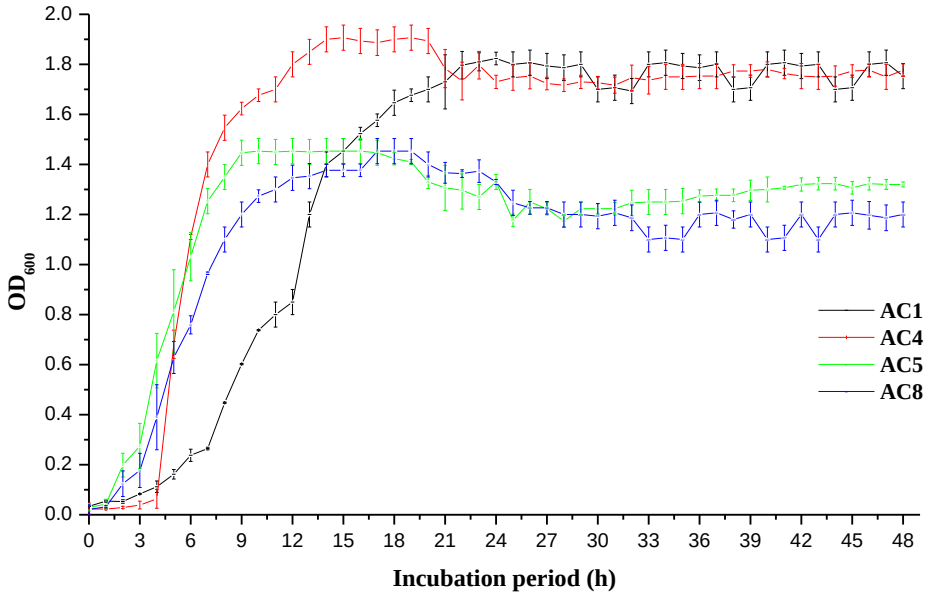


Figure 2.4. Growth profiles of the individual isolates of AC consortium in LB medium. Error bars represent standard deviation from the mean, n = 3.

2.3.5. Confirmation of methylotrophy of the isolates of AC consortium

2.3.5.1. Methanol utilization by the AC consortium and its individual isolates

Ability of the AC consortium and its 4 individual isolates to utilize methanol as the sole carbon source was checked in terms of their growth and COD reduction. As shown in Figure 2.5, all the individual isolates were able to utilize methanol completely in 120 h, while the AC consortium utilized methanol completely in 96 h. Thus, it implied that the isolates of AC consortium showed higher activity in terms of COD reduction when they were present together as compared to their individual activity.

2.3.5.2. Utilization of methanol present in DNR effluent by the individual isolates of AC consortium

The ability of the isolates of AC consortium to use methanol as the sole carbon source was also determined during incubation in DNR effluent by GC. The methanol concentration of DNR effluent of 2549.96 mg/l was reduced to 1.97 mg/l by isolate AC1, 0.70 mg/l by isolate AC4, 1.56 mg/l by isolate AC5 and 0.00 mg/l by isolate AC8 (Figure 2.6). More than 95 % methanol biodegradation was obtained using the isolates AC1 (99 %), AC4 (99 %), AC5 (99 %) and AC8 (100 %). Thus, the isolates of AC consortium were confirmed as methylotrophs since they were able to utilize methanol which is a single carbon source.

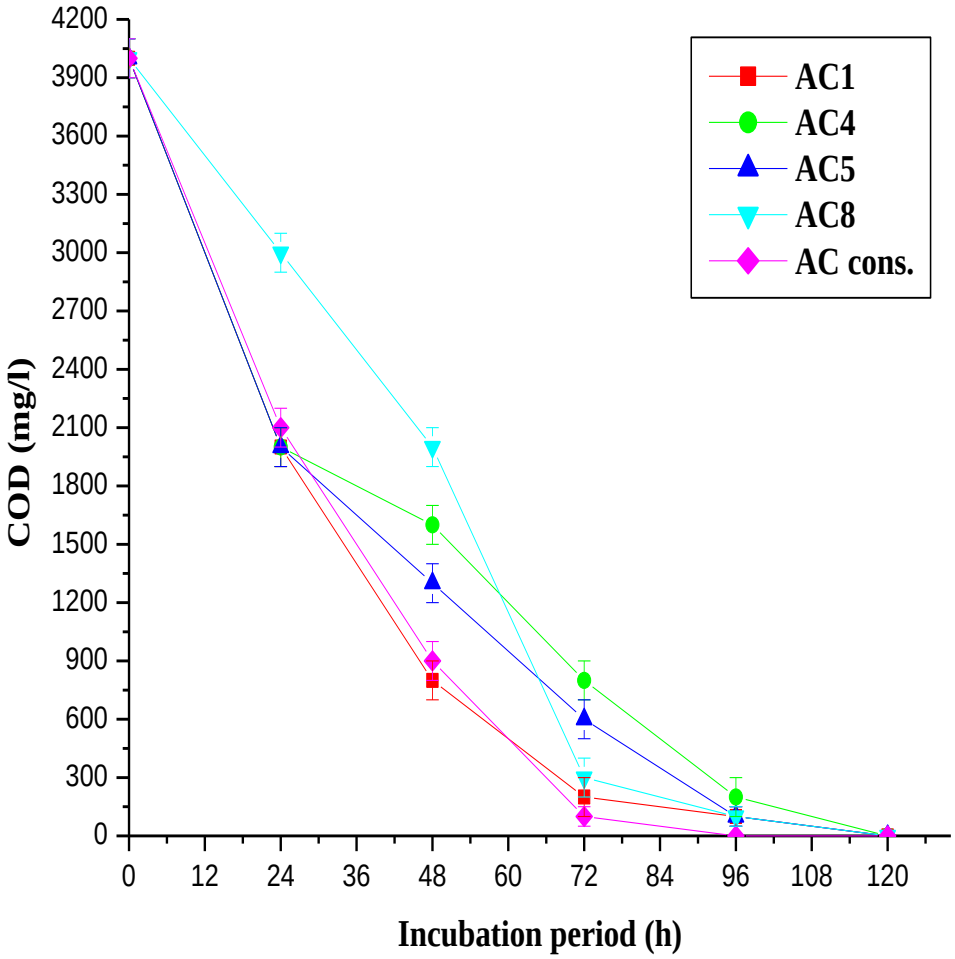
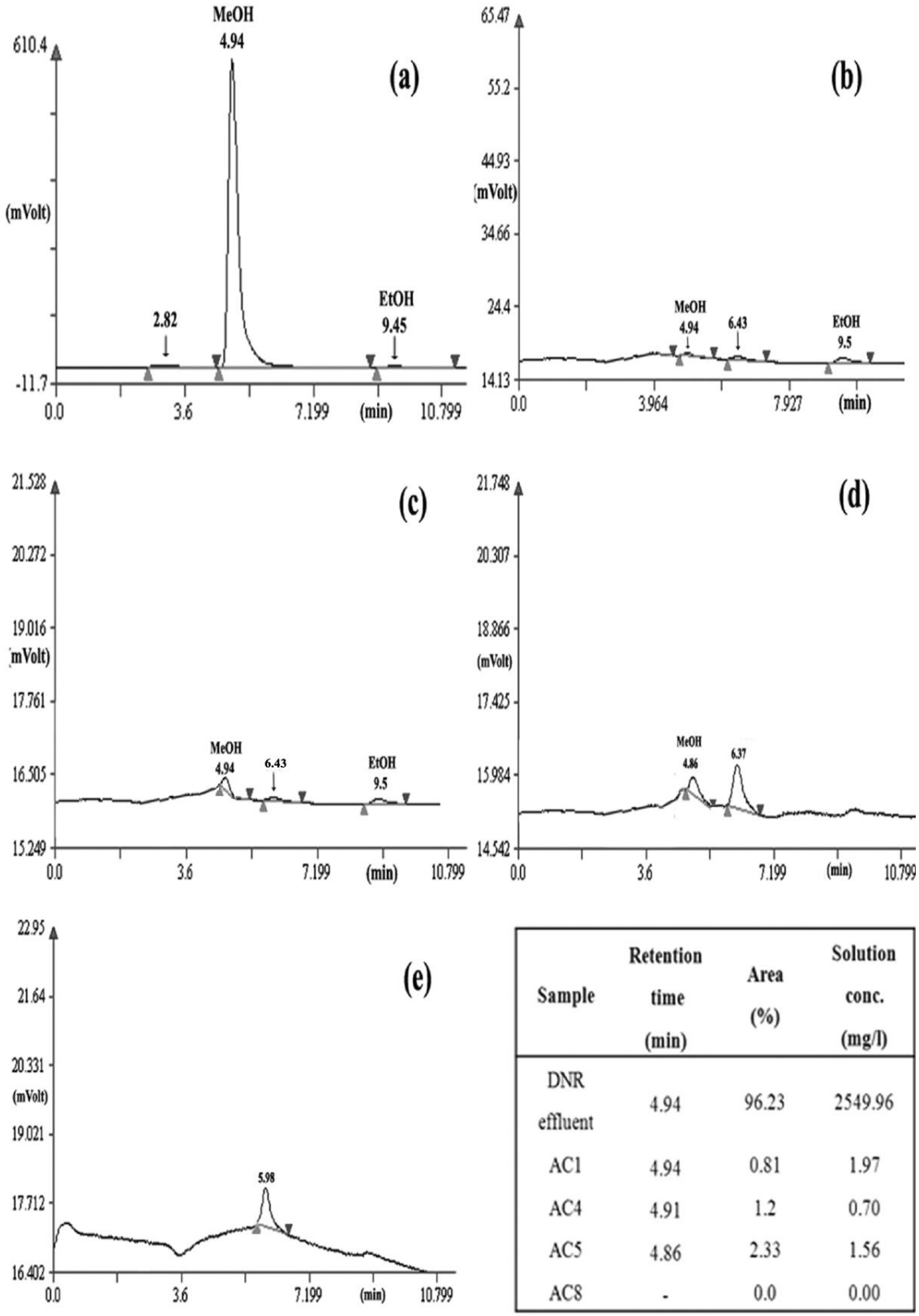


Figure 2.5. Methanol utilization ability of the AC consortium and its individual isolates. Error bars represent standard deviation from the mean, n = 3.



Sample	Retention time (min)	Area (%)	Solution conc. (mg/l)
DNR effluent	4.94	96.23	2549.96
AC1	4.94	0.81	1.97
AC4	4.91	1.2	0.70
AC5	4.86	2.33	1.56
AC8	-	0.0	0.00

Figure 2.6. Gas chromatograms depicting utilization of methanol present in DNR effluent by the isolates of AC consortium. (a) Uninoculated control, (b) AC1, (c) AC4, (d) AC5 and (e) AC8

2.3.5.3. *mxaF* gene detection in the isolates of AC consortium

The *mxaF* gene has been traditionally used as a genetic marker for environmental detection of methanol-oxidizing capability of methylotrophs (McDonald *et al*., 2008). Expression of *mxaF* gene is a promising biomarker of *in situ* denitrification activity on methanol in mixed-culture engineered wastewater treatment processes (Lu *et al*., 2011). Suzuki *et al*. (2009) used *mxaF* specific primers to identify methylotrophic strains which can utilize methanol as a sole source of carbon. Hence, *mxaF* gene is widely used as a biomarker to identify methylotrophic bacteria present in different habitats.

In the present study, the isolates of AC consortium were further authenticated for methylotrophy by PCR amplification of the partial *mxaF* gene using genomic DNA of the individual isolates. The results confirmed the presence of about 550 bp sized partial *mxaF* gene in all the isolates of AC consortium (Figure 2.7).

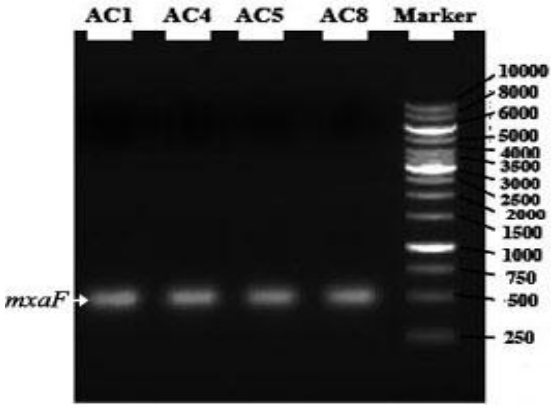


Figure 2.7. PCR amplification of *mxaF* gene in the isolates of AC consortium.

2.3.6. Polyphasic identification

2.3.6.1. Biochemical characterization of the isolates of AC consortium

The isolates of AC consortium were identified phenotypically using biochemical tests (Table 2.3). Thus, isolate AC1 was identified as *Bordetella petrii*, isolate AC4 as *Bacillus licheniformis*, isolate AC5 as *Salmonella subterranea* and isolate AC8 as *Pseudomonas stutzeri*.

2.3.6.2. Identification of the isolates of AC consortium by 16S rRNA gene sequencing

The members of AC consortium were also identified by 16S rRNA gene sequencing. Accordingly, isolate AC1 showed 98 % sequence similarity to *B. petrii*, isolate AC4 showed 99 % sequence similarity to *B. licheniformis*, isolate AC5 showed

98 % sequence similarity to *S. subterranea* and isolate AC8 showed 98 % sequence similarity to *P. stutzeri*.

Thus, isolate AC1 was named as *B. petrii* AC1, isolate AC4 as *B.licheniformis* AC4, isolate AC5 as *S. subterranea* AC5 and isolate AC8 as *P. stutzeri* AC8 based on their phenotypic characteristics and 16S rRNA gene sequence analysis.

B. petrii is the only environmental species hitherto found among the otherwise host-restricted and pathogenic members of the genus *Bordetella*. Its metabolic versatility may enable *B. petrii* to thrive in very different ecological niches (von Wintzingerode *et al*., 2001). *B. petrii* strains were repeatedly found in environmental samples, e.g. microbial consortia degrading aromatic compounds (Bianchi *et al*., 2005; Wang *et al*., 2007).

B. licheniformis strains are widely distributed in the environment; they are common in most soils and dominate in nutrient poor soils such as moorland and deserts. They are used for the manufacture of several industrial products and, in particular, extracellular enzymes such as amylase and proteases (de Boer *et al*., 1994).

S. subterranea is reported to be a new species of genus *Salmonella*. It is better adapted to living in soils than most of its enteric relatives. Shelobolina *et al*. (2004) described the isolation of a facultatively anaerobic, acid tolerant, *S. subterranea* strain FRCl, from a low pH, nitrate- and uranium-contaminated sediment at the Natural and Accelerated Bioremediation Research (NABIR) Field Research Center in Oak Ridge, Tennesse, USA.

P. stutzeri is distributed widely in the environment, occupying diverse ecological niches. It has been demonstrated that *P. stutzeri* is also distributed in wastewater. Like other *Pseudomonas* species (e.g. *P. putida*), *P. stutzeri* is involved in environmentally important metabolic activities. Some of its major tasks are metal cycling and degradation of biogenic and xenobiotic compounds (Lalucat *et al*., 2006).

B. licheniformis and *P. stutzeri* have been reported as methylotrophic in nature (Anesti *et al*., 2005; Bulygina *et al*., 1990). However, to our knowledge, no reports are available to date characterizing *B. petrii* and *S. subterranea* as methylotrophs. Hence, this is the first report of methylotrophic *B. petrii* and *S. subterranea*.

Table 2.3. Biochemical characteristics of the isolates of AC consortium

Test	AC1	***Bordetella petrii*[a]**	AC4	***Bacillus licheniformis*[b]**	AC5	***Salmonella subterranea*[c]**	AC8	***Pseudomonas stutzeri*[d]**
Gram staining	Gram negative	Gram negative	Gram positive	Gram positive	Gram negative	Gram negative	Gram negative	Gram negative
Endospore staining	ND	ND	Terminal	Terminal	ND	ND	ND	ND
Glucose fermentation	-	-	+	+	+	+	ND	ND
Citrate utilization	+	+	ND	ND	-	-	-	-
Triple sugar iron test:								
Slant	Alkaline	Alkaline	Acidic	Acidic	ND	ND	ND	ND
Butt	No change	No change	Acidic	Acidic	ND	ND	ND	ND
Gas production	-	-	+	+	ND	ND	ND	ND
H_2S production	-	-	-	-	-	-	ND	ND
Catalase	+	+	+	+	ND	ND	+	+
Oxidase	+	+	+	+	ND	ND	+	+
Growth on Mac Conkey's agar	+	+	ND	ND	ND	ND	ND	ND
Growth on LB with:								
8 % NaCl	ND	ND	+	+	ND	ND	ND	ND
12 % NaCl	ND	ND	-	-	ND	ND	ND	ND
ONPG	ND	ND	ND	ND	+	+	ND	ND
Lysine utilization	ND	ND	ND	ND	-	-	ND	ND
Ornithine utilization	ND	ND	ND	ND	-	+	ND	ND
Urease	ND	ND	ND	ND	-	-	ND	ND
Phenylalanine deaminase	ND	ND	ND	ND	-	-	ND	ND

Nitrate reduction	ND	ND	ND	ND	+	+	ND	ND
Voges Proskauer	ND	ND	ND	ND	-	-	+	+
Methyl red	ND	ND	ND	ND	+	+	-	-
Indole	ND	ND	ND	ND	+	+	-	-
Malonate utilization	ND	ND	ND	ND	-	-	ND	ND
Esculin hydrolysis	ND	ND	ND	ND	+	-	ND	ND
Acid production from:								
Arabinose	ND	ND	ND	ND	+	+	ND	ND
Xylose	ND	ND	ND	ND	+	+	ND	ND
Adonitol	ND	ND	ND	ND	-	-	ND	ND
Rhamnose	ND	ND	ND	ND	+	+	ND	ND
Cellobiose	ND	ND	ND	ND	+	-	ND	ND
Melibiose	ND	ND	ND	ND	-	-	ND	ND
Raffinose	ND	ND	ND	ND	-	+/-	ND	ND
Trehalose	ND	ND	ND	ND	+	-	ND	ND
Lactose	ND	ND	ND	ND	-	+	ND	ND

ND – Not Determined, [a] von Winzingerode *et al*. (2001), [b] Suthar *et al*. (2009), [c] Shelobolina *et al*. (2004), [d] Lalucat *et al*. (2006)

2.3.7. Phylogenetic analysis

Phylogenetic analysis of the isolates of AC consortium and other related sequences from public databases was performed using 1,000 bases of the 16S rRNA gene for comparison. The results obtained indicated that *B. petrii* AC1 was phylogenetically most closely related to *B. petrii* AJ870969, *B. licheniformis* AC4 to *B. licheniformis* EU256500, *S. subterranea* AC5 to *S. subterranea* AY373829 and *P. stutzeri* AC8 to *P. stutzeri* GU339239 (Figure 2.8). *B. petrii* AJ870969 was the first clinical isolate (Fry *et al*., 2005) of the only environmental species of the genus *Bordetella* (von Wintzingerode *et al*., 2001). *B. licheniformis* EU256500 was isolated from an air-dried sausage from Beijing, China. *S. subterranea* AY373829 was isolated from a low-pH, nitrate- and U(VI)-contaminated subsurface sediment at the Natural and Accelerated Bioremediation Research Field Research Center in Oak Ridge, Tennessee, USA (Shelobolina *et al*., 2004). *P. stutzeri* GU339239 was isolated from the Rajkot industrial estate, polluted with oil spills from coconut-extracting industry. Hence, it can be observed that *B. petrii*, *B. licheniformis*, *S. subterranea* and *P. stutzeri* are widely distributed in nature.

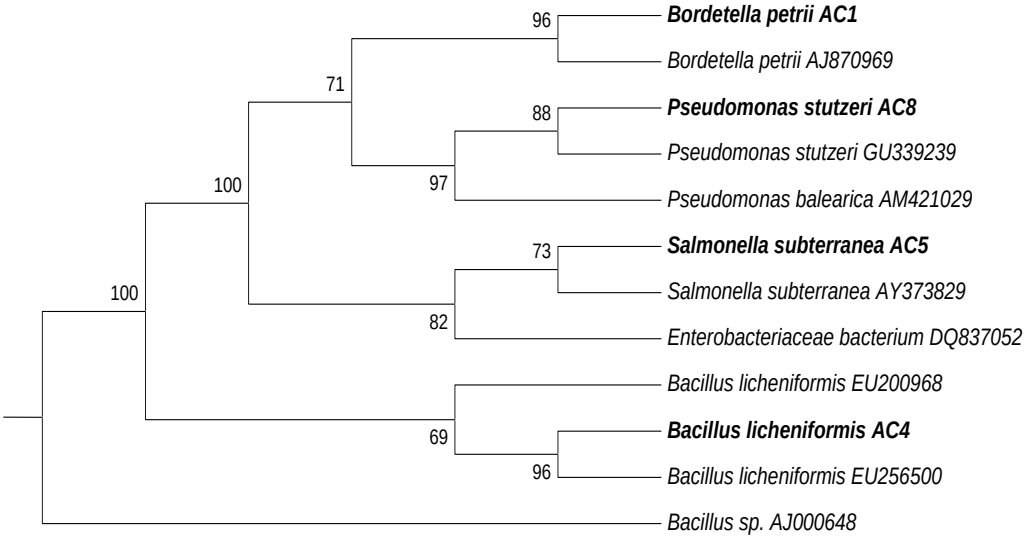


Figure 2.8. Phylogenetic analysis of the isolates of AC consortium. The tree was constructed by distance analysis with Jukes-Cantor correction. Numbers next to branches indicate bootstrap values.

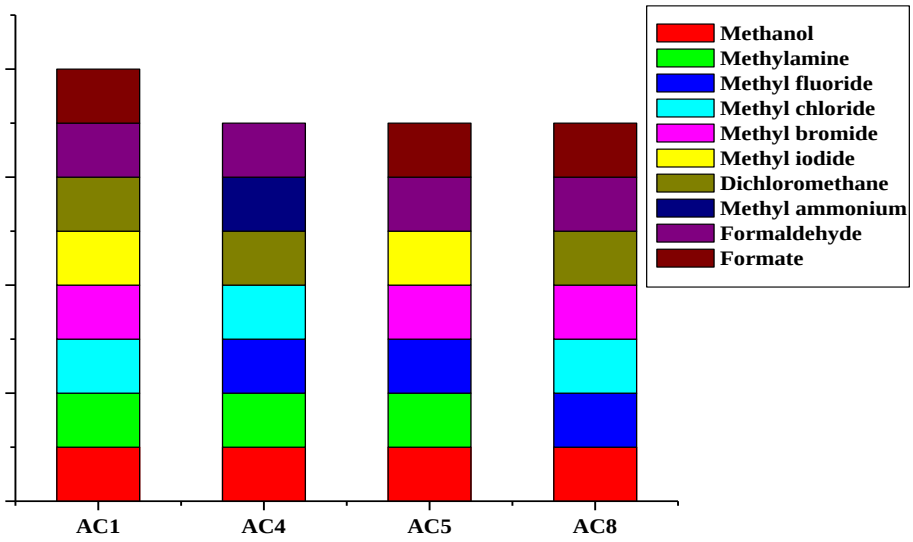
2.3.8. Carbon substrate utilization

Methylotrophic bacteria utilize a variety of different one-carbon compounds including methane, methanol, methylated amines, halomethanes and methylated compounds containing sulfur, and multi-carbon compounds such as dimethyl ether, dimethylamine, pyruvate and succinate (Hanson and Hanson, 1996; Skovran *et al*., 2010). Carbon substrate utilization profile will give an insight into the metabolic diversity of the isolates as wider metabolic diversity broadens the potential of the isolates and, in turn, the consortium in utilizing a variety of industrial effluents. Hence, carbon substrate utilization ability of the isolates of AC consortium was studied.

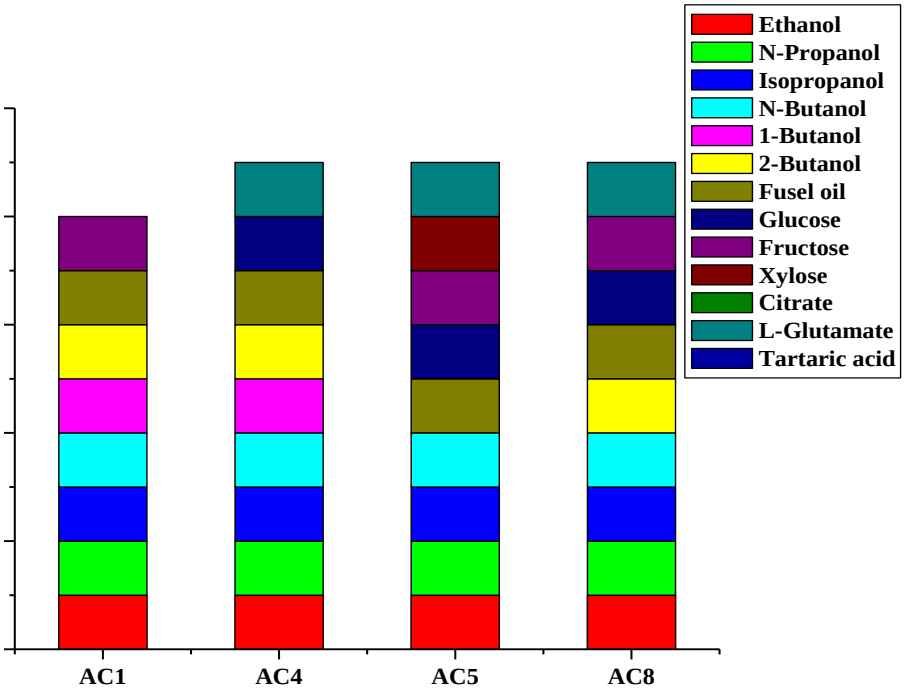
2.3.8.1. Differential carbon substrate utilization pattern among the isolates of AC consortium

The metabolic diversity of the individual isolates of AC consortium was studied in terms of their ability to utilize a variety of single carbon and multi carbon substrates. Among the 10 single carbon substrates provided as sole carbon source, *B. petrii* AC1 was the best of all the 4 isolates in that it showed growth on 8 out of 10 substrates, while *B. licheniformis* AC4, *S. subterranea* AC5 and *P. stutzeri* AC8 could grow on 7 out of 10 substrates (Figure 2.9(a)). All the isolates showed growth on methanol and formaldehyde. *B. licheniformis* AC4 was the only isolate to grow on methyl ammonium; methyl amine, methyl bromide, methyl chloride and methyl fluoride were utilized by at least 3 of the 4 isolates, while methyl iodide was a growth substrate only for *B. petrii* AC1 and *S. subterranea* AC5. Generally, all the single carbon substrates were used for growth by the members of AC consortium put together. Among the multi carbon substrates tested as sole carbon sources, ethanol, N-propanol, isopropanol, N-butanol and fusel oil were growth substrates for all the isolates of AC consortium; while 2-butanol, glucose, fructose and L-glutamate served as substrates for at least 3 out of the 4 members of AC consortium (Figure 2.9(b)). Xylose was utilized for growth only by *S. subterranea* AC5. The members of AC consortium being facultative methylotrophs were able to utilize a variety of single and multi carbon substrates for growth equally well and, therefore, would be suitable for biodegradation studies.

In a similar study, the carbon substrate utilization profile of pink pigmented facultative methylotrophic isolates, obtained from cotton, maize and sunflower leaf samples, was analyzed and compared with that of standard species (Balachandar *et al*., 2008). Likewise, the comparison of differential carbon-substrate utilization pattern of the isolates of AC consortium demonstrated their broad metabolic diversity and, therefore, their promising biotreatment potential.



(a)



(b)

Figure 2.9. (a) Single and (b) multi carbon substrate utilization pattern of the isolates of AC consortium

2.3.8.2. Alcohol utilization pattern of AC consortium and its individual isolates

Metabolic diversity of the individual isolates of AC consortium, further studied in terms of their ability to utilize a variety of industrially important alcohols, demonstrated that the AC consortium and its individual members could grow on all the alcohols tested. *B. petrii* AC1, *P. stutzeri* AC8 and AC consortium showed higher utilization of methanol, ethanol, 1-propanol and 2-propanol; *B. licheniformis* AC4 showed higher utilization of methanol and ethanol, and *S. subterranea* AC5 showed higher utilization of methanol, ethanol and 1-propanol as compared to other alcohols tested (Figure 2.10). Piveteau *et al*. (2001) have reported alcohol degradation by methylotrophic bacterium, *B. cepacia* CIP I-2052. All the members of AC consortium could utilize a wide range of alcohols, among which methanol, ethanol, 1-propanol and 2-propanol were utilized to a greater extent. Thus, these results were important in confirming the nature of the isolates as facultative methylotrophs.

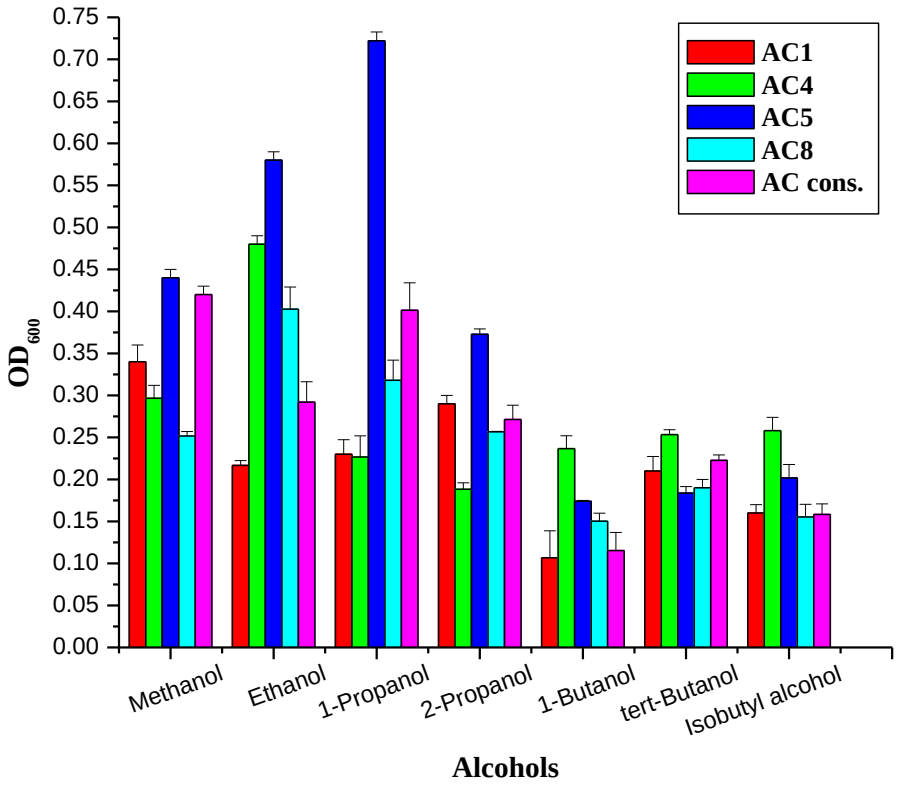


Figure 2.10. Alcohol utilization by the AC consortium and its individual isolates. Error bars represent standard deviation from the mean, n = 3.

2.3.8.3. Biodegradation of different carbon substrates and xenobiotics by AC consortium

Having studied the substrate utilization profile of the isolates of AC consortium, the biodegradation ability of the AC consortium was further checked on different carbon substrates and xenobiotics in terms of their growth and COD reduction. It was observed that out of the 15 substrates used in this study, the AC consortium showed complete degradation of acetate, succinate and pyruvate at 100 %; methanol, ethanol, propanol, 2-propanol, 2-butanol and acetone were maximally utilized at > 50 %; whereas butanol, formaldehyde, *tert*-amyl alcohol, benzene, xylene and toluene were utilized at < 50 % (Figure 2.11). Thus, it was observed that the biodegradation ability of the AC consortium differed from substrate to substrate. Moreover, the AC consortium was able to degrade all of the xenobiotics tested, viz. TAA, benzene, xylene and toluene.

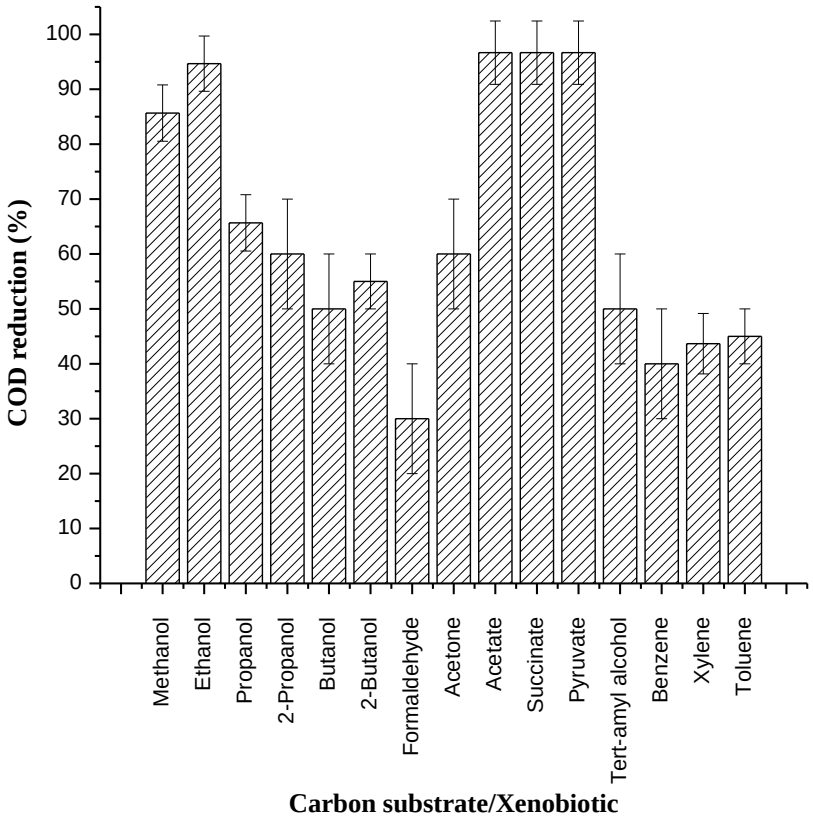


Figure 2.11. Carbon substrate/xenobiotic biodegradation ability of the AC consortium in terms of COD reduction. Error bars represent standard deviation from the mean, n = 3.

The ability of methylotrophic *B. cepacia* CIP I-2052 to use different carbon substrates as the sole carbon and energy source was determined by Piveteau *et al*. (2001), showing that it could not utilize primary and secondary alcohols, except methanol, and acetone. *Rhodococcus wratislaviensis* IFP 2016 was able to completely degrade benzene, toluene, ethylbenzene and xylene (Auffret *et al*., 2009). In comparison to these reports, the AC consortium could readily utilize all the carbon substrates/xenobiotics tested. Hence, it can be concluded that the AC consortium has a broad biodegradation potential.

2.3.9. MDH estimation in the isolates of AC consortium

MDH is the first enzyme of the serine as well as RuMP pathways used as a marker for methylotrophs. MDH was isolated from the isolates of AC consortium by following the method of Liu *et al*. (2006). The MDH present in the isolates of AC consortium was in the range of 0.16 - 0.56 units/ml with its specific activity in the range of 0.48 - 0.87 units/mg (Table 2.4).

Eshraghi (2007) reported NAD^+-dependent MDH activity of 0.11 units/mg from methylotrophic *Pseudonocardia* sp. 381 isolated from compost. Liu *et al*. (2006) reported MDH activity of 0.25 units/mg from *Methylobacterium extorquens* AM1. Thus, MDH activities of the isolates of AC consortium were higher than those reported.

Table 2.4. MDH activity of the isolates of AC consortium

Isolate	MDH (units/ml)	Specific activity (units/mg)
B. petrii AC1	0.56	0.87
B. licheniformis AC4	0.31	0.71
S. subterranea AC5	0.16	0.48
P. stutzeri AC8	0.24	0.62

2.3.10. Methanol tolerance of the isolates of AC consortium

2.3.10.1. Exposure to vapor phase methanol on solid media

The isolates of AC consortium were able to grow on MM2 agar, observed in terms of cell colonies, after 120 h exposure to 2 g/l methanol supplied in the

desiccator system. 300 cfu of *B. petrii* AC1, 42 cfu of *B. licheniformis* AC4, 200 cfu of *S. subterranea* AC5 and 80 cfu of *P. stutzeri* AC8 were observed on the methanol exposed plates. The results obtained indicated that the isolates of AC consortium could readily utilize vapor phase methanol, as is the observation of Gutierrez (1999).

2.3.10.2. Exposure to pre-solubilized concentrations of methanol in liquid media

Methanol grown cells of the isolates of AC consortium were found capable of tolerating exposure to shock concentrations of up to 1.2 g/l methanol, as observed by an increase in their growth (OD_{600}) after exposure to concentrations up to 1.2 g/l (Figure 2.12).

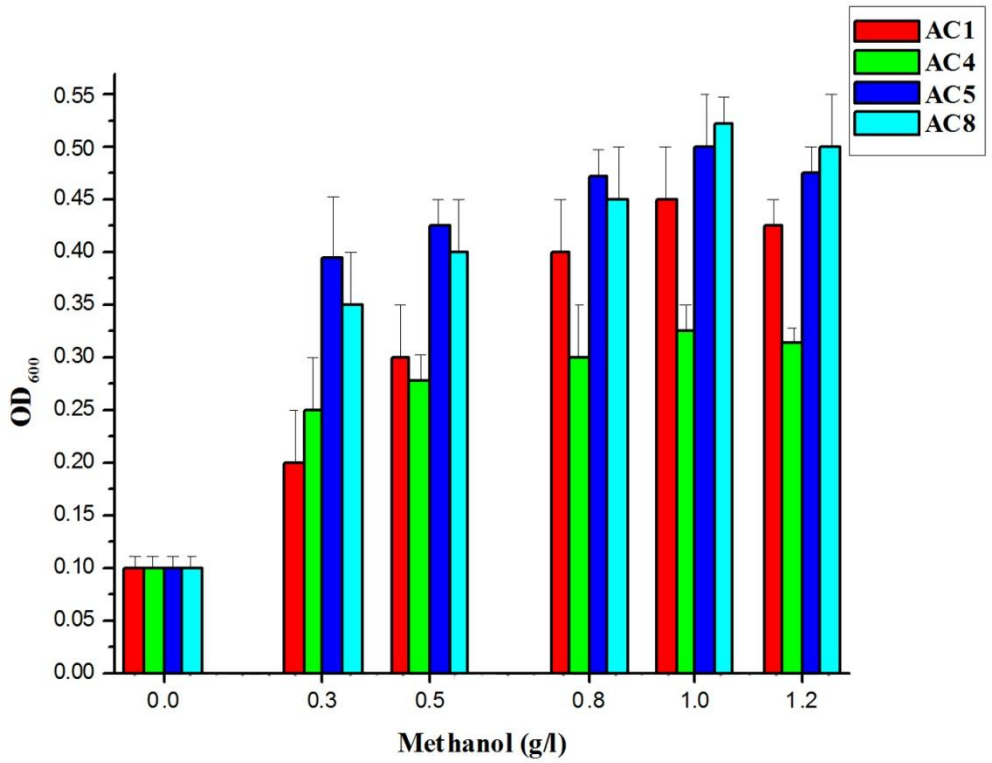


Figure 2.12. Growth of the isolates of AC consortium after 120 h exposure to step concentrations of pre-solubilized methanol in liquid medium

Hence, the isolates of AC consortium could tolerate methanol up to 1.2 g/l, which is a highly toxic concentration of methanol (Skovran *et al.*, 2010), thus, implying that the AC consortium could be used for treatment of industrial effluents containing high concentrations of methanol.

The isolates of AC consortium, viz. *B. petrii* AC1, *B. licheniformis* AC4, *S. subterranea* AC5 and *P. stutzeri* AC8, were fast-growing which grew well together in the consortium. They were confirmed as methylotrophs both phenotypically and genotypically. The methylotrophic isolates of the species, *B. petrii* and *S. subterranea*, were reported here for the first time. The isolates of AC consortium showed a broad metabolic diversity which included many xenobiotics. They could tolerate high concentrations of methanol due to their high MDH activity. In conclusion, all the 4 isolates comprising the AC consortium were found to possess the potential of being applied for wastewater treatment and, hence, were selected for the further studies.

2.4. References

Anesti, V., McDonald, I.R., Ramaswamy, M., Wade, W.G., Kelly, D.P., Wood, A.P., 2005. Isolation and molecular detection of methylotrophic bacteria occurring in the human mouth. *Environmental microbiology*, 7, 1227-1238.

Anthony, C., 1982. The biochemistry of methylotrophs, Academic Press, London.

Anthony, C., Williams, P., 2003. The structure and mechanism of methanol dehydrogenase. *Biochimica et Biophysica Acta (BBA)-Proteins & Proteomics*, 1647, 18-23.

Arfman, N., Watling, E.M., Clement, W., van Oosterwijk, R.J., de Vries, G.E., Harder, W., Attwood, M.M., Dijkhuizen, L., 1989. Methanol metabolism in thermotolerant methylotrophic *Bacillus* strains involving a novel catabolic NAD-dependent methanol dehydrogenase as a key enzyme. *Archives of Microbiology*, 152, 280-288.

Auffret, M., Labbe, D., Thouand, G., Greer, C.W., Fayolle-Guichard, F., 2009. Degradation of a mixture of hydrocarbons, gasoline, and diesel oil additives by *Rhodococcus aetherivorans* and *Rhodococcus wratislaviensis*. *Applied and environmental microbiology*, 75, 7774-7782.

Balachandar, D., Raja, P., Sundaram, S.P., 2008. Genetic and metabolic diversity of pink-pigmented facultative methylotrophs in phyllosphere of tropical plants. *Brazilian Journal of Microbiology*, 39, 68-73.

Baytshtok, V., Kim, S., Yu, R., Park, H., Chandran, K., 2008. Molecular and biokinetic characterization of methylotrophic denitrification using nitrate and

nitrite as terminal electron acceptors. *Water Science and Technology*, 58, 359-365.

Bianchi, F., Careri, M., Mustat, L., Malcevschi, A., Musci, M., 2005. Bioremediation of Toluene and Naphthalene: development and Validation of a GC-FID Method for Their Monitoring. *Annali di chimica*, 95, 515-524.

de Boer, A.S., Priest, F., Diderichsen, B., 1994. On the industrial use of *Bacillus licheniformis*: a review. *Applied Microbiology and Biotechnology*, 40, 595-598.

Bulygina, E.S., Galchenko, V.F., Govorukhina, N.I., Netrusov, A.I., Nikitin, D.I., Trotsenko, Y.A., Chumakov, K.M., 1990. Taxonomic studies on methylotrophic bacteria by 5S ribosomal RNA sequencing. *Journal of general microbiology*, 136, 441-446.

Chistoserdova, L., 2011. Modularity of methylotrophy, revisited. *Environmental microbiology*, 13, 2603-2622.

Chongcharoen, R., Smith, T.J., Flint, K.P., Dalton, H., 2005. Adaptation and acclimatization to formaldehyde in methylotrophs capable of high-concentration formaldehyde detoxification. *Microbiology*, 151, 2615-2622.

Coats, E.R., Loge, F.J., Wolcott, M.P., Englund, K., McDonald, A.G., 2007. Synthesis of polyhydroxyalkanoates in municipal wastewater treatment. *Water Environment Research*, 79, 2396-2403.

Crowther, G., 2004. Alcohol consumption as a way of life: the metabolism of methylotrophic bacteria. Lidstrom Lab, Washington.

Doronina, N.V., Trotsenko, Y.A., Tourova, T.P., Kuznetsov, B.B., Leisinger, T., 2000. *Methylopila helvetica* sp. nov. and *Methylobacterium dichloromethanicum* sp. nov. - Novel Aerobic Facultatively Methylotrophic Bacteria Utilizing Dichloromethane. *Systematic and applied microbiology*, 23, 210-218.

Eller, G., Kruger, M., Frenzel, P., 2005. Comparing field and microcosm experiments: a case study on methano- • and methyl-trophic bacteria in paddy soil. *FEMS microbiology ecology*, 51, 279-291.

Eshraghi, S., 2007. NAD^+-Dependent Methanol Dehydrogenase from One-Carbon Compound Utilizer Thermoactinomycete. *Journal of Biological Sciences*, 7, 205-209.

Fayolle, F., Hernandez, G., Le Roux, F., Vandecasteele, J.-P., 1998. Isolation of two aerobic bacterial strains that degrade efficiently ethyl t-butyl ether (ETBE). *Biotechnology letters*, 20, 283-286.

Fry, N.K., Duncan, J., Malnick, H., Warner, M., Smith, A.J., Jackson, M.S., Ayoub, A., 2005. *Bordetella petrii* clinical isolate. *Emerging infectious diseases*, 11, 1131.

Grafstrom, R.C., Fornace, A.J., Autrup, H., Lechner, J.F., Harris, C.C., 1983. Formaldehyde damage to DNA and inhibition of DNA repair in human bronchial cells. *Science*, 220, 216-218.

Gutierrez, J.A., 1999. Mechanisms Conferring a *Rhodococcus* Species with High Resistance to Benzene. University of New South Wales.

Hamer, G., 1997. Microbial consortia for multiple pollutant biodegradation. *Pure and applied chemistry*, 69, 2343-2356.

Hanson, R.S., Hanson, T.E., 1996. Methanotrophic bacteria. *Microbiological reviews*, 60, 439-471.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., Williams, S.T., 1994. Bergey's Manual of determinative bacteriology, ninth ed. Williams & Wilkins Press, USA, pp. 71-561.

Kalyuzhnaya, M.G., Zabinsky, R., Bowerman, S., Baker, D.R., Lidstrom, M.E., Chistoserdova, L., 2006. Fluorescence in situ hybridization-flow cytometry-cell sorting-based method for separation and enrichment of type I and type II methanotroph populations. *Applied and environmental microbiology*, 72, 4293-4301.

Lalucat, J., Bennasar, A., Bosch, R., Garcia-Valdes, E., Palleroni, N.J., 2006. Biology of *Pseudomonas stutzeri*. *Microbiology and Molecular Biology Reviews*, 70, 510-547.

Liu, Q., Kirchhoff, J.R., Faehnle, C.R., Viola, R.E., Hudson, R.A., 2006. A rapid method for the purification of methanol dehydrogenase from *Methylobacterium extorquens*. *Protein expression and purification*, 46, 316-320.

Lu, H., Nuruzzaman, F., Ravindhar, J., Chandran, K., 2011. Alcohol dehydrogenase expression as a biomarker of denitrification activity in activated sludge using methanol and glycerol as electron donors. *Environmental microbiology*, 13, 2930-2938.

MacFaddin, J.F., 1980. Biochemical Tests for Identification of Medical Bacteria, second ed. Williams and Wilkins, Baltimore.

de Marco, P., Pacheco, C.C., Figueiredo, A.R., Moradas-Ferreira, P., 2004. Novel pollutant-resistant methylotrophic bacteria for use in bioremediation. *FEMS microbiology letters*, 234, 75-80.

McDonald, I.R., Kenna, E.M., Murrell, J.C., 1995. Detection of methanotrophic bacteria in environmental samples with the PCR. *Applied and environmental microbiology*, 61, 116-121.

McDonald, I.R., Bodrossy, L., Chen, Y., Murrell, J.C., 2008. Molecular ecology techniques for the study of aerobic methanotrophs. *Applied and environmental microbiology*, 74, 1305-1315.

Miller, L.G., Kalin, R.M., McCauley, S.E., Hamilton, J.T.G., Harper, D.B., Millet, D.B., Oremland, R.S., Goldstein, A.H., 2001. Large carbon isotope fractionation associated with oxidation of methyl halides by methylotrophic bacteria. *Proceedings of the National Academy of Sciences*, 98, 5833-5837.

Nunn, D.N., Day, D., Anthony, C., 1989. The second subunit of methanol dehydrogenase of *Methylobacterium extorquens* AM1. *Biochemical Journal*, 260, 857-862.

Pillai, P., Archana, G., 2008. Hide depilation and feather disintegration studies with keratinolytic serine protease from a novel *Bacillus subtilis* isolate. *Applied Microbiology and Biotechnology*, 78, 643-650.

Piveteau, P., Fayolle, F., Vandecasteele, J.P., Monot, F., 2001. Biodegradation of *tert*-butyl alcohol and related xenobiotics by a methylotrophic bacterial isolate. *Applied Microbiology and Biotechnology*, 55, 369-373.

Shelobolina, E.S., Sullivan, S.A., O'Neill, K.R., Nevin, K.P., Lovley, D.R., 2004. Isolation, characterization, and U (VI)-reducing potential of a facultatively anaerobic, acid-resistant Bacterium from Low-pH, nitrate-and U (VI)-contaminated subsurface sediment and description of *Salmonella subterranea* sp. nov. *Applied and environmental microbiology*, 70, 2959-2965.

Skovran, E., Crowther, G.J., Guo, X., Yang, S., Lidstrom, M.E., 2010. A systems biology approach uncovers cellular strategies used by *Methylobacterium extorquens* AM1 during the switch from multi-to single-carbon growth. *PLoS One*, 5, e14091.

Srinandan, C.S., Jadav, V., Cecilia, D., Nerurkar, A.S., 2010. Nutrients determine the spatial architecture of *Paracoccus* sp. biofilm. *Biofouling*, 26, 449-459.

Suthar, H., Hingurao, K., Desai, A., Nerurkar, A., 2009. Selective plugging strategy based Microbial Enhanced Oil Recovery using *Bacillus licheniformis* TT33. *Journal of Microbiology and Biotechnology*, 19, 1230-1237.

Suzuki, R., Lisdiyanti, P., Komagata, K., Uchimura, T., 2009. *MxaF* gene, a gene encoding alpha subunit of methanol dehydrogenase in and false growth of acetic acid bacteria on methanol. *The Journal of general and applied microbiology*, 55, 101-110.

Tani, A., Sahin, N., 2013. *Methylobacterium haplocladii* sp. nov., and *Methylobacterium brachythecii* sp. nov., isolated from bryophytes. *International journal of systematic and evolutionary microbiology*.

Tomar, M., 1999. Determination of organic constituents, in: Hauserman, B., Wood, S. (Eds.), Quality assessment of water and wastewater. CRC press, Florida, pp. 183-212.

Wang, F., Grundmann, S., Schmid, M., Dorfler, U., Roherer, S., Charles Munch, J., Hartmann, A., Jiang, X., Schroll, R., 2007. Isolation and characterization of 1, 2, 4-trichlorobenzene mineralizing *Bordetella* sp. and its bioremediation potential in soil. *Chemosphere*, 67, 896-902.

Waturangi, D., Marko, N., Suhartono, M.T., 2011. Purification of methanol dehydrogenase from mouth methylotrophic bacteria of tropical region. *Malaysian Journal of Microbiology*, 7, 226-229.

Widdel, F., 2007. Theory and measurement of bacterial growth. *Di dalam Grundpraktikum Mikrobiologie*, 4, 1-11.

von Wintzingerode, F., Schattke, A., Siddiqui, R.A., Rosick, U., Gobel, U.B., Gross, R., 2001. *Bordetella petrii* sp. nov., isolated from an anaerobic bioreactor, and emended description of the genus *Bordetella*. *International journal of systematic and evolutionary microbiology*, 51, 1257-1265.

Zhang, M., Lidstrom, M.E., 2003. Promoters and transcripts for genes involved in methanol oxidation in *Methylobacterium extorquens* AM1. *Microbiology*, 149, 1033-1040.

Chapter 3
Biodegradation of xenobiotics by the AC consortium

3.1. Introduction

Increasing pollution of the environment by xenobiotic compounds has provoked the need for understanding the impact of these toxic compounds on microbial populations, the catabolic degradation pathway of xenobiotics and upgrade in bioremediation processes. Metabolic pathways and specific operon systems have been found in diverse but limited groups of microbes that are responsible for the transformation of xenobiotic compounds. Adaptation of native microbial community to xenobiotic substrates is, thus, crucial for any mineralization to occur in polluted environment (Ojo, 2007). Biotransformation of xenobiotic compounds in natural environment has been studied by Sinha *et al*. (2009) to understand the microbial ecology, physiology and evolution for their potential in bioremediation. *Comamonas testosteroni* (Boon *et al*., 2000; Kharoune *et al*., 2001b), anaerobic *Chloroflexi* (Yan *et al*., 2009), *Acinetobacter baumannii* CA2, *Pseudomonas putida* CA16 and *Klebsiella* sp. CA17 (Vangnai and Petchkroh, 2007) are some of the specially isolated bacteria performing xenobiotic degradation and utilization as growth substrates.

Methylotrophs have been reported to degrade a variety of xenobiotic compounds found in nature (Bhatt *et al*., 2007; de Marco *et al*., 2004). *Pseudomonas*, *Hyphomicrobium* and *Methylobacterium* strains have been shown to possess the enzymes for xenobiotic degradation (Loffler and Muller, 1991). Doronina *et al*. (2000) have reported *Methylopila helvetica* and *Methylobacterium dichloromethanicum* utilizing dichloromethane (DCM), methanol and methylamine as well as a variety of polycarbon compounds. *Burkholderia cepacia* CIP I-2052, a methylotroph isolated from an activated sludge sample, can utilize *tert*-butyl alcohol (TBA) as its sole carbon and energy source (Piveteau *et al*., 2001).

Several reports as stated above suggest that the biodegradation potential of methylotrophs is commendable. In the light of the versatility of the AC consortium and its members in growing on a variety of compounds reported in chapter 2, it was of interest to study their xenobiotic biodegradation potential in detail. The xenobiotics selected for the studies have been divided into 2 types: soluble and insoluble xenobiotics. Methyl *tert*-butyl ether (MTBE) and its intermediate, TBA, have been chosen from the former, whereas 1,2-Dichloroethane (DCE) and its intermediate, 2-chloroethanol (CE), have been chosen from the latter for biodegradation studies with the AC consortium.

Chapter 3A

Methyl *tert*-Butyl Ether biodegradation by the AC consortium

3A.1. Introduction

Developed to improve combustibility and reduce emissions of toxic air pollutants, methyl *tert*-butyl ether (MTBE) is currently the most common oxygenate in gasoline. Its extensive use, spills and leakages from fuel tanks have led to widespread MTBE pollution of surface water, groundwater and soils (Munoz-Castellanos *et al*., 2006). Its concentration ranges from ng to mg/l in sites affected by point sources (Barcelo, 2007). MTBE is highly water-soluble (51 g/l), influences the taste and odor of water at low concentration (40-70 µg/l) (Rosell *et al*., 2003), and is also regarded as a potential human carcinogen (Squillace *et al*., 1996). Rosell *et al*. (2003) showed that MTBE is recalcitrant in nature, even 5 years were not long enough to completely eliminate this compound.

Microbial degradation of MTBE has been studied previously. The bacteria *Achromobacter xylosoxidans* MCM1/1, *Enterobacter cloacae* MCM2/1, and *Ochrobactrum anthropi* MCM5/1 and the fungus *Exophiala dermatitidis* MCM3/4 showed high levels of MTBE biodegradation (Barbera *et al*., 2011). Salanitro *et al*. (1994) isolated a mixed bacterial culture capable of degrading MTBE. Several cultures, such as *Methylibium petroleiphilum* PM1, *Hydrogenophaga flava* ENV735, *Mycobacterium austroafricanum* IFP 2012 (Chen *et al*., 2007), *Rubrivivax gelatinosus* PM1 (Deeb *et al*., 2001) and *Hydrogenophaga flava* ENV 735 (Hatzinger *et al*., 2001) are able to use MTBE as sole carbon and energy source. Short alkane-oxidizing bacteria have been reported to co-metabolically oxidize MTBE (Garnier *et al*., 2000). Fortin *et al*. (2001) have discussed the characteristics of a consortium degrading MTBE in liquid cultures, while Kane *et al*. (2001) have reported MTBE biodegradation by microcosms having different origins. Various strains of the actinomycete, *Rhodococcus*, viz., *Rhodococcus* sp. (Mo *et al*., 1997), *Rhodococcus aetherivorans* (Goodfellow *et al*., 2004), *Rhodococcus* sp. strain EH831 (Lee and Cho, 2009), *Rhodococcus wratislaviensis* IFP 2016 and *Rhodococcus aetherivorans* IFP 2017 (Auffret *et al*., 2009) were reported to degrade MTBE.

Among the methylotrophs degrading MTBE, *Mycobacterium austroafricanum* IFP 2012 (Fayolle *et al*., 2003; Francois *et al*., 2002; Lopes Ferreira *et al*., 2006a) has been studied in detail. Lopes Ferreira *et al*. (2006b) have characterized a cluster of genes specifically involved in the MTBE biodegradation. Propane-oxidizing bacteria

(Steffan *et al*., 1997) and *Mycobacterium vaccae* JOB5 (Smith *et al*., 2003) are able to degrade MTBE by co-metabolism.

Because of its undesirable and ecologically harmful effects, MTBE removal has become a public health and environmental concern (Corcho *et al*., 2000). Biodegradation is the most cost effective and feasible approach for MTBE removal, as can be inferred from the studies available in literature (Barbera *et al*., 2011). Considering its place as a pollutant and xenobiotic, very few strains capable of growing only on MTBE as sole carbon source have been isolated, whereas MTBE is co-metabolized in many cases (Chen *et al*., 2007; Farrokhi and Ahmadizad, 2009; Francois *et al*., 2002). Studies regarding its biotreatment at bench scale are also sparse. AC consortium and its individual isolates were able to degrade MTBE appreciably among other xenobiotics. Hence, MTBE degradation ability of the AC consortium and its isolates was undertaken in this chapter. Furthermore, TBA, an important intermediate in MTBE degradation, was also taken as a substrate for degradation studies by the AC consortium.

3A.2. Materials and Methods

3A.2.1. Soluble xenobiotic biodegradation

The ability of the isolates of AC consortium to utilize a variety of xenobiotics was checked in terms of their growth on individual xenobiotics. A colony was inoculated in 5 ml PNB medium, incubated at 37 ^{o}C for 12 h at 180 rpm and the cell pellet obtained was suspended in 1 ml MM2 medium. 0.2 ml of this suspension was inoculated in 9.8 ml MM2 medium containing (per liter) KNO_3, 1.0 g; K_2HPO_4, 1.0 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.02 g; $MnCl_2.4H_2O$, 0.002 g; $NaMoO_4.2H_2O$, 0.001g; $FeSO_4.7H_2O$, 0.05 g; Yeast extract, 0.5 g, pH, 7.0 (Srinandan *et al*., 2010) supplemented with 0.5 % (v/v) xenobiotic as its sole carbon source. The tubes were incubated at 37 ^{o}C at 180 rpm for 120 h. Growth of the isolates was measured in terms of their OD at 600 nm. The soluble xenobiotics used for this study included: *tert*-amyl alcohol (TAA), methylamine, CE, *tert*-amyl methyl ether (TAME), MTBE, methacrylic acid, epichlorohydrin, trimethylamine hydrochloride (TMAH) and allyl chloride (Dijkhuizen *et al*., 1978; Piveteau *et al*., 2001).

3A.2.2. MTBE biodegradation

3A.2.2.1. MTBE biodegradation by AC consortium and its individual isolates

To analyze the MTBE degradation efficiency of the AC consortium, 2 % inoculum was inoculated in 9.8 ml MM2 medium supplemented with 1 % (v/v) MTBE as its sole carbon source. The tubes were incubated at 37 ˚C at 180 rpm for 120 h.

Growth of the isolates on MTBE was measured in terms of their OD at 600 nm and MTBE biodegradation in terms of Chemical Oxygen Demand (COD) (Tomar, 1999).

3A.2.2.2. Effect of cations on growth of AC consortium and its individual isolates on MTBE

Effect of different cations of the MM2 medium on MTBE biodegradation was checked in terms of growth (OD_{600}) of the isolates of AC consortium in MM2 medium containing 1 % (v/v) MTBE supplemented with: (a) $MgSO_4.7H_2O$, 0.2 g/l; $CaCl_2.2H_2O$, 0.02 g/l; $MnCl_2.4H_2O$, 0.002 g/l, $NaMoO_4.2H_2O$, 0.001 g/l, and $FeSO_4.7H_2O$, 0.05 g/l; (b) $MgSO_4.7H_2O$, 0.2 g/l; (c) $CaCl_2.2H_2O$, 0.02 g/l; (d) $MnCl_2.4H_2O$, 0.002 g/l; (e) $NaMoO_4.2H_2O$, 0.001 g/l, (f) $FeSO_4.7H_2O$, 0.05 g/l, and (g) no cations (Piveteau *et al*., 2001). The experimental procedure described in section 3A.2.2.1 was followed.

3A.2.2.3. Effect of magnesium concentration on the growth of AC consortium and its individual isolates on MTBE

Effect of different concentrations of Mg^{2+}, viz. 0.0, 0.2, 0.3, 0.4 and 0.5 g/l, on MTBE biodegradation by AC consortium and its individual isolates was checked in terms of their growth on MM2 medium with Mg^{2+} as the only significant cation (Piveteau *et al*., 2001). The experimental procedure described in section 3A.2.2.1 was followed.

3A.2.2.4. Influence of increasing concentration of MTBE on the growth of AC consortium and its individual isolates

Different concentrations of MTBE, viz. 1.5, 3.0, 4.5 and 7.0 g/l, were supplemented in MM2 medium to check their effect on the growth of AC consortium and its individual isolates (Piveteau *et al*., 2001). The modified MM2 medium used composed of (per liter): K_2HPO_4, 1.0 g; KNO_3, 1.0 g; Yeast extract, 0.5 g, and $MgSO_4$, 0.3 g. The experimental procedure described in section 3A.2.2.1 was followed.

3A.2.2.5. Effect of different combinations of the isolates of AC consortium on growth on MTBE

Different combinations of AC consortium were designed and used to check their effect on MTBE utilization, as compared to the AC consortium and its individual

isolates. These combinations included (a) AC consortium without *Bordetella petrii* AC1, (b) AC consortium without *Bacillus licheniformis* AC4, (c) AC consortium without *Salmonella subterranea* AC5 and (d) AC consortium without *Pseudomonas stutzeri* AC8. 9.8 ml MM2 medium supplemented with MTBE at the concentration of 7.4 g/l was inoculated with 2 % inoculum. The experimental procedure described in section 3A.2.2.1 was followed.

3A.2.2.6. MTBE utilization by the AC consortium and its individual isolates in optimized medium

MTBE utilization by the AC consortium and its individual isolates after optimization of different parameters was checked in terms of their growth and COD reduction (Tomar, 1999). The experimental procedure described in section 3A.2.2.1 was followed. Utilization of MTBE was measured by gas chromatography (Thermo, GC Trace Ultra) equipped with HP-5 capillary column. 1 µl of each sample was used for injection. The oven temperature was kept at 35 ^{o}C for 3 min and programmed at 10 ^{o}C/min to 160 ^{o}C. The injector and detector temperatures were 230 ^{o}C and 250 ^{o}C respectively. The carrier gas was helium and the flow rate was 3 ml/min (Chen *et al*., 2007).

3A.2.3. TBA biodegradation

3A.2.3.1. TBA biodegradation by AC consortium and its individual isolates

The ability of the isolates of AC consortium to utilize TBA was checked by inoculating 2 % inoculum in 9.8 ml MM2 medium supplemented with 1 % (v/v) TBA as its sole carbon source. The tubes were incubated at 37 ˚C at 180 rpm for 120 h. Growth of the isolates was measured in terms of their OD at 600 nm and TBA degradation in terms of COD (Tomar, 1999).

3A.2.3.2. Effect of different cations on growth of AC consortium and its individual isolates on TBA

Effect of different cations, supplemented in the MM2 medium, on TBA utilization was checked in terms of the growth of AC consortium and its individual isolates on TBA in different nutrient conditions (Piveteau *et al*., 2001). The cations used included: (a) $MgSO_4.7H_2O$, 0.2 g/l; (b) $CaCl_2.2H_2O$, 0.02 g/l; (c) $MnCl_2.4H_2O$,

0.002 g/l; (d) $NaMoO_4.2H_2O$, 0.001 g/l; (e) $FeSO_4.7H_2O$, 0.05 g/l; (f) no cations and (g) $MgSO_4.7H_2O$, 0.2 g/l; $CaCl_2.2H_2O$, 0.02 g/l; $MnCl_2.4H_2O$, 0.002 g/l; $NaMoO_4.2H_2O$, 0.001 g/l, $FeSO_4.7H_2O$, 0.05 g/l. The experimental procedure described in section 3A.2.3.1 was followed.

3A.2.3.3. Effect of cations concentration on growth of AC consortium and its individual isolates on TBA

Effect of different concentrations of cations, viz. 0.5, 1, 2, 3 and 4 % (w/v), on TBA utilization by AC consortium and its individual isolates was checked in terms of their growth on MM2 medium in the presence of all cations. The initial cation concentration used was considered as 1 % and accordingly other concentrations were calculated. The experimental procedure described in section 3A.2.3.1 was followed.

3A.2.3.4. Influence of increasing concentration of TBA on growth of AC consortium and its individual isolates

Effect of different concentrations of TBA, viz. 3.9, 5.4, 7.8 and 9.3 g/l, on the growth of AC consortium and its individual isolates was studied (Piveteau *et al*., 2001). The modified MM2 medium contained all cations at the concentration of 4 %. The experimental procedure described in section 3A.2.3.1 was followed.

3A.2.3.5. TBA utilization by the AC consortium and its individual isolates in optimized medium

TBA utilization by the AC consortium after optimization of different parameters was checked in terms of their growth and COD reduction (Tomar, 1999). The experimental procedure described in section 3A.2.3.1 was followed. Residual concentration of TBA was measured by gas chromatography (Thermo, GC Trace Ultra) equipped with a flame ionization detector and HP-5 capillary column (15 m x 0.53 mm). 0.4 μl of the sample was injected. The starting temperature was 40 ^{o}C, which was maintained for 2 min, increased by 20 ^{o}C/min to 90 ^{o}C and then increased by 40 ^{o}C/min to 260 ^{o}C. The carrier gas was helium and the flow rate was 7 ml/min (Kharoune *et al*., 2001a).

3A.2.4. GC-MS analysis of MTBE biodegradation by AC consortium

9.8 ml optimized MM2 medium with MTBE at the concentration of 7.4 g/l was inoculated with 2 % inoculum. The tubes were incubated at 37 ^{o}C at 180 rpm for 120 h. 1 ml of the culture was centrifuged at 10,000 rpm for 5 min and the cell free supernatant was used for detection of MTBE and its intermediate products by gas chromatography/mass spectrometry (6890N network GC system/5973 network mass selective detector; Agilent Technologies, Wilmington, DE). Gas chromatography was equipped with HP-5MS capillary column (0.25 mm x 30 m x 0.25 m) (J&W Scientific, USA). 1 µl of each sample was used for injection. The oven temperature was kept at 35 ^{o}C for 3 min and programmed at 10 ^{o}C/min to 160 ^{o}C. The carrier gas was helium and the flow rate was 1 ml/min. The mass spectrometry was operated in electron impact mode at 70 eV. The interface and ion source temperatures were 280 ^{o}C and 230 ^{o}C respectively (Chen *et al*., 2007).

3A.2.5. Reactor studies

3A.2.5.1. Treatability studies

Synthetic effluent used for the treatability and reactor studies contained (per liter): K_2HPO_4, 1.0 g; KNO_3, 1.0 g; Yeast extract, 0.5 g; $MgSO_4$, 0.3 g, and MTBE, 7.4 g. The AC consortium was prepared by inoculating a loopful of each isolate in 5 ml LB medium and incubating at 37 ^{o}C at 180 rpm for 24 h. 2 % inoculum was inoculated into 100 ml synthetic effluent in a 250 ml Erlenmeyer flask and incubated at 37 ^{o}C for 24 h with constant stirring at 100 rpm. 1 ml of the synthetic effluent was collected at intervals of 1 h and centrifuged at 10,000 rpm for 5 min. The supernatant was collected and used as a sample for COD estimation (Tomar, 1999).

3A.2.5.2. Batch reactor studies

The flask level treatability studies were scaled up to 5 l batch reactor (Figure 3A.1). The reactor (20 cm x 16 cm x 26 cm), made up of 4 mm thick acrylic, was connected to a ‘V’ shaped settling tank (18 cm x 16 cm x 26 cm) attached at an angle of 45 ^{o} to the aeration tank. Of the total 10 l volume, the working volume of the reactor was 5 l and the head-space was 5 l. The reactor was sealed and no external aeration was provided considering the volatile nature of MTBE. The AC consortium was prepared by inoculating 1 ml of each culture in 100 ml LB medium and

incubating at 37 ^{o}C at 180 rpm for 24 h. 2 % inoculum was inoculated into 5 l synthetic effluent (described in section 3A.2.5.1) in the reactor and incubated at 37 ^{o}C for 96 h with constant stirring at 300 rpm. 1 ml of the effluent was collected at intervals of 6 h and centrifuged at 10,000 rpm for 5 min. The supernatant was used as a sample for COD estimation (Tomar, 1999).

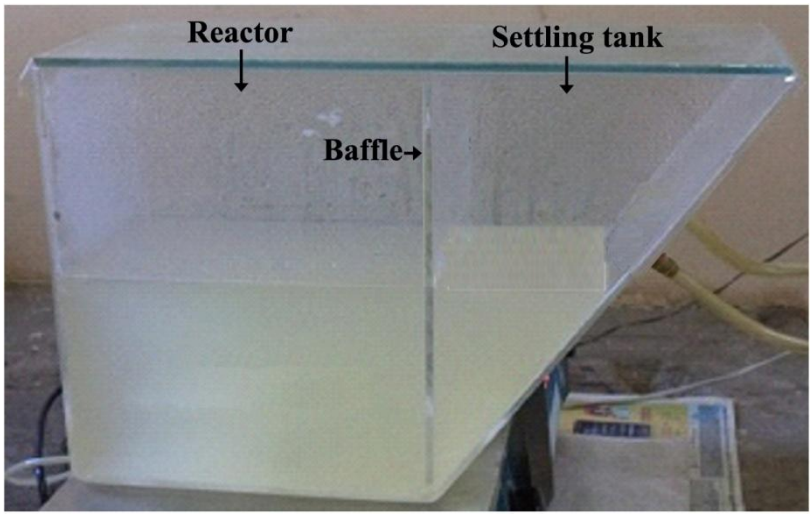


Figure 3A.1. A 5 l batch reactor with a settling tank separated by a baffle

3A.2.5.3. Continuous reactor studies

The process was further scaled up to a 5 l continuous reactor (Figure 3A.1) connected to an influent tank and an effluent tank. The inoculum preparation and reactor conditions were identical to those mentioned in section 3A.2.5.2. The HRT was kept 72 h for the synthetic effluent. The reactor was run for 15 d, first 3 d in batch mode, followed by next 12 d in continuous mode. 1 ml of the synthetic effluent was collected at intervals of 24 h and centrifuged at 10,000 rpm for 5 min. The supernatant was used as a sample for COD estimation (Tomar, 1999).

3A.2.6. Statistical analysis

Student's t-Test was applied to evaluate the effect of different cations, cation concentrations and MTBE/TBA concentrations on the MTBE/TBA biodegradation ability of AC consortium and two-way ANOVA was applied to evaluate these effects on the individual isolates. It was assumed that the original data followed a normal distribution. All statistical analyses were performed using GraphPad Prism 5.0 software (San Diego, CA) (Barbera *et al*., 2011).

3A.3. Results and Discussion

Methylotrophs have been reported to degrade a variety of xenobiotics. Dechlorination of DCM by facultative methylotrophic bacteria like *Pseudomonas* strains, *Hyphomicrobium* strains and several *Methylobacterium* sp. strains is reported to be catalyzed by inducible glutathione *S*-transferases (Bhatt *et al*., 2007). *Methylobacterium extorquens* PM1, *Methylophilus methylotrophus* EHg7, *M. extorquens* Mi1 and *Methylobacterium fujisawaense* F5.4 have been reported to tolerate MTBE (de Marco *et al*., 2004). *B. cepacia* CIP I-2052, a methylotrophic bacterial isolate, has been reported to degrade TBA and TAA (Piveteau *et al*., 2001). As methylotrophs have been credited with the ability to degrade diverse xenobiotics in the literature, in order to assess the xenobiotic biodegradation potential of AC consortium the ensuing studies were undertaken.

3A.3.1. Soluble xenobiotic utilization by AC consortium and its individual isolates

Ability of the isolates of AC consortium to utilize soluble xenobiotics was checked in terms of their growth on a variety of xenobiotics. They could grow on all the xenobiotics tested. The AC consortium and its individual isolates showed good growth on TAA, CE, TAME, MTBE, TMAH and allyl chloride (0.15 – 0.7 OD_{600}); moderate growth on methylamine (0.07 – 0.11 OD_{600}), and poor growth on methacrylic acid and epichlorohydrin (0.03 – 0.06 OD_{600}) (Figure 3A.2). *B. petrii* AC1 showed higher utilization of CE, MTBE, TMAH and allyl chloride as compared to other xenobiotics; *B. licheniformis* AC4 showed higher utilization of TAME, MTBE and allyl chloride; *S. subterranea* AC5 showed higher utilization of MTBE; *P. stutzeri* AC8 showed higher utilization of MTBE and allyl chloride, while the AC consortium showed higher utilization of CE, TAME, MTBE, TMAH and allyl chloride. However, MTBE was maximally utilized by the AC consortium and its individual isolates (0.29 – 0.7 OD_{600}) (Figure 3A.2). Hence, MTBE was selected for the further studies.

In comparison to those reported, the isolates of AC consortium biodegraded all the xenobiotics tested, implying that they were versatile in xenobiotic biodegradation. As observed in Figure 3A.2, the AC consortium showed maximum growth on MTBE

as compared to other xenobiotics. Hence, MTBE was the best growth substrate for AC consortium and its individual isolates.

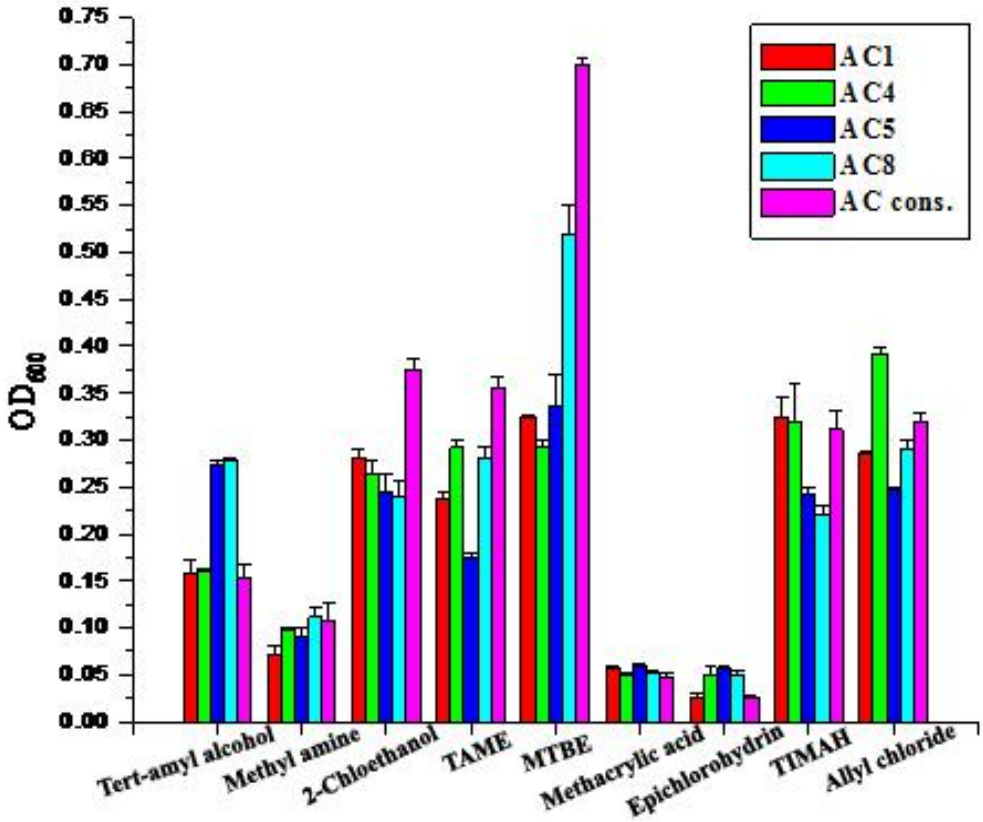


Figure 3A.2. Growth of the AC consortium and its individual isolates on soluble xenobiotics. Error bars represent standard deviation from the mean, n = 3.

3A.3.2. MTBE biodegradation

MTBE is a widely used fuel ether, which has become a soil and water contaminant (Barbera *et al*., 2011). Bioremediation is often proposed as the most promising alternative after wastewater treatment. However, MTBE biodegradation appears to be quite different from the biodegradation of usual gasoline contaminants such as benzene, toluene, ethyl benzene and xylene (BTEX) (Fortin *et al*., 2001). MTBE has been shown to biodegrade under aerobic and co-metabolic conditions (Farrokhi and Ahmadizad, 2009). MTBE being a toxic xenobiotic and hazardous pollutant of groundwater, its biodegradation studies are important. Methylotrophs are implicated in MTBE biodegradation. Notably, most of the studies cited in literature are by individual pure cultures (Barbera *et al*., 2011; Chen *et al*., 2007; Francois *et al*., 2002). Hamer (1997) suggested that the performance of a microbial consortium is

better as compared to individual strain performance. In the present study, the isolates of AC consortium could biodegrade MTBE maximally as compared to the other soluble xenobiotics tested. Considering the fact that MTBE is degraded by only few bacteria, as seen in the literature, it was selected for further studies so as to evaluate the potential of AC consortium in MTBE biodegradation.

3A.3.2.1. MTBE biodegradation by AC consortium and its individual isolates

MTBE was supplemented as the sole carbon source in MM2 medium to study its utilization by members of the AC consortium. *B. petrii* AC1 reduced the COD of MTBE containing medium from 700 mg/l to 50 mg/l, *B. licheniformis* AC4 to 100 mg/l, *S. subterranea* AC5 to 50 mg/l and *P. stutzeri* AC8 to 50 mg/l, while the AC consortium could grow effectively on MTBE and also reduce its COD from 700 mg/l to below detection limit in 120 h (Figure 3A.3). Thus, the AC consortium was more effective than its individual members in COD reduction, indicating its higher potential to biodegrade MTBE.

Bacterial biodegradation of MTBE has been previously reported. *Rhodococcus aetherivorans* IFP 2017 showed significant MTBE degradation (Auffret *et al*., 2009). The resting cells of *Achromobacter xylosoxidans* MCM1/1 showed 78 % MTBE biodegradation (Barbera *et al*., 2011). *Methylibium petroleiphilum* PM1 is a methylotroph distinguished by its ability to completely metabolize MTBE (Kane *et al*., 2007). Chen *et al*. (2007) reported degradation of MTBE by resting cells of *M. petroleiphilum* PM1 in poor nutrition solution. Likewise, all the 4 isolates of AC consortium biodegraded MTBE efficiently.

3A.3.2.2. Effect of cations on growth of AC consortium and its individual isolates on MTBE

Cations have been reported to have significant effect on MTBE biodegradation. The MTBE degradation activity of *M. petroleiphilum* PM1 was enhanced by 4.65-fold when 1mM of Ba^{2+} was added in the growth medium (Chen *et al*., 2007). MTBE biodegradation by *Ochrobactrum cytisi* was stimulated at low concentrations of Zn^{2+} and Mn^{2+} but inhibited at high concentrations of Zn^{2+} and Mn^{2+}, and at low concentration of Ni^{2+} (Lin *et al*., 2007). Hence, the effect of different cations of MM2 medium, viz., Mg^{2+}, Ca^{2+}, Mn^{2+}, Na^{+} and Fe^{2+} on MTBE utilization by the isolates of AC consortium was studied. Out of all the cations tested,

Mg^{2+} supported maximum utilization of MTBE by AC consortium and its individual isolates (Figure 3A.4). The growth of *B. petrii* AC1 increased by 2.29-fold when Mg^{2+} alone was supplemented to MM2 medium, that of *B. licheniformis* AC4 by 2.16-fold, that of *P. stutzeri* AC8 by 2.66-fold and that of AC consortium by 3.98-fold. ANOVA analysis, performed at a significance level of 0.05, indicated that Mg^{2+} had a significant effect on MTBE utilization by *B. petrii* AC1, *B. licheniformis* AC4, *P. stutzeri* AC8 and the AC consortium with a p-value < 0.001, while all cations had a significant effect on MTBE utilization by *S. subterranea* AC5 with a p-value < 0.05. Mg^{2+} supplementation to the MM2 medium was statistically significant for the growth of AC consortium and its individual isolates with p-values of 0.03, 0.045 and 0.033 (Student's t-Test) over Mn^{2+}, Na^{+} and Fe^{2+} respectively. Therefore, ANOVA and Student's t-Test proved that Mg^{2+} had a statistically significant effect on MTBE biodegradation by the AC consortium and its individual isolates.

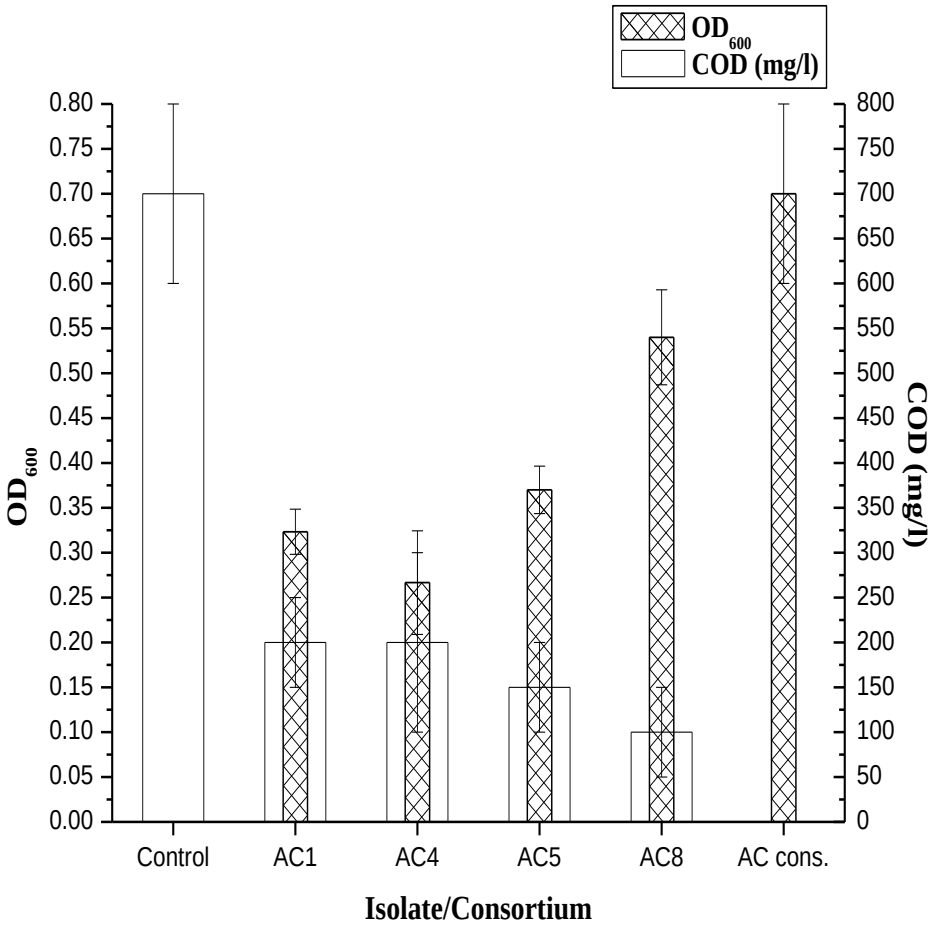


Figure 3A.3. MTBE biodegradation by AC consortium and its individual isolates. Error bars represent standard deviation from the mean, n = 3.

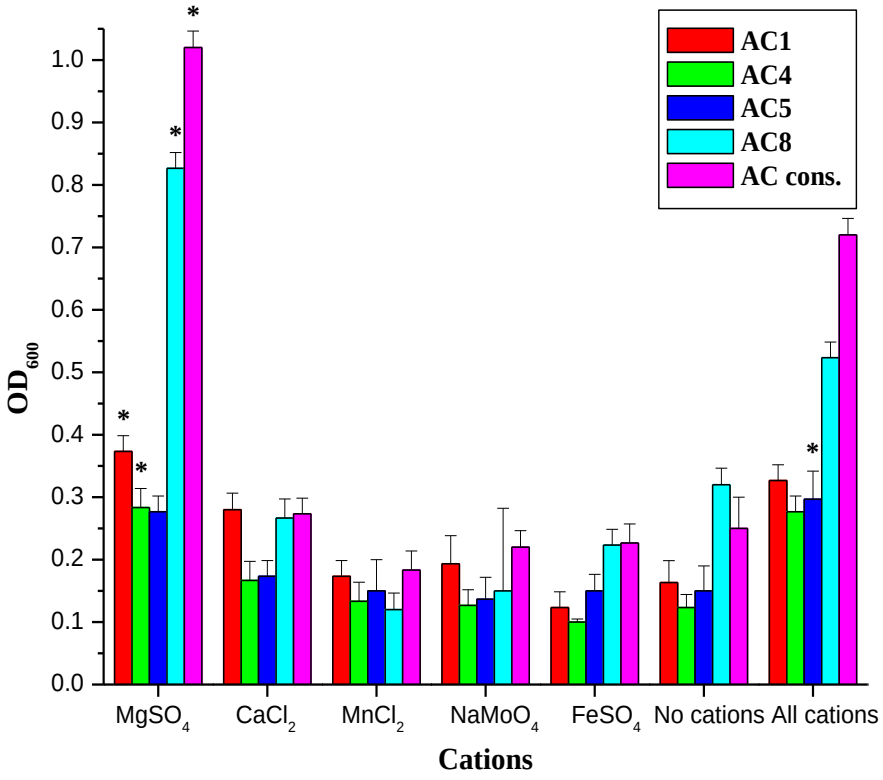


Figure 3A.4. Effect of cations on the growth of AC consortium and its individual isolates on MTBE (asterisk indicates that the growth is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

3A.3.2.3. Effect of magnesium concentration on growth of AC consortium and its individual isolates on MTBE

The influence of different concentrations of Mg^{2+} on MTBE utilization by the AC consortium and its individual members indicated that Mg^{2+} at the concentration of 0.3 g/l was optimal for the AC consortium as well as its individual isolates, except *S. subterranea* AC5 that showed maximum growth on MTBE at the Mg^{2+} concentration of 0.2 g/l (Figure 3A.5). However, the AC consortium and its individual isolates were able to utilize MTBE effectively in presence of all the concentrations tested. *B. petrii* AC1 and *B. licheniformis* AC4 showed a statistically significant growth (ANOVA) on MTBE in the presence of Mg^{2+} at 0.3 g/l at a significance level of 0.05, with a p-value < 0.01; *P. stutzeri* AC8 showed the same with a p-value < 0.05, while the AC consortium showed a more statistically significant growth with a p-value < 0.001. *S. subterranea* AC5 showed a statistically significant growth on MTBE in the presence

of Mg^{2+} at 0.2 g/l with a p-value < 0.05. The Student's t-Test further confirmed that Mg^{2+} at 0.3 g/l was statistically significant for the growth of AC consortium and its individual isolates with a p-value of 0.04 over Mg^{2+} at 0 g/l. Hence, 0.3 g/l of Mg^{2+} was selected for the further studies.

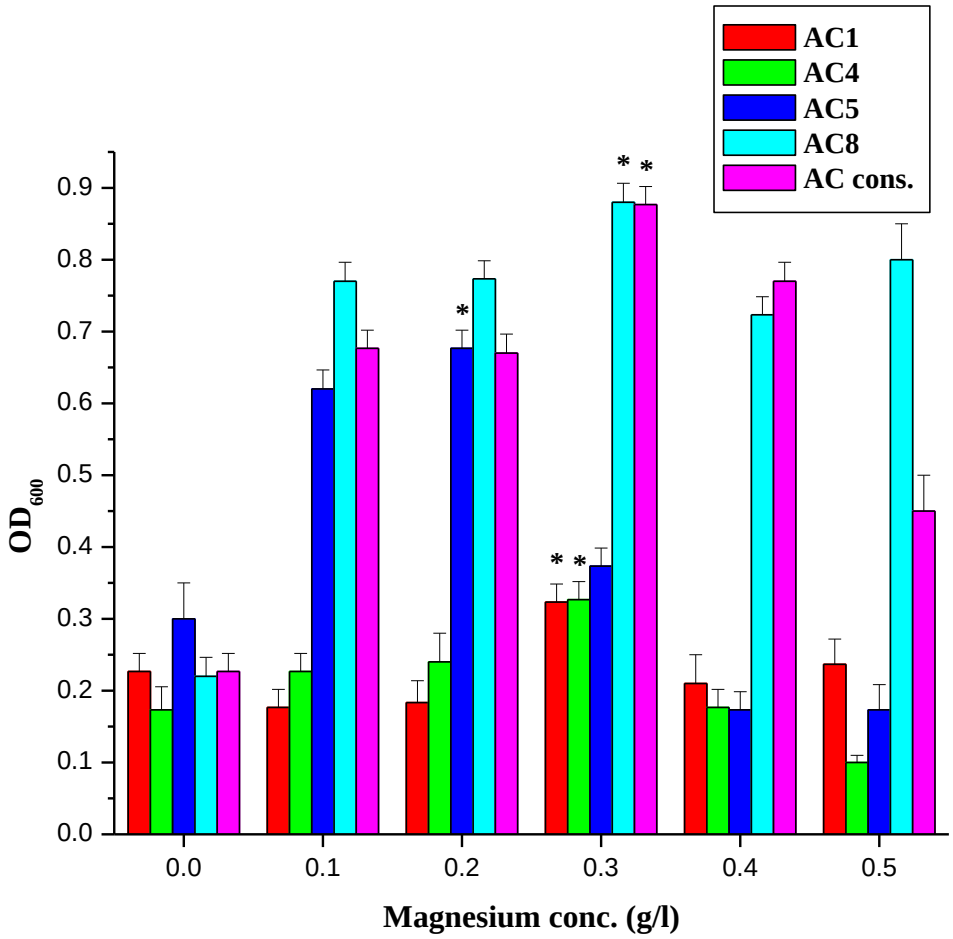


Figure 3A.5. Effect of Mg^{2+} concentration on the growth of AC consortium and its individual isolates on MTBE (asterisk indicates that the growth is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

3A.3.2.4. Influence of increasing concentration of MTBE on the growth of AC consortium and its individual isolates

To assess the resilience of the AC consortium, the influence of different concentrations of MTBE on the growth of AC consortium and its individual isolates was checked. All the AC isolates showed maximum growth at the MTBE concentration of 4.5 g/l, while the AC consortium showed maximum growth at higher

MTBE concentration of 7.0 g/l (Figure 3A.6). *P. stutzeri* AC8 showed comparable growth with the AC consortium in all the concentrations of MTBE tested, while the other 3 isolates grew moderately. *B. petrii* AC1 showed a statistically significant growth (ANOVA) on MTBE at the concentration of 4.5 g/l at a significance level of 0.05, with a p-value < 0.05, while *B. licheniformis* AC4, *S. subterranea* AC5 and *P. stutzeri* AC8 showed a statistically significant growth with a p-value < 0.01. The AC consortium showed a statistically significant growth on MTBE at the concentration of 7.0 g/l with a p-value < 0.05.

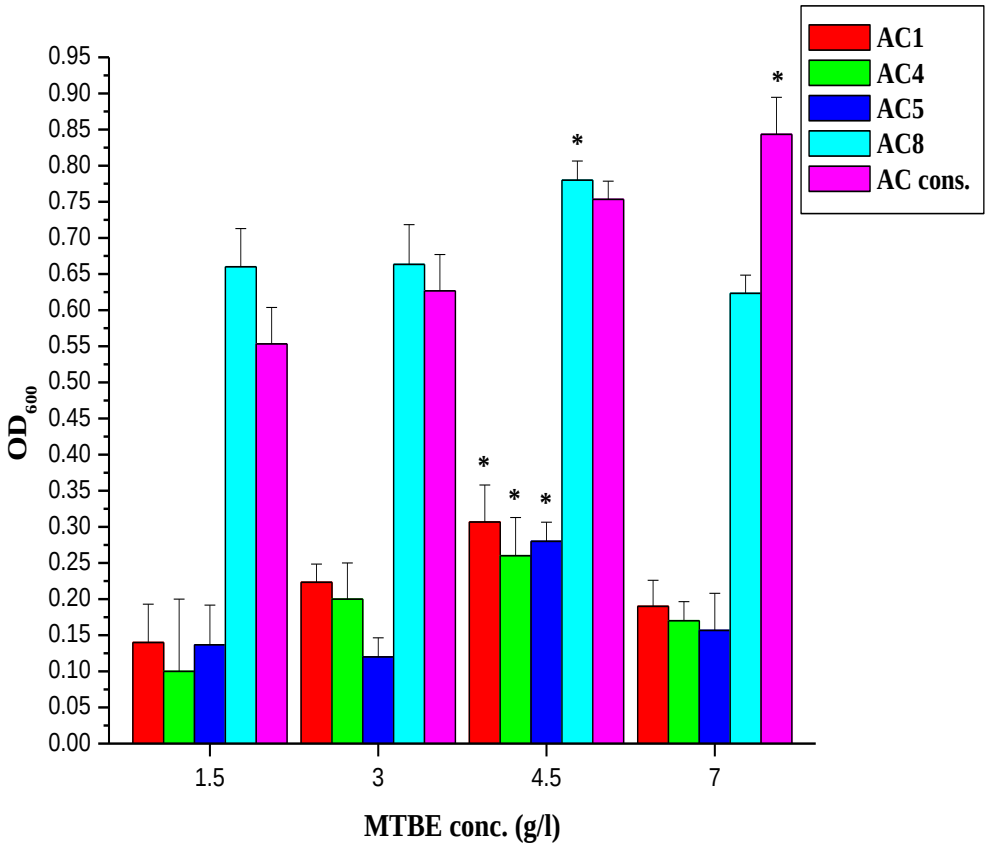


Figure 3A.6. Effect of MTBE concentration on the growth of AC consortium and its individual isolates (asterisk indicates that the growth is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

MTBE biodegradation ability of different microorganisms has been reported in literature. The bacteria *Achromobacter xylosoxidans* MCM1/1, *Enterobacter cloacae* MCM2/1 and *Ochrobactrum anthropi* MCM5/1 and the fungus *Exophiala*

dermatitidis MCM3/4 were able to degrade MTBE up to the concentration of 0.4 g/l (Barbera *et al*., 2011). A microbial consortium isolated from activated sludges was capable of degrading MTBE at concentrations up to 1 g/l (Farrokhi and Ahmadizad, 2009). An enriched bacterial consortium, derived from an old environmental MTBE spill, could grow on MTBE with concentration up to 0.5 g/l (Liu *et al*., 2009). In comparison to the previous reports, it was observed in the present study that the AC consortium was able to grow on higher concentrations of MTBE, i.e. 7 g/l, 7 times than that reported by Farrokhi and Ahmadizad (2009).

3A.3.2.5. Effect of different combinations of the isolates of AC consortium on growth on MTBE

In order to understand the contribution of the individual isolates of AC consortium, this study was conducted where MTBE utilization ability of different combinations of AC consortium was assessed. The AC consortium and *P. stutzeri* AC8 showed similar and maximum growth on MTBE as compared to the other isolates, viz. *B. petrii* AC1, *B. licheniformis* AC4 and *S. subterranea* AC5. It was particularly observed that when the individual isolates grouped together as AC consortium they showed higher growth on MTBE and thereby higher biodegradation potential (Figure 3A.7). Absence of *P. stutzeri* AC8 from the AC consortium affected the growth of AC consortium the most, while the deletion of *B. petrii* AC1 also affected the consortium growth to certain extent. However, deletion of *B. licheniformis* AC4 and *S. subterranea* AC5 from the AC consortium did not have much effect on its MTBE utilization. *P. stutzeri* AC8 is the dominant isolate of AC consortium so far as MTBE biodegradation is concerned.

Previous studies have proved higher potential of a bacterial consortium towards MTBE biodegradation as compared to individual bacterial biodegradation. An aerobic microbial consortium, isolated from activated sludges, showed efficient MTBE biodegradation (Farrokhi and Ahmadizad, 2009). A microbial consortium enriched by Fortin *et al*. (2001) was capable of degrading MTBE. An enriched bacterial consortium derived from an old environmental MTBE spill was reported to degrade MTBE when provided as a sole carbon and energy source (Liu *et al*., 2009). Thus, it can be concluded here that all the isolates comprising the AC consortium contributed variably towards its degradation potential and absence of any one of them affected the performance of the AC consortium.

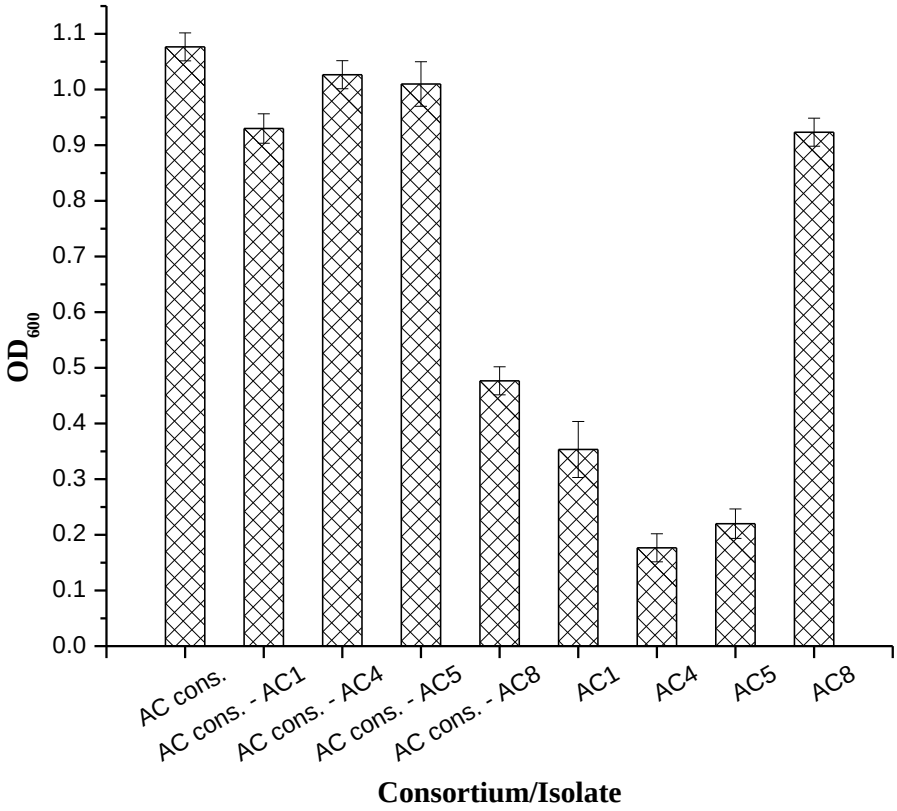


Figure 3A.7. Effect of different combinations of AC consortium on growth on MTBE. Error bars represent standard deviation from the mean, n = 3.

3A.3.2.6. MTBE utilization by the AC consortium and its individual isolates in optimized medium

After optimization of parameters like cations, cation concentration and MTBE concentration, MTBE utilization by the isolates and AC consortium was checked. It was found that the growth of AC consortium and its individual isolates in the optimized MM2 medium increased as compared to that obtained in the original medium (Figure 3A.8). The isolates and AC consortium reduced the COD of the MM2 medium containing MTBE to below permissible limit.

The GC analysis showed that the initial MTBE concentration of 7.4 g/l was reduced to 0.13 g/l by *B. petrii* AC1, 0.14 g/l by *B. licheniformis* AC4, 0.15 g/l by *S. subterranea* AC5, 0.12 g/l by *P. stutzeri* AC8 and 0.12 g/l by AC consortium (Figure 3A.9). 98 % MTBE biodegradation was obtained using the AC consortium as well as its individual isolates in 120 h. Thus, it is implied that all the members of AC consortium contributed equally towards efficient MTBE biodegradation.

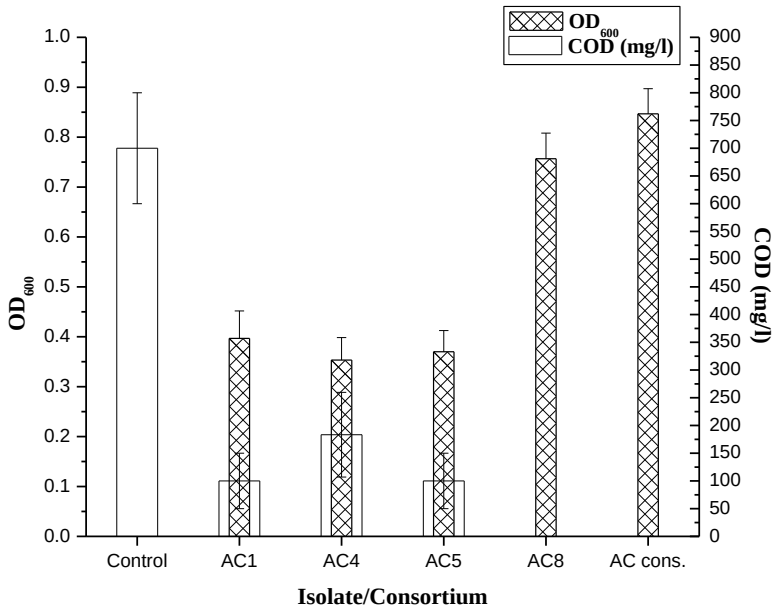
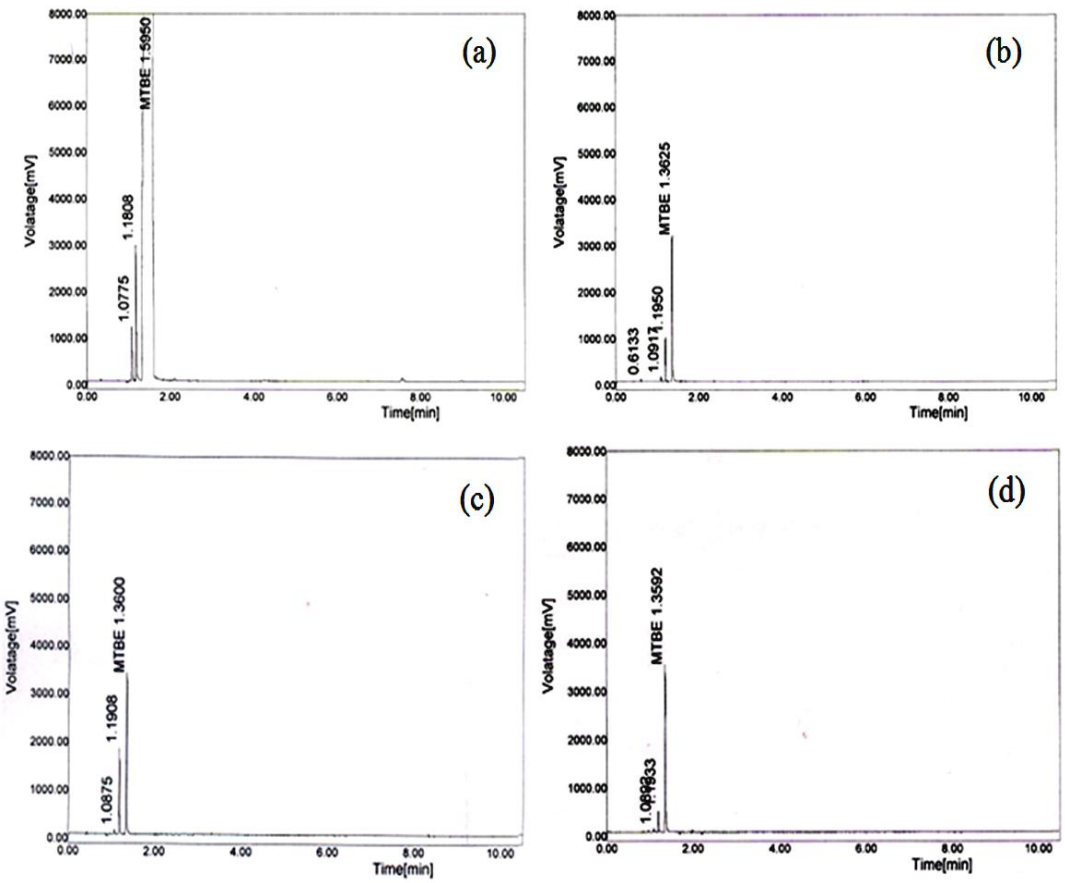
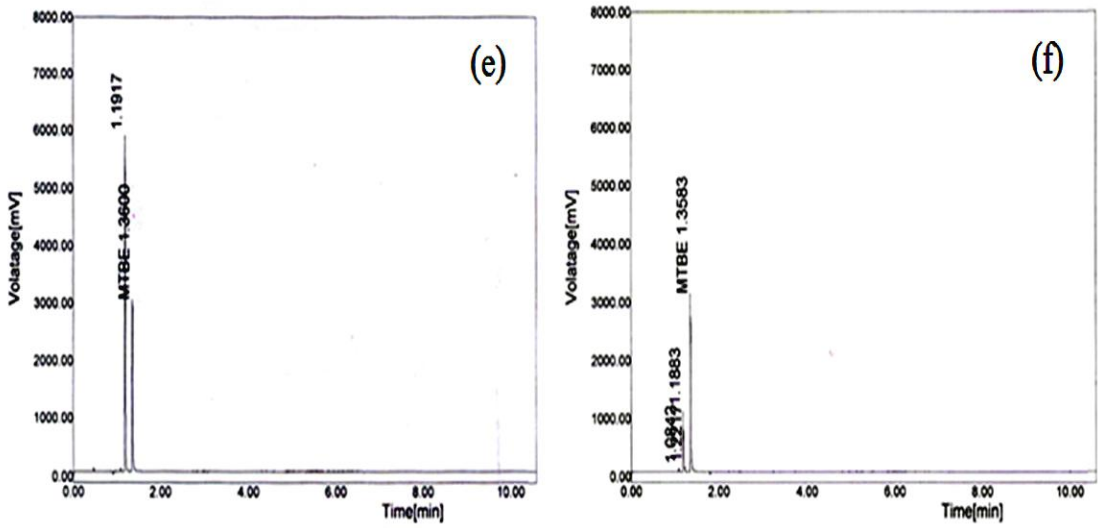


Figure 3A.8. MTBE utilization by the AC consortium and its individual isolates in optimized medium. Error bars represent standard deviation from the mean, n = 3.





Sample No.	Sample	Retention time (min)	Area (mV*s)	MTBE conc. (g/l)
a	Control	1.59	204392.24	7.4
b	*B. petrii* AC1	1.36	3552.19	0.13
c	*B. licheniformis* AC4	1.36	3786.56	0.14
d	*S. subterranea* AC5	1.36	4039.66	0.15
e	*P. stutzeri* AC8	1.36	3302.82	0.12
f	AC consortium	1.36	3426.41	0.12

Figure 3A.9. Gas chromatograms depicting MTBE utilization by the AC consortium and its individual isolates. (a) Uninoculated control, (b) *B. petrii* AC1, (c) *B. licheniformis* AC4, (d) *S. subterranea* AC5, (e) *P. stutzeri* AC8 and (f) AC consortium

3A.3.3. TBA biodegradation

MTBE integrates with 1 oxygen atom by monooxygenase and produces TBF, which is quickly hydrolyzed to TBA, and subsequently degraded to isopropanol, acetone and acetaldehyde through dehydrogenase enzymes (Chen *et al*., 2007). TBA is also a potential fuel oxygenate. TBA has been reported to be metabolized more slowly than MTBE (Steffan *et al*., 1997). The degradation of MTBE into TBA by pure strains in processes of co-metabolism linked in particular to degradation of hydrocarbons has been reported (Hernandez-Perez *et al*., 2001). Hence, TBA is an important metabolic intermediate of MTBE to be studied with respect to its biodegradation by the AC consortium. TBA biodegradation by AC consortium would

also indicate that TBA degradation is not the limiting step in its MTBE metabolism. Therefore, the following studies were undertaken.

3A.3.3.1. TBA biodegradation by the AC consortium and its individual isolates

TBA supplemented as the sole carbon source in MM2 medium was utilized by the members of AC consortium appreciably (Figure 3A.10). *B. petrii* AC1 reduced the COD of TBA containing medium from 550 mg/l to 100 mg/l, *B. licheniformis* AC4 to 250 mg/l, *S. subterranea* AC5 to 100 mg/l and *P. stutzeri* AC8 to 100 mg/l, while the AC consortium could grow effectively on TBA and also reduce its COD to below detection limit in 120 h, indicating its higher potential to biodegrade TBA.

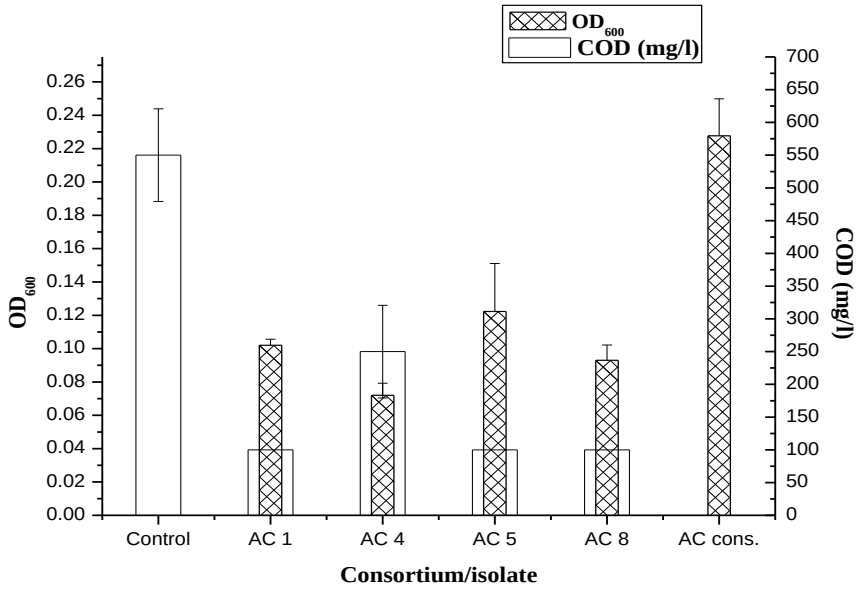


Figure 3A.10. TBA biodegradation by the AC consortium and its individual isolates. Error bars represent standard deviation from the mean, n = 3.

A number of bacteria have been reported to degrade TBA. *B. cepacia* CIP I-2052, a methylotrophic bacterial isolate, was able to grow on TBA as the sole source of carbon and energy (Piveteau *et al*., 2001). *M. petroleiphilum* PM1 degraded TBA faster than MTBE (Chen *et al*., 2007). Fayolle *et al*. (2003) reported that the same monooxygenase was responsible for the oxidation of both MTBE and TBA, with a low affinity for TBA in *M. austroafricanum* IFP 2012. Likewise, all the 4 isolates of AC consortium were able to biodegrade TBA. However, TBA utilization by AC consortium was higher than its individual isolates.

3A.3.3.2. Effect of different cations on the growth of AC consortium and its individual isolates on TBA

The MM2 medium supplemented with the cations, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+} and Na^{+}, enhanced the growth of AC consortium and its individual members on TBA (Figure 3A.11). Presence of all the cations in MM2 medium supported the maximum utilization of TBA by the AC consortium and its members. In case of *B. petrii* AC1, Mn^{2+} and Fe^{2+}, while in case of *B. licheniformis* AC4 and *S. subterranea* AC5, Mg^{2+} and Fe^{2+} influenced their growth positively. Growth was low in case of all the individual isolates and AC consortium in the absence of all the cations. *B. petrii* AC1 showed a statistically significant growth (ANOVA) on TBA in the presence of all cations at a significance level of 0.05, with a p-value < 0.01, while *B. licheniformis* AC4, *S. subterranea* AC5, *P. stutzeri* AC8 and the AC consortium showed the same with a p-value < 0.05. Student's t-Test further confirmed that all the cations together were statistically significant for the growth of AC consortium and its individual isolates with p-values of 0.006, < 0.0001, 0.004, 0.0014, 0.0005 and < 0.0001 over Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Na^{+} and no cations respectively. Hence, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+} and Na^{+} together had a statistically significant effect on MTBE biodegradation by the individual isolates as well as AC consortium.

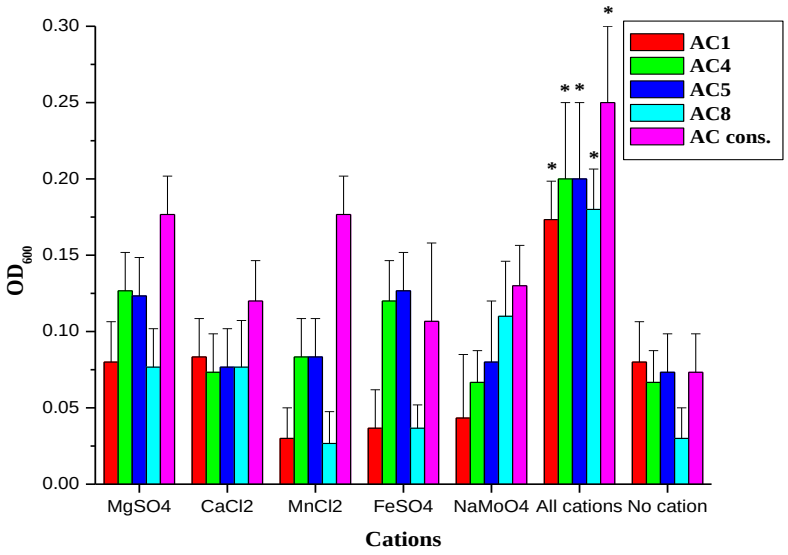


Figure 3A.11. Effect of different cations on growth of the AC consortium and its individual isolates on TBA (asterisk indicates that the growth is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

Piveteau *et al*. (2001) has reported that Co^{2+} had a stimulatory effect on the growth of *B. cepacia* CIP I-2052 and its ability to degrade TBA. However, in the present study, it was observed that Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+} and Na^{+} had a significant effect on the growth of the AC consortium and its individual isolates on TBA.

3A.3.3.3. Effect of different concentrations of all cations on the growth of AC consortium and its individual isolates on TBA

Different concentrations of cations were supplemented to the MM2 medium to check for the cation concentration supporting highest utilization of TBA by the AC consortium. It was observed that the isolates as well as AC consortium showed maximum growth and, hence, TBA degradation at 4 % cation concentration (Figure 3A.12). *B. petrii* AC1, *B. licheniformis* AC4, *P. stutzeri* AC8 and the AC consortium showed a more statistically significant growth (ANOVA) on TBA in the presence of all cations at 4 % concentration at a significance level of 0.05, with a p-value < 0.001, while *S. subterranea* AC5 showed a statistically significant growth with a p-value < 0.01. Student's t-Test also proved that all the cations at 4 % concentration were statistically significant for the growth of AC consortium and its individual isolates on TBA with p-values of < 0.0001, < 0.0001, < 0.0001 and 0.0005 over 0.5, 1, 2 and 3 % cation concentrations respectively. Hence, all cations supplemented at the concentration of 4 % were used for the further studies.

3A.3.3.4. Influence of increasing concentration of TBA on the growth of AC consortium and its individual isolates

Different concentrations of TBA were added in MM2 medium supplemented with all cations at the concentration of 4 %, in order to check their effect on the growth of the isolates and AC consortium. *B. petrii* AC1 and *B. licheniformis* AC4 showed maximum growth at the TBA concentration of 7.8 g/l and 5.4 g/l respectively, while *S. subterranea* AC5, *P. stutzeri* AC8 and AC consortium showed maximum growth at the TBA concentration of 3.9 g/l (Figure 3A.13). The AC consortium and its isolates could tolerate the TBA concentration up to 9.3 g/l. ANOVA indicated that *P. stutzeri* AC8 and the AC consortium showed a more statistically significant growth on TBA at the concentration of 3.9 g/l at a significance level of 0.05, with a p-value < 0.001, and *S. subterranea* AC5 showed a statistically significant growth with a p-value < 0.01. *B. petrii* AC1 showed a statistically significant growth on TBA at the

concentration of 7.8 g/l with a p-value < 0.001, while *B. licheniformis* AC4 showed a statistically significant growth on TBA at the concentration of 5.4 g/l with a p-value < 0.05.

TBA biodegradation ability of a variety of microorganisms has been studied. *B. cepacia* CIP I-2052 could degrade TBA up to the concentration of 6 g/l (Piveteau *et al*., 2001). *M. austroafricanum* IFP 2012 was able to grow on TBA at the concentration of 1 g/l (Francois *et al*., 2002). In comparison to the earlier reported studies, in the present study, the isolates of AC consortium were able to grow on higher concentration of TBA, i.e. 7.8 g/l.

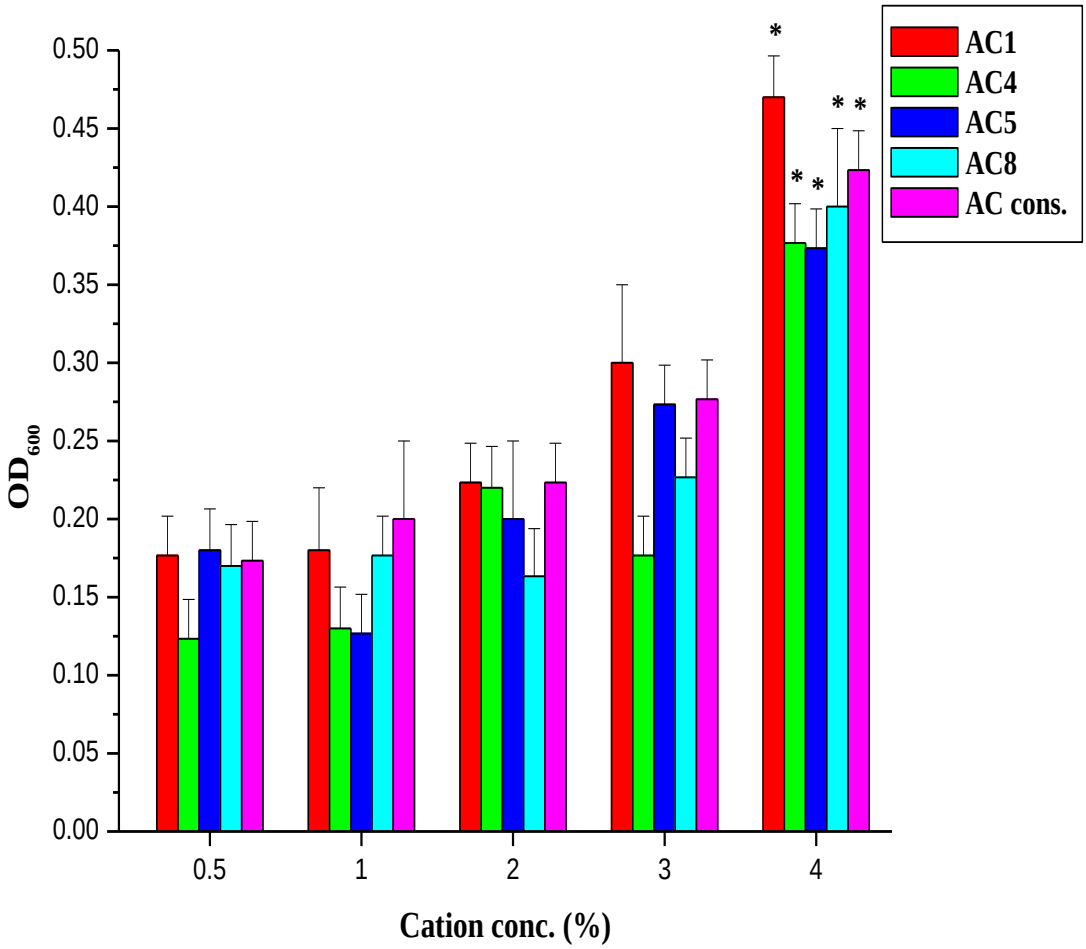


Figure 3A.12. Effect of cations concentration on the growth of AC consortium and its individual isolates on TBA (asterisk indicates that the growth is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

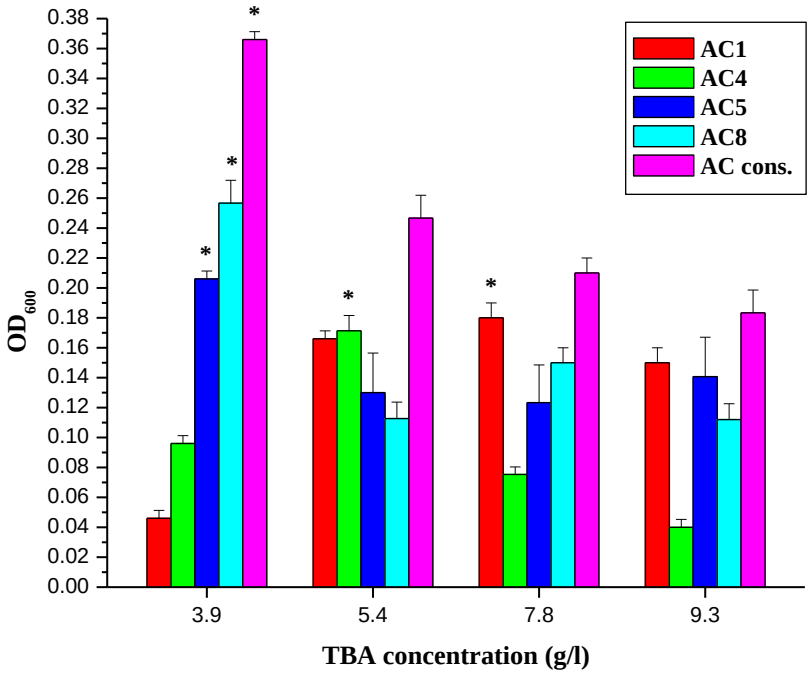


Figure 3A.13. Effect of TBA concentration on the growth of AC consortium and its individual isolates (asterisk indicates that the growth is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

3A.3.3.5. TBA utilization by the AC consortium and its individual isolates in optimized medium

After optimization of parameters like cations, cation concentration and TBA concentration, TBA utilization by the isolates and AC consortium was checked in terms of their growth and COD reduction. Growth and COD reduction ability of all the isolates showed considerable increase as compared to that without optimization. The isolates and AC consortium reduced the COD of the medium containing TBA to below permissible limit (Figure 3A.14).

The GC analysis showed that the initial TBA concentration of 7.75 g/l was reduced to 0.47 g/l by *B. petrii* AC1, 0.53 g/l by *B. licheniformis* AC4, 0.54 g/l by *S. subterranea* AC5, 0.49 g/l by *P. stutzeri* AC8 and 0.47 g/l by AC consortium (Figure 3A.15). 93 % TBA biodegradation was obtained using *B. licheniformis* AC4 and *S. subterranea* AC5, while 94 % TBA biodegradation was obtained using *B. petrii* AC1, *P. stutzeri* AC8 and AC consortium in 120 h. Thus, it can be implied that all the members of AC consortium contributed equally towards efficient TBA biodegradation.

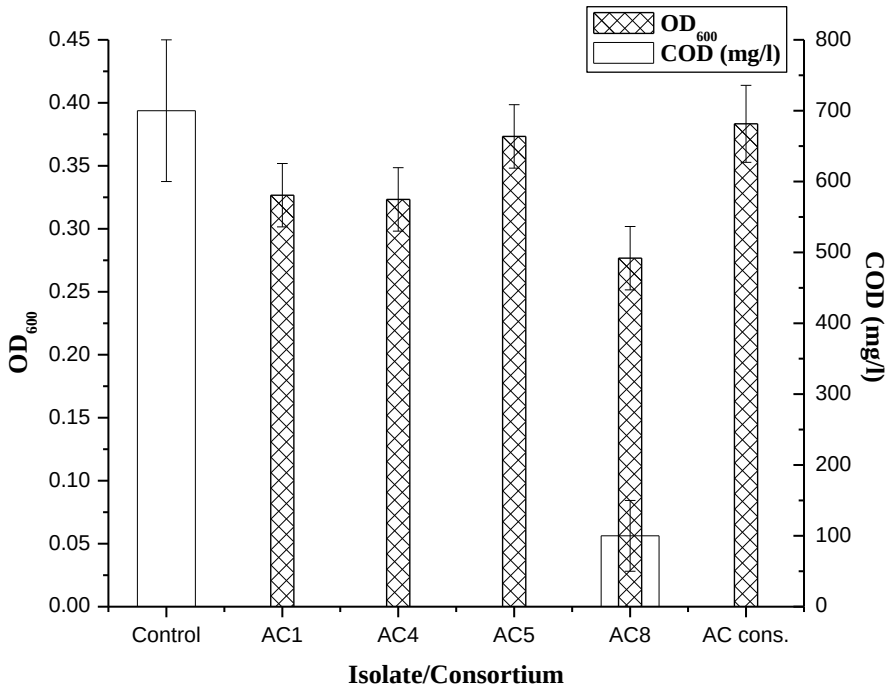
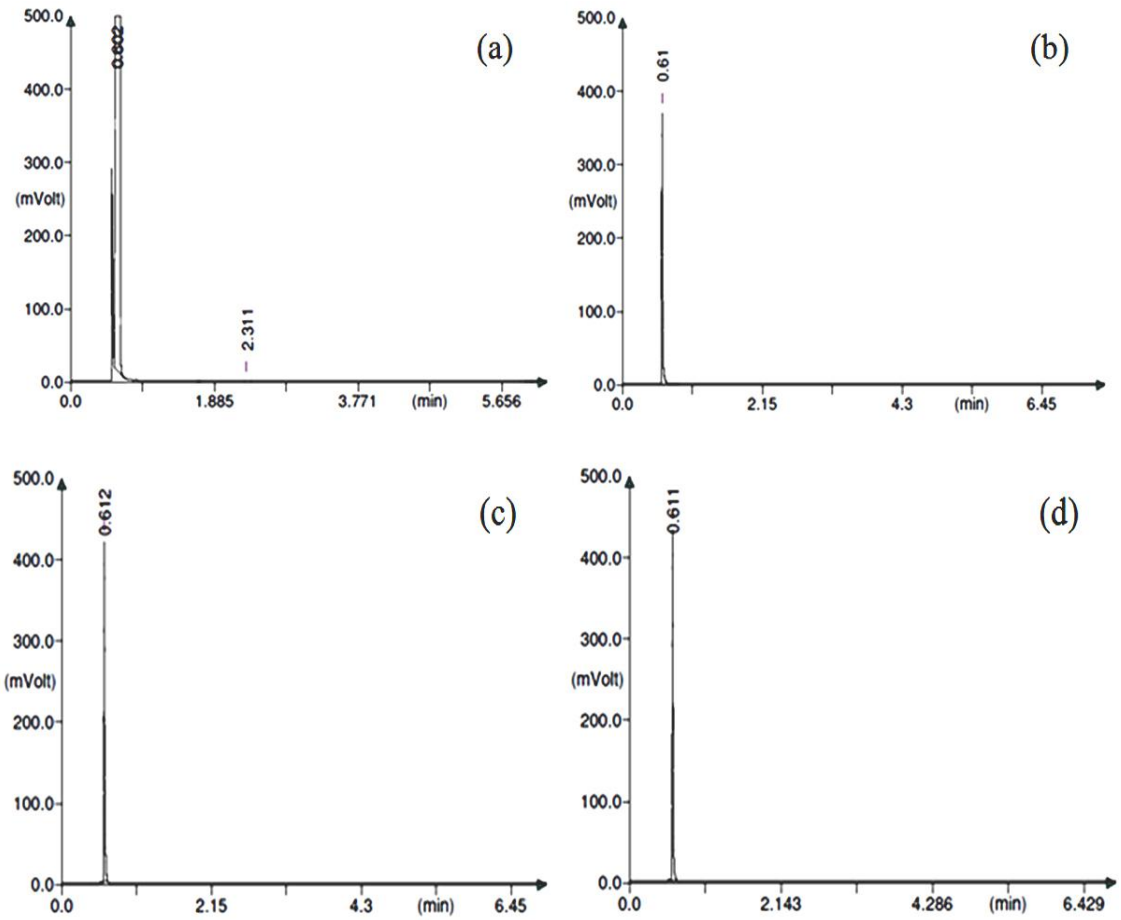
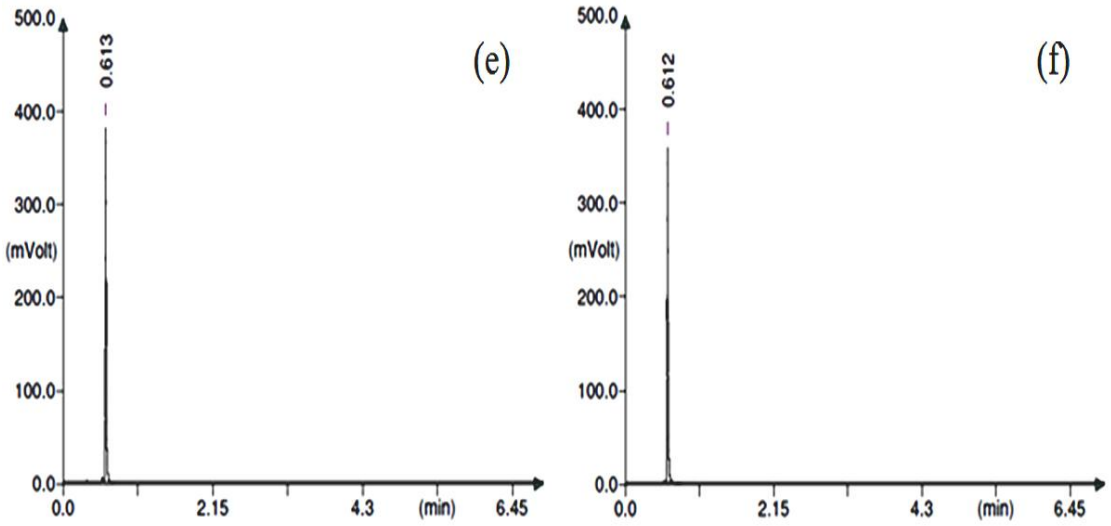


Figure 3A.14. TBA utilization by the AC consortium and its individual isolates in optimized medium. Error bars represent standard deviation from the mean, n = 3.





Sample No.	Sample	Retention time (min)	Area (.1*uV*sec)	TBA conc. (g/l)
a	Control	0.60	61453140	7.75
b	*B. petrii* AC1	0.61	3773684	0.47
c	*B. licheniformis* AC4	0.61	4209816	0.53
d	*S. subterranea* AC5	0.61	4282192	0.54
e	*P. stutzeri* AC8	0.61	3882741	0.49
f	AC consortium	0.61	3755994	0.47

Figure 3A.15. Gas chromatograms depicting TBA utilization by the AC consortium and its individual isolates. (a) Uninoculated control, (b) *B. petrii* AC1, (c) *B. licheniformis* AC4, (d) *S. subterranea* AC5, (e) *P. stutzeri* AC8 and (f) AC consortium

3A.3.4. Intermediate products of MTBE on biodegradation by the AC consortium

MTBE biodegradation ability of the AC consortium was checked in order to analyze its complete degradation using GC-MS. The peaks obtained in GC analysis (Figure 3A.16(a & c)) were subjected to mass spectrometry. The results obtained were compared with the GC-MS of the uninoculated control medium. Figure 3A.16(b) shows the mass spectrum of the peak obtained at a retention time of 2.243 min in the gas chromatogram. This predominant peak was identified as MTBE on the basis of m/z 73 (Chen *et al*., 2007). No such peak was obtained on MTBE biodegradation by the AC consortium (Figure 3A.16(c)). Thus, the results

of GC-MS analysis indicated that MTBE was completely utilized by the AC consortium and neither TBA nor TBF were accumulated on its biodegradation (Figure 3A.16), indicating that TBA and TBF are also degraded by the AC consortium.

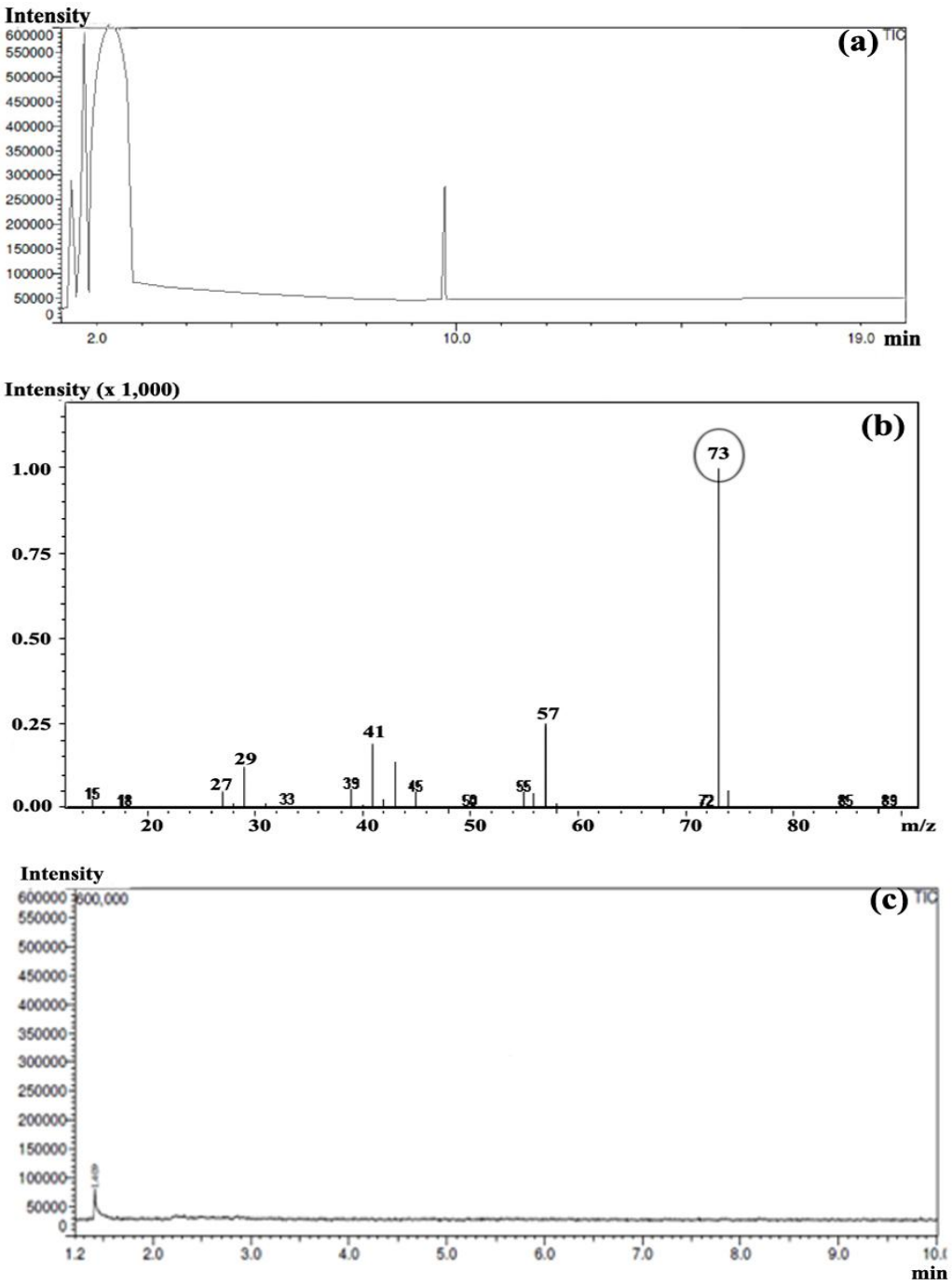


Figure 3A.16. GC-MS of MTBE biodegradation by the AC consortium. (a) GC of uninoculated control medium, (b) Mass spectrum of the predominant peak in GC obtained in (a), and (c) GC of the AC consortium inoculated MTBE containing medium.

TBA was not detected on aerobic biodegradation of MTBE by the indigenous microorganisms using an upflow fixed-bed reactor (Kharoune *et al*., 2001a). On the contrary, traces of TBA were detected during MTBE degradation by an enriched bacterial consortium derived from an old environmental MTBE spill (Liu *et al*., 2009). In the present study, the AC consortium was able to biodegrade MTBE completely without the accumulation of its degradative intermediates, TBA and TBF.

3A.3.5. Reactor studies

Microorganisms have been previously used for the biotreatment of wastewaters containing MTBE. *M. petroleiphilum* PM1 degraded MTBE present in groundwater at a high rate (Chen *et al*., 2007). MTBE biodegradation was investigated by Wilson *et al*. (2002) using a continuously stirred tank reactor with biomass retention operated under aerobic conditions. In order to check the potential of AC consortium for treatment of MTBE containing effluents, reactor studies were carried out using synthetic effluent containing MTBE as the sole carbon source.

3A.3.5.1. COD reduction of MTBE containing synthetic effluent by the AC consortium at flask level

A 100 ml reactor system containing synthetic effluent supplemented with MTBE as the sole carbon source was developed. The AC consortium reduced the initial COD of the MTBE containing synthetic effluent from 650 mg/l to below detection limit in 5 h (Figure 3A.17).

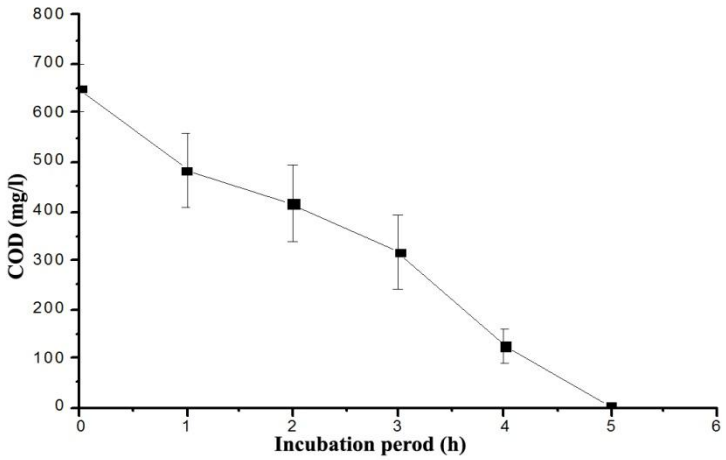


Figure 3A.17. COD reduction of MTBE containing synthetic effluent by the AC consortium at flask level. Error bars represent standard deviation from the mean, n = 3.

3A.3.5.2. COD reduction of MTBE containing synthetic effluent by the AC consortium at reactor level

The treatability studies were scaled up to 5 l batch reactor level in order to check the potential of AC consortium to treat the synthetic effluent at a larger scale in terms of its COD reduction. The AC consortium reduced the initial COD of the synthetic effluent from 950 mg/l to below detection limit in 78 h (Figure 3A.18(a)). The process was further scaled up to a 5 l continuous reactor level. The AC consortium reduced the initial COD of the synthetic effluent from 1000 mg/l to below detection limit in 10 d (Figure 3A.18(b)).

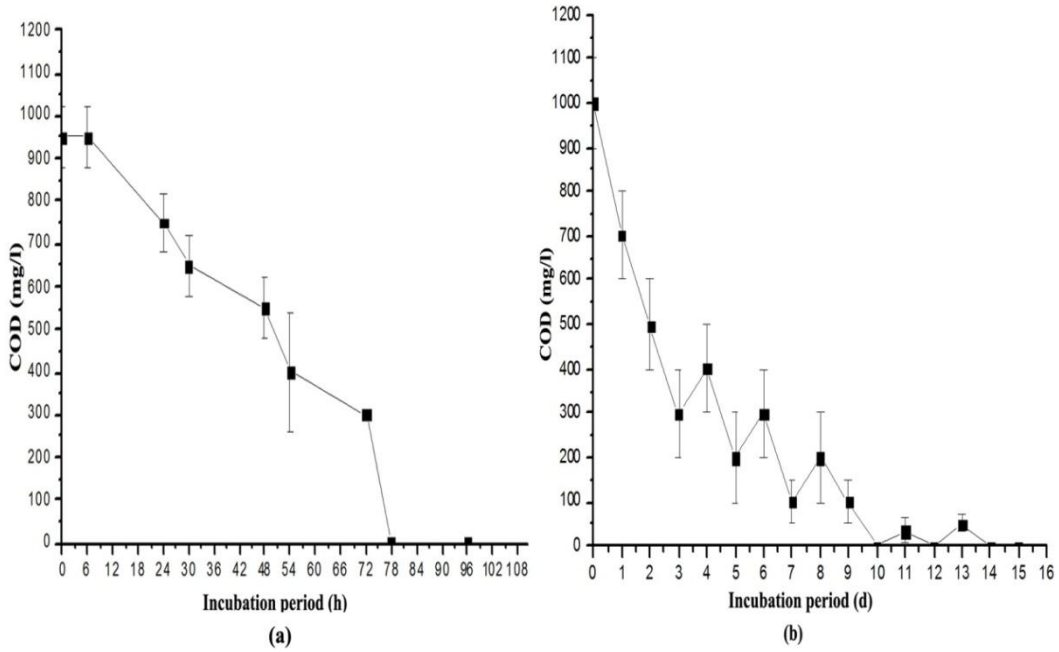


Figure 3A.18. COD reduction of MTBE containing synthetic effluent by the AC consortium in (a) batch and (b) continuous reactor. Error bars represent standard deviation from the mean, n = 3.

Reactor studies have been carried out previously for MTBE biodegradation. *Ochrobactrum cytisi*, acclimatized in a 1.5 l bioreactor fed with MTBE as sole carbon source was capable of degrading MTBE (Lin *et al.*, 2007). Kharoune *et al*. (2001a) demonstrated that a microbial consortium, isolated from an unleaded gasoline-polluted soil, effectively degraded MTBE at a rate of 53 mg/l/d in batch experiment and at 75 ± 2 mg/l/d in an upflow fixed-bed reactor. Wilson *et al*. (2002) showed that MTBE at the concentration of 43 mg/l was biodegraded in 48 h in a batch reactor. As compared to these reports, the AC consortium degraded MTBE completely at higher rates of 292

mg/l/d and 100 mg/l/d in batch and continuous reactors respectively. Thus, the AC consortium can serve as an efficient microbial seed for biotreatment of MTBE containing industrial effluents.

Chapter 3B
1,2-Dichloroethane biodegradation by the AC consortium

3B.1. Introduction

1,2-Dichloroethane (DCE) is an organochloride with the molecular formula $C_2H_2Cl_2$. DCE is widely used in industry, commonly for the production of vinyl chloride. DCE is produced annually in a volume larger than that of any other industrial halogenated chemical (Janssen *et al*., 1989). The widespread use of DCE in a variety of products such as TCE and tetrachloroethane, and manufacturing processes has made it to be frequently encountered in most sites contaminated with organic chemicals (Hage and Hartmans, 1999). DCE is toxic (especially by inhalation due to its high vapor pressure), highly flammable, carcinogenic and a potential mutagen. Its 50-year half-life in anoxic aquifers makes it a perennial pollutant and health risk that is very expensive to treat conventionally, requiring a method of bioremediation (De Wildeman *et al*., 2003). One strategy to reduce the environmental impact of such hazardous compounds is to implement point source treatment technologies, preventing dilution of other uncontaminated streams by dealing with the hazardous waste close to its point of emission (Freitas do Santos and Livingston, 1995).

DCE is susceptible to both abiotic and biological transformation and its aerobic (Olaniran *et al*., 2009) and anaerobic biodegradation (van der Zaan *et al*., 2009) have been reported. The presence of heavy-metal ions at high concentrations can result in inhibition of the activity of microorganisms involved in degradation of organics (Roane *et al*., 2001). Olaniran *et al*. (2009) investigated the impact of lead and mercury on DCE degradation and found that the presence of heavy metals has a negative impact on DCE degradation by bacteria. Due to the recalcitrance of most of the chlorinated compounds, relatively few bacterial strains have been identified as possessing the capability to mineralize them (Pieper and Reineke, 2000). *P. stutzeri* strain JJ is able to grow on CE under denitrifying conditions (Dijk *et al*., 2003). *Xanthobacter autotrophicus* GJ10 showed efficient degradation of synthetic wastewater containing DCE (Baptista *et al*., 2006).

The application of these specific strains to industrial situations can be difficult, as typical operating conditions, such as non-sterile long-term operation and dynamic waste production regimens, can be challenging (Koutinas *et al*., 2006). Being an insoluble xenobiotic, DCE degrading microorganisms have been reported sparingly. Also, the efficiency of a microbial consortium is always higher in comparison to individual strains (Hamer, 1997). Hence, in the present study, the AC consortium, already studied for MTBE and TBA biodegradation, was used for the biodegradation

of DCE. Studies carried out in this chapter deal with the biodegradation of DCE and its metabolic intermediate, 2-chloroethanol (CE), by the AC consortium and its individual isolates.

3B.2. Materials and Methods

3B.2.1. Insoluble xenobiotic biodegradation

The ability of the isolates of AC consortium to biodegrade different insoluble xenobiotics was checked in terms of their growth on these xenobiotics in vapor phase. A test tube (10 cm x 1 cm), containing 1 ml xenobiotic, was inserted into a test tube (15 cm x 2 cm) containing 9.8 ml MM2 medium (as described in section 3A.2.1) inoculated with 2 % inoculum (Figure 3B.1). The tubes were incubated at 37 ^{o}C at 180 rpm for 120 h. Growth of the isolates was measured in terms of their OD at 600 nm. The insoluble xenobiotics used for this study included: dibromoethane, DCE, TCE, 3-chloroaniline, 4-chloroaniline and dichlorobenzene (DCB) (Dolfing *et al*., 1993; Gisi *et al*., 1998).

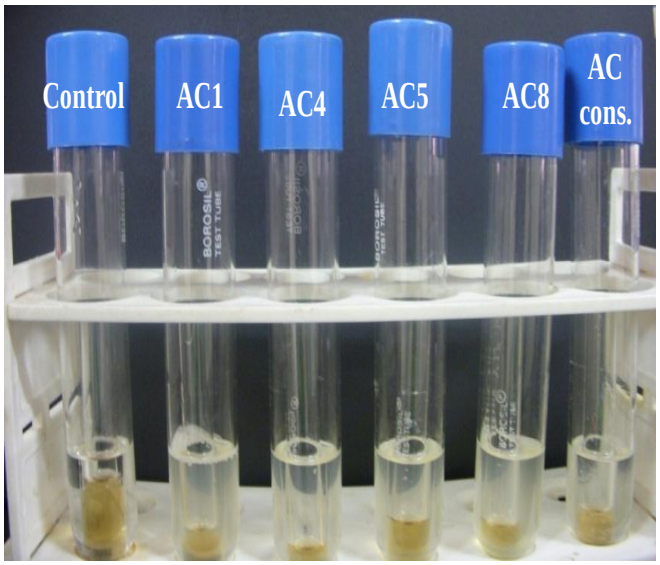


Figure 3B.1. Experimental set-up to study growth of the isolates of AC consortium on insoluble xenobiotics

3B.2.2. DCE biodegradation

3B.2.2.1. DCE biodegradation by the AC consortium and its individual isolates

Ability of the AC consortium and its individual isolates to utilize DCE was checked in terms of their growth and DCE degradation. 2 % inoculum was inoculated

in 9.8 ml MM2 medium supplemented with 1 ml DCE as its sole carbon source as described in section 3B.2.1. The tubes were incubated at 37 ^{o}C at 180 rpm for 120 h. Growth of the isolates was measured in terms of their OD at 600 nm and DCE utilization in terms of COD (Tomar, 1999).

3B.2.2.2. Effect of different cations on the growth of AC consortium and its individual isolates on DCE

Effect of different cations on DCE utilization was checked in terms of the growth of AC consortium and its individual isolates in MM2 medium supplemented with: (a) $MgSO_4.7H_2O$, 0.2 g/l; (b) $CaCl_2.2H_2O$, 0.02 g/l; (c) $MnCl_2.4H_2O$, 0.002 g/l; (d) $NaMoO_4.2H_2O$, 0.001 g/l; (e) $FeSO_4.7H_2O$, 0.5 g/l; (f) no cation and (g) $MgSO_4.7H_2O$, 0.2 g/l; $CaCl_2.2H_2O$, 0.02 g/l; $MnCl_2.4H_2O$, 0.002 g/l; $NaMoO_4.2H_2O$, 0.001 g/l, and $FeSO_4.7H_2O$, 0.05 g/l (Lin *et al*., 2007; Piveteau *et al*., 2001). The experimental procedure described in section 3B.2.2.1 was followed.

3B.2.2.3. Effect of magnesium concentration on the growth of AC consortium and its individual isolates on DCE

Effect of different concentrations of Mg^{2+}, viz. 0.0, 0.1, 0.2, 0.4, 0.5, 0.6 and 0.8 g/l, on DCE utilization by the AC consortium and its individual isolates was checked in terms of their growth in MM2 medium containing Mg^{2+} as the only cation (Piveteau *et al*., 2001). The experimental procedure described in section 3B.2.2.1 was followed.

3B.2.2.4. Influence of increasing concentration of DCE on the growth of AC consortium and its individual isolates

Different concentrations of DCE, viz. 2.5, 6.25, 8.75 and 12.5 g/l, were supplemented in MM2 medium to check their effect on the growth of AC consortium and its individual isolates (Piveteau *et al*., 2001). The modified MM2 medium used composed of (per liter): K_2HPO_4, 1.0 g; KNO_3, 1.0 g; Yeast extract, 0.5g, and $MgSO_4$, 0.5 g. The experimental procedure described in section 3B.2.2.1 was followed.

3B.2.2.5. Effect of different combinations of the isolates of AC consortium on growth on DCE

Different combinations of AC consortium were designed and used to check their effect on DCE utilization as compared to the AC consortium and its individual isolates. These combinations included (a) AC consortium without *B. petrii* AC1, (b) AC consortium without *B. licheniformis* AC4, (c) AC consortium without *S. subterranea* AC5 and (d) AC consortium without *P. stutzeri* AC8. 9.8 ml MM2 medium supplemented with DCE at the concentration of 12.56 g/l was inoculated with 2 % inoculum. The experimental procedure described in section 3B.2.2.1 was followed.

3B.2.2.6. DCE utilization by the AC consortium and its individual isolates in optimized medium

DCE utilization by the AC consortium and its individual isolates after optimization of different parameters was checked in terms of their growth and COD reduction (Tomar, 1999). 9.8 ml MM2 medium supplemented with DCE at the concentration of 12.56 g/l was inoculated with 2 % inoculum. The experimental procedure described in section 3B.2.2.1 was followed. The DCE concentration, in order to estimate the amount utilized as a gas, was analyzed using gas chromatography (Thermo, GC Trace Ultra) with a flame ionization detector and a column (15 m x 0.53 mm) with an HP-5 stationary phase. DCE present as gas was analyzed by directly injecting 0.5 ml of sample into the GC. The oven temperature was set at 40 ^{o}C, which was maintained for 2 min, increased by 20 ^{o}C/min to 90 ^{o}C, and then increased by 40 ^{o}C/min to 260 ^{o}C. The injector and detector temperatures were 230 ^{o}C and 250 ^{o}C respectively. The carrier gas was helium and the flow rate was 3 ml/min (Baptista *et al*., 2006).

3B.2.3. CE biodegradation

3B.2.3.1. CE biodegradation by AC consortium and its individual isolates

Ability of the AC consortium and its individual isolates to biodegrade CE was checked in terms of their growth on CE and its degradation. 2 % inoculum was inoculated in 9.8 ml MM2 medium supplemented with 1 % (v/v) CE as its sole carbon source. The tubes were incubated at 37 ^{o}C at 180 rpm for 120 h. Growth of the

isolates was measured in terms of their OD at 600 nm and CE utilization in terms of COD (Tomar, 1999).

3B.2.3.2. Effect of different cations on the growth of AC consortium and its individual isolates on CE

Effect of different cations, supplemented in the MM2 medium containing 1 % (v/v) CE, on CE utilization by the AC consortium and its individual isolates was checked in terms of their growth (Piveteau *et al*., 2001). The cations used included: (a) $MgSO_4.7H_2O$, 0.2 g/l; (b) $CaCl_2.2H_2O$, 0.02 g/l; (c) $MnCl_2.4H_2O$, 0.002 g/l; (d) $NaMoO_4.2H_2O$, 0.001 g/l; (e) $FeSO_4.7H_2O$, 0.05 g/l; (f) no cations, and (g) $MgSO_4.7H_2O$, 0.2 g/l; $CaCl_2.2H_2O$, 0.02 g/l; $MnCl_2.4H_2O$, 0.002 g/l; $NaMoO_4.2H_2O$, 0.001 g/l, $FeSO_4.7H_2O$, 0.05 g/l. The experimental procedure described in section 3B.2.3.1 was followed.

3B.2.3.3. Effect of cations concentration on the growth of AC consortium and its individual isolates on CE

Effect of different concentrations of cations, viz. 0.5, 1, 2, 3 and 4 %, on CE utilization by the AC consortium and its individual isolates was checked in terms of their growth in MM2 medium in the presence of all cations. The initial cation concentration used was considered as 1 % and accordingly other concentrations were calculated. The experimental procedure described in section 3B.2.3.1 was followed.

3B.2.3.4. Influence of increasing concentration of CE on the growth of AC consortium and its individual isolates

Effect of different concentrations of CE, viz. 6.0, 8.4, 12.0 and 14.4 g/l, on the growth of AC consortium and its individual isolates was studied to evaluate their tolerance of CE (Piveteau *et al*., 2001). The experimental procedure described in section 3B.2.3.1 was followed.

3B.2.3.5. CE utilization by AC consortium and its individual isolates in optimized medium

CE utilization by the AC consortium and its individual isolates after media optimization was checked in terms of their growth and COD reduction (Tomar, 1999). 9.8 ml MM2 medium supplemented with CE at the concentration of 12.0 g/l was

inoculated with 2 % inoculum. The experimental procedure described in section 3B.2.3.1 was followed. Residual concentration of CE was measured by gas chromatography (Thermo, GC Trace Ultra) equipped with a flame ionization detector and a capillary column (15 m x 0.53 mm) packed with HP-5. 0.4 µl of the sample was injected. The starting temperature was 40 ^{o}C, which was maintained for 2 min, then increased by 20 ^{o}C/min to 90 ^{o}C and further increased by 40 ^{o}C/min to 260 ^{o}C. The carrier gas was helium and the flow rate was 7 ml/min (Kharoune *et al*., 2001a).

3B.2.4. GC-MS analysis of DCE biodegradation by the AC consortium

9.8 ml MM2 medium supplemented with DCE at the concentration of 12.56 g/l was inoculated with 2 % inoculum. The tubes were incubated at 37 ^{o}C at 180 rpm for 120 h. The culture obtained was centrifuged at 10,000 rpm for 5 min and the cell free supernatant was analyzed by GC-MS (6890N network GC system/5973 network mass selective detector; Agilent Technologies, Wilmington, DE). The column was a capillary column (0.25 mm x 30 m x 0.25 m). 1 µl of each sample was used for injection. The carrier gas was helium and the flow rate was 1 ml/min. The mass spectrometer was operated at an electron beam energy of 70 eV and a source temperature of 180 ^{o}C. The temperature program used was 3 min at 50 ^{o}C followed by 10 ^{o}C/min to 250 ^{o}C (van den Wijngaard *et al*., 1989).

3B.2.5. Statistical analysis

Student's t-Test was applied to evaluate the effect of different cations, cation concentrations and DCE/CE concentrations on the DCE/CE biodegradation ability of the AC consortium and two-way ANOVA was applied to evaluate their effects on its individual isolates. It was assumed that the original data followed a normal distribution. All statistical analyses were performed using GraphPad Prism 5.0 software (San Diego, CA) (Barbera *et al*., 2011).

3B.3. Results and Discussion

3B.3.1. Insoluble xenobiotic utilization by the AC consortium and its individual isolates

The selection of microorganisms able to grow on xenobiotic compounds is the first problem to solve in a biodegradation process. Xenobiotic degradative capacities

of microorganisms are analyzed based on their acclimatization period (Cook *et al*., 1983). Microbial selection on xenobiotic compounds that are poorly soluble in water and/or toxic to growing microbial cells often requires extremely long acclimatization periods (Haigler *et al*., 1988; Spain and Nishino, 1987). This, in itself, becomes a limiting step in the degradation process.

The AC consortium and its individual members could grow on and utilize all the xenobiotics tested in their vapor phase, except 4-chloroaniline where growth of all the isolates and AC consortium was lowest (0.01 – 0.04 OD_{600}) (Figure 3B.2). However, the AC consortium and its individual isolates showed maximum utilization of DCE (0.33 – 0.52 OD_{600}). Growth of the isolates and AC consortium was moderate in DCB and 3-chloroaniline (0.13 – 0.35 OD_{600}), whereas low in dibromoethane and TCE (0.06 – 0.21 OD_{600}). As DCE was the best growth substrate for the AC consortium, it was selected for the further studies.

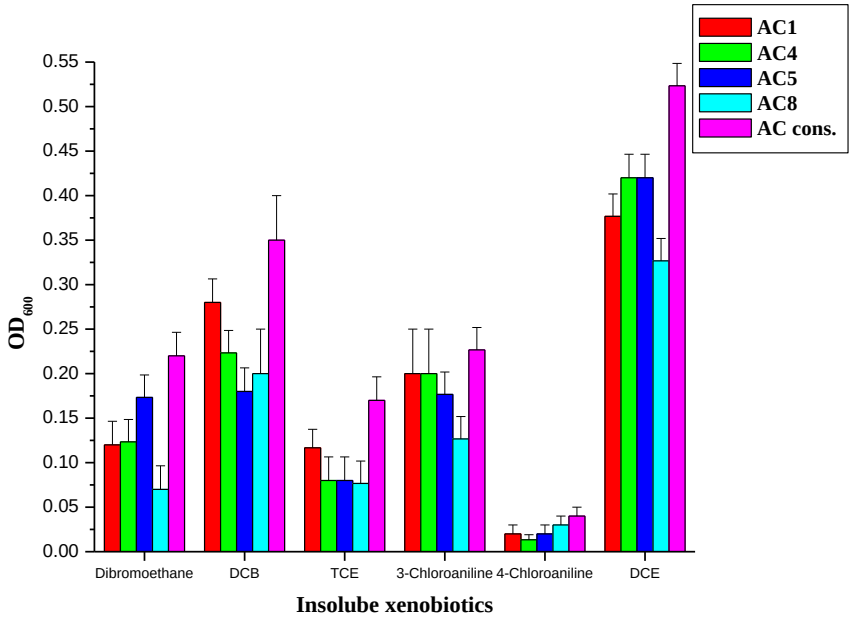


Figure 3B.2. Growth of the AC consortium and its individual isolates on insoluble xenobiotics. Error bars represent standard deviation from the mean, n = 3.

3B.3.2. DCE biodegradation

DCE, a potential mutagen and carcinogen, is commonly introduced into the environment through its industrial and agricultural use (Olaniran *et al*., 2009). Out of all the insoluble xenobiotics tested, DCE was selected for the further studies since the AC consortium as well as its individual isolates showed its maximum utilization.

3B.3.2.1. DCE biodegradation by the AC consortium and its individual isolates

DCE utilization by AC consortium and its individual isolates was measured in terms of their growth and COD reduction (Figure 3B.3). *B. petrii* AC1 reduced the COD of DCE containing medium from 750 mg/l to 150 mg/l, *B. licheniformis* AC4 and *S. subterranea* AC5 to 100 mg/l, and *P. stutzeri* AC8 to 200 mg/l, while the AC consortium could grow well on DCE as well as reduce its COD from 750 mg/l to below detection limit in 120 h.

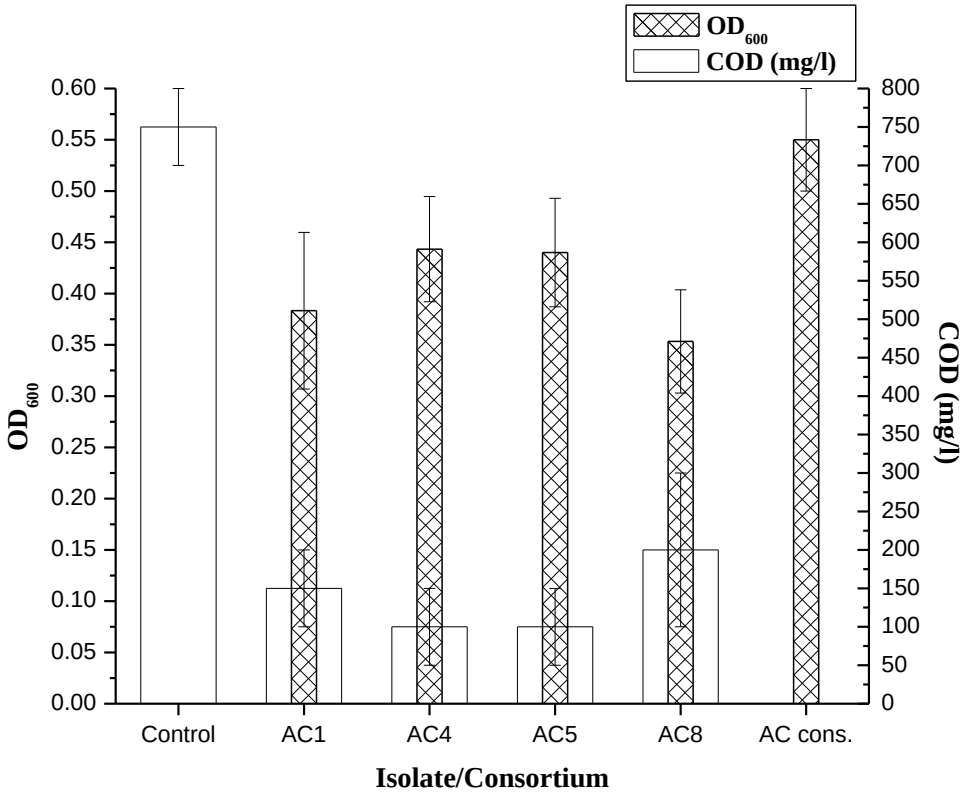


Figure 3B.3. DCE biodegradation by the AC consortium and its individual isolates. Error bars represent standard deviation from the mean, n = 3.

A number of microorganisms have been reported to degrade DCE. *X. autotrophicus* GJ10 was able to utilize DCE as sole carbon source for growth (Janssen *et al*., 1985). It also showed efficient degradation of synthetic wastewater containing DCE (Baptista *et al*., 2006). Olaniran *et al*. (2009) have successfully demonstrated the potential of indigenous microbial populations in co-contaminated loam and clay soils to degrade DCE under aerobic conditions. Likewise, the isolates of AC consortium could also biodegrade DCE effectively.

3B.3.2.2. Effect of different cations on the growth of AC consortium and its individual isolates on DCE

Cations play diverse physiologically important roles within a bacterial cell like detoxification of reactive oxygen species, stabilization of macromolecules within the cell and as co-factors for enzymes. These enzymes may be involved in the degradation of complex substrates such as xenobiotics (Zaharik and Finlay, 2004). Biostimulation and treatment additives increased DCE degradation by some bacterial isolates indigenous to contaminated sites in South Africa, with the best degradation observed upon addition of glucose and a combination of diphosphate salt and sodium chloride (Olaniran *et al*., 2009).

The effect of different cations of MM2 medium on DCE utilization by the AC consortium and its individual members was checked. Out of all the cations, viz. Mg^{2+}, Ca^{2+}, Mn^{2+}, Na^{+} and Fe^{2+}, tested, Mg^{2+} supported maximum utilization of DCE by the individual isolates and AC consortium (Figure 3B.4). Ca^{2+}, Mn^{2+} and Fe^{2+} increased the growth of *B. licheniformis* AC4 noticeably (0.23 – 0.35 OD_{600}), while in case of the other isolates the growth increase was not so pronounced (0.17 - 0.23 OD_{600}). *B. petrii* AC1 and *B. licheniformis* AC4 showed a statistically significant growth (ANOVA) on DCE in the presence of Mg^{2+} at a significance level of 0.05, with a p-value < 0.05, while *S. subterranea* AC5, *P. stutzeri* AC8 and the AC consortium showed a more statistically significant growth with a p-value < 0.001. Student's t-Test indicated that Mg^{2+} was statistically significant for the growth of AC consortium and its individual isolates on DCE with p-values of 0.037, 0.0017, 0.001, 0.0015, 0.0002 and 0.0031 over Ca^{2+}, Mn^{2+}, Na^{+}, Fe^{2+}, no cations and all cations respectively. ANOVA and Student's t-Test proved that Mg^{2+} had a statistically significant effect on DCE biodegradation by the individual isolates as well as AC consortium. Hence, it was observed that Mg^{2+} supplementation alone is sufficient to enhance the performance of the AC consortium in DCE degradation.

3B.3.2.3. Effect of magnesium concentration on the growth of AC consortium and its individual isolates on DCE

The effect of different concentrations of Mg^{2+} on DCE utilization by AC consortium and its individual members, when analyzed, showed that the AC consortium and its individual isolates were able to degrade DCE effectively in the presence of all the Mg^{2+} concentrations tested (Figure 3B.5). However, the AC

consortium and its individual isolates showed maximum DCE utilization at the concentration of 0.5 g/l. ANOVA showed that *B. petrii* AC1 showed a statistically significant growth on DCE in the presence of Mg^{2+} at 0.5 g/l at a significance level of 0.05, with a p-value < 0.01; *B. licheniformis* AC4, *P. stutzeri* AC8 and the AC consortium showed the same with a p-value < 0.05, and *S. subterranea* AC5 showed a more statistically significant growth with a p-value < 0.001. Student's t-Test also proved that Mg^{2+} at 0.5 g/l was statistically significant for the growth of AC consortium and its individual isolates on DCE with p-values of 0.0035, 0.027, 0.039, 0.0076 and 0.0043 over Mg^{2+} at 0, 0.1, 0.2, 0.6 and 0.8 g/l respectively. Hence, Mg^{2+} at 0.5 g/l was used for the further studies.

3B.3.2.4. Influence of increasing concentration of DCE on the growth of AC consortium and its individual isolates

Out of all the DCE concentrations, viz. 2.5, 6.25, 8.75 and 12.5 g/l, checked, it was observed that the AC consortium and its individual isolates showed maximum growth on DCE at 12.5 g/l (Figure 3B.6). *B. petrii* AC1, *S. subterranea* AC5 and *P. stutzeri* AC8 showed a statistically significant growth (ANOVA) on DCE at 12.5 g/l at a significance level of 0.05, with a p-value < 0.01, while *B. licheniformis* AC4 and the AC consortium showed a more statistically significant growth with a p-value < 0.001. Student's t-Test further confirmed that DCE at the concentration of 12.5 g/l was statistically significant for the growth of AC consortium and its individual isolates with p-values of 0.0001, < 0.0001 and < 0.0001 over DCE at 2.5, 6.25 and 8.75 g/l respectively. Hence, DCE at the concentration of 12.5 g/l was selected for the further studies.

DCE biodegradation ability of a variety of microorganisms has been reported previously. *X. autotrophicus* GJ10 could efficiently degrade DCE at the concentration of 3 g/l (Baptista *et al*., 2006). In comparison to this, the isolates of AC consortium degraded DCE at a higher concentration of 12.5 g/l. In the present study, the AC consortium showed tolerance to high concentration of DCE and degraded DCE in a simple mineral solution containing K_2HPO_4, KNO_3, $MgSO_4$ and yeast extract. Like *Pseudomonas* sp. strain DCA1 (Hage and Hartmans, 1999), the AC consortium does not require additional organic nutrients, such as vitamins, for optimal growth, as is the case with many others reported (Janssen *et al*., 1985).

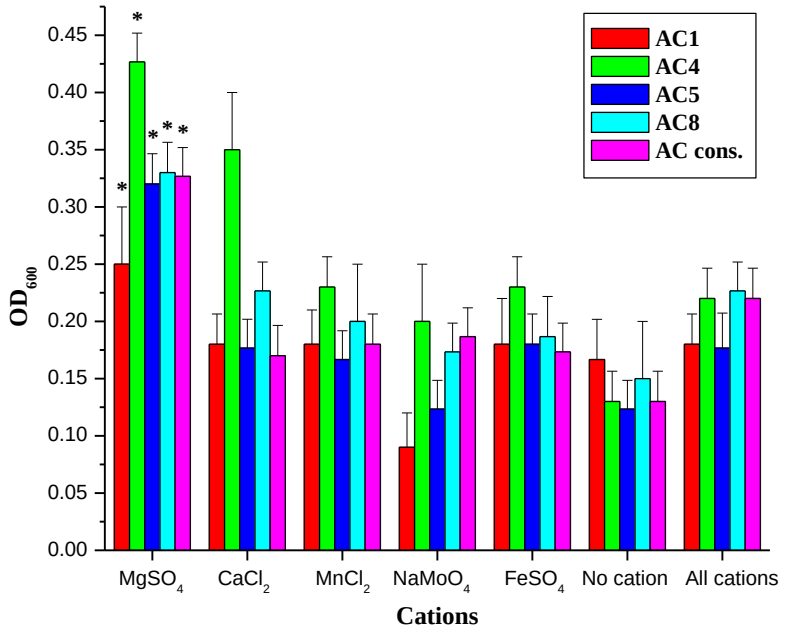


Figure 3B.4. Effect of different cations on the growth of AC consortium and its individual isolates on DCE (asterisk indicates that the growth is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

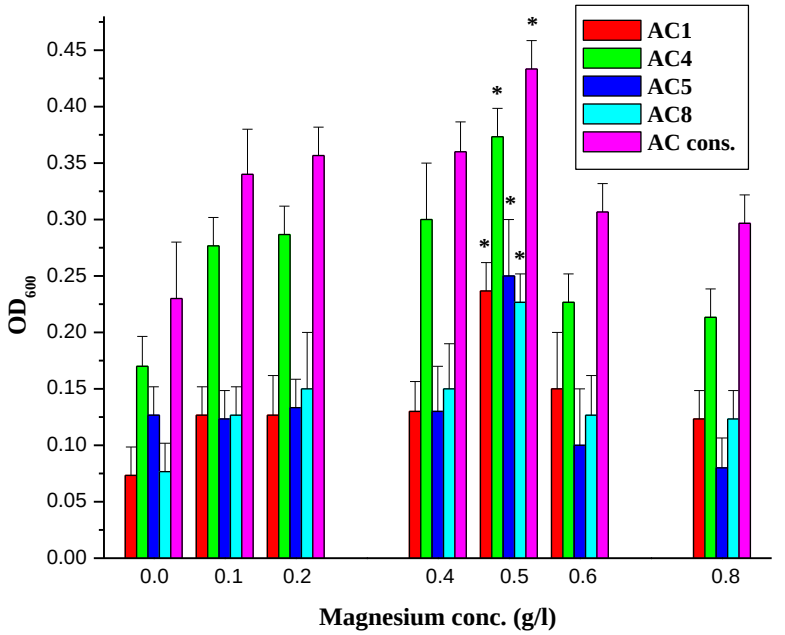


Figure 3B.5. Effect of Mg^{2+} concentration on the growth of AC consortium and its individual isolates on DCE (asterisk indicates that the growth is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

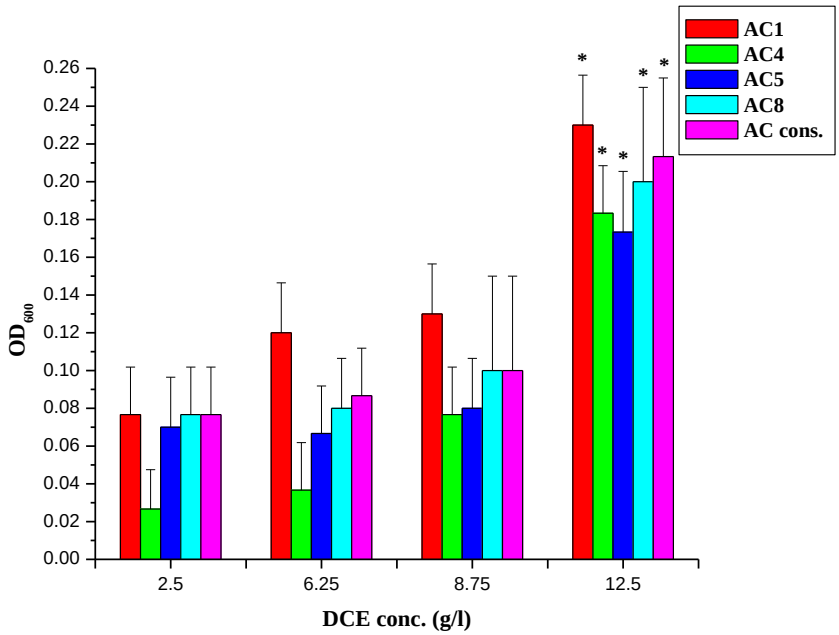


Figure 3B.6. Effect of DCE concentration on the growth of AC consortium and its individual isolates (asterisk indicates that the growth is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

3B.3.2.5. Effect of different combinations of the isolates of AC consortium on growth on DCE

Different combinations of the isolates of AC consortium were used to study their effect on DCE utilization (Figure 3B.7). The AC consortium showed maximum growth on DCE, while the individual isolates also showed considerable growth. The growth of AC consortium was highest at 0.23 OD_{600}, while the individual isolates showed growth in the range of 0.17 – 0.19 OD_{600}, which was lower than the AC consortium. In comparison to the AC consortium, the combinations in which one of its members was eliminated showed a sizeable decrease in their growth on DCE. Thus, the presence of all the isolates in the AC consortium was important for effective biodegradation of DCE.

3B.3.2.6. DCE utilization by the AC consortium and its individual isolates in optimized medium

After optimization of parameters like cations, cation concentration and DCE concentration, enhancement in DCE utilization by the isolates and AC consortium

was checked in terms of their growth and COD reduction. Growth of all the isolates showed considerable increase (Figure 3B.8) as compared to that without optimization (Figure 3B.3). The COD reduction ability of the isolates of AC consortium also increased. *B. petrii* AC1 reduced the COD of the DCE containing medium from 750 mg/l to 100 mg/l, *S. subterranea* AC5 to 50 mg/l, and *B. licheniformis* AC4, *P. stutzeri* AC8 and AC consortium to below detection limit.

The GC analysis showed that the initial DCE concentration of 12.56 g/l was reduced to 6.06 g/l by *B. petrii* AC1, 6.97 g/l by *B. licheniformis* AC4, 5.58 g/l by *S. subterranea* AC5, 5.48 g/l by *P. stutzeri* AC8 and 4.97 g/l by AC consortium (Figure 3B.9). 52 % of DCE biodegradation was obtained using *B. petrii* AC1, 45 % using *B. licheniformis* AC4, 56 % using *S. subterranea* AC5 and *P. stutzeri* AC8, and 60 % using AC consortium in 120 h. Thus, it can be implied that all the members of AC consortium were efficient in DCE biodegradation.

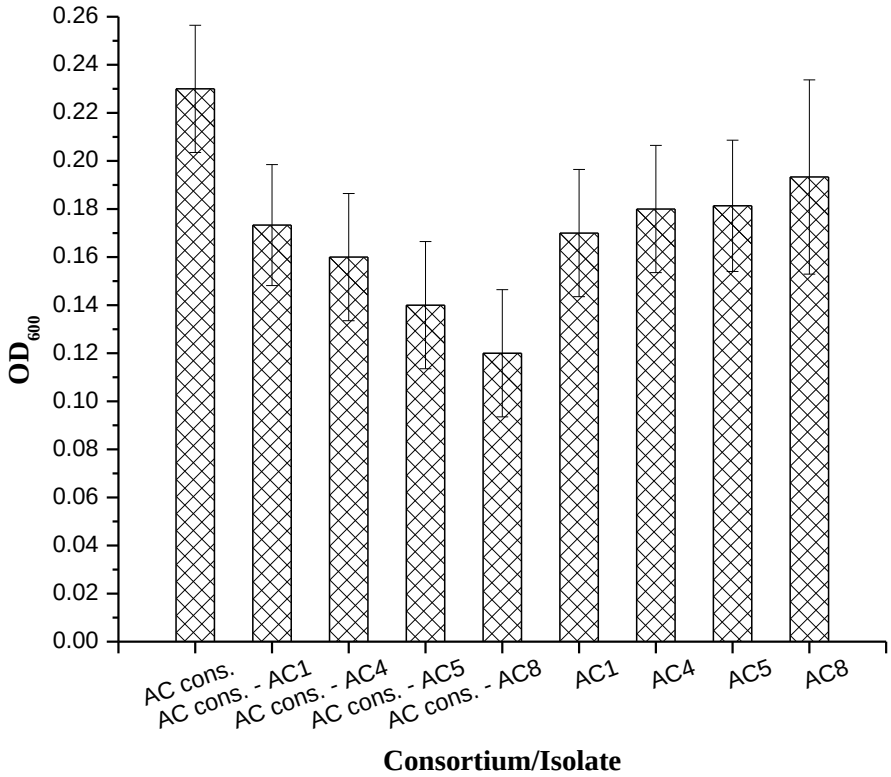


Figure 3B.7. Effect of different combinations of AC consortium on growth on DCE. Error bars represent standard deviation from the mean, n = 3.

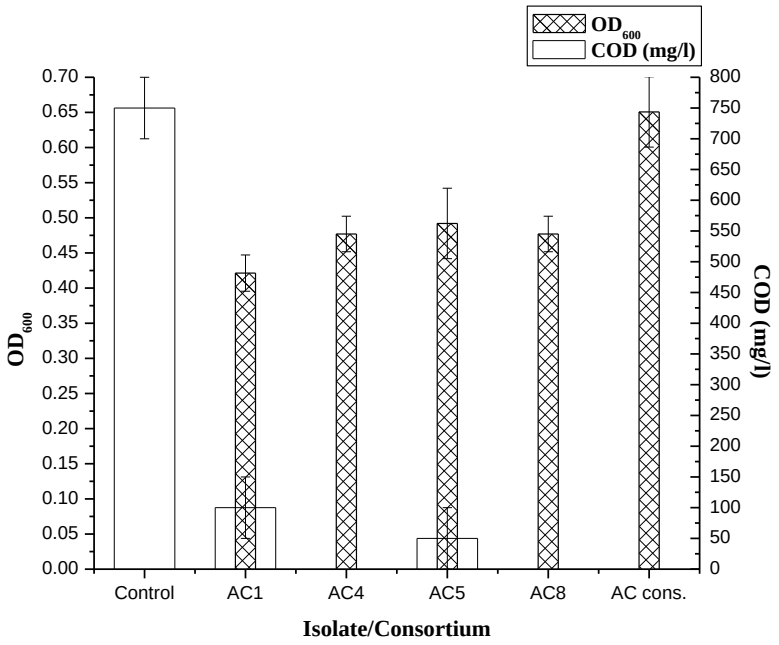
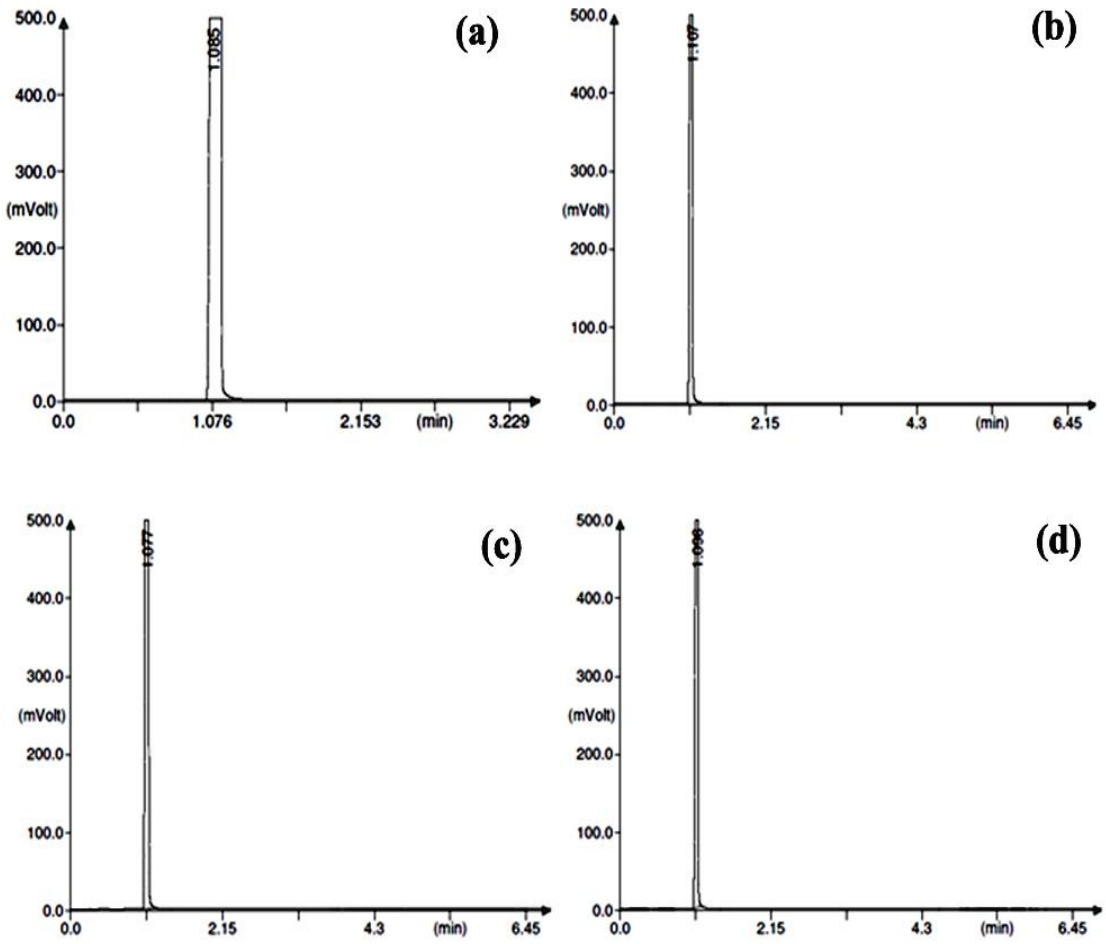
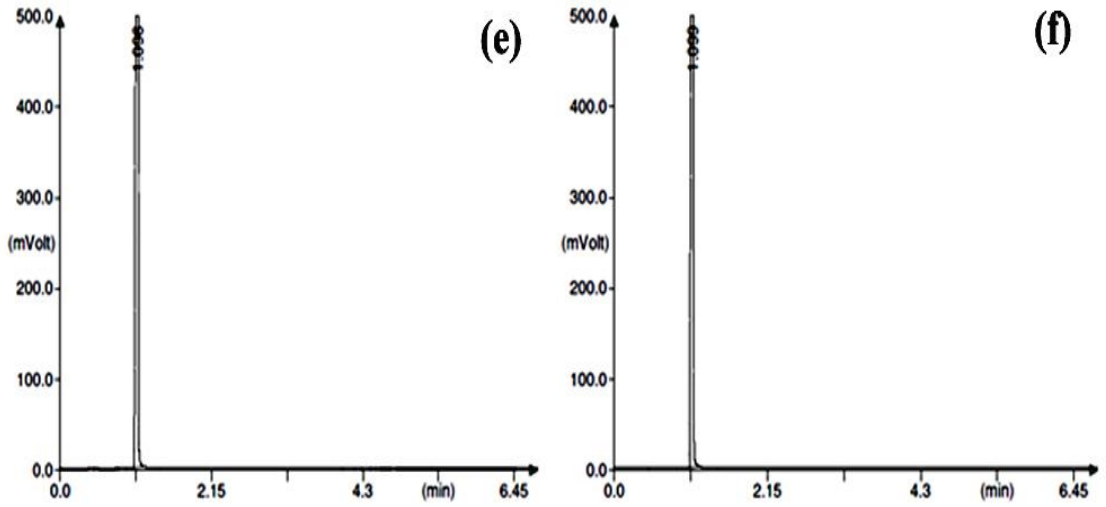


Figure 3B.8. DCE utilization by the AC consortium and its individual isolates in optimized medium. Error bars represent standard deviation from the mean, n = 3.





Sample No.	Sample	Retention time (min)	Area (.1*uV*sec)	DCE conc. (g/l)
a	Control	1.08	71671360	12.56
b	*B. petrii* AC1	1.11	34576890	6.06
c	*B. licheniformis* AC4	1.10	39790260	6.97
d	*S. subterranea* AC5	1.10	31854860	5.58
e	*P. stutzeri* AC8	1.10	31285160	5.48
f	AC consortium	1.08	28347310	4.97

Figure 3B.9. Gas chromatograms depicting DCE utilization by the AC consortium and its individual isolates. (a) Uninoculated control, (b) *B. petrii* AC1, (c) *B. licheniformis* AC4, (d) *S. subterranea* AC5, (e) *P. stutzeri* AC8 and (f) AC consortium

3B.3.3. CE biodegradation

CE is the first metabolic intermediate of DCE. CE is converted to chloroacetaldehyde to chloroacetate, which is converted to glycolate which finally enters the central metabolic pathways (Dijk *et al*., 2004). Just as TBA degradation might be the limiting step in MTBE metabolism (Fayolle *et al*., 2003), whether CE degradation might as well be the limiting step in DCE metabolism was checked in the further studies.

3B.3.3.1. CE biodegradation by the AC consortium and its individual isolates

CE utilization by the AC consortium and its individual isolates was measured in terms of growth and COD (Figure 3B.10). The results indicated that all the isolates and AC consortium could grow on CE. *P. stutzeri* AC8 showed maximum growth on CE, but reduced its COD from 1000 mg/l to 250 mg/l only. *B. petrii* AC1 reduced the COD of CE containing medium from 1000 mg/l to 50 mg/l, *B. licheniformis* AC4 and *S. subterranea* AC5 to 100 mg/l, and AC consortium to below detection limit in 120 h. Hence, the members of AC consortium were able to biodegrade CE, with the AC consortium showing highest biodegradation potential.

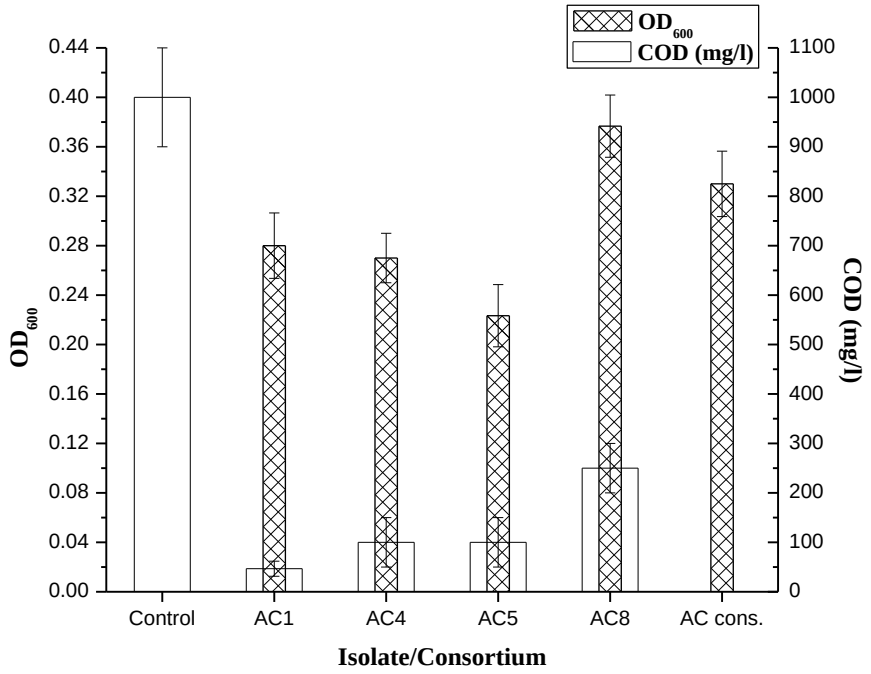


Figure 3B.10. CE biodegradation by the AC consortium and its individual isolates. Error bars represent standard deviation from the mean, n = 3.

Dijk *et al*. (2003) have reported that *P. stutzeri* strain JJ is able to grow on CE under denitrifying conditions. The pathway of CE degradation in the denitrifying *P. stutzeri* strain JJ was investigated by Dijk *et al*. (2004) and found to be the same as in aerobic bacteria that degrade CE. There are not many reports on CE biodegradation. Hence, the exhibition of CE degradation capacity of the AC consortium showed that this was not the limiting step in DCE biodegradation and the AC consortium possessed the ability to degrade DCE beyond CE.

3B.3.3.2. Effect of different cations on the growth of AC consortium and its individual isolates on CE

Effect of different cations, viz. Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+} and Na^{+}, on CE utilization by the AC consortium and its individual members was checked (Figure 3B.11). Out of all the nutrient conditions tested, it was shown that the addition of Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+} and Na^{+} together in the MM2 medium had a stimulatory effect on the growth of AC consortium and its members on CE. In cases where Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+} and Na^{+} were supplemented individually, the AC consortium and its isolates did not show appreciable increase in growth as compared to the condition where no cation was supplemented. *B. petrii* AC1 and *B. licheniformis* AC4 showed a statistically significant growth (ANOVA) on CE in the presence of all cations at a significance level of 0.05, with a p-value < 0.001; *S. subterranea* AC5 and *P. stutzeri* AC8 showed the same with a p-value < 0.05, and the AC consortium showed the same with a p-value < 0.01. Student's t-Test also proved that the presence of all the cations together was statistically significant for the growth of AC consortium and its individual isolates with a p-value < 0.0001 over Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Na^{+} and no cations. Hence, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+} and Na^{+} present together had a statistically significant effect on CE biodegradation by the individual isolates as well as AC consortium.

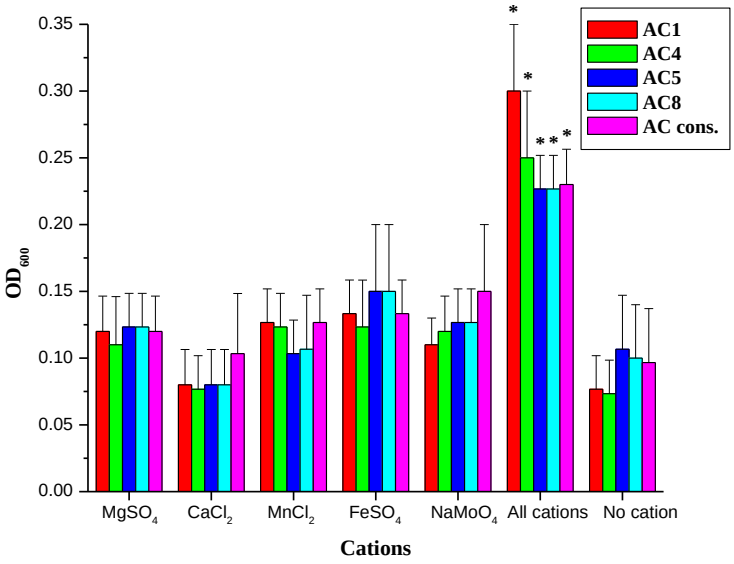


Figure 3B.11. Effect of different cations on the growth of AC consortium and its individual isolates on CE (asterisk indicates that the growth is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

3B.3.3.3. Effect of cations concentration on the growth of AC consortium and its individual isolates on CE

The effect of different concentrations of Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+} and Na^{+} on CE utilization by AC consortium and its individual members was checked and it was observed that the isolates as well as AC consortium were able to degrade CE effectively in the presence of all the cation concentrations tested (Figure 3B.12). However, all the members and AC consortium showed maximum growth in the presence of all cations at the concentration of 4 %. *B. petrii* AC1, *S. subterranea* AC5 and the AC consortium showed a more statistically significant growth (ANOVA) on CE in the presence of all cations at the concentration of 4 % at a significance level of 0.05, with a p-value < 0.001; *B. licheniformis* AC4 showed a statistically significant growth with a p-value < 0.05, and *P. stutzeri* AC8 showed the same with a p-value < 0.01. All the cations at the concentration of 4 % were statistically significant for the growth of AC consortium and its individual isolates with p-values of < 0.0001, < 0.0001, 0.002 and 0.0014 (Student's t-Test) over 0.5, 1, 2 and 3 % concentrations respectively. Hence, 4 % cation concentration was selected for the further studies.

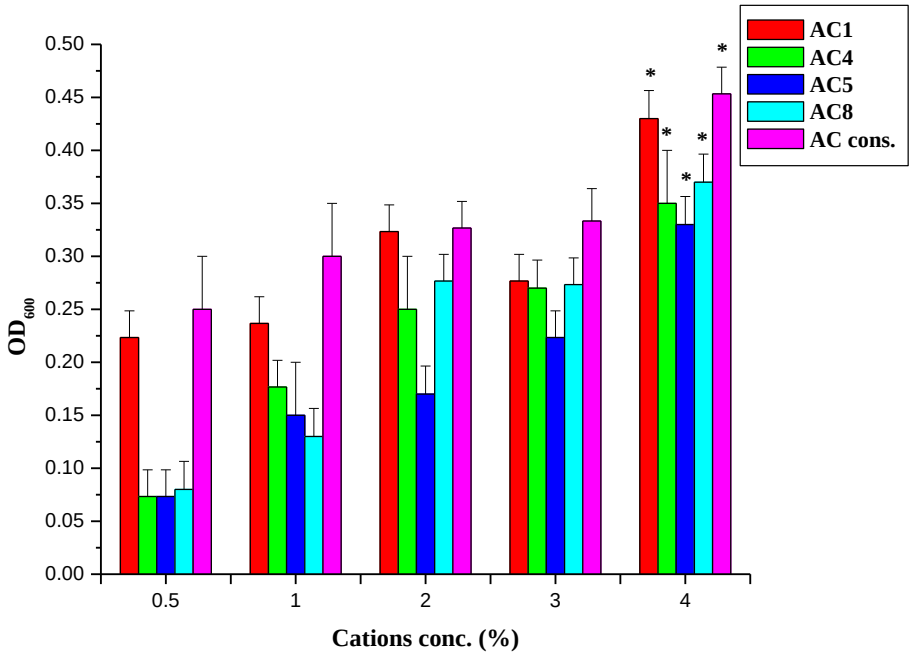


Figure 3B.12. Effect of cations concentration on the growth of AC consortium and its individual isolates on CE (asterisk indicates that the growth is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

3B.3.3.4. Influence of increasing concentration of CE on the growth of AC consortium and its individual isolates

The effect of different concentrations of CE on the growth of the AC consortium and its individual isolates was studied. *B. petrii* AC1, *S. subterranea* AC5, *P. stutzeri* AC8 and AC consortium showed maximum growth at 6.0 g/l concentration of CE, while *B. licheniformis* AC4 showed maximum growth at 8.4 g/l CE concentration (Figure 3B.13). However, as the concentrations increased, the growth of the isolates on CE decreased considerably. *B. petrii* AC1 showed a statistically significant growth (ANOVA) on CE at 6.0 g/l concentration at a significance level of 0.05, with a p-value < 0.01, and *S. subterranea* AC5 and *P. stutzeri* AC8 showed the same with a p-value < 0.05. CE at 6.0 g/l concentration was reported to be the most significant (p-value < 0.001) for its utilization by the AC consortium. *B. licheniformis* AC4 showed a statistically significant growth on CE at 8.4 g/l concentration with a p-value < 0.05. CE at the concentration of 6.0 g/l was statistically significant for the growth of AC consortium and its individual isolates with p-values of 0.022, 0.0074 and 0.0049 (Student's t-Test) over 8.4, 12.0 and 14.4 g/l CE concentrations respectively.

P. stutzeri strain JJ was reported to degrade CE at the concentration of 0.8 g/l (Dijk *et al.*, 2004). In comparison, the isolates of AC consortium degraded CE at a higher concentration of 8.4 g/l in the present study.

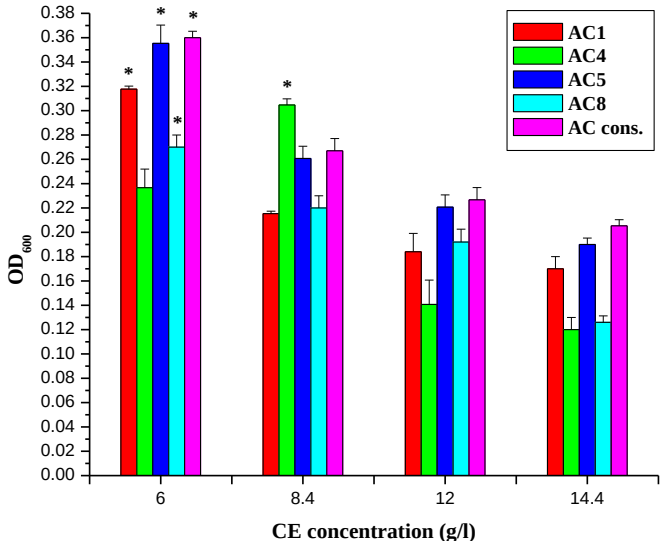


Figure 3B.13. Effect of CE concentration on growth of AC consortium and its individual isolates (asterisk indicates that the growth is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

3B.3.3.5. CE utilization by the AC consortium and its individual isolates in optimized medium

After optimization of parameters like cations, cation concentration and CE concentration, CE utilization by the isolates and AC consortium was checked in terms of their growth and COD reduction. Growth of all the isolates showed considerable increase (Figure 3B.14) as compared to that without optimization (Figure 3B.10). The individual isolates as well as AC consortium reduced the COD of the CE containing medium to below detection limit in 120 h (Figure 3B.14).

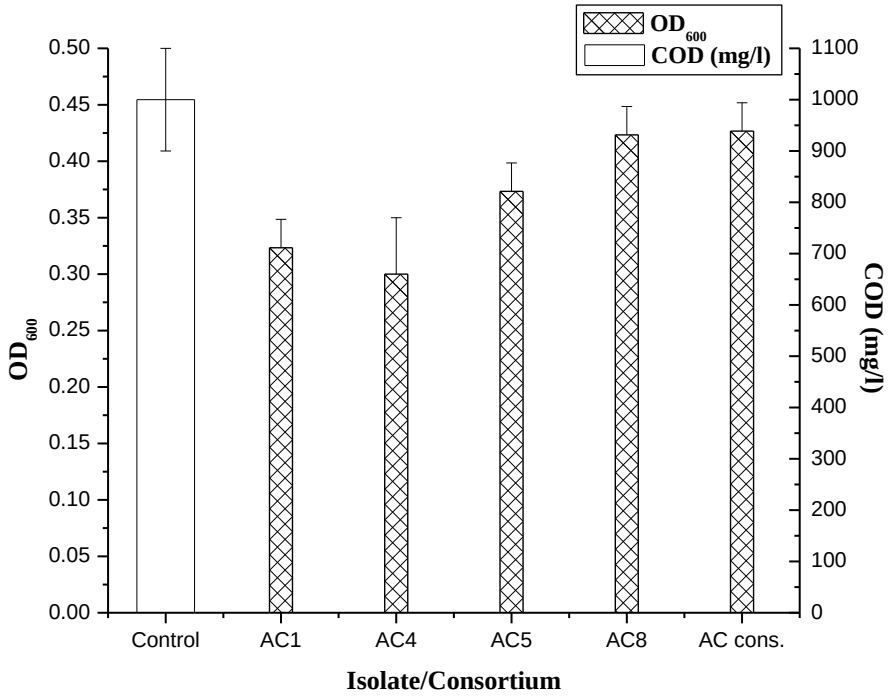
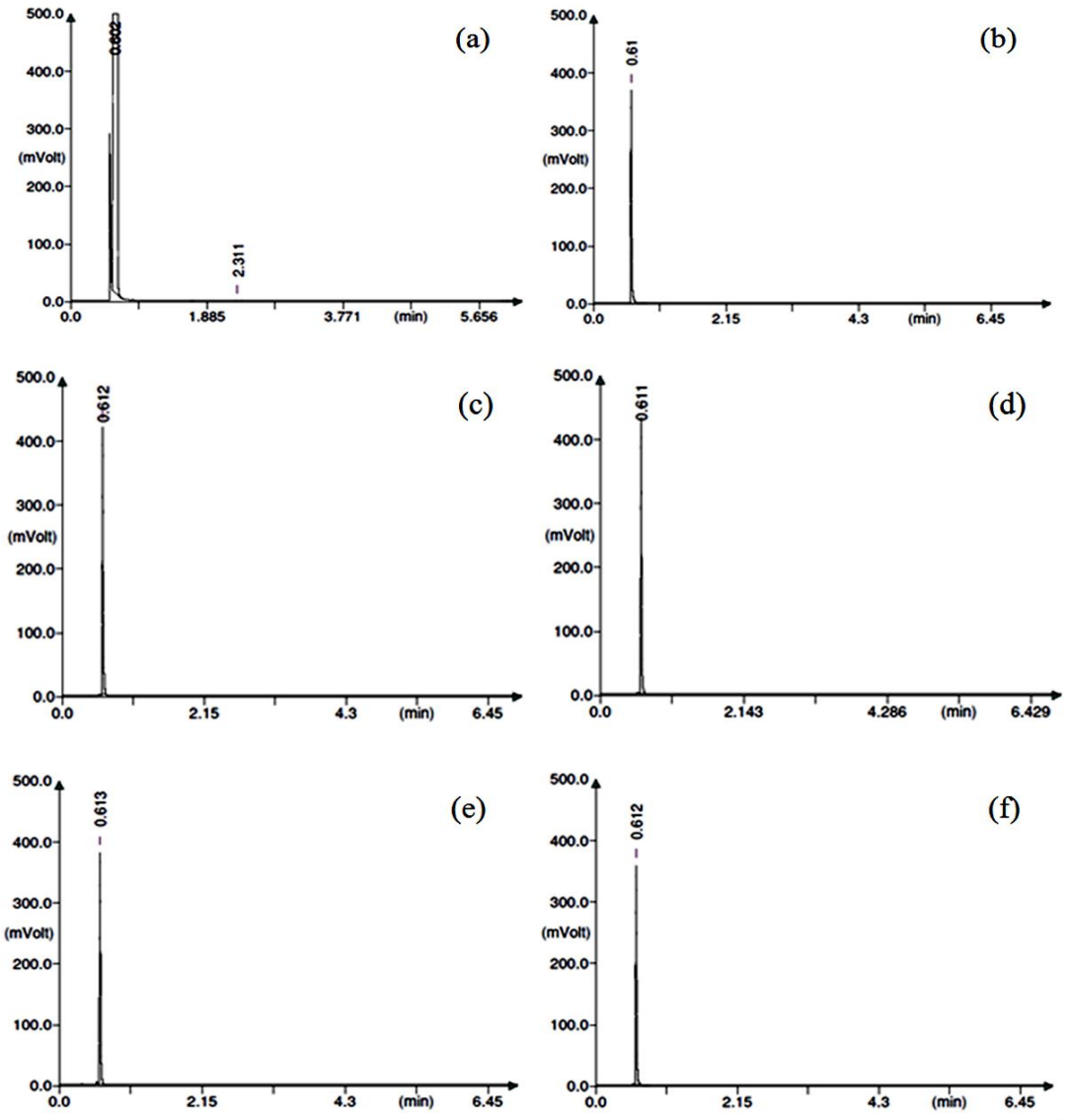


Figure 3B.14. CE utilization by the AC consortium and its individual isolates in optimized medium. Error bars represent standard deviation from the mean, n = 3.

The GC analysis showed that the initial CE concentration of 8.4 g/l was reduced to 0.44 g/l by *B. petrii* AC1, 0.35 g/l by *B. licheniformis* AC4, 0.35 g/l by *S. subterranea* AC5, 0.46 g/l by *P. stutzeri* AC8 and 0.34 g/l by AC consortium (Figure 3B.15). 95 % CE biodegradation was obtained using *B. petrii* AC1 and *P. stutzeri* AC8, and 96 % using *B. licheniformis* AC4, *S. subterranea* AC5 and AC consortium in 120 h. Thus, it can be implied that all the members of AC consortium contributed equally towards efficient CE biodegradation.



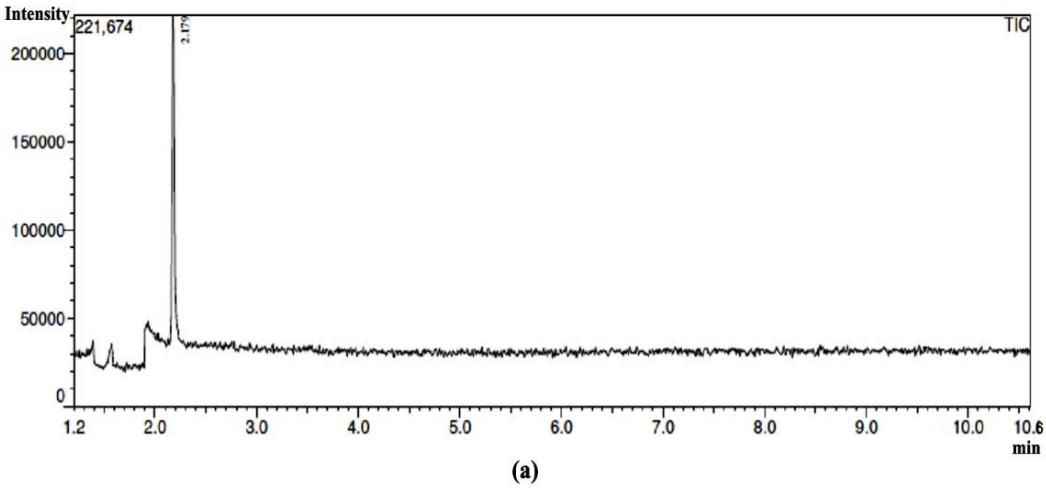
Sample No.	Sample	Retention time (min)	Area (.1*uV*sec)	CE concentration (g/l)
A	Control	1.38	56939310	8.4
B	*B. petrii* AC1	1.20	2973562	0.44
C	*B. licheniformis* AC4	1.20	2348268	0.35
D	*S. subterranea* AC5	1.20	2391545	0.35
E	*P. stutzeri* AC8	1.21	3122361	0.46
F	AC consortium	1.20	2299069	0.34

Figure 3B.15. Gas chromatograms depicting CE utilization by the AC consortium and its individual isolates. (a) Uninoculated control, (b) *B. petrii* AC1, (c) *B. licheniformis* AC4, (d) *S. subterranea* AC5, (e) *P. stutzeri* AC8 and (f) AC consortium

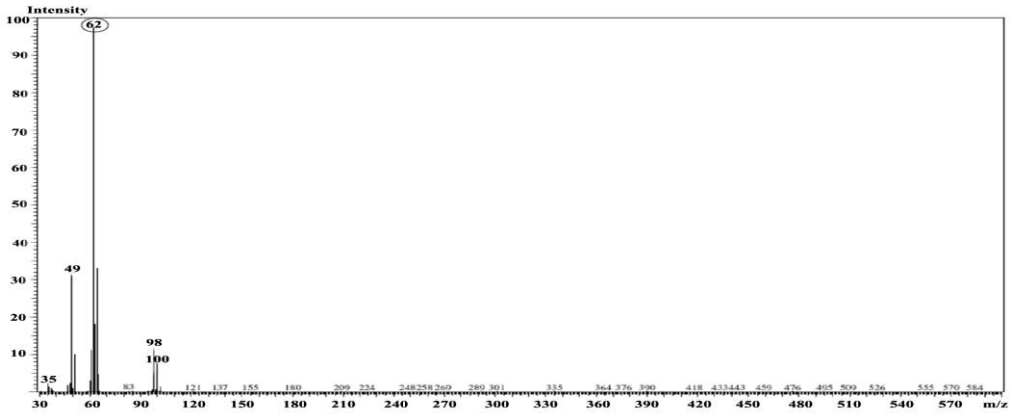
3B.3.4. Intermediate products of DCE on biodegradation by the AC consortium

DCE degradation ability of the AC consortium was analyzed using GC-MS. The peaks obtained by GC (Figure 3B.16(a & c)) were subjected to mass spectrometry. The results obtained were compared with the GC-MS of uninoculated control medium. Figure 3B.16(b) shows the mass spectrum of the peak obtained at a retention time of 2.179 min in the gas chromatogram. This predominant peak was identified as DCE on the basis of the m/z 62 (Mena-Benitez *et al*., 2008). This peak disappeared on DCE biodegradation by the AC consortium (Figure 3B.16(c)), indicating its complete degradation. The results of GC-MS analysis indicated that the DCE was completely utilized by the AC consortium and CE, the first metabolic intermediate of DCE biodegradation, was not accumulated during DCE biodegradation (Figure 3B.16).

Therefore, it can be implied that the AC consortium was effective in biodegradation of DCE and its intermediate, CE.



(a)



(b)

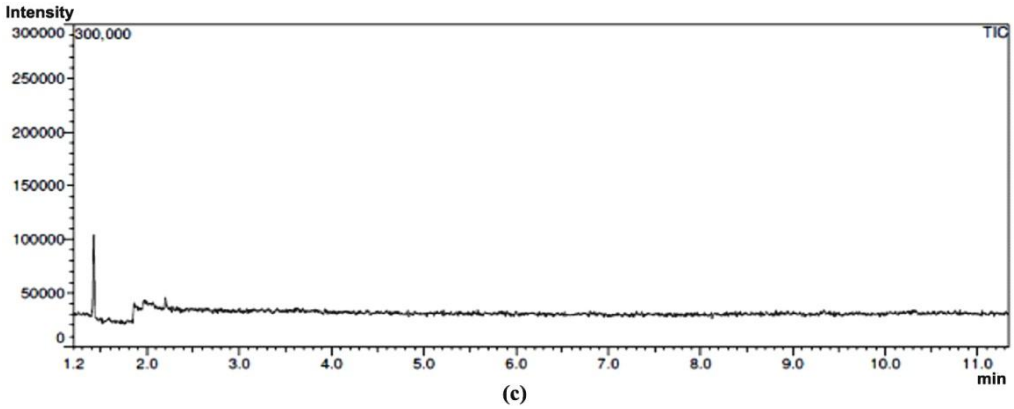


Figure 3B.16. GC-MS of DCE biodegradation by the AC consortium. (a) GC of uninoculated control medium, (b) MS of the predominant peak obtained at the retention time of 2.18 min in the GC in (a), and (c) GC of the DCE supplemented medium inoculated with the AC consortium.

The AC consortium could utilize MTBE, TBA, DCE and CE as sole sources of carbon and energy. The results obtained with the degradation studies of MTBE, TBA, DCE and CE indicate that the AC consortium shows promising potential for effective bioremediation of xenobiotics. Considering the spectrum of xenobiotic degradation of the AC consortium, it offers promising opportunities for treatment of effluents generated from various chemical industries, which are studied in the subsequent chapter. In conclusion, the capacity of AC consortium to degrade some of the toxic xenobiotics was determined. To our knowledge, the AC consortium is first such consortium with a broad range of xenobiotic degradation ability.

3.2. References

Auffret, M., Labbe, D., Thouand, G., Greer, C.W., Fayolle-Guichard, F., 2009. Degradation of a mixture of hydrocarbons, gasoline, and diesel oil additives by *Rhodococcus aetherivorans* and *Rhodococcus wratislaviensis*. *Applied and environmental microbiology*, 75, 7774-7782.

Baptista, I.I.R., Peeva, L.G., Zhou, N.-Y., Leak, D.J., Mantalaris, A., Livingston, A.G., 2006. Stability and performance of *Xanthobacter autotrophicus* GJ10 during 1, 2-dichloroethane biodegradation. *Applied and environmental microbiology*, 72, 4411-4418.

Barbera, M.J., Mateo, E., Monkaityte, R., Constanti, M., 2011. Biodegradation of methyl *tert*-butyl ether by newly identified soil microorganisms in a simple mineral solution. *World Journal of Microbiology and Biotechnology*, 27, 813-821.

Barcelo, D., 2007. Fuel oxygenates. Springer cop, Berlin, New York.

Bhatt, P., Kumar, M.S., Mudliar, S., Chakrabarti, T., 2007. Biodegradation of chlorinated compounds - a review. *Critical Reviews in Environmental Science and Technology*, 37, 165-198.

Boon, N., Goris, J., De Vos, P., Verstraete, W., Top, E.M., 2000. Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain, I2gfp. *Applied and environmental microbiology*, 66, 2906-2913.

Chen, J., Chen, D., Zhong, W., Zhang, J., Chen, X., 2007. Biodegradation of methyl *tert*-butyl ether by *Methylibium petroleiphilum* PM1 in poor nutrition solution. *Journal Of Environmental Science And Health Part A*, 42, 2123-2129.

Cook, A.M., Grossenbacher, H., Hutter, R., 1983. Isolation and cultivation of microbes with biodegradative potential. *Experientia*, 39, 1191-1198.

Corcho, D., Watkinson, R.J., Lerner, D.N., 2000. Cometabolic degradation of MTBE by a cyclohexane-oxidising bacteria. Second International Conference on Remediation of Chlorinated and Racalcitrant Compounds, pp. 183-189.

Deeb, R.A., Hu, H.-Y., Hanson, J.R., Scow, K.M., Alvarez-Cohen, L., 2001. Substrate interactions in BTEX and MTBE mixtures by an MTBE-degrading isolate. *Environmental science & technology*, 35, 312-317.

Dijk, J.A., Stams, A.J.M., Schraa, G., Ballerstedt, H., De Bont, J.A.M., Gerritse, J., 2003. Anaerobic oxidation of 2-chloroethanol under denitrifying conditions by

Pseudomonas stutzeri strain JJ. *Applied microbiology and biotechnology*, 63, 68-74.

Dijk, J.A., Gerritse, J., Schraa, G., Stams, A.J.M., 2004. Degradation pathway of 2-chloroethanol in *Pseudomonas stutzeri* strain JJ under denitrifying conditions. *Archives of microbiology*, 182, 514-519.

Dijkhuizen, L., Knight, M., Harder, W., 1978. Metabolic regulation in *Pseudomonas oxalaticus* OX1. *Archives of microbiology*, 116, 77-83.

Dolfing, J., van den Wijngaard, A.J., Janssen, D.B., 1993. Microbiological aspects of the removal of chlorinated hydrocarbons from air. *Biodegradation*, 4, 261-282.

Doronina, N.V., Trotsenko, Y.A., Tourova, T.P., Kuznetsov, B.B., Leisinger, T., 2000. *Methylopila helvetica* sp. nov. and *Methylobacterium dichloromethanicum* sp. nov. - Novel Aerobic Facultatively Methylotrophic Bacteria Utilizing Dichloromethane. *Systematic and applied microbiology*, 23, 210-218.

Farrokhi, M., Ahmadizad, S., 2009. Effects Of Stimulator Substances On Aerobic Methyl *tert*-Butyl Ether Biodegradation By Microbial Consortium. *Iranian Journal of Environmental Health Science & Engineering*, 6, 81-88.

Fayolle, F., Francois, A., Garnier, L., Godefroy, D., Mathis, H., Piveteau, P., Monot, F., 2003. Limitations in MTBE biodegradation. *Oil & gas science and technology*, 58, 497-504.

Fortin, N.Y., Morales, M., Nakagawa, Y., Focht, D.D., Deshusses, M.A., 2001. Methyl *tert*-butyl ether (MTBE) degradation by a microbial consortium. *Environmental Microbiology*, 3, 407-416.

Francois, A., Mathis, H., Godefroy, D., Piveteau, P., Fayolle, F., Monot, F., 2002. Biodegradation of methyl *tert*-butyl ether and other fuel oxygenates by a new strain, *Mycobacterium austroafricanum* IFP 2012. *Applied and environmental microbiology*, 68, 2754-2762.

Freitas do Santos, L., Livingston, A.G., 1995. Novel membrane bioreactor for detoxification of VOC wastewaters: biodegradation of 1,2-dichloroethane. *Water Research*, 29, 179-194.

Garnier, P.M., Auria, R., Augur, C., Revah, S., 2000. Cometabolic biodegradation of methyl *tert*-butyl ether by a soil consortium: effect of components present in gasoline. *The Journal of general and applied microbiology*, 46, 79-84.

Gisi, D., Willi, L., Traber, H., Leisinger, T., Vuilleumier, S., 1998. Effects of bacterial host and dichloromethane dehalogenase on the competitiveness of methylotrophic bacteria growing with dichloromethane. *Applied and environmental microbiology*, 64, 1194-1202.

Goodfellow, M., Jones, A.L., Maldonado, L.A., Salanitro, J., 2004. *Rhodococcus aetherivorans* sp. nov., A New Species that Contains Methyl *t*-butyl Ether-Degrading Actinomycetes. *Systematic and applied microbiology*, 27, 61-65.

Hage, J.C., Hartmans, S., 1999. Monooxygenase-mediated 1, 2-dichloroethane degradation by *Pseudomonas* sp. strain DCA1. *Applied and environmental microbiology*, 65, 2466-2470.

Haigler, B.E., Nishino, S.F., Spain, J.C., 1988. Degradation of 1, 2-dichlorobenzene by a *Pseudomonas* sp. *Applied and environmental microbiology*, 54, 294-301.

Hamer, G., 1997. Microbial consortia for multiple pollutant biodegradation. *Pure and applied chemistry*, 69, 2343-2356.

Hatzinger, P.B., McClay, K., Vainberg, S., Tugusheva, M., Condee, C.W., Steffan, R.J., 2001. Biodegradation of methyl *tert*-butyl ether by a pure bacterial culture. *Applied and environmental microbiology*, 67, 5601-5607.

Hernandez-Perez, G., Fayolle, F., Vandecasteele, J.-P., 2001. Biodegradation of ethyl *t*-butyl ether (ETBE), methyl *t*-butyl ether (MTBE) and *t*-amyl methyl ether (TAME) by *Gordonia terrae*. *Applied microbiology and biotechnology*, 55, 117-121.

Janssen, D.B., Scheper, A., Dijkhuizen, L., Witholt, B., 1985. Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. *Applied and environmental microbiology*, 49, 673-677.

Janssen, D.B., Pries, F., Van der Ploeg, J., Kazemier, B., Terpstra, P., Witholt, B., 1989. Cloning of 1, 2-dichloroethane degradation genes of *Xanthobacter autotrophicus* GJ10 and expression and sequencing of the *dhlA* gene. *Journal of bacteriology*, 171, 6791-6799.

Kane, S.R., Beller, H.R., Legler, T.C., Koester, C.J., Pinkart, H.C., Halden, R.U., Happel, A.M., 2001. Aerobic Biodegradation of Methyl *tert*-Butyl Ether by Aquifer Bacteria from Leaking Underground Storage Tank Sites. *Applied and environmental microbiology*, 67, 5824-5829.

Kane, S.R., Chakicherla, A.Y., Chain, P.S.G., Schmidt, R., Shin, M.W., Legler, T.C., Scow, K.M., Larimer, F.W., Lucas, S.M., Richardson, P.M., 2007. Whole-

genome analysis of the methyl *tert*-butyl ether-degrading *beta*-proteobacterium *Methylibium petroleiphilum* PM1. *Journal of bacteriology*, 189, 1931-1945.

Kharoune, M., Pauss, A., Lebeault, J.M., 2001a. Aerobic biodegradation of an oxygenates mixture: ETBE, MTBE and TAME in an upflow fixed-bed reactor. *Water Research*, 35, 1665-1674.

Kharoune, M., Kharoune, L., Lebeault, J.M., Pauss, A., 2001b. Isolation and characterization of two aerobic bacterial strains that completely degrade ethyl *tert*-butyl ether (ETBE). *Applied Microbiology and Biotechnology*, 55, 348-353.

Koutinas, M., Martin, J., Peeva, L.G., Mantalaris, A., Livingston, A.G., 2006. An oil-absorber-bioscrubber system to stabilize biotreatment of pollutants present in waste gas. Fluctuating loads of 1, 2-dichloroethane. *Environmental science & technology*, 40, 595-602.

Lee, E.-H., Cho, K.-S., 2009. Effect of substrate interaction on the degradation of methyl *tert*-butyl ether, benzene, toluene, ethylbenzene, and xylene by *Rhodococcus* sp. *Journal of hazardous materials*, 167, 669-674.

Lin, C.-W., Cheng, Y.-W., Tsai, S.-L., 2007. Influences of metals on kinetics of methyl *tert*-butyl ether biodegradation by *Ochrobactrum cytisi*. *Chemosphere*, 69, 1485-1491.

Liu, H., Yan, J., Wang, Q., Karlson, U.G., Zou, G., Yuan, Z., 2009. Biodegradation of methyl *tert*-butyl ether by enriched bacterial culture. *Current microbiology*, 59, 30-34.

Loffler, F., Muller, R., 1991. Identification of 4-chlorobenzoyl-coenzyme A as intermediate in the dehalogenation catalysed by 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3. *FEBS letters*, 290, 224-226.

Lopes Ferreira, N.L., Maciel, H., Mathis, H., Monot, F., Fayolle-Guichard, F., Greer, C.W., 2006a. Isolation and characterization of a new *Mycobacterium austroafricanum* strain, IFP 2015, growing on MTBE. *Applied microbiology and biotechnology*, 70, 358-365.

Lopes Ferreira, N.L., Labbe, D., Monot, F., Fayolle-Guichard, F., Greer, C.W., 2006b. Genes involved in the methyl *tert*-butyl ether (MTBE) metabolic pathway of *Mycobacterium austroafricanum* IFP 2012. *Microbiology*, 152, 1361-1374.

de Marco, P., Pacheco, C.C., Figueiredo, A.R., Moradas-Ferreira, P., 2004. Novel pollutant-resistant methylotrophic bacteria for use in bioremediation. *FEMS microbiology letters*, 234, 75-80.

Mena-Benitez, G.L., Gandia-Herrero, F., Graham, S., Larson, T.R., McQueen-Mason, S.J., French, C.E., Rylott, E.L., Bruce, N.C., 2008. Engineering a Catabolic Pathway in Plants for the Degradation of 1,2-Dichloroethane[1[OA]]. *Plant Physiology*, 147, 1192–1198.

Mo, K., Lora, C.O., Wanken, A.E., Javanmardian, M., Yang, X., Kulpa, C.F., 1997. Biodegradation of methyl *t*-butyl ether by pure bacterial cultures. *Applied microbiology and biotechnology*, 47, 69-72.

Munoz-Castellanos, L.N., Torres-Munoz, J.V., Keer-Rendon, A., Manzanares-Papayanopoulos, L.I., Nevarez-Moorillon, G.V., 2006. Aerobic biodegradation of methyl *tert*-butyl ether (MTBE) by pure bacterial cultures isolated from contaminated soil. *World Journal of Microbiology and Biotechnology*, 22, 851-855.

Ojo, O.A., 2007. Molecular strategies of microbial adaptation to xenobiotics in natural environment. *Biotechnology and Molecular Biology Review*, 2, 001-013.

Olaniran, A.O., Balgobind, A., Pillay, B., 2009. Impacts of heavy metals on 1, 2-dichloroethane biodegradation in co-contaminated soil. *Journal of Environmental Sciences*, 21, 661-666.

Pieper, D.H., Reineke, W., 2000. Engineering bacteria for bioremediation. *Current Opinion in Biotechnology*, 11, 262-270.

Piveteau, P., Fayolle, F., Vandecasteele, J.P., Monot, F., 2001. Biodegradation of *tert*-butyl alcohol and related xenobiotics by a methylotrophic bacterial isolate. *Applied microbiology and biotechnology*, 55, 369-373.

Roane, T.M., Josephson, K.L., Pepper, I.L., 2001. Dual-bioaugmentation strategy to enhance remediation of cocontaminated soil. *Applied and environmental microbiology*, 67, 3208-3215.

Rosell, M., Lacorte, S., Ginebreda, A., Barcelo, D., 2003. Simultaneous determination of methyl *tert*-butyl ether and its degradation products, other gasoline oxygenates and benzene, toluene, ethylbenzene and xylenes in Catalonian groundwater by purge-and-trap-gas chromatography-mass spectrometry. *Journal of Chromatography A*, 995, 171-184.

Salanitro, J.P., Diaz, L.A., Williams, M.P., Wisniewski, H.L., 1994. Isolation of a bacterial culture that degrades methyl *t*-butyl ether. *Applied and environmental microbiology*, 60, 2593-2596.

Sinha, S., Chattopadhyay, P., Pan, I., Chatterjee, S., Chanda, P., Bandyopadhyay, D., Das, K., Sen, S.K., 2009. Microbial transformation of xenobiotics for environmental bioremediation. *African Journal of Biotechnology*, 8, 6016-6027.

Smith, C.A., O'Reilly, K.T., Hyman, M.R., 2003. Characterization of the initial reactions during the cometabolic oxidation of methyl *tert*-butyl ether by propane-grown *Mycobacterium vaccae* JOB5. *Applied and environmental microbiology*, 69, 796-804.

Spain, J.C., Nishino, S.F., 1987. Degradation of 1, 4-dichlorobenzene by a *Pseudomonas* sp. *Applied and environmental microbiology*, 53, 1010-1019.

Squillace, P.J., Zogorski, J.S., Wilber, W.G., Price, C.V., 1996. Preliminary assessment of the occurrence and possible sources of MTBE in groundwater in the United States, 1993-1994. *Environmental science & technology*, 30, 1721-1730.

Srinandan, C.S., Jadav, V., Cecilia, D., Nerurkar, A.S., 2010. Nutrients determine the spatial architecture of *Paracoccus* sp. biofilm. *Biofouling*, 26, 449-459.

Steffan, R.J., McClay, K., Vainberg, S., Condee, C.W., Zhang, D., 1997. Biodegradation of the gasoline oxygenates methyl *tert*-butyl ether, ethyl *tert*-butyl ether, and *tert*-amyl methyl ether by propane-oxidizing bacteria. *Applied and environmental microbiology*, 63, 4216-4222.

Tomar, M., 1999. Quality assessment of water and wastewater. CRC press, Florida, USA.

Vangnai, A.S., Petchkroh, W., 2007. Biodegradation of 4-chloroaniline by bacteria enriched from soil. *FEMS microbiology letters*, 268, 209-216.

van den Wijngaard, A.J., Janssen, D.B., Witholt, B., 1989. Degradation of epichlorohydrin and halohydrins by bacterial cultures isolated from freshwater sediment. *Journal of general microbiology*, 135, 2199-2208.

De Wildeman, S., Diekert, G., Van Langenhove, H., Verstraete, W., 2003. Stereoselective microbial dehalorespiration with vicinal dichlorinated alkanes. *Applied and environmental microbiology*, 69, 5643-5647.

Wilson, G.J., Pruden, A., Suidan, M.T., Venosa, A.D., 2002. Biodegradation kinetics of MTBE in laboratory batch and continuous flow reactors. *Journal of environmental engineering*, 128, 824-829.

Yan, J., Rash, B.A., Rainey, F.A., Moe, W.M., 2009. Isolation of novel bacteria within the *Chloroflexi* capable of reductive dechlorination of 1, 2, 3-trichloropropane. *Environmental Microbiology*, 11, 833-843.

van der Zaan, B., de Weert, J., Rijnaarts, H., de Vos, W.M., Smidt, H., Gerritse, J., 2009. Degradation of 1,2-dichloroethane by microbial communities from river sediment at various redox conditions. *Water research*, 43, 3207-3216.

Zaharik, M.L., Finlay, B.B., 2004. Mn^{2+} and bacterial pathogenesis. *Frontiers in Bioscience*, 9, 1035-1042.

Chapter 4
Biotreatment of different industrial effluents using AC consortium

4.1. Introduction

Fertilizer industry is one of the major water consuming industries, responsible for water and soil pollution of considerable magnitude. Industrial effluents are constantly adding up toxic substances into the ground water reservoir at a very high rate, especially in industrial zones (Singh *et al*., 2006). Owing to a rapid increase in the manufacture of fertilizers, there has been a substantial increase in liquid waste, which is traditionally discharged either onto open land or into nearby watercourses, causing varying degrees of pollution of air, water and soil (Mishra and Singh, 1987). Most of the effluent is disposed of in the natural system, which is unable to assimilate such a large quantity. Due to the high organic load, stagnating effluent at low-lying sites acts as a great source of environmental pollution, leading to a number of health hazards. The continuous use of large quantities of chemical fertilizers in intensive cropping systems results in various problems and unfavorable soil conditions (Sharma *et al*., 2008). Highly variable physico-chemical nature and composition of industrial effluent necessitates its treatment before introducing into the ecosystem or before recycling (George *et al*., 1997).

Globally pesticides are toxic to humans and reach them through food chain. Most of the pesticides are non-biodegradable because of their molecular structure with stable internal bonds. Pesticide pollution of natural waters has become a pervasive problem. Wastewaters from pesticide manufacturing industries originate from cleaning activities after batch operation during the synthesis processes. They may contain toxic organics and pesticide residues which pose a threat to the quality of surface and groundwater. The overall impact of a pesticide depends on its behaviour in the environment, its toxicity and the amounts applied. It has been reported that an increasing amount of the pesticide residues may be present in the soil and these can ultimately be leached to aquifer levels and contaminate the groundwater or may be carried away by runoff waters and soil erosion in natural water resources including rivers (Sherma, 1995). Treatment of concentrated wastewater is often difficult because of microbial toxicity and mass transfer limitations (Babu *et al*., 2011).

Unlike microbially mediated production processes, microbially mediated environmental protection and restoration processes involve process cultures comprising multiple microbial consortia and it is the consortium performance, rather than individual strain performance, that is critical as far as both process efficiency and

economics are concerned. Biotreatment processes involve multiple substrates (pollutants) and highly complex mixed microbial cultures. Furthermore, biotreatment processes function in the continuous flow mode, frequently under unsteady state conditions, and involve multiple biogeochemical cycles (Hamer, 1997).

Reports are available on consortia and methylotrophs individually with respect to wastewater treatment. *Methylobacterium thiocyanatum* strain BIP, able to use formaldehyde, methanol and a range of multi-carbon compounds as its principal growth substrates, has been used for industrial remediation of formaldehyde-containing effluents (Chongcharoen *et al*., 2005). *Methanomethylovorans thermophila* sp. nov. strain L2FAWT, a thermophilic, obligately methylotrophic, methanogenic archaeon, was isolated from a thermophilic laboratory-scale upflow anaerobic sludge blanket reactor fed with methanol as the carbon and energy source (Jiang *et al*., 2005). Coats *et al*. (2007) have reported PHAs production with a mixed microbial consortium indigenous to an activated sludge process on carbon present in municipal wastewaters. Wakelin *et al*. (2008) investigated the effects of wastewater treatment plant (WWTP) discharge on the ecology of bacterial communities in the sediment of a small, low-gradient stream in South Australia.

Many industrial effluents are not amenable to biotreatment due to presence of recalcitrant and toxic carbon compounds. Industrial effluents having high COD are environmentally hazardous. It is important to investigate the suitability of an effluent to a biological process before designing such a process. For this purpose, BOD and COD are correlated. BOD_5/COD ratio is called Biodegradability Index, which varies from 0.4 to 0.8 for domestic wastewaters. The industries follow a thumb rule where if BOD/COD ratio is > 0.6, then the waste is fairly biodegradable and can be effectively treated biologically. If BOD/COD ratio is between 0.3 and 0.6, then seeding is required to treat it biologically. If BOD/COD ratio is < 0.3, then it cannot be treated biologically (Srinivas, 2008).

Considering the versatility of the AC consortium in growing on diverse xenobiotics and utilizing some like MTBE, DCE, TBA and CE appreciably, it was thought relevant to evaluate the potential of AC consortium in especially treating effluents having biodegradability index in the range of 0.3 – 0.6, discharged from different industries. Therefore, this chapter deals with the utilization of AC consortium for treatment of different industrial effluents at a bench scale reactor level.

4.2. Materials and Methods

4.2.1. Wastewater composition

The industrial effluents selected for the biotreatment studies included: DNR effluent obtained from the denitrifying reactor of a fertilizer company, Bharuch, Gujarat, India; ECO effluent obtained from common effluent treatment plant (CETP), Ankleshwar industrial estate, Gujarat, India; COR effluent obtained from a pesticide company, Ankleshwar, Gujarat, India, and UPL effluent obtained from the final effluent treatment plant of a chemical company, Ankleshwar, Gujarat, India. The biodegradability indices of all the 4 effluents were in the range of 0.3 – 0.6.

4.2.2. Treatability studies

These studies were carried out with the AC consortium at flask level. A consortium of the selected AC isolates was prepared by inoculating a loopful of each isolate together into 5 ml PNB medium and incubating at 37 ^{o}C for 12 h at 180 rpm. 2 % inoculum was inoculated into 100 ml freshly procured industrial effluent and incubated at 37 ^{o}C for 24 h with constant stirring at 100 rpm. 1 ml of the effluent was collected at intervals of 1 h and centrifuged at 10,000 rpm for 5 min. The supernatant was collected and used as a sample for COD estimation.

For confirming that the inoculated AC consortium was responsible for the treatment of the 4 selected effluents, 2 types of experiments were carried out. In the first experiment, the treatability parameters of the effluent inoculated with AC consortium were compared with that of the control (uninoculated effluent). In the second experiment, morphological characters and antibiotic resistance patterns of the individual isolates of AC consortium were compared with the predominant isolates obtained after treatability studies of the respective effluent. The antibiotic sensitivity pattern of the isolates of AC consortium was checked by screening on different antibiotic discs to identify their antibiotic resistance for the preparation of selective Mueller Hinton agar plates (MacFaddin, 1980). Himedia octodiscs, viz. G-I-plus, G-III-plus, G-VII-plus and G-VIII-plus for Gram positive bacteria; G-III-minus, G-VII-minus, G-VIII-minus and G-XII-minus for Gram negative bacteria, and Combi 1, 61 and 510 for both Gram positive and Gram negative bacteria, were used. Aliquots obtained from the treatability studies flasks inoculated with the AC consortium were drawn at 0 h and 72 h, serially diluted and spread on antibiotic selective agar plates

containing the respective antibiotics against which the individual isolates of AC consortium showed resistance. The plates were incubated at 37 ^{o}C for 24 h and growth of the isolates was measured in terms of cfu/ml.

4.2.2.1. COD estimation by dichromate reflux method (Tomar, 1999)

The 500 ml refluxing flasks were cleaned with chromic acid. A few cleaned and dried glass beads were kept in each refluxing flask. 0.4 g $HgSO_4$ was added to each flask. A 20 ml sample or an aliquot diluted to 20 ml with double distilled water (DDW) was added in each flask and swirled. A flask was prepared as blank with 20 ml DDW. Exactly 10 ml of 0.25 N $K_2Cr_2O_7$ was added to each flask and swirled. 30 ml of concentrated H_2SO_4 was carefully added to each flask. The reflux mixture was thoroughly mixed before heating. All the flasks were placed on a hot plate. The condensers were connected and cold water was circulated through them. A smooth flow of water was maintained. The hot plate was turned on to the highest temperature setting. When the solution started boiling, the timer was set for exactly 2 h. The hot plate was turned off and the samples were allowed to cool down to room temperature, keeping all the connections intact. The condensers were washed with 40 ml DDW and the flasks were removed from the condensers. The refluxing solutions were diluted with DDW to make a final volume of 140 ml. The solution was again cooled to room temperature.

0.5 ml of ferroin indicator was added in each flask containing cold refluxing sample/blank. It was titrated against 0.10 N $Fe(NH_4)_2(SO_4)_2.6H_2O$. The endpoint was indicated by a color change from blue-green to reddish-brown.

COD of the effluent was calculated using the following formula:

$$\text{mg COD/l} = \frac{(A-B) \times N \times 8000}{\text{Volume of sample, ml}}$$

Where,

A = Volume of $Fe(NH_4)_2(SO_4)_2.6H_2O$ used for blank, ml

B = Volume of $Fe(NH_4)_2(SO_4)_2.6H_2O$ used for sample, ml

N = Normality of $Fe(NH_4)_2(SO_4)_2.6H_2O$

4.2.3. Batch reactor studies

The reactor used in this study was a 6 l batch reactor made of 4 mm thick glass (Figure 4.1). It consisted of an aeration tank (30 cm x 25 cm x 20 cm) and a settling

tank (7 cm x 25 cm x 20 cm) at an angle of 30 ^{o} from the aeration tank. The working volume of the reactor was 6 l and the headspace volume was 5 l. The reactor was run in a well ventilated room at ambient temperature. A consortium of the selected AC isolates was prepared by inoculating a loopful of each isolate together into 120 ml PNB and incubating at 37 ^{o}C for 12 h at 180 rpm. 2 % inoculum was inoculated into 6 l freshly procured industrial effluent in the reactor and incubated at 37 ^{o}C for 96 h with constant aeration and stirring at 300 rpm. 1 ml of the effluent was collected at intervals of 6 h and centrifuged at 10,000 rpm for 5 min. The supernatant was collected and used as a sample for COD estimation (section 4.2.2.1). An uninoculated reactor was also run for each effluent as a control reactor to assess the oxidation of the effluent carbon by the indigenous microflora of the respective effluents.

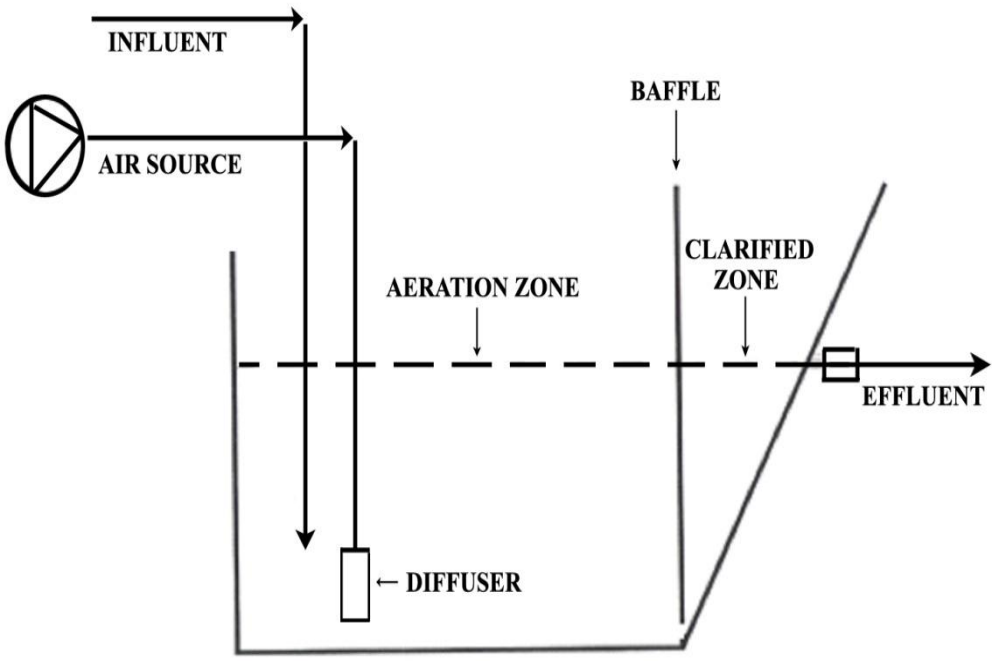


Figure 4.1. Schematic diagram of a suspended reactor constituting of an aeration zone, a clarified zone and an aeration source

4.2.3.1. Estimation of different environmental parameters

Environmental parameters like BOD, pH, OD_{600}, mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), sludge volume index (SVI) and food-to-microorganisms (F/M) ratio of the industrial effluents were also estimated.

4.2.3.1.1. BOD estimation by dissolved oxygen dilution method (Tomar, 1999)

Samples of wastewater were prepared by placing various incremental portions of the industrial effluent in BOD bottles with aerated distilled water. The aerated distilled water contained phosphate buffer, $MgSO_4$, $CaCl_2$ and $FeCl_3$. A bottle filled with only aerated distilled water was used as a blank. The bottles were completely filled, sealed without entrapment of any air bubbles and incubated for 5 d at 20 ^oC in a BOD incubator. The dissolved oxygen (DO) level was measured by the azide modification iodometric method. The relation between the oxygen consumed and the volume of sample taken was used to calculate BOD.

To the sample collected in a 385 ml BOD bottle, 2.57 ml $MnSO_4$ solution was added, followed by 2.57 ml alkaline iodide azide reagent, well below the surface of the liquid. The bottles were stoppered with care to exclude air bubbles and mixed by inverting several times. When the precipitates settled down, 2.57 ml concentrated H_2SO_4 solution was added in a clear supernatant by allowing the acid to run down. The contents were mixed by gentle inversion until the precipitates were completely dissolved. 100 ml of the sample was titrated against N/80 $Na_2S_2O_3$ solution till it gave yellow color. 1 % starch was added which gave blue color to the solution and titrated again until the blue color disappeared.

BOD of the effluent was calculated using the following formula:

mg BOD/l = (A - B) x dilution factor

Where,

A = Volume of $Na_2S_2O_3$ used for blank, ml

B = Volume of $Na_2S_2O_3$ used for sample, ml

4.2.3.1.2. MLSS estimation (Maiti, 2004)

A glass fiber filter disk, rough side up, was placed in a filter container and placed in the filtration apparatus. The filter was wetted with a small volume of distilled water. 100 ml portion of the well-mixed sample was filtered. The measured volume was filtered by applying suction for about 3 min after the filtration was complete. The filter paper was removed from the filtration apparatus and transferred to an aluminium-weighing disk as support. It was dried for at least 1 h in the range of 103 to 105 ^oC in hot air oven, cooled in desiccator to balance temperature and weighed. The cycle of drying, cooling, desiccating and weighing was repeated until a constant weight was obtained.

MLSS of the effluent was calculated using the following formula:

$$\text{MLSS, mg/l} = \frac{(A - B) \times 1000}{\text{Sample volume, ml}}$$

Where,

A = Weight of filter paper + dried residue, mg

B = Weight of filter paper, mg

4.2.3.1.3. MLVSS estimation (Maiti, 2004)

The filter paper containing MLSS residues was put in a muffle furnace at 550 ^{o}C. The furnace temperature was raised before inserting the sample. 15 to 20 min ignition was required for 200 mg residues. The dish was allowed to cool partially in air until most of the heat had been dissipated. It was transferred to desiccator for final cooling in dry atmosphere and weighed as soon as it had cooled to room temperature. The cycle of igniting, cooling, desiccating and weighing was repeated until constant weight was obtained or until weight change was less than 4 % or 0.5 mg, whichever was less.

MLVSS of the effluent was calculated using the following formula:

$$\text{MLVSS, mg/l} = \frac{(A - B) \times 1000}{\text{Sample volume, ml}}$$

Where,

A = weight of residue + dish before ignition, mg

B = weight of residue + dish after ignition, mg

4.2.3.1.4. SVI estimation (Maiti, 2004)

MLSS concentration of well-mixed sample of suspension was determined. The suspension was poured in 1 l graduated measuring cylinder. Settling was allowed for 30 min. The volume occupied by suspension was noted in ml.

SVI of the effluent was calculated using the following formula:

$$\text{SVI, ml/g} = \frac{\text{Settled sludge volume (ml/l)}}{\text{MLSS (mg/l)}} \times 1000$$

4.2.3.1.5. F/M Ratio calculation (Maiti, 2004)

BOD_5 concentration was measured by standard method. MLVSS in the aeration tank and HRT of the system were calculated. F/M ratio was calculated by using the following formula and expressed in per day (i.e. 1/d).

$$\text{F/M ratio} = \frac{S_o \cdot Q}{V \cdot X} = \frac{S_o}{\theta \cdot X}$$

Where,

S_o = Influent BOD_5 or COD concentration, mg/l

X = MLVSS, mg/l

V = Volume of aeration tank, m^3

Q = Influent wastewater flow rate, m^3/d

$\theta = (V/Q)$ = Hydraulic retention time (HRT), d

F/M = Food-to-microorganisms ratio, d^{-1}

4.2.4. Continuous reactor studies

The process was further scaled up to a 6 l continuous reactor consisting of an aeration tank, an influent tank, an effluent tank and an aeration source (Figure 4.1). The HRT was kept 60 h for DNR effluent, 96 h for COR effluent and 72 h for ECO and UPL effluents. 1 ml of the effluent was collected at intervals of 24 h and centrifuged at 10,000 rpm for 5 min. The supernatant was collected and used as a sample for COD estimation (section 4.2.2.1). Other environmental parameters like BOD, pH, OD_{600}, MLSS, MLVSS, SVI and F/M ratio of the industrial effluents were also estimated (section 4.2.3.1).

4.2.5. Pilot-scale reactor studies

The process was further scaled up to a pilot scale 2000 l continuous reactor consisting of an aeration tank, an influent tank, an effluent tank and a surface agitator. This study was carried out with the DNR effluent. The HRT was kept 62 h for the DNR effluent. Sample of the effluent was collected at intervals of 24 h and used for COD estimation (section 4.2.2.1). Other environmental parameters like MLSS and MLVSS of the DNR effluent were also estimated (section 4.2.3.1).

4.2.6. Statistical analysis

One-way ANOVA was applied to evaluate the biotreatment efficiency of the AC consortium for the 4 selected industrial effluents at flask, batch and continuous reactor levels. Student's t-Test was applied to evaluate the COD reduction ability of the AC consortium in comparison with that of the indigenous microflora of the

respective effluents. It was assumed that the original data followed a normal distribution. All statistical analyses were performed using Origin 6.0 software (OriginLab Corporation, Northampton, MA, USA) (Barbera *et al*., 2011).

4.3. Results and Discussion

According to the Central Pollution Control Board, Ministry of Environment and Forest, Government of India, New Delhi, India, the environmental standards for discharge of effluents from fertilizer industries include: Suspended Solids (SS) - 100 mg/1, pH - 6.5-8.0, BOD - 30 mg/l, COD - 250 mg/l; the environmental standards for discharge of effluents from pesticide industries include: SS - 100 mg/l, pH - 6.5-8.5, BOD - 30 mg/l, COD - 100 mg/l, and the environmental standards for discharge of effluents from CETPs include: SS – 100 mg/l, pH – 5.5-9.0, BOD – 30 mg/l, COD – 250 mg/l (EPA Notification [S. O. 844(E) November 19, 1986]). Since in the case of DNR, ECO, COR and UPL effluents, difficulty was experienced in complying to the environmental standards for their discharge, as they were treated in CETPs or plants run on indigenous microflora, these effluents were selected for their efficient biotreatment by the AC consortium.

The COD and BOD values of the freshly procured industrial effluents were estimated to calculate their biodegradability indices (Table 4.1). Accordingly, the BOD/COD ratios of the 4 effluents were found to be in the range of 0.3 – 0.43, implying that a special microbial seed needs to be developed for their biodegradation, since if the BOD/COD ratio of an industrial effluent is between 0.3 – 0.6, then a special microbial seed is required for its biotreatment (Srinivas, 2008). Therefore, the versatility of AC consortium to act as a special microbial seed for biotreatment of these 4 effluents was studied.

Table 4.1. Biodegradability indices of the 4 industrial effluents used in the reactor studies

Parameter	Reactor											
	Flask				Batch				Continuous			
	DNR	ECO	COR	UPL	DNR	ECO	COR	UPL	DNR	ECO	COR	UPL
COD (mg/l)	770	1000	1600	1600	1600	950	1850	1900	1300	1100	2000	1800
BOD (mg/l)	331	370	656	624	488	284	741	744	538	432	616	588
BOD/COD ratio	0.43	0.37	0.41	0.39	0.3	0.3	0.4	0.39	0.41	0.39	0.31	0.33

4.3.1. Treatability studies

In the flask level treatability studies, the AC consortium was used for COD reduction of the DNR, ECO, COR and UPL effluents. It reduced the initial COD of DNR effluent from 770 mg/l to below detection limit in 8 h, that of ECO effluent from 1000 mg/l to below detection limit in 9 h, that of COR effluent from 1600 mg/l to below detection limit in 12 h and that of UPL effluent from 1600 mg/l to below detection limit in 8 h (Figure 4.2) with the COD removal rates of 96.25, 111.11, 133.33 and 200 mg/l/h for DNR, ECO, COR and UPL effluents respectively. As compared to the AC consortium, the indigenous microflora of the DNR effluent (not inoculated with the AC consortium) reduced its COD to 400 mg/l, that of ECO effluent to 500 mg/l, that of COR effluent to 800 mg/l and that of UPL effluent to 700 mg/l in 12 h (Figure 4.2). The AC consortium showed a statistically significant COD reduction (Student's t-Test) of DNR effluent at a significance level of 0.05, with a p-value of 0.005; of ECO and COR effluents with a p-value of 0.003, and of UPL effluent with a p-value of 0.002 as compared to the indigenous microflora of the respective effluents. In case of all the effluents, the indigenous microflora was unable to treat the effluents effectively as it could be seen that even though it reduced the effluent COD, the permissible limit was never attained. The AC consortium was found to be more suitable for DNR, ECO and UPL effluents, as it reduced their initial COD to below permissible limit within 9 h.

The DNR effluent contained discharges from a fertilizer company. Broadly nitrate fertilizers are manufactured in this company and, therefore, it contains high ammonia and nitrates. The ECO effluent is from a CETP which receives the industrial effluent from Ankleshwar, Jhagadia and Panoli industrial estates, where diverse types of chemical industries are situated. The UPL effluent is likely to contain diverse pollutants as its company manufactures dyes, pigments, chemicals, textiles and pharmaceuticals. The COR effluent is likely to possess pesticide remnants as the industry manufactures pesticides, fertilizers, insecticides, fungicides and insecticides. The COD of COR effluent was brought down to below permissible limit by the AC consortium in 12 h. In all probability, the COR effluent is likely to contain recalcitrant or difficult to degrade pollutants, hence, it took longer time to reach the permissible limit. The fact that the COR effluent was treatable by AC consortium suggests that it has a wider potential in treating effluents containing not easily biodegradable carbon. The results of the treatability studies indicate that the AC consortium was suitable to biodegrade the carbon substrates and other oxidizable matter from all the selected effluents of diverse nature.

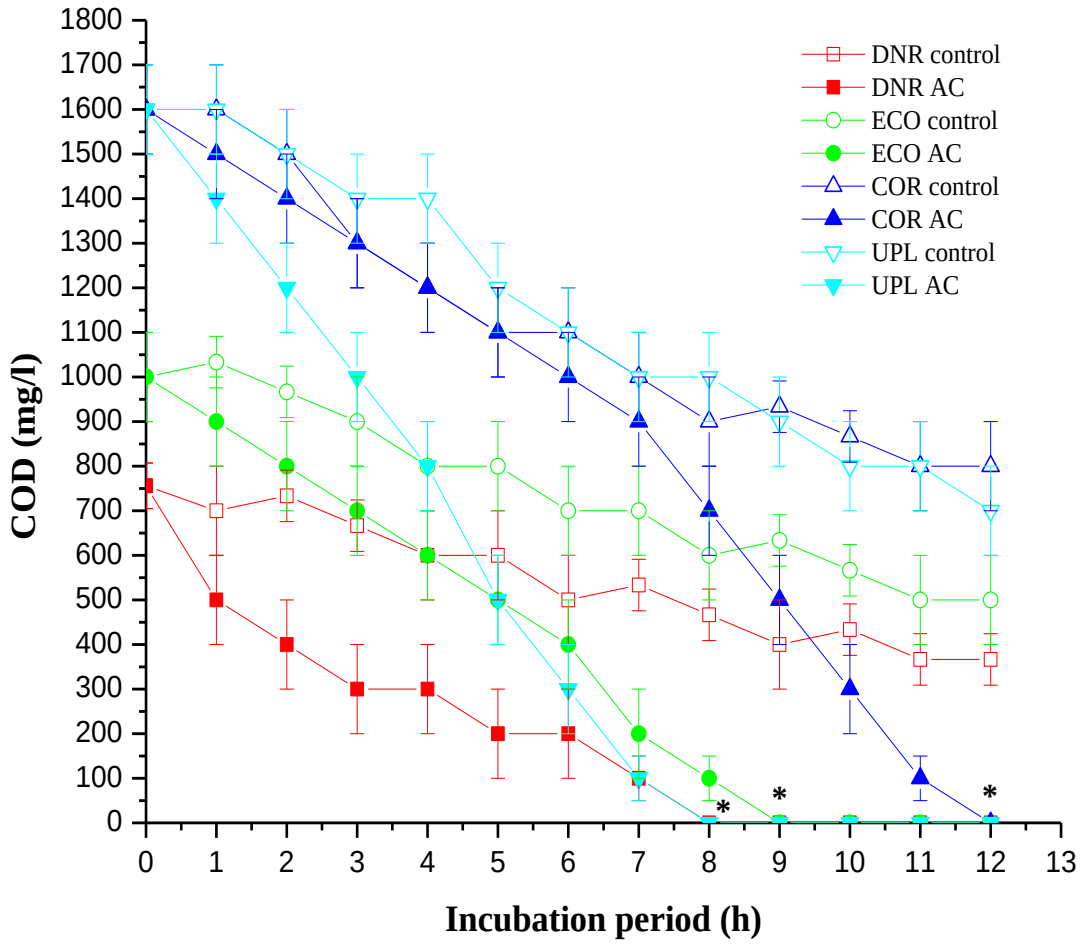


Figure 4.2. Treatability studies for COD reduction of DNR, ECO, COR and UPL effluents by the AC consortium at flask level (asterisk indicates that the COD reduction is statistically significant at a significance level of 0.05). Open symbols represent the COD of DNR, ECO, COR and UPL effluents uninoculated with the AC consortium (control reactor). Error bars represent standard deviation from the mean, n = 3.

Antibiotic sensitivity of the isolates of AC consortium was checked in order to analyze their antibiotic resistance. It was observed that *Bordetella petrii* AC1 showed resistance to co-trimoxazole, amoxycillin and tetracycline; *Bacillus licheniformis* AC4 to ampicillin; *Salmonella subterranea* AC5 to bacitracin, and *Pseudomonas stutzeri* AC8 to polymyxin and cephalosporin (Table 4.2(a)). From this, a specific antibiotic resistance pattern was obtained for the 4 isolates of AC consortium, which was used to check the presence of AC consortium in the inoculated DNR effluent after 72h of incubation. The identical profile of antibiotic resistance and morphological characteristics of the predominant isolates obtained after 72 h from the DNR effluent indicated that the isolates of AC consortium could survive and grow well in the effluent (Table 4.2(a)). Based on this exclusive antibiotic resistance, specific antibiotic selective agar plates were prepared using amoxycillin (10 µg/ml), ampicillin (25 µg/ml), bacitracin (10 µg/ml) and cephalosporin (30 µg/ml) for *B. petrii* AC1, *B. licheniformis* AC4, *S. subterranea* AC5 and *P. stutzeri* AC8 respectively. At 0 h, the colony counts of *B. petrii* AC1, *B. licheniformis* AC4, *S. subterranea* AC5 and *P. stutzeri* AC8 were obtained (Table 4.2(b)). It was noted that after 72 h, in addition to the colonies of the inoculated isolates of AC consortium, as observed from their distinct colony morphologies, colonies of the indigenous microflora of the DNR effluent were also observed at lower dilutions; however, only the colonies of the inoculated isolates of the AC consortium were obtained at higher dilutions. As described earlier in chapter 2 (Figure 2.4), the individual isolates of AC consortium demonstrated similar growth profiles in LB medium, therefore, it was envisaged that they should grow together in the DNR effluent as well. Further, it was observed that the isolates grew together and were maintained at the end of the treatability studies in the same ratio in which they were inoculated, as is evident from the colony counts of isolate AC1* (*B. petrii* AC1), isolate AC4* (*B. licheniformis* AC4), isolate AC5* (*S. subterranea* AC5) and isolate AC8* (*P. stutzeri* AC8) (Table 4.2(b)). Thus, it was established that the isolates were maintained as a consortium as they did not eliminate each other and were present in equal proportions. Moreover, it can be concluded that they remained numerically dominant throughout the studies as compared to the indigenous microflora of the DNR effluent, leading to its efficient COD reduction.

Table 4.2(a). Comparison of antibiotic sensitivity of the isolates of AC consortium and the predominant isolates obtained in the treatability studies of the DNR effluent

Antibiotic	***B. petrii* AC1**	**Isolate AC1***	***B. licheniformis* AC4**	**Isolate AC4***	***S. subterranea* AC5**	**Isolate AC5***	***P. stutzeri* AC8**	**Isolate AC8***
Co-Trimoxazole	R	R	S	S	S	S	S	S
Amoxycillin	R	R	S	S	S	S	S	S
Tetracycline	R	R	S	S	S	S	S	S
Ampicillin	S	S	R	R	S	S	S	S
Bacitracin	S	S	S	S	R	R	S	S
Polymyxin	S	S	S	S	S	S	R	R
Cephalosporin	S	S	S	S	S	S	R	R

S - Sensitive, R – Resistant * - Predominant isolates obtained at 72 h

Table 4.2(b). Growth of the isolates of AC consortium and the predominant isolates obtained in the treatability studies of the DNR effluent on selective agar plates

Isolate	**Selective agar plate**	**Growth (cfu/ml)**
B. petrii AC1	Amoxycillin	6.06×10^7
Isolate AC1*		5.84×10^6
B. licheniformis AC4	Ampicillin	2.25×10^6
Isolate AC4*		1.68×10^5
S. subterranea AC5	Bacitracin	2.34×10^7
Isolate AC5*		1.76×10^6
P. stutzeri AC8	Cephalosporin	8.01×10^6
Isolate AC8*		7.65×10^5

* - Predominant isolates obtained at 72 h

Microbial consortia have been more successful as compared to individual microorganisms in wastewater treatment as reported in literature. Durai *et al*. (2010) showed aerobic digestion of tannery industry wastewater using mixed microbial consortium in terms of 74 % COD reduction. Tannery saline wastewater obtained from a CETP near Chennai, India, was treated with salt tolerant mixed bacterial consortia showing appreciable biodegradation with 80 % COD reduction in particular at 8 % salinity level (Sivaprakasam *et al*., 2008). Bioremediation of olive oil mill wastewater (OMWW) was conducted using bacterial mixture of the phenol degrading bacteria, *Azotobacter vinelandii*, *P*. *fluorescens* and *P*. *putida* (Omer, 2012). Methylotrophs have also been previously used successfully in wastewater treatment. Coats *et al*. (2007) used 2 l flask reactors operated under anaerobic/aerobic and aerobic-only modes for synthesizing PHA by a mixed microbial consortium, while reducing soluble COD by approximately 62 to 71 %. As compared to these reports, the AC consortium showed 100 % COD reduction of the 4 effluents selected. As the nature of all the 4 effluents selected for the study was diverse containing a variety of pollutants, the treatability studies establish the versatility of the AC consortium and specifically its methylotrophic members.

4.3.2. Reactor studies

Significant environmental parameters of wastewater treatment were studied in batch and continuous reactor studies. Apart from COD; BOD, pH, MLSS, MLVSS, SVI and F/M ratio are also important in wastewater biotreatment. BOD determines the amount of organic carbon in an effluent that is biodegradable. The minimum BOD value for the discharge of effluents is 30 mg/l. pH of the environment is also a key factor in the growth of microorganisms. Generally, the optimum pH for bacterial growth is in the range of 6.5 – 7.5. For carbon removal, pH in the range of 6.0 – 9.0 is tolerable, while optimal performance occurs near a neutral pH. The mixture of solids resulting from combining recycled sludge with influent wastewater in the bioreactor is termed MLSS and MLVSS. The MLSS and MLVSS values of the discharge effluent should be below 100 mg/l. The SVI is an index of the settleability of the sludge in wastewater treatment. A desired SVI value of 100 ml/g is considered a good settling sludge. The F/M ratio is a process parameter which describes the degree of starvation

of microorganisms. Typical value for the F/M ratio of the discharge effluent is 0.04/d (Tchobanoglous *et al*., 2003).

The positive results of the treatability studies indicated that the 4 effluents were amenable to biotreatment and that too by a special microbial seed, as the indigenous microflora of the 4 effluents was unable to treat them efficiently (Figure 4.2). After the confirmation of treatability in flask level studies, bench scale batch reactors were run with proper monitoring of the environmental parameters. Here, an attempt was made to achieve desirable values of the other environmental parameters along with COD removal by the AC consortium.

4.3.2.1. Batch reactor studies

The flask level process was scaled up to a 6 l batch reactor level (Figure 4.3) with the 4 selected industrial effluents. The biological treatment process used for the reactor studies was a complete mix activated sludge process. The process was hastened in the batch reactor as compared to the flask level treatability studies. The AC consortium was able to reduce the initial COD of DNR effluent from 1600 mg/l to below detection limit in 60 h, that of ECO effluent from 950 mg/l to below detection limit in 72 h, that of COR effluent from 1850 mg/l to below detection limit in 96 h and that of UPL effluent from 1900 mg/l to below detection limit in 72 h (Figure 4.4) with the COD removal rates of 640, 316.67, 462.5 and 633.33 mg/l/d for DNR, ECO, COR and UPL effluents respectively. As compared to the AC consortium, the indigenous microflora of the DNR effluent reduced its COD to 700 mg/l, that of ECO effluent to 300 mg/l, that of COR effluent to 800 mg/l and that of UPL effluent to 900 mg/l in 96 h (Figure 4.4). The AC consortium showed a statistically significant COD reduction (Student's t-Test) of DNR, ECO and UPL effluents at a significance level of 0.05, with a p-value of 0.002, and of COR effluent with a p-value of 0.001 as compared to the indigenous microflora of the respective effluents. The statistical significance (p-value $\leq$ 0.002) of the COD reduction by AC consortium in the batch reactor was higher in comparison to that in the flask level treatability studies (p-value $\leq$ 0.005), indicating a stronger effect of AC consortium in effluent treatment at a larger scale. Even though COD reduction was observed in the control reactors (reactors not inoculated with the AC consortium), it took longer time and the reduction was incomplete as the COD at the end of the reactor run was well above the permissible limits. This clearly demonstrates the potential of the AC consortium as a special microbial seed for treatment of these effluents.

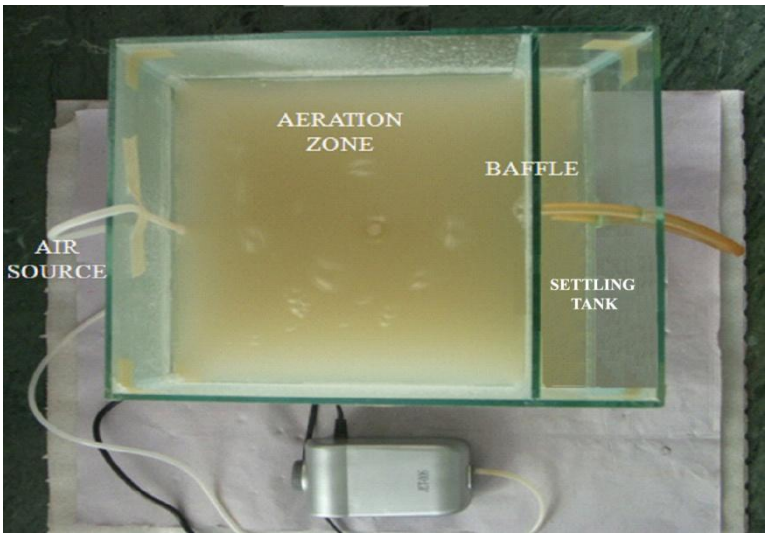


Figure 4.3. A 6 l bench scale reactor consisting an aeration tank, a setting tank, a baffle and an aeration source

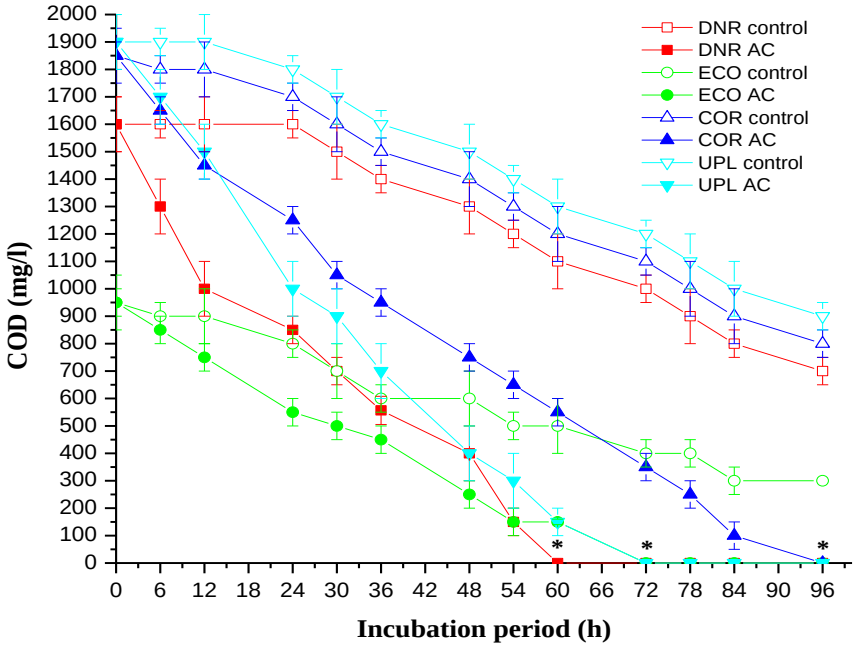


Figure 4.4. COD reduction of DNR, ECO, COR and UPL effluents by the AC consortium in batch reactor (asterisk indicates that the COD reduction is statistically significant at a significance level of 0.05). Open symbols represent the COD of DNR, ECO, COR and UPL effluents uninoculated with the AC consortium (control reactor). Error bars represent standard deviation from the mean, n = 3.

Environmentally important parameters like BOD, pH, OD_{600}, MLSS, MLVSS, SVI and F/M ratio of all the 4 effluents were also estimated for their efficient biotreatment (Table 4.3). BOD of the effluents was reduced in the range of 20 – 27 mg/l, pH in the range of 7.0 – 7.6, MLSS and MLVSS in the range of 103 – 132 mg/l, SVI in the range of 198-208 ml/g and F/M ratio in the range of 0.03 – 0.05. BOD of the desired value was obtained at the end of each reactor, implying that biodegradable organic carbon was almost negligible at the end of the reactor study. pH of the discharge effluent was in the neutral range, implying that the environmental conditions were favorable for bacterial growth. MLVSS indicated that adequate active biomass was developed in the 4 reactors. SVI is a standard measure of settleability and compactness of the activated sludge solids. In case of AC consortium, the SVI values were nearing 200 ml/g in all the 4 effluents, which is above the desirable range of 80 – 150 ml/g (Tchobanoglous *et al*., 2003). The reason for the higher SVI values was that the reactor was run in a batch mode and, hence, the sludge generated during the entire reactor run was accumulated in the reactor itself. The F/M ratio of 0.03 – 0.05 obtained here implied that the food required for the microorganisms was almost exhausted at the end of the reactor runs.

The degradation of captan (fungicide) containing wastewater was evaluated by Ghaly and Dave (2012) using soil microbes under batch mode of operation. 100 % captan degradation was achieved after 5 d in the batch bioreactor. *Pseudomonas*, designated as IES-*Ps*-1, was used to assess its potential for pesticide removal from industrial wastewater using the activated sludge (Jilani and Khan, 2013). Under aerobic culture conditions using mechanical aerators, almost complete removal of cypermethrin at 20 mg/l in terms of 97 % COD reduction occurred in 48 h. Baytshtok *et al*. (2008) have reported that the methylotrophic bacteria in activated sludge metabolized methanol in a sequencing batch denitrifying reactor (SBDR). Methylotrophic denitrifying communities in activated sludge showed combined nitrogen and phosphorus removal in a sequencing batch reactor (Hallin *et al*., 2006). Similarly, the AC consortium showed efficient biotreatment of 4 different kinds of industrial effluents at batch scale reactor level, implying its broad biotreatment potential.

Table 4.3. Environmental parameters of DNR, ECO, COR and UPL effluents in batch reactor

Parameter	Time Interval (h)							
	DNR effluent		ECO effluent		COR effluent		UPL effluent	
	0	60	0	72	0	96	0	72
BOD (mg/l)	488.71 (14.65)	27.48 (1.54)	284.71 (14.65)	25.48 (1.54)	741.71 (14.36)	20.48 (1.47)	744.71 (11.65)	27.48 (1.46)
pH	7.68 (0.23)	6.98 (0.13)	7.78 (0.42)	7.09 (0.32)	8.78 (0.22)	7.59 (0.47)	6.78 (0.32)	7.03 (0.23)
OD_{600}	0.00 (0.00)	2.48 (0.35)	0.00 (0.00)	2.04 (0.35)	0.00 (0.00)	2.54 (0.31)	0.00 (0.00)	2.84 (0.53)
MLSS (mg/l)	205.33 (5.22)	132.33 (12.24)	195.33 (3.22)	132.53 (2.24)	190.33 (3.28)	122.33 (2.25)	185.33 (3.36)	122.33 (2.29)
MLVSS (mg/l)	187.0 (11.70)	123.67 (8.43)	178.0 (7.70)	113.67 (6.98)	187.03 (5.70)	114.67 (6.08)	168.0 (7.17)	103.67 (6.38)
SVI (ml/g)	87.39 (2.65)	207.74 (8.23)	96.39 (0.65)	207.54 (8.13)	86.39 (0.54)	197.74 (8.03)	93.39 (0.45)	203.71 (8.01)
F/M ratio	1.09 (0.03)	0.04 (0.01)	1.03 (0.02)	0.05 (0.04)	1.05 (0.03)	0.03 (0.02)	1.04 (0.02)	0.04 (0.03)

Each value indicates average and standard deviation (in parentheses) of 3 independent experiments. For each effluent, the first column describes the parameters at the start of the reactor and the second column describes the parameters at the end of the reactor.

4.3.2.2. Continuous reactor studies

The process was further scaled up to a 6 l continuous reactor level (Figure 4.5) with the 4 selected industrial effluents. The HRTs for the 4 effluents were decided on the basis of the time taken by the AC consortium to reduce the COD of the respective effluents to below detection limit in the batch reactor. The AC consortium reduced the initial COD of DNR effluent from 1300 mg/l to below detection limit in 15 d, that of ECO effluent from 1100 mg/l to below detection limit in 9 d, that of COR effluent from 2000 mg/l to below detection limit in 10 d and that of UPL effluent from 1800 mg/l to below detection limit in 10 d (Figure 4.6) with the COD removal rates of 86.67, 122.22, 200 and 180 mg/l/d for DNR, ECO, COR and UPL effluents respectively. As compared to the AC consortium, the indigenous microflora of the DNR effluent in the control reactor reduced its COD to 500 mg/l, that of ECO effluent to 300 mg/l, that of COR effluent to 700 mg/l and that of UPL effluent to 600 mg/l in 15 d (Figure 4.6).

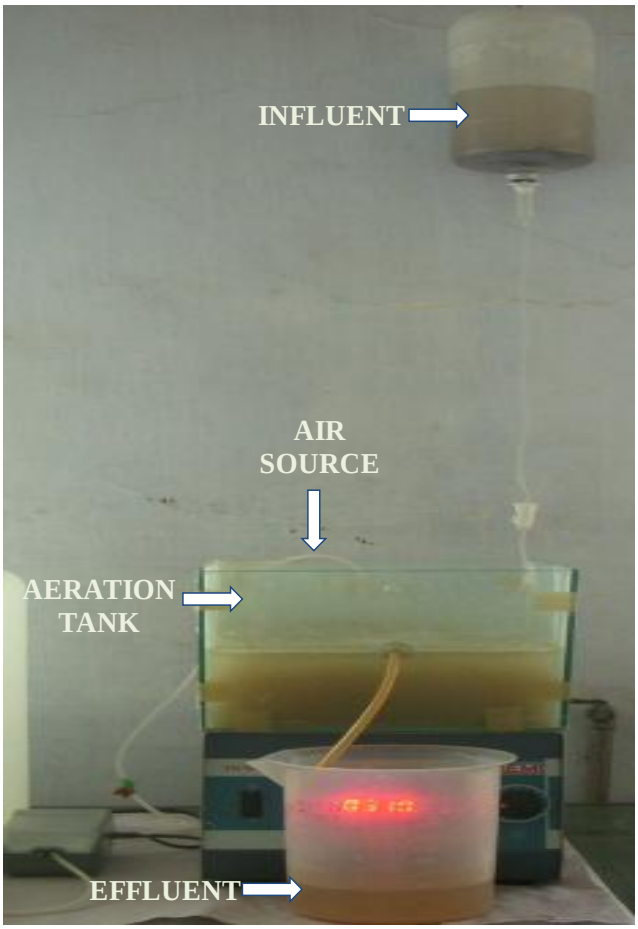


Figure 4.5. A 6 l bench scale continuous reactor consisting of an aeration tank, an influent tank, an effluent tank and an aeration source

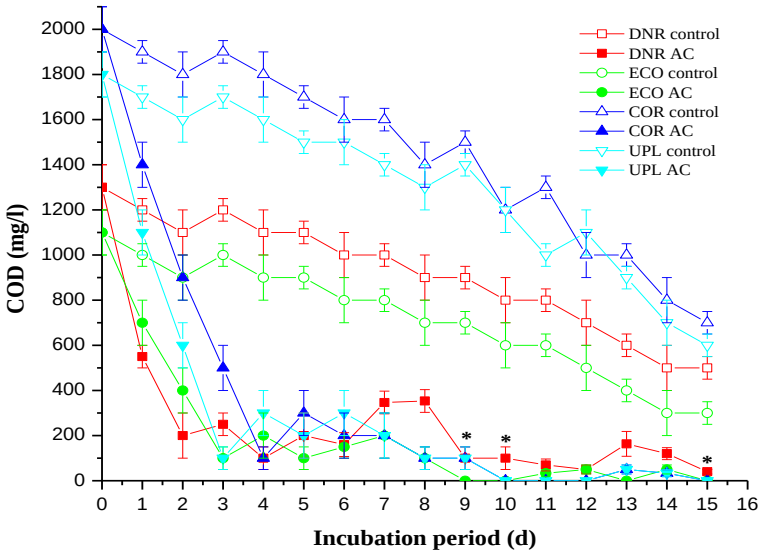


Figure 4.6. COD reduction of DNR, ECO, COR and UPL effluents by the AC consortium in continuous reactor (asterisk indicates that the COD reduction is statistically significant at a significance level of 0.05). Open symbols represent the COD of DNR, ECO, COR and UPL effluents uninoculated with the AC consortium (control reactor). Error bars represent standard deviation from the mean, n = 3.

The AC consortium showed a statistically significant COD reduction (Student's t-Test) of DNR effluent at a significance level of 0.05, with a p-value of 0.003; of ECO effluent with a p-value of 0.007, and of COR and UPL effluents with a p-value of 0.002 as compared to the indeginous microflora of the respective effluents. COD reduction in the AC consortium seeded reactors was found to reach the permissible limit of 100 mg/l within the first 3 – 4 d and was maintained thereafter to the attained value (Figure 4.6). However, the control reactors were found to run very inefficiently with the indigenous microflora as their COD was reduced maximally to 300 mg/l even after 15 d, which was well above the permissible limit. Thus, the AC consortium, and not the indigenous microflora, was responsible for efficiently reducing the COD of DNR, ECO, COR and UPL effluents to below detection limit in minimum time period, as confirmed by the Student's t-Test.

Environmentally important parameters like BOD, pH, OD_{600}, MLSS, MLVSS, SVI and F/M ratio of DNR, ECO, COR and UPL effluents were also estimated for their efficient biotreatment (Tables 4.4 - 4.7). In case of DNR effluent, the initial BOD of 539 mg/l was reduced by the AC consortium to 27 mg/l, implying that the

biodegradable organic carbon was almost exhausted at the end of the reactor (Table 4.4). The pH was maintained at neutral throughout the reactor study enabling the optimum performance of AC consortium. The MLSS and MLVSS values were reduced to 129 and 98 mg/l respectively representing the active biomass present in the reactor at the end of the study. The SVI was reduced from 93 to 36 ml/g, implying good settleability of the activated sludge. An initial F/M ratio of 1.04 was reduced to 0.03 implying that almost all the food required for the microorganisms was exhausted.

In case of ECO effluent, the initial BOD of 433 mg/l was reduced by the AC consortium to 23 mg/l, implying efficient biodegradation of the organic carbon present in the effluent (Table 4.5). The pH was maintained at neutral throughout the reactor study. The MLSS and MLVSS values were reduced to 131 and 103 mg/l respectively, showing the desirable values for effluent discharge. The SVI was reduced from 83 to 38 ml/g, suggesting that the activated sludge had good settling property. The F/M ratio was reduced from 1.07 to 0.04, implying that almost negligible carbon was present in the effluent to be used by the microorganisms.

In case of COR effluent, its initial BOD of 617 mg/l was reduced by the AC consortium to 24 mg/l, implying that efficient BOD removal was obtained at the end of the reactor study (Table 4.6). The pH was maintained at neutral throughout the reactor study. The MLSS and MLVSS values were reduced to 140 and 103 mg/l respectively, showing the active biomass in the reactor at the end of the study. The SVI was reduced from 93 to 43 ml/g, representing the good settling sludge. The initial F/M ratio of 1.03 was reduced to 0.03, implying efficient carbon removal at the end of the reactor study.

In case of UPL effluent, its initial BOD of 588 mg/l was reduced by the AC consortium to 25 mg/l, implying 96 % BOD removal at the end of the reactor (Table 4.7). The pH was maintained at neutral throughout the reactor study. The MLSS and MLVSS values were reduced to 141 and 93 mg/l, implying that they approximately met the minimum permissible standards. The SVI was reduced from 88 to 37 ml/g, implying that there was no sludge bulking at the end of the reactor study. The initial F/M ratio of 1.06 was reduced to 0.04, implying that the organic matter in the reactor was almost nil as compared to the active biomass at the end of the experiment. Thus, the results obtained indicated that the important operating parameters of the 4 selected effluents were reduced to below permissible limit by the AC consortium.

Table 4.4. Environmental parameters of DNR effluent in continuous reactor

Parameter	Time Interval (h)						
	0	**60**	**120**	**180**	**240**	**300**	**360**
BOD (mg/l)	538.67 (28.34)	27.29 (2.43)	26.67 (2.34)	27.29 (1.24)	26.67 (2.98)	27.29 (1.09)	26.67 (2.87)
pH	7.34 (0.64)	6.61 (0.37)	7.37 (0.36)	7.34 (0.59)	6.60 (0.49)	6.64 (0.57)	7.39 (0.61)
OD_{600}	0.00 (0.00)	2.33 (0.13)	1.51 (0.22)	3.35 (0.07)	1.65 (0.07)	2.47 (0.03)	1.65 (0.15)
MLSS (mg/l)	217.14 (12.03)	125.81 (17.96)	124.48 (15.39)	173.26 (12.03)	130.85 (13.33)	182.81 (12.33)	128.84 (16.33)
MLVSS (mg/l)	181.00 (11.03)	118.82 (21.07)	121.17 (11.30)	103.07 (9.48)	108.63 (8.11)	100.84 (5.79)	97.82 (8.26)
SVI (ml/g)	93.44 (2.33)	35.91 (3.79)	24.47 (3.22)	34.50 (3.18)	35.59 (2.60)	57.17 (4.12)	36.48 (4.19)
F/M ratio	1.04 (0.08)	0.04 (0.02)	0.04 (0.01)	0.05 (0.02)	0.04 (0.02)	0.04 (0.01)	0.03 (0.01)

Each value indicates average and standard deviation (in parentheses) of 3 independent experiments. The parameters are estimated at different time intervals of HRT (60 h).

Table 4.5. Environmental parameters of ECO effluent in continuous reactor

Parameter	Time Interval (h)			
	0	**72**	**144**	**216**
BOD (mg/l)	432.67 (26.34)	25.29 (2.43)	23.67 (2.35)	22.67 (2.08)
pH	7.84 (0.23)	7.01 (0.47)	7.27 (0.31)	6.80 (0.19)
OD_{600}	0.00 (0.00)	2.53 (0.16)	1.81 (0.12)	2.65 (0.07)
MLSS (mg/l)	207.14 (2.03)	125.81 (7.96)	124.48 (5.39)	130.85 (3.33)
MLVSS (mg/l)	184.08 (10.03)	112.82 (11.07)	112.17 (6.30)	102.63 (8.15)
SVI (ml/g)	83.44 (2.97)	53.91 (3.37)	42.47 (1.22)	37.59 (2.75)
F/M ratio	1.07 (0.03)	0.05 (0.01)	0.04 (0.02)	0.05 (0.02)

Each value indicates average and standard deviation (in parentheses) of 3 independent experiments. The parameters are estimated at different time intervals of HRT (72 h).

Table 4.6. Environmental parameters of COR effluent in continuous reactor

Parameter	Time Interval (h)			
	0	**96**	**192**	**288**
BOD (mg/l)	616.67 (26.43)	28.29 (2.03)	28.67 (2.53)	24.42 (2.35)
pH	8.84 (0.32)	7.09 (0.37)	7.37 (0.18)	7.34 (0.53)
OD_{600}	0.00 (0.00)	2.33 (0.06)	1.91 (0.14)	2.51 (0.17)
MLSS (mg/l)	217.14 (2.33)	194.81 (7.56)	170.48 (5.87)	140.26 (6.18)
MLVSS (mg/l)	204.08 (9.03)	172.82 (10.07)	152.17 (6.03)	103.07 (9.89)
SVI (ml/g)	93.44 (2.67)	63.91 (3.57)	52.47 (3.22)	43.27 (2.08)
F/M ratio	1.03 (0.04)	0.06 (0.02)	0.04 (0.01)	0.03 (0.01)

Each value indicates average and standard deviation (in parentheses) of 3 independent experiments. The parameters are estimated at different time intervals of HRT (96 h).

Table 4.7. Environmental parameters of UPL effluent in continuous reactor

Parameter	Continuous Reactor				
	0	**72**	**144**	**216**	**288**
BOD (mg/l)	588.37 (16.34)	29.25 (2.32)	25.67 (2.52)	26.42 (2.12)	24.67 (2.80)
pH	6.84 (0.23)	7.03 (0.17)	7.26 (0.19)	7.18 (0.35)	7.08 (0.21)
OD_{600}	0.00 (0.00)	2.73 (0.26)	2.81 (0.15)	2.53 (0.16)	2.76 (0.17)
MLSS (mg/l)	201.14 (2.32)	195.81 (7.29)	132.48 (5.34)	185.26 (6.27)	140.85 (3.02)
MLVSS (mg/l)	178.08 (8.03)	102.82 (10.07)	123.17 (6.23)	108.07 (7.98)	92.63 (8.41)
SVI (ml/g)	88.44 (2.87)	62.91 (3.67)	52.47 (1.81)	44.27 (2.02)	36.59 (2.57)
F/M ratio	1.06 (0.03)	0.06 (0.02)	0.05 (0.01)	0.03 (0.01)	0.04 (0.02)

Each value indicates average and standard deviation (in parentheses) of 3 independent experiments. The parameters are estimated at different time intervals of HRT (72 h).

Sharifi-Yazdi *et al*. (2001) reported biological treatment of chemical factory wastewater by the activated sludge unit which showed 85 % COD reduction of the effluent in 30 d. Ghaly and Dave (2012) developed a safe and effective in the farm biological treatment using soil microbes for low level agricultural pesticide wastewater which achieved a pesticide degradation efficiency of 89.6 % after 10 d in the continuous bioreactor. The application of biological treatment to remove bulk COD from different types of pesticide solution led to more than 95 % COD removal in 50 d using the indigenous microflora when wastewater was fed to the bioreactor (Lafi and Al-Qodah, 2006). In the present study, the AC consortium treated 4 different kinds of industrial effluents efficiently within shorter duration than that reported, implying its broad potential to treat various kinds of industrial effluents.

Table 4.8 summarizes the COD reduction ability of the AC consortium at batch and continuous reactor levels. It was observed that the COD reduction ability of the AC consortium was appreciably higher than that of the indigenous microflora of the respective industrial effluents. In the flask level treatability studies, the AC consortium showed 100 % COD reduction of DNR effluent in 8 h, ECO effluent in 9 h, COR effluent in 12 h and UPL effluent in 8 h, as compared to 48, 50, 50 and 56 % COD reduction of DNR, ECO, COR and UPL effluents respectively in 12 h by their indigenous microflora. In the batch reactor, the AC consortium showed 100 % COD reduction of DNR effluent in 60 h, ECO effluent in 72 h, and COR and UPL effluents in 96 h, as compared to 56, 68, 57 and 57 % COD reduction of DNR, ECO, COR and UPL effluents respectively in 96 h by their indigenous microflora. In the continuous reactor, the AC consortium showed 100 % COD reduction of DNR effluent in 15 d, ECO effluent in 9 d, and COR and UPL effluents in 10 d, as compared to 62, 73, 65 and 67 % COD reduction of DNR, ECO, COR and UPL effluents respectively in 15 d by their indigenous microflora. Thus, the AC consortium is a suitable microbial seed for biotreatment of a variety of industrial effluents.

One-way ANOVA was performed for the comparison of difference in % COD reduction obtained in control and AC consortium seeded flask, batch and continuous reactors. A p-value of 0.005 indicated that there was a significant difference in the observed means of % COD reduction in all the 3 reactors at 95 % level of significance (Table 4.9). The results obtained indicated that the values obtained for difference in % COD reduction of the 4 different effluents at flask, batch and continuous reactor levels were significantly different within the effluents of each level and between the

levels. This implied that COD reduction by the AC consortium was significant in all the 4 effluents and its COD reduction ability differed significantly for the 4 effluents at flask, batch and continuous reactor levels.

Table 4.8. Summary of the COD reduction obtained in flask, batch and continuous reactor studies with DNR, ECO, COR and UPL effluents

Reactor	**Flask**				**Batch**				**Continuous**			
Effluent	**DNR**	**ECO**	**COR**	**UPL**	**DNR**	**ECO**	**COR**	**UPL**	**DNR**	**ECO**	**COR**	**UPL**
% COD reduction in control reactor*	48.05 (12)	50 (12)	50 (12)	56.25 (12)	56.25 (96)	68.42 (96)	56.75 (96)	56.63 (96)	61.54 (15)	72.73 (15)	65 (15)	66.67 (15)
% COD reduction by AC consortium	100 (8)	100 (9)	100 (12)	100 (8)	100 (60)	100 (72)	100 (96)	100 (96)	100 (15)	100 (9)	100 (10)	100 (10)
Difference in % COD reduction	51.95	50	50	43.75	43.75	31.58	43.25	43.37	38.46	27.27	35	33.33

Each value indicates the average of 3 independent experiments. The values in parenthesis indicate the time taken to reduce the COD of the 4 effluents in h (flask and batch) and d (continuous). *Control reactor contained effluents uninoculated with the AC consortium.

Table 4.9. One-Way ANOVA for comparison of difference in % COD reduction obtained in control and AC consortium seeded flask, batch and continuous reactors

Data	**Mean**	**Variance**	**N**
Flask level	48.92	12.75	4
Batch reactor	40.49	35.31	4
Continuous reactor	33.51	21.90	4
F = 10.2145 and p = 0.0048 At the 0.05 level, the means are significantly different			

The desirable BOD value of 30 mg/l for discharge of effluents was obtained at the end of each reactor study; the pH obtained was in the fixed range of 5.5 – 9.0; the MLSS and MLVSS values were reduced approximately to 100 mg/l; the SVI obtained

was approximately 100 ml/g, and the F/M ratio was lower than the stipulated value of 0.04 (Tchobanoglous *et al*., 2003). Thus, the AC consortium was able to treat DNR, ECO, COR and UPL effluents efficiently so as to match the environmental standards for discharge of industrial effluents. Hence, it can be concluded that the AC consortium is a very efficient and versatile microbial seed for treating a variety of industrial effluents to an eco-friendly level.

4.3.3. Pilot-scale reactor studies

The process was further scaled up to a pilot scale 2000 l continuous reactor level with DNR effluent. Here, the AC consortium reduced the initial COD of DNR effluent from about 1892 mg/l to below permissible limit and maintained it over a period of 120 d (Figure 4.7). Other environmentally important parameters like MLSS and MLVSS of the DNR effluent were also estimated for its efficient biotreatment (Figure 4.7). The desirable MLSS and MLVSS values of 100 mg/l were obtained for most of the reactor run, implying that an active biomass was maintained in the reactor throughout the experiment. Hence, the AC consortium is an efficient microbial seed for biotreatment of DNR effluent at an upgraded scale.

In a similar study carried out by Hallin *et al*. (2006), methanol added to the denitrification process in activated sludge increased the capacity of the methylotrophic denitrifiers in the sludge to utilize both methanol and formate for denitrification in a 2400 l pilot-scale plant. A wastewater treatment facility, Shanghai, China, having wastewater containing a large variety of persistent organic materials, including petroleum hydrocarbons, halogenated hydrocarbons, nitroaromatic compounds, organic dyes and heavy metal ions, showed COD removal efficiency at pilot-scale in the range of 78.9 - 93.1 % when used along with the Zero-Valent Iron reactor during a monitoring period of 132 d (Ma and Zhang, 2008). Ratcliffe *et al*. (2006) showed successful operation of the powdered activated carbon system achieving over 80 % COD reduction of the chemical industry wastewater, also containing phenols and ammonia, coming from the MBBR and reducing the ammonia to < 1 ppm during the steady operations. In comparison to these reports, the AC consortium showed up to 100 % COD reduction of DNR effluent at pilot-scale.

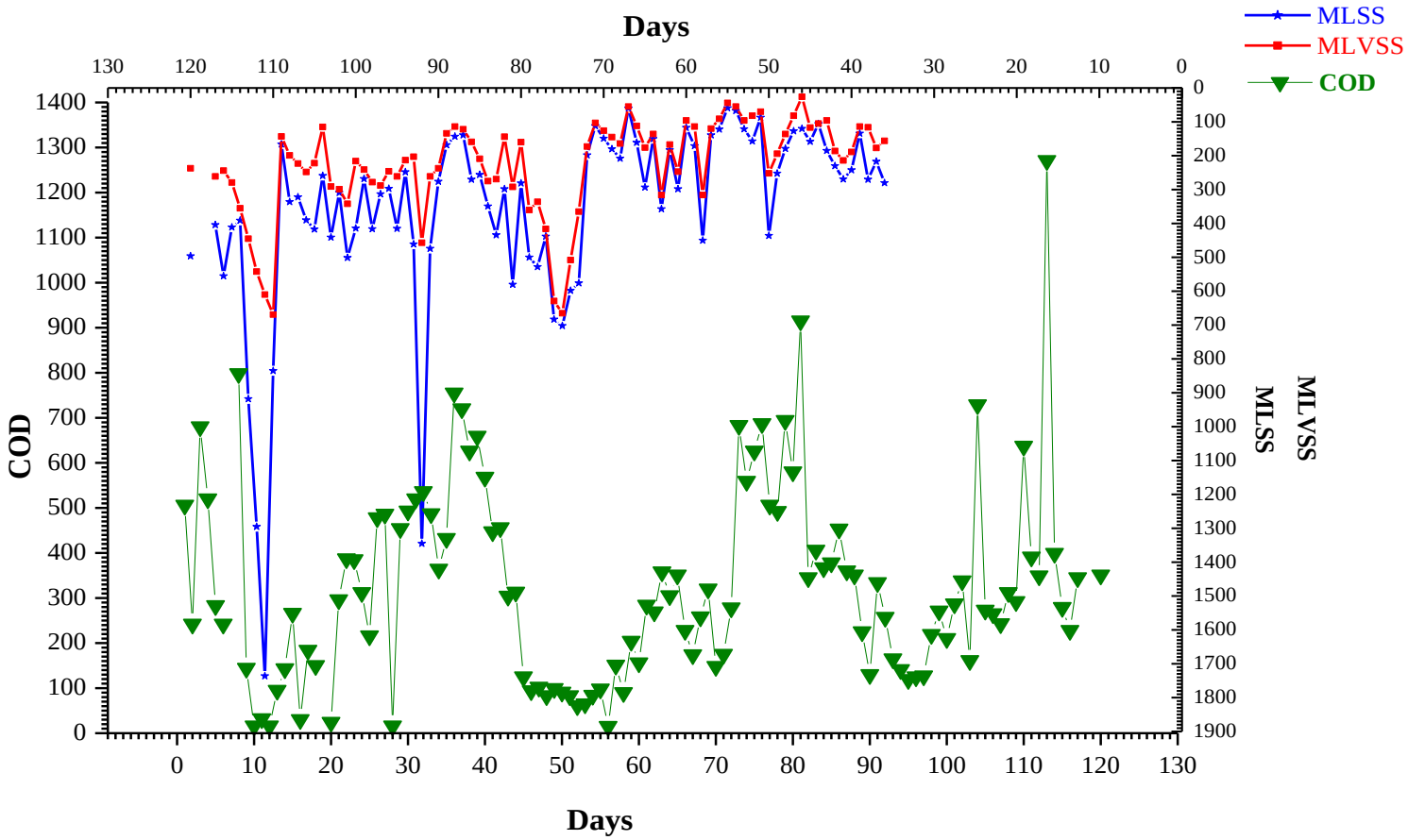


Figure 4.7. Biotreatment of DNR effluent by the AC consortium in a pilot-scale reactor; profiles of COD, MLSS and MLVSS in a 2000 l continuous reactor

In conclusion, the individual isolates of AC consortium were maintained as a consortium during the biotreatment studies. The AC consortium showed efficient biotreatment of 4 industrial effluents obtained from fertilizer, chemical and pesticide industries and CETP by lowering their COD to below permissible limit at flask, batch and continuous reactor levels. Optimum range for each of the operating variables like COD, BOD, pH, MLSS, MLVSS, SVI and F/M ratio was obtained by the AC consortium in the reactors run with these effluents. Therefore, formation of the 4 member AC consortium of methylotrophic bacteria has led to the development of an efficient mixture of bacteria capable of degrading a variety of industrial effluents containing environmentally hazardous pollutants generated from their respective industries.

4.4. References

Babu, B.R., Meera, K.M.S., Venkatesan, P., 2011. Removal of pesticides from wastewater by electrochemical methods - A comparative approach. *Sustainable Environment Research*, 21, 401-406.

Barbera, M.J., Mateo, E., Monkaityte, R., Constanti, M., 2011. Biodegradation of methyl *tert*-butyl ether by newly identified soil microorganisms in a simple mineral solution. *World Journal of Microbiology and Biotechnology*, 27, 813-821.

Baytshtok, V., Kim, S., Yu, R., Park, H., Chandran, K., 2008. Molecular and biokinetic characterization of methylotrophic denitrification using nitrate and nitrite as terminal electron acceptors. *Water Science and Technology*, 58, 359-365.

Chongcharoen, R., Smith, T.J., Flint, K.P., Dalton, H., 2005. Adaptation and acclimatization to formaldehyde in methylotrophs capable of high-concentration formaldehyde detoxification. *Microbiology*, 151, 2615-2622.

Coats, E.R., Loge, F.J., Wolcott, M.P., Englund, K., McDonald, A.G., 2007. Synthesis of polyhydroxyalkanoates in municipal wastewater treatment. *Water Environment Research*, 79, 2396-2403.

Durai, G., Rajamohan, N., Karthikeyan, C., Rajasimman, M., 2010. Kinetics studies on biological treatment of tannery wastewater using mixed culture. *International Journal of Chemical and Biological Engineering*, 3, 105-109.

George, S., Chellapandian, M., Sivasankar, B., Jayaraman, K., 1997. A new process for the treatment of fertilizer effluent using immobilized urease. *Bioprocess Engineering*, 16, 83-85.

Ghaly, A.E., Dave, D., 2012. Kinetics of biological treatment of low level pesticide wastewater. *American Journal of Environmental Sciences*, 8, 424-432.

Hallin, S., Throback, I.N., Dicksved, J., Pell, M., 2006. Metabolic profiles and genetic diversity of denitrifying communities in activated sludge after addition of methanol or ethanol. *Applied and environmental microbiology*, 72, 5445-5452.

Hamer, G., 1997. Microbial consortia for multiple pollutant biodegradation. *Pure and applied chemistry*, 69, 2343-2356.

Jiang, B., Parshina, S.N., Van Doesburg, W., Lomans, B.P., Stams, A.J.M., 2005. *Methanomethylovorans thermophila* sp. nov., a thermophilic, methylotrophic methanogen from an anaerobic reactor fed with methanol. *International journal of systematic and evolutionary microbiology*, 55, 2465-2470.

Jilani, S., Khan, M.A., 2013. Biological Treatment of Industrial Wastewater Using Biosimulator. *Middle-East Journal of Scientific Research*, 13, 124-132.

Lafi, W.K., Al-Qodah, Z., 2006. Combined advanced oxidation and biological treatment processes for the removal of pesticides from aqueous solutions. *Journal of hazardous materials*, 137, 489-497.

Ma, L., Zhang, W.-X., 2008. Enhanced biological treatment of industrial wastewater with bimetallic zero-valent iron. *Environmental Science & Technology*, 42, 5384-5389.

MacFaddin, J.F., 1980. Biochemical Tests for Identification of Medical Bacteria, second ed. Williams and Wilkins, Baltimore.

Maiti, S.K., 2004. Handbook of Methods in Environmental Studies - Water and Wastewater Analysis, vol. 1. Oxford Book Company, New Delhi, India.

Mishra, L.C., Singh, K.K., 1987. Pollution effects of fertilizer factory effluent on growth and development of corn and rice seedlings. *Conservation & recycling*, 10, 27-35.

Omer, A.M., 2012. Production of Organic Biofertilizer from Olive Mill Waste Water. *Australian Journal of Basic and Applied Sciences*, 6, 654-663.

Ratcliffe, M., Rogers, C., Merdinger, M., Prince, J., Mabuza, T., Johnson, C.H., 2006. Treatment of High Strength Chemical Industry Wastewater using Moving Bed Biofilm Reactor (MBBR) and Powdered Activated Carbon (PAC)

Technology. *Proceedings of the Water Environment Federation*, 2006, 1677-1694.

Sharifi-Yazdi, M.K., Azimi, C., Khalili, M.B., 2001. Study of the Biological Treatment of Industrial Waste Water by the Activated Sludge Unit. *Iranian Journal of Public Health*, 30, 87-90.

Sharma, P., Dhillon, K.S., Dhillon, S.K., 2008. Impact of distillery effluent application along with inorganic fertilizers on dry matter yield and mineral composition of rice. *Acta Agronomica Hungarica*, 56, 187-201.

Sherma, J., 1995. Pesticides. *Analytical Chemistry*, 67, 1R-20R.

Singh, P.P., Mall, M., Singh, J., 2006. Impact of fertilizer factory effluent on seed germination, seedling growth and chlorophyll content of gram (*Cicer aeritenum*). *Journal of Environmental Biology*, 27, 153-156.

Sivaprakasam, S., Mahadevan, S., Sekar, S., Rajakumar, S., 2008. Biological treatment of tannery wastewater by using salt-tolerant bacterial strains. *Microbial cell factories*, 7, 15.

Srinivas, T., 2008. Environmental biotechnology. New Age International, New Delhi, India.

Tchobanoglous, G., Burton, F.L., Metcalf & Eddy, Stensel, H.D., 2003. Suspended growth biological treatment processes, in: Tchobanoglous, G., Burton, F.L., Stensel, H.D. (Eds.), Wastewater Engineering Treatment and Reuse, 4th edn, McGraw-Hill, Inc., New York, USA, pp. 659-886.

Tomar, M., 1999. Quality assessment of water and wastewater. CRC press, Florida, USA.

Wakelin, S.A., Colloff, M.J., Kookana, R.S., 2008. Effect of wastewater treatment plant effluent on microbial function and community structure in the sediment of a freshwater stream with variable seasonal flow. *Applied and environmental microbiology*, 74, 2659-2668.

Chapter 5

Development of a bench scale Moving Bed Biofilm Reactor (MBBR) for COD reduction of industrial effluents by AC consortium

5.1. Introduction

The MBBR was developed in Norway in the late 1980s and early 1990s. It is covered by several patents and has been a huge success world-wide for treatment of municipal and industrial wastewaters (Rusten *et al*., 2006). Contrary to most biofilm reactors, the MBBR utilizes the whole tank volume for biomass growth. MBBR is self-cleaning and has low head loss (Rusten *et al*., 2000). Unlike the activated sludge reactor, it does not need any sludge recycle. This is achieved by having the biomass grow on carriers that move freely in the water volume of the reactor and that are kept within the reactor volume by a sieve arrangement at the reactor outlet. The reactor may be used for aerobic, anoxic or anaerobic processes. One important advantage of the MBBR is that the filling fraction of biofilm carriers in the reactor may be subject to preferences. In order to be able to move the carrier suspension freely, it is recommended that filling fractions should be below 70 % (Rusten *et al*., 2006). Bioreactors with up to 60 % of the working volume occupied by carriers have been studied and shown to perform effectively (Maurer *et al*., 2001).

The movement of small carrier elements within a reactor is produced by coarse bubble aeration in aerobic applications and mechanical mixing in anoxic applications. The biofilm carrier elements are made of high density polyethylene and are shaped like small cylinder (10 mm x 7 mm) with a cross inside the cylinder and longitudinal fins on the outside (Johnson *et al*., 2000). The reactor volume is totally mixed and consequently there is no dead or unused space in the reactor. Different reactor shapes can be used and the MBBR process is ideal for upgrading of overloaded treatment plants or for converting unused tanks into biofilm reactors. 2 Kaldnes MBBR™ full scale treatment plants were commissioned by Johnson *et al*. (2000) in 1999, following successful pilot tests which generated the design data. The MBBR plants were incorporated into each facility as roughing reactors ahead of existing activated sludge processes.

Attributes which differentiate MBBRs from other biological wastewater treatment systems have been identified as: simplicity, low space requirement and no sludge separation requirement for effective operation (advantages over activated sludge); low head loss, no channeling or requirement for backwashing (advantages over fixed-bed biofilters/trickling filters); large surface area for

colonization and high specific biomass activity; protection of slow growing microorganisms from excessive abrasive removal (advantage over turbulent reactor biofilm systems such as fluidized beds); versatility; ability for retrofitting into existing tank volumes and/or addition to existing treatment systems to improve overall performance (Gapes and Keller, 2009). Biofilm-internal and external mass transfer resistance was investigated by Gapes and Keller (2009) in laboratory-scale nitrifying MBBR, demonstrating the importance of these factors for MBBR.

Biofilm processes have proved to be very reliable for tertiary nitrification, because of the higher volumetric loading rates that can be applied and the low solids build-up in the reactor. This allows the use of smaller reactors usually with no need of tertiary settling tanks. KMT® biofilm carriers have already been successfully used for nitrification (Bonomo *et al.*, 2000). The hydrodynamic behavior and denitrification capacity of downflow self-cleaning MBBR using floating packed-bed carriers were investigated by Dupla *et al*. (2006). A mathematical model of nitrification and denitrification in a moving-fixed bed biofilm reactor was developed by Lin (2008) to describe ammonium utilization by nitrifying biomass as well as nitrate and organic carbon utilization by denitrifying biomass respectively. The MBBR has been successfully used in the biological treatment of different effluents, such as the wastewater from pulp and paper industry, refineries, lactic industry, and in municipal wastewater treatment plants, as well as in different conditions like mesophilic and thermophilic environments (Moreno-Andrade *et al*., 2009).

The MBBR has definite advantages over the suspended reactor as discussed above. Excess sludge treatment and disposal currently represents a rising challenge for wastewater treatment plants. An advantage of the MBBR is its easy management (no clogging, no rotating shafts and no sludge recycle). For this reason, the MBBR can be considered an interesting solution when a biological plant needs to be upgraded (Andreottola *et al*., 2000). In order to improve the activated sludge process, MBBR was developed as an efficient replacement of the existing activated sludge tanks. In this chapter, to evaluate the suitability of the AC consortium for MBBR, the biofilm development studies were performed initially to assess the biofilm forming ability of AC consortium and their results were used to scale up to MBBR.

5.2. Materials and Methods

5.2.1. Biofilm development

5.2.1.1. Biofilm formation by the isolates of AC consortium on a microscopic glass slide

Biofilm forming ability of the isolates of AC consortium was checked on a microscopic glass slide. The medium used for this study was PNB medium (Chapter 2, section 2.2.1). 5 % inoculum was inoculated in 100 ml PNB medium in a 250 ml Erlenmeyer flask containing a microscopic glass slide. The flasks were incubated at 37 ^{o}C for 96 h at 50 rpm. The biofilm obtained on the glass slide was stained with 1 % crystal violet and observed under a brightfield microscope (Olympus CX41) at 100 X magnification (Jadhav *et al*., 2005).

5.2.1.2. Biofilm forming ability of the isolates of AC consortium using microtiter plate biofilm assay

Biofilm forming ability of the isolates of AC consortium was analyzed by microtiter plate biofilm assay (Srinandan *et al*., 2010). The medium used for this study was AMS medium, containing (per liter) $MgSO_4$, 1.0 g; $CaCl_2$, 0.2 g; $FeCl_3$, 4.0 mg; NH_4Cl, 0.5 g; Trace element solution, 0.5 ml; Phosphate buffer, 20.0 ml; Methanol, 5.0 ml, pH, 6.8. The phosphate buffer solution constituted of a mixture of KH_2PO_4 and $Na_2HPO_4.12H_2O$ at a concentration of 15 g in 300 ml D/W at pH 6.8 (Whittenbury *et al*., 1970). 1 ml of AMS medium was inoculated with 2 % inoculum in a 24-well polystyrene microtiter plate. After the incubation period of 72 h, the suspended growth of the isolates in the wells was measured in terms of their OD at 600 nm. Thereafter, the wells were rinsed 3 times with 1 ml of sterile D/W to remove any adhering planktonic cells. The biofilm was then stained with 1 ml of 1 % crystal violet for 45 min, rinsed 3 times with 1 ml of D/W and destained with 70 % ethanol for 15 min. Absorbance of the solution was measured at 595 nm. Absorbance of the samples was normalized with that of the uninoculated control.

5.2.1.3. Biofilm forming ability of the isolates of AC consortium on biofilm carriers

Biofilm forming ability of the isolates of AC consortium was checked on commercially available Kaldnes type K1 biofilm carriers. The characteristics of K1 biofilm carriers (depicted in Figure 5.1(b)) are listed in Table 5.1. AC consortium was

prepared by inoculating a loopful of each isolate together into a test tube containing 5 ml PNB medium and incubating at 37 ^{o}C for 12 h at 180 rpm. 5 % inoculum was inoculated into 100 ml DNR effluent (obtained from the denitrifying reactor of a fertilizer company) in a 250 ml Erlenmeyer flask, containing 30 % (v/v) biofilm carriers (Jing *et al*., 2009), and incubated at 37 ^{o}C for 10 d with constant aeration. The biofilm formed on the carriers was observed visually in comparison to the unused carriers.

Table 5.1. Typical characteristics of Kaldnes type K1 biofilm carriers (Rusten *et al*., 2006)

Parameter	K1 biofilm carrier
Chemical nature	Polyethylene
Diameter (mm)	9.1
Length (mm)	7.2
Bulk density (kg/m^3)	150
Specific biofilm surface area (m^2/m^3)	500
Specific biofilm surface area at 60 % fill (m^2/m^3)	300

5.2.2. Effect of different parameters on biofilm formation

The individual constituents of AMS medium were optimized to support highest biofilm formation by the AC consortium and its individual isolates using microtiter plate biofilm assay. The effects of inoculum size, inoculum age and incubation period on biofilm formation were also checked.

5.2.2.1. Influence of methanol concentration on biofilm formation

A colony of each isolate, viz. *Bordetella petrii* AC1, *Bacillus licheniformis* AC4, *Salmonella subterranea* AC5 and *Pseudomonas stutzeri* AC8, was inoculated together in a test tube containing 5 ml LB medium and incubated at 37 ^{o}C for 24 h at 180 rpm. Thereafter, it was centrifuged at 10,000 rpm for 5 min and the cell pellet obtained was suspended in 1 ml AMS medium which was used as an inoculum. The concentration of methanol was changed from 5 ml/l to 1 ml/l in the AMS medium. 20 µl of the inoculum was inoculated in a microtiter plate well containing 980 µl AMS medium and incubated at 37 ^{o}C for 72 h under static condition. The procedure for quantifying the suspended growth and biofilm colorimetrically was the same as described in section 5.2.1.2.

5.2.2.2. Effect of different carbon, nitrogen and phosphorus sources on the biofilm forming ability of the AC consortium and its individual isolates

Potassium acetate, sodium succinate and methanol were used as different carbon sources, NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$ were used as different nitrogen sources, and KH_2PO_4 + Na_2HPO_4, KH_2PO_4 + K_2HPO_4, Na_2HPO_4 and KH_2PO_4 were used as different phosphorus sources (Srinandan *et al*., 2010). The experimental procedure described in section 5.2.2.1 was followed.

5.2.2.3. Effect of different concentrations of the selected carbon, nitrogen and phosphorus sources on the biofilm forming ability of the AC consortium and its individual isolates

Influence of different concentrations of potassium acetate, NH_4Cl and KH_2PO_4 + Na_2HPO_4 on the biofilm forming ability of the AC consortium and its individual isolates was checked using microtiter plate biofilm assay. The concentrations of potassium acetate used included 4, 6, 8 and 10 g/l; those of NH_4Cl included 0.2, 0.5, 0.7 and 1 g/l, and those of KH_2PO_4 + Na_2HPO_4 included 15, 20, 25 and 30 ml/l. The experimental procedure described in section 5.2.2.1 was followed.

5.2.2.4. Effect of different concentrations of macronutrients on the biofilm forming ability of the AC consortium and its individual isolates

Influence of different concentrations of $CaCl_2$, $MgSO_4$ and $FeCl_3$ on the biofilm forming ability of the individual isolates and AC consortium was checked using microtiter plate biofilm assay. The different concentrations of $CaCl_2$ used included 0.1, 0.2, 0.3 and 0.4 g/l; those of $MgSO_4$ included 0.5, 1.0, 1.5 and 2.0 g/l, and those of $FeCl_3$ included 2, 4, 6 and 8 mg/l. The experimental procedure described in section 5.2.2.1 was followed.

5.2.2.5. Effect of physiological parameters on the biofilm forming ability of the AC consortium and its individual isolates

Influence of inoculum size, inoculum age and incubation period on the biofilm forming ability of the AC consortium and its individual isolates was checked using microtiter plate biofilm assay. Different inoculum sizes used included 1, 2, 3 and 4 %; different ages of the inoculum included 12, 24, 48 and 72 h, and different incubation

periods included 72, 96 and 120 h. The experimental procedure described in section 5.2.2.1 was followed.

5.2.3. Biofilm forming ability of the AC consortium and its members in optimized AMS medium

Based on the above mentioned studies, the components of AMS medium were optimized to support highest biofilm formation by the isolates of AC consortium. The optimized AMS medium contained (per liter) potassium acetate, 8.0 g; NH_4Cl, 1.0 g; Phosphate buffer (KH_2PO_4 + Na_2HPO_4), 25.0 ml; $MgSO_4$, 1.5 g; $CaCl_2$, 0.4 g; $FeCl_3$, 6.0 mg; Trace element solution, 0.5 ml, pH, 6.8. The experimental procedure described in section 5.2.2.1 was followed.

5.2.4. Biofilm forming ability of the AC consortium and its individual isolates in industrial effluent

A colony of isolates of the AC consortium was inoculated in a test tube containing 5 ml LB medium and incubated at 37 ^oC for 24 h at 180 rpm. Thereafter, it was centrifuged at 10,000 rpm for 5 min and the cell pellet obtained was suspended in 1 ml DNR effluent which was used as an inoculum. 30 µl of the inoculum was inoculated in a microtiter plate well containing 970 µl of DNR effluent and incubated at 37 ^oC for 120 h under static condition. The procedure for quantifying the suspended growth and biofilm colorimetrically was the same as described in section 5.2.1.2.

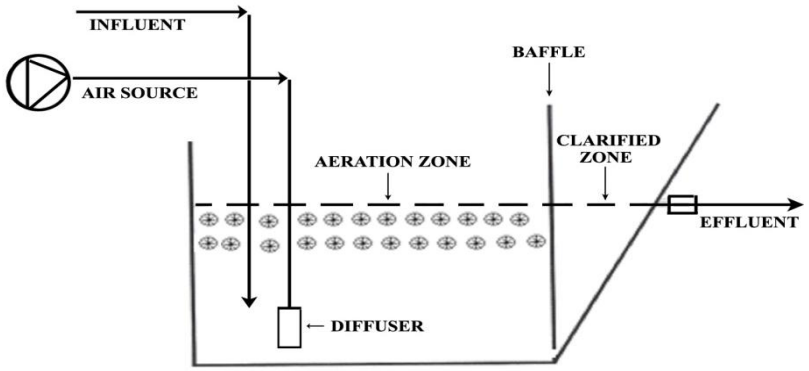
5.2.5. MBBR studies

5.2.5.1. Treatability studies

Ability of the AC consortium to treat the DNR effluent using MBBR was first checked at flask level. AC consortium was prepared by inoculating a loopful of each isolate together into 5 ml PNB medium and incubating at 37 ^oC for 24 h at 180 rpm. 3 % inoculum was inoculated into 100 ml freshly procured DNR effluent, containing 60 % (v/v) biofilm carriers (Rusten *et al*., 2006), and incubated at 37 ^oC for 120 h with constant aeration. 1 ml of the effluent was collected at intervals of 12 h and centrifuged at 10,000 rpm for 5 min. The supernatant was collected and used as a sample for COD estimation (Tomar, 1999).

5.2.5.2. Biotreatment of DNR effluent by the AC consortium using MBBR

The 6 l MBBR used for this study constituted of an aeration tank, a settling tank and an aeration source (Figure 5.1(a)). Kaldnes type K1 biofilm carriers, as depicted in Figure 5.1(b), were used in these studies. AC consortium was prepared by inoculating a loopful of each isolate together into 200 ml PNB medium and incubating at 37 ^{o}C for 24 h at 180 rpm. 3 % inoculum was inoculated into 6 l freshly procured DNR effluent, containing (a) 60 % (v/v) (Rusten *et al*., 2006) and (b) 30 % (v/v) (Jing *et al*., 2009) biofilm carriers, in the reactor and incubated at 37 ^{o}C for 7 d with constant aeration and stirring at 300 rpm. 1 ml of the effluent was collected at intervals of 24 h and centrifuged at 10,000 rpm for 5 min. The supernatant of the effluent was used as a sample for COD estimation (Tomar, 1999). The DNR effluent was removed after an interval of 7 d, constituting 1 run, and replaced by fresh DNR effluent. Likewise, 3 subsequent runs were carried out where the biofilm containing carriers were recycled, while the DNR effluent was entirely changed after every run.



(a)

(b)

Figure 5.1. (a) Schematic diagram of a MBBR constituting of an aeration zone, a clarified zone, an aeration source and biofilm carriers (b) Kaldnes type K1 biofilm carriers

The amount of biomass attached on the biofilm carriers was determined using 20 carriers randomly selected from the MBBR (Jing *et al*., 2009). The biofilm carriers were separated from the DNR effluent and dried at 105 ^{o}C. The dried samples were weighed in order to determine the total mass (M_t) which included the carrier mass (M_c) and the fixed biomass. After the biomass was washed off, the cleaned carriers were weighed and the amount of biomass attached to the 20 carriers (BS_{20}) was calculated according to the following equation:

$$BS_{20} = M_t - M_c$$

Additionally, the amount of biomass in the reactor (BS) was calculated by the following equation: When the filling ratio (FR) is 100 %, there would be 560 biofilm carriers flowing in unit volume of the reactor.

$$BS = BS_{20} \times \frac{560L^{-1}}{20} \times FR$$

5.2.5.3. Biotreatment of different industrial effluents using MBBR

The industrial effluents selected for the MBBR studies (previously used in the suspended reactor studies carried out in Chapter 4) included: DNR, ECO, COR and UPL effluents (freshly procured). The reactor used was a 6 l continuous reactor comparising of an aeration tank, an influent tank, an effluent tank and an aeration source (Figure 5.1(a)). The inoculum was prepared by inoculating 1 l AMS medium in a 2 l Erlenmeyer flask containing Kaldnes type K1 biofilm carriers (1 l, v/v) with 3 % AC consortium grown in LB medium and incubating at 37 ^{o}C for 120 h under aerobic conditions. After the incubation period, the biofilm carriers were inserted into the MBBR containing 5 l DNR effluent in order to make up the working volume of the reactor to 6 l. 1 ml of the effluent was collected at intervals of 24 h and centrifuged at 10,000 rpm for 5 min. The supernatant of the effluent was used as a sample for COD estimation (Tomar, 1999). All the 4 industrial effluents were treated consecutively. At the end of the reactor run, a biofilm carrier was visualized using Environmental Scanning Electron Microscopy (ESEM, Model XL-30, Philips, Netherlands) for the presence of bacterial biofilm.

5.2.6. Statistical analysis

Student's t-Test was applied to evaluate the effect of different chemical and physiological parameters on the biofilm forming ability of the AC consortium and two-way ANOVA was applied to evaluate their effects on the bifilm forming ability

of the individual isolates of AC consortium. It was assumed that the original data followed a normal distribution. All statistical analyses were performed using GraphPad Prism 5.0 software (San Diego, CA) (Barbera *et al*., 2011).

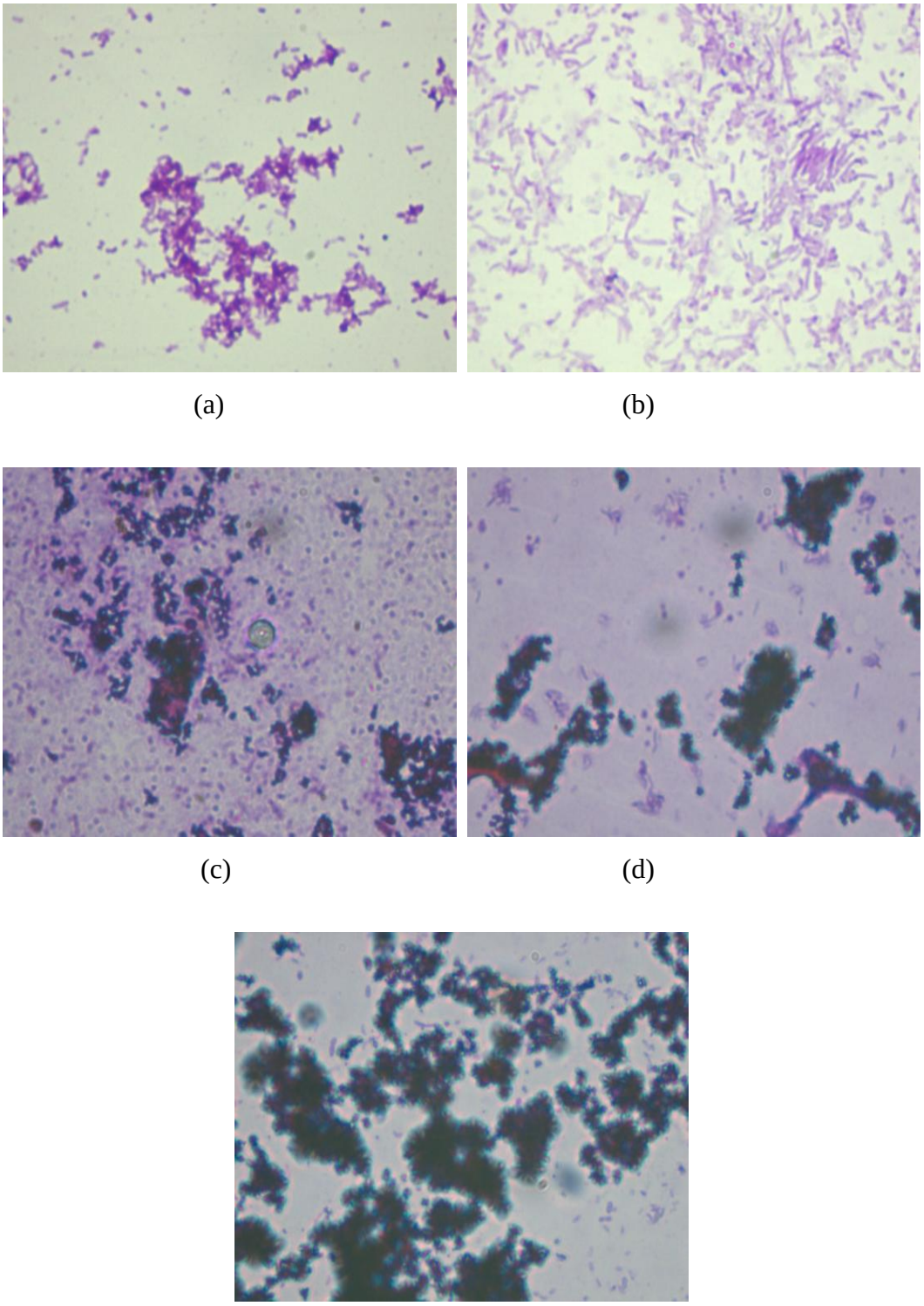
5.3. Results and Discussion

MBBR has several advantages over suspended wastewater treatment processes, the most important one being that it does not require any sludge recycle (Rusten *et al*., 2006). MBBR has been successfully used in the biological treatment of different kinds of effluents (Moreno-Andrade *et al*., 2009). Many activated sludge processes have been upgraded to MBBR, as it is easy to modify the existing facility (Johnson *et al*., 2000). This can prove to be particularly advantageous where the processes involve development of a special microbial seed, as in the case of AC consortium. The AC consortium with its broad biotreatment potential can be developed suitably for the MBBR process. With this idea, studies were carried out to develop a bench-scale MBBR system for the treatment of industrial effluents using AC consortium in this chapter.

5.3.1. Biofilm development

5.3.1.1. Biofilm formation by the isolates of AC consortium on a microscopic glass slide

Biofilm forming ability of the isolates of AC consortium was checked on a microscopic glass slide (Jadhav *et al*., 2005). All the isolates showed different stages of biofilm formation on microscopic examination of the glass slides, in spite of being of the same age of 96 h. In case of *B. petrii* AC1, the attached cell number increased marking the initiation of microcolonies (Figure 5.2(a)); the cells of *B. licheniformis* AC4 were attached to the substratum (Figure 5.2(b)); the cells of *S. subterranea* AC5 and *P. stutzeri* AC8 showed increase in the microcolony size (Figure 5.2(c & d)), and in case of AC consortium, complete biofilm formation was observed (Figure 5.2(e)). Therefore, it could be concluded that the AC isolates formed a complete biofilm when present as a consortium. The results gave an initial indication that the AC consortium can form a biofilm on glass surface.



(a) (b)

(c) (d)

(e)

Figure 5.2. Biofilm formation by (a) *B. petrii* AC1, (b) *B. licheniformis* AC4, (c) *S. subterranea* AC5, (d) *P. stutzeri* AC8 and (e) AC consortium on a microscopic glass slide observed under a brightfield microscope at 100 X magnification

5.3.1.2. Biofilm forming ability of the isolates of AC consortium using microtiter plate biofilm assay

Microtiter plate assay was standardized to assess the biofilm forming ability of the AC consortium and its individual isolates on a synthetic substratum, like polystyrene in case of the microtiter plate. The ability of the isolates of AC consortium to form a biofilm was checked in AMS medium using crystal violet staining (section 5.2.1.2). More the crystal violet absorption, more is the biofilm formed. Accordingly, *B. licheniformis* AC4 and *P. stutzeri* AC8 showed highest biofilm formation, while *B. petrii* AC1 showed least. The AC consortium showed a comparable biofilm formation to *B. licheniformis* AC4 and *P. stutzeri* AC8 (Figure 5.3). Thus, the isolates of AC consortium showed biofilm forming ability on a polystyrene microtiter plate.

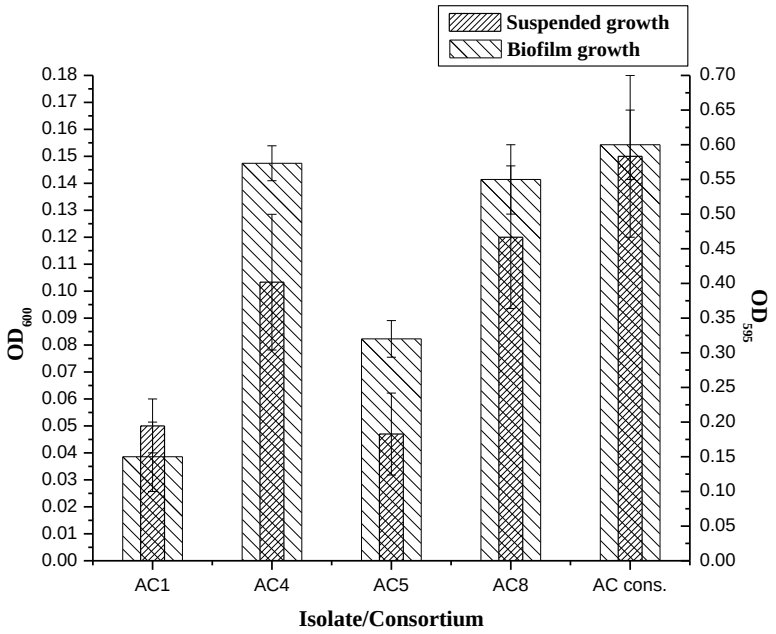


Figure 5.3. Biofilm forming ability of the isolates of AC consortium in AMS medium using microtiter plate biofilm assay. Error bars represent standard deviation from the mean, n = 3.

5.3.1.3. Biofilm forming ability of the isolates of AC consortium on biofilm carriers

The commercially available Kaldnes type K1 biofilm carriers are made up of polyethylene. Since the AC consortium demonstrated biofilm forming ability on

microtiter plates, the same was checked on the synthetic carriers. Biofilm forming ability of the AC consortium when checked on the biofilm carriers in DNR effluent (Figure 5.4(a)), it was observed that the AC consortium formed a strong biofilm on these carriers (Figure 5.4(b)). Biofilm formation was higher on the inner surfaces of the carriers as compared to their outer surface. Hence, the Kaldnes type K1 biofilm carriers were selected for the MBBR studies.

(a) (b)

Figure 5.4. (a) Kaldnes type K1 biofilm carrier and (b) Biofilm development by AC consortium on Kaldnes type K1 biofilm carrier

5.3.2. Effect of different parameters on biofilm formation

5.3.2.1. Effect of methanol concentration on biofilm formation by the AC consortium and its individual isolates

Being methylotrophs, the effect of methanol as a carbon substrate was checked on biofilm formation of the isolates of AC consortium. The concentration of methanol was decreased from 0.5 % to 0.1 % (v/v) in the AMS medium. As a result, the growth of isolates increased slightly on decreasing methanol concentration. Thereafter, the methanol concentration was again increased to 0.5 % and yeast extract was added as a growth factor at 0.01 %. The results showed that better growth and biofilm formation of the isolates and AC consortium were obtained when methanol was supplemented with a growth factor in the form of yeast extract as shown in Figure 5.5.

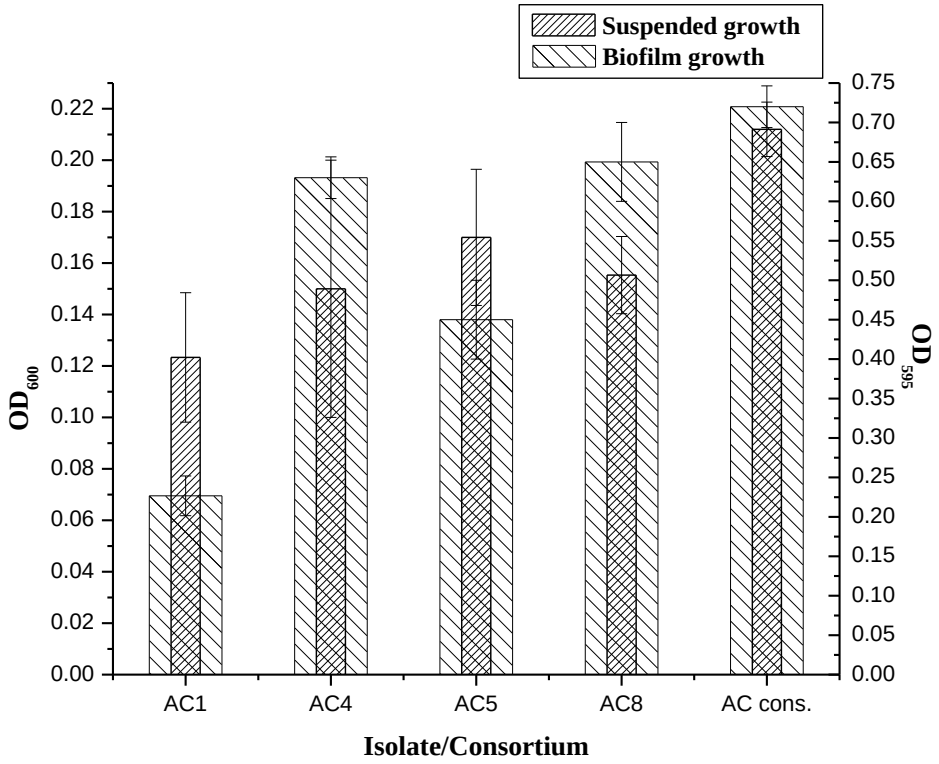


Figure 5.5. Comparison of biofilm formation by the AC consortium and its individual isolates at 0.5 % methanol concentration. Error bars represent standard deviation from the mean, n = 3.

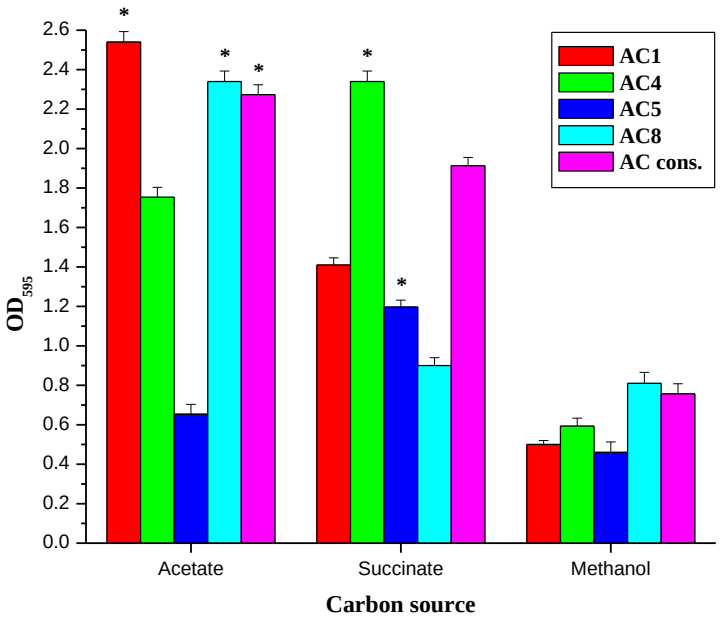
5.3.2.2. Effect of different carbon, nitrogen and phosphorus sources on biofilm forming ability of the AC consortium and its individual isolates

Biofilm forming ability of the AC consortium and its individual isolates was checked with respect to different sources of carbon, nitrogen and phosphorus. Out of potassium acetate, sodium succinate and methanol used as the carbon sources, potassium acetate supported highest biofilm formation by the AC consortium and its individual isolates (Figure 5.6(a)). At a significance level of 0.05 (ANOVA), the biofilm formation by *B. petrii* AC1 and *P. stutzeri* AC8 (p-value < 0.001) was 10 times more statistically significant in the presence of potassium acetate as compared to that by the AC consortium (p-value < 0.01). *B. licheniformis* AC4 and *S. subterranea* AC5 showed a statistically significant biofilm formation (ANOVA) in the presence of sodium succinate with a p-value < 0.001. As shown in Figure 5.6(a), most of the isolates and AC consortium showed higher biofilm formation in the presence of potassium acetate, hence, significance of potassium acetate was also

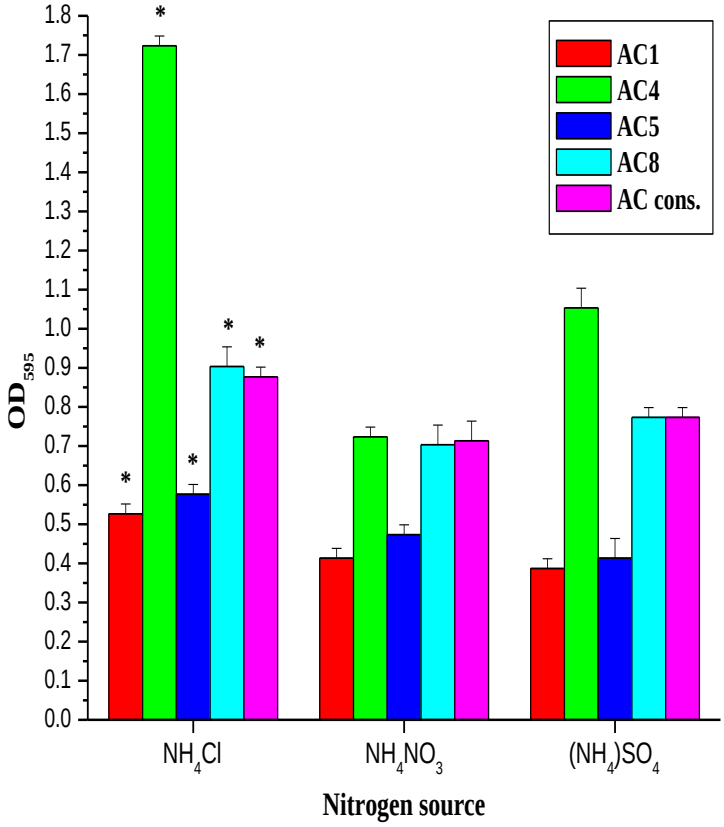
proved by the Student's t-Test, which showed that potassium acetate was statistically significant for biofilm formation by the AC consortium and its individual isolates with a p-value of 0.021 over methanol.

Out of NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$ used as the nitrogen sources, NH_4Cl supported highest biofilm formation by the AC consortium and its individual isolates (Figure 5.6(b)). *B. petrii* AC1, *S. subterranea* AC5 and the AC consortium showed a statistically significant biofilm formation (ANOVA) in the presence of NH_4Cl at a significance level of 0.05, with a p-value < 0.01, while *B. licheniformis* AC4 and *P. stutzeri* AC8 showed a more statistically significant biofilm formation with a p-value < 0.001.

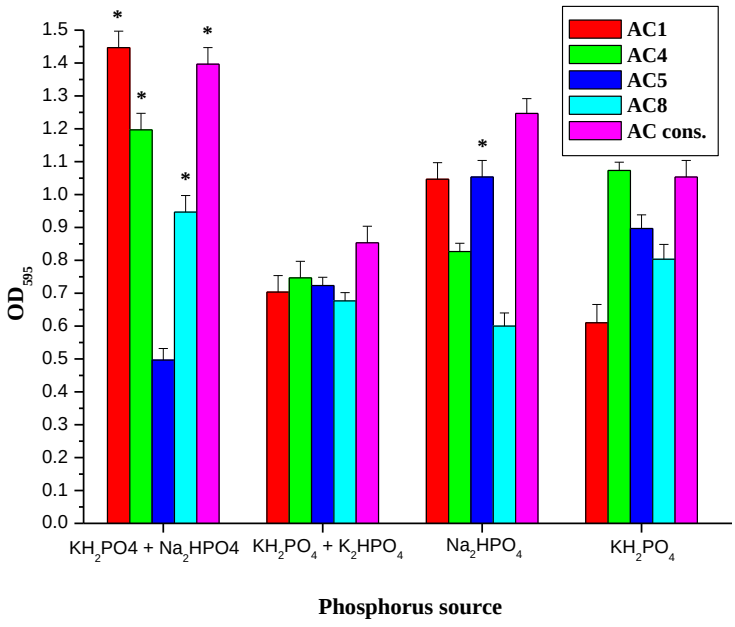
Out of $KH_2PO_4 + Na_2HPO_4$, $KH_2PO_4 + K_2HPO_4$, Na_2HPO_4 and KH_2PO_4 used as the phosphorus sources, $KH_2PO_4 + Na_2HPO_4$ supported highest biofilm formation by the AC consortium and its individual isolates (Figure 5.6(c)). *B. petrii* AC1 and the AC consortium showed a more statistically significant biofilm formation (ANOVA) in the presence of $KH_2PO_4 + Na_2HPO_4$ at a significance level of 0.05, with a p-value < 0.001, as compared to *B. licheniformis* AC4 and *P. stutzeri* AC8 which showed a statistically significant biofilm formation with a p-value < 0.01. *S. subterranea* AC5 showed a statistically significant biofilm formation in the presence of Na_2HPO_4 with a p-value < 0.001.



(a)



(b)



(c)

Figure 5.6. Effect of (a) carbon, (b) nitrogen and (c) phosphorus sources on the biofilm forming ability of the AC consortium and its individual isolates (asterisk indicates that the biofilm formation is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

Therefore, potassium acetate, NH_4Cl and KH_2PO_4 + Na_2HPO_4 were used as the carbon, nitrogen and phosphorus sources respectively in AMS medium for the further experiments.

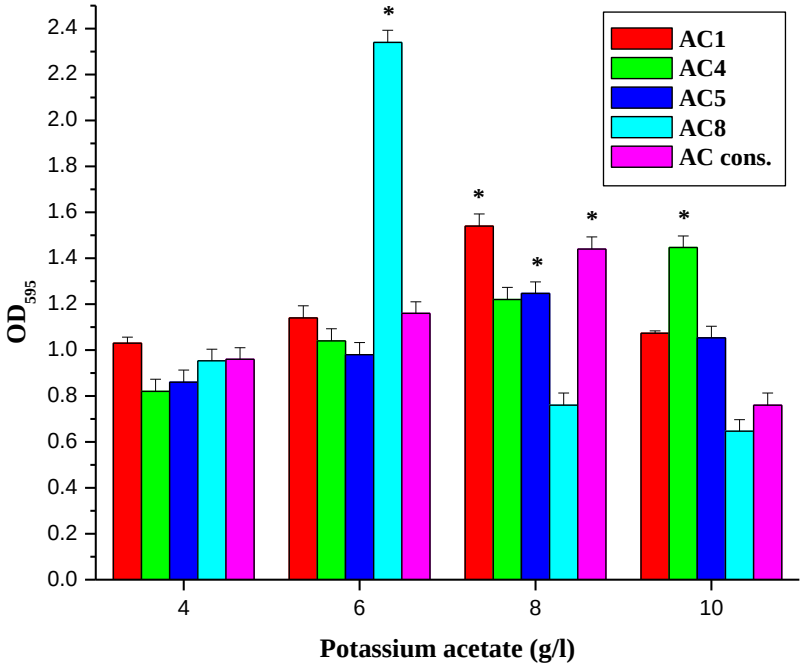
5.3.2.3. Effect of different concentrations of the selected carbon, nitrogen and phosphorus sources on biofilm forming ability of the AC consortium and its individual isolates

The effects of different concentrations of potassium acetate, NH_4Cl and KH_2PO_4 + Na_2HPO_4 were checked on biofilm forming ability of the AC consortium and its individual isolates using microtiter plate biofilm assay. Out of the different concentrations of potassium acetate, the concentration of 8 g/l supported highest biofilm formation by the AC consortium and its individual isolates (Figure 5.7(a)). *B. petrii* AC1, *S. subterranea* AC5 and the AC consortium showed a statistically significant biofilm formation (ANOVA) in the presence of potassium acetate at the concentration of 8 g/l at a significance level of 0.05, *B. licheniformis* AC4 showed a statistically significant biofilm formation in the presence of potassium acetate at the concentration of 10 g/l and *P. stutzeri* AC8 showed a statistically significant biofilm formation in the presence of potassium acetate at the concentration of 6 g/l with a p-value < 0.001.

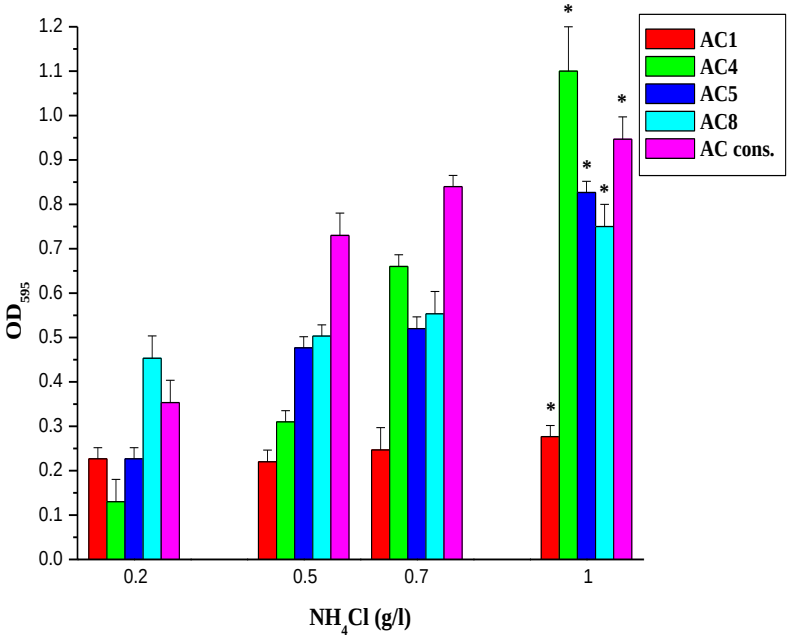
Out of the different concentrations of NH_4Cl, the concentration of 1 g/l supported highest biofilm formation by the AC consortium and its individual isolates (Figure 5.7(b)). At a significance level of 0.05 (ANOVA), *B. petrii* AC1 (p-value < 0.01); *B. licheniformis* AC4, *S. subterranea* AC5 and *P. stutzeri* AC8 (p-value < 0.001), and the AC consortium (p-value < 0.05) showed a statistically significant biofilm formation in the presence of NH_4Cl at the concentration of 1.0 g/l. The Student's t-Test also proved that NH_4Cl at the concentration of 1.0 g/l was statistically significant for the biofilm formation by AC consortium and its individual isolates with a p-value of 0.021 over 0.2 g/l concentration.

Out of the different concentrations of KH_2PO_4 + Na_2HPO_4, the concentration of 25 ml/l supported highest biofilm formation by the AC consortium and its individual isolates (Figure 5.7(c)). *B. petrii* AC1, *B. licheniformis* AC4 and *P. stutzeri* AC8 showed a statistically significant biofilm formation (ANOVA) in the presence of KH_2PO_4 + Na_2HPO_4 at the concentration of 25 ml/l at a significance level of 0.05,

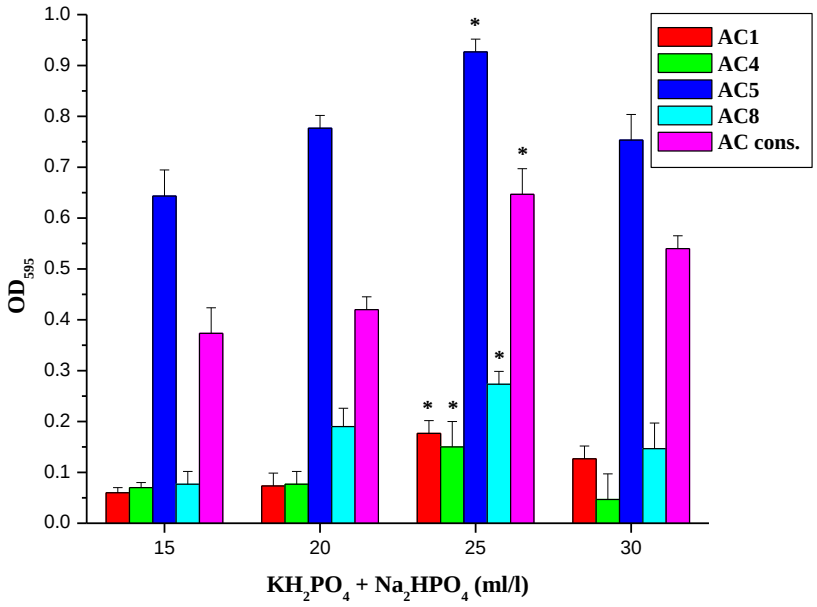
with a p-value < 0.05; *S. subterranea* AC5 showed a more statistically significant biofilm formation with a p-value < 0.001, and the AC consortium showed the same with a p-value < 0.01. Therefore, potassium acetate, NH_4Cl and KH_2PO_4 + Na_2HPO_4 at the concentrations of 8 g/l, 1 g/l and 25 ml/l respectively were supplemented in AMS medium for the further experiments.



(a)



(b)



(c)

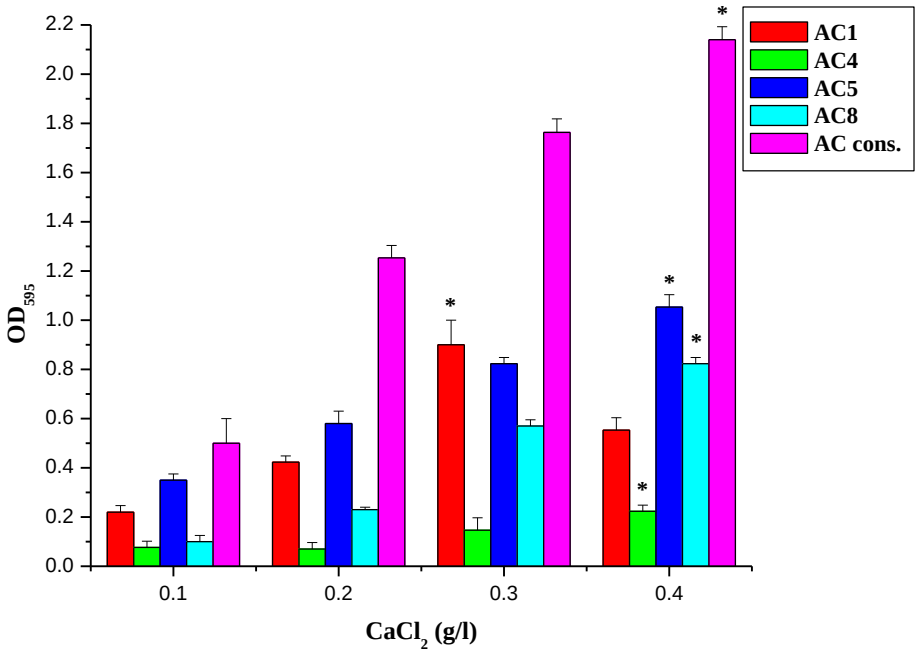
Figure 5.7. Influence of different concentrations of (a) potassium acetate, (b) NH_4Cl and (c) KH_2PO_4 + Na_2HPO_4 on the biofilm forming ability of the isolates of AC consortium (asterisk indicates that the biofilm formation is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

5.3.2.4. Effect of different concentrations of macronutrients on biofilm forming ability of the AC consortium and its individual isolates

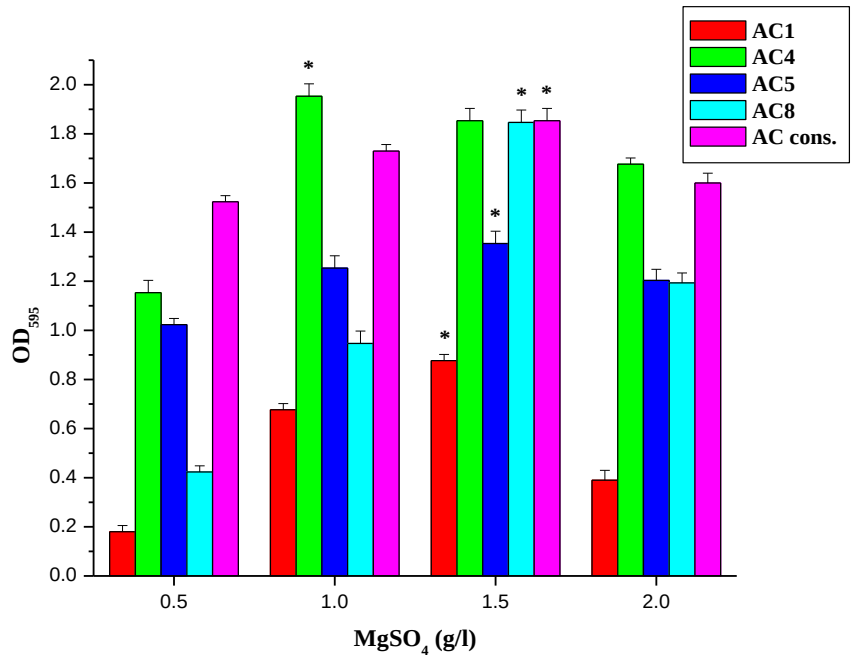
The effects of different concentrations of macronutrients, viz. $CaCl_2$, $MgSO_4$ and $FeCl_3$, were checked on biofilm forming ability of the AC consortium and its individual isolates. Out of the different concentrations of $CaCl_2$, the concentration of 0.4 g/l supported highest biofilm formation by the AC consortium and its individual isolates (Figure 5.8(a)). *B. petrii* AC1 showed a statistically significant biofilm formation (ANOVA) in the presence of $CaCl_2$ at the concentration of 0.3 g/l at a significance level of 0.05, with a p-value < 0.001. *B. licheniformis* AC4 showed a statistically significant biofilm formation in the presence of $CaCl_2$ at the concentration of 0.4 g/l with a p-value < 0.01, while *S. subterranea* AC5, *P. stutzeri* AC8 and the AC consortium showed a more statistically significant biofilm formation with a p-value < 0.001.

Out of the different concentrations of $MgSO_4$, the concentration of 1.5 g/l supported highest biofilm formation by the AC consortium and its individual isolates (Figure 5.8(b)). At a significance level of 0.05 (ANOVA), *B. petrii* AC1 and *P. stutzeri* AC8 showed a more statistically significant biofilm formation in the presence of $MgSO_4$ at the concentration of 1.5 g/l with a p-value < 0.001; *S. subterranea* AC5 showed a statistically significant biofilm formation with a p-value < 0.05, while the AC consortium showed the same with a p-value < 0.01. *B. licheniformis* AC4 showed a statistically significant biofilm formation in the presence of $MgSO_4$ at the concentration of 1.0 g/l with a p-value < 0.05.

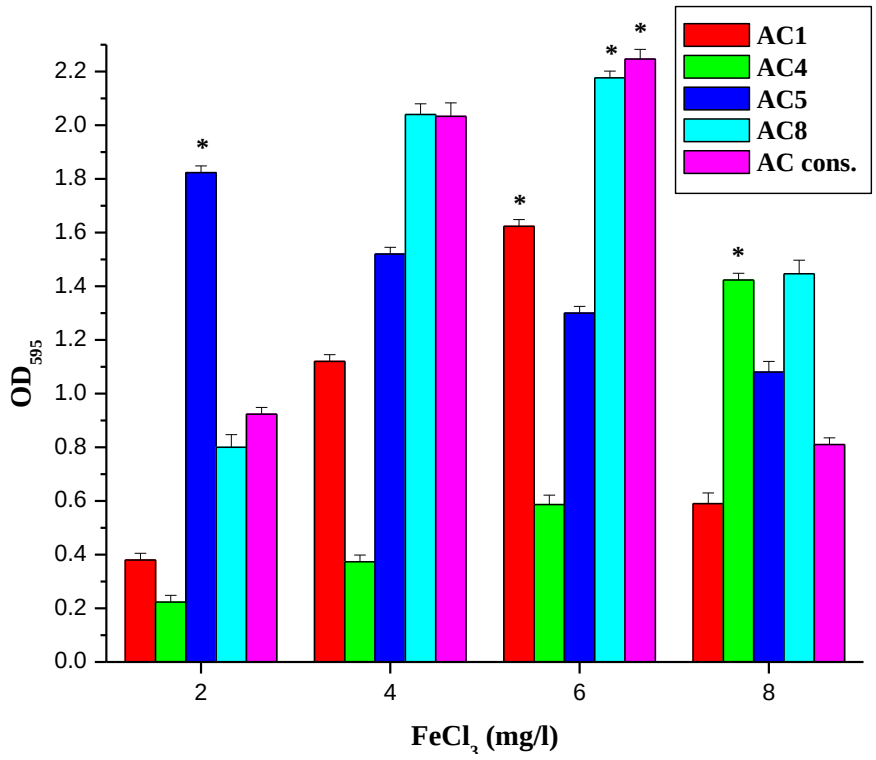
Out of the different concentrations of $FeCl_3$, the concentration of 6 mg/l supported highest biofilm formation by the AC consortium and its individual isolates (Figure 5.8(c)). At a significance level of 0.05 (ANOVA), a statistically significant biofilm formation was shown by *B. petrii* AC1, *P. stutzeri* AC8 and the AC consortium in the presence of $FeCl_3$ at the concentration of 6 mg/l; by *B. licheniformis* AC4 in the presence of $FeCl_3$ at the concentration of 8 mg/l, and by *S. subterranea* AC5 in the presence of $FeCl_3$ at the concentration of 2 mg/l with a p-value < 0.001. Therefore, $CaCl_2$, $MgSO_4$ and $FeCl_3$ at the concentrations of 0.4 g/l, 1.5 g/l and 6 mg/l respectively were supplemented in AMS medium for the further experiments.



(a)



(b)



(c)

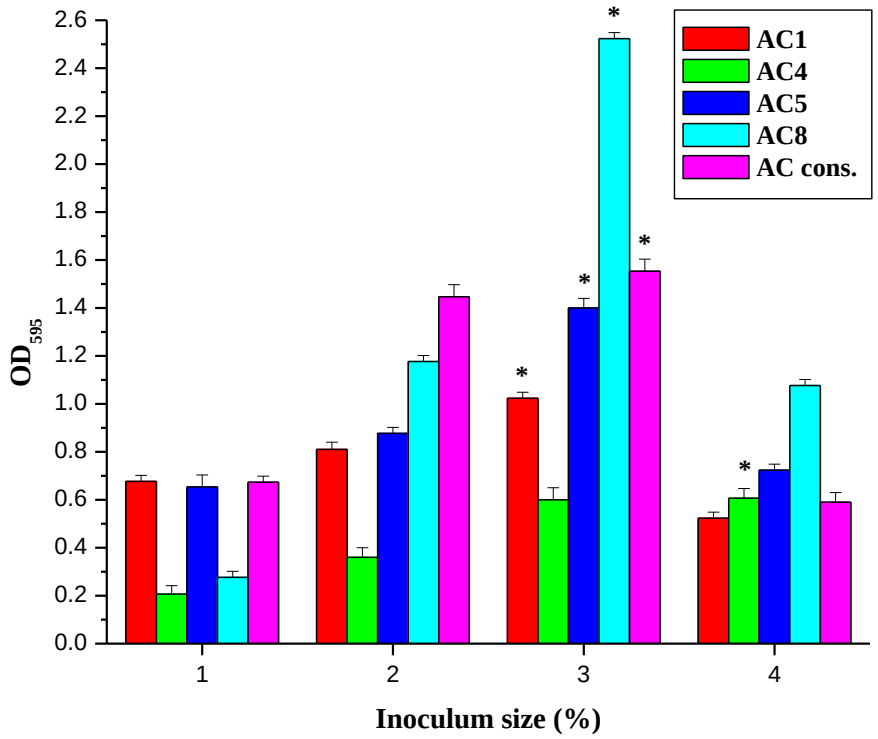
Figure 5.8. Influence of different concentrations of (a) $CaCl_2$, (b) $MgSO_4$ and (c) $FeCl_3$ on biofilm forming ability of the AC consortium and its individual isolates (asterisk indicates that the biofilm formation is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

5.3.2.5. Effect of physiological parameters on biofilm forming ability of the AC consortium and its individual isolates

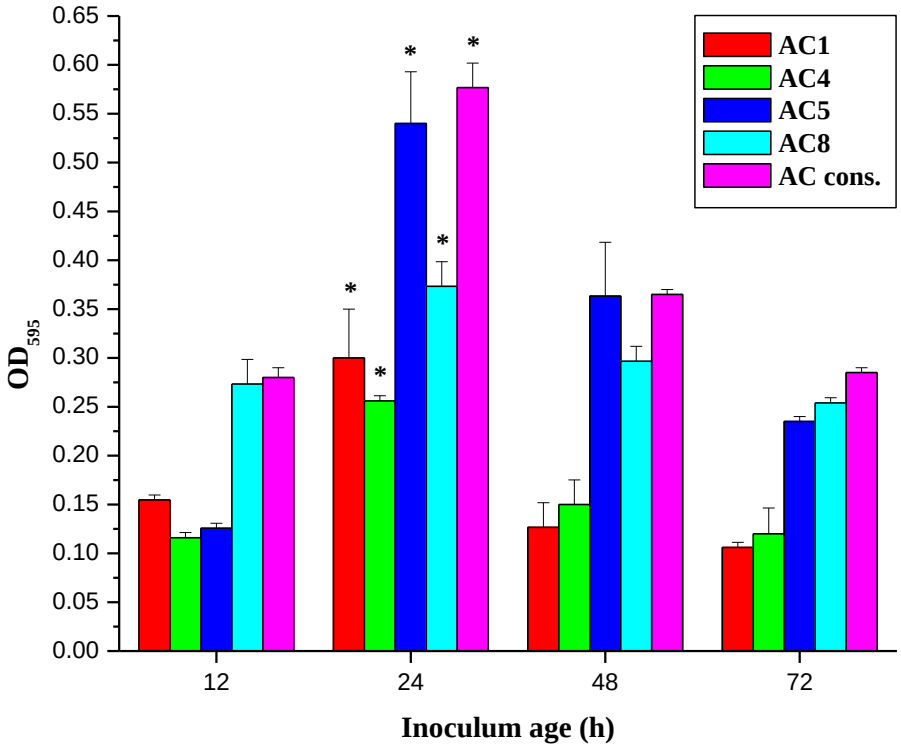
The effects of different physiological parameters, viz. inoculum size, inoculum age and incubation period, were checked on biofilm forming ability of the AC consortium and its individual isolates. Out of the different inoculum sizes, 3 % inoculum supported highest biofilm formation by the AC consortium and its individual isolates (Figure 5.9(a)). 3 % inoculum of *B. petrii* AC1, *S. subterranea* AC5 and *P. stutzeri* AC8 showed a more statistically significant biofilm formation (ANOVA) at a significance level of 0.05, with a p-value < 0.001, and 3 % inoculum of the AC consortium showed a statistically significant biofilm formation with a p-value < 0.01; while 4 % inoculum of *B. licheniformis* AC4 showed the same with a p-value < 0.001. The Student's t-Test confirmed that 3 % inoculum was statistically significant for the biofilm formation by AC consortium and its individual isolates with a p-value of 0.046 over 1 % inoculum.

Out of the different inoculum ages, 24 h old inoculum supported highest biofilm formation by the AC consortium and its individual isolates (Figure 5.9(b)). At a significance level of 0.05 (ANOVA), 24 h old inoculum of *B. petrii* AC1, *S. subterranea* AC5 and the AC consortium (p-value < 0.001); *B. licheniformis* AC4 (p-value < 0.01), and *P. stutzeri* AC8 (p-value < 0.05) showed a statistically significant biofilm formation. The Student's t-Test further confirmed that 24 h old inoculum was statistically significant for the biofilm formation by AC consortium and its individual isolates with p-values of 0.008 and 0.01 over 12 h and 72 h old inoculums respectively.

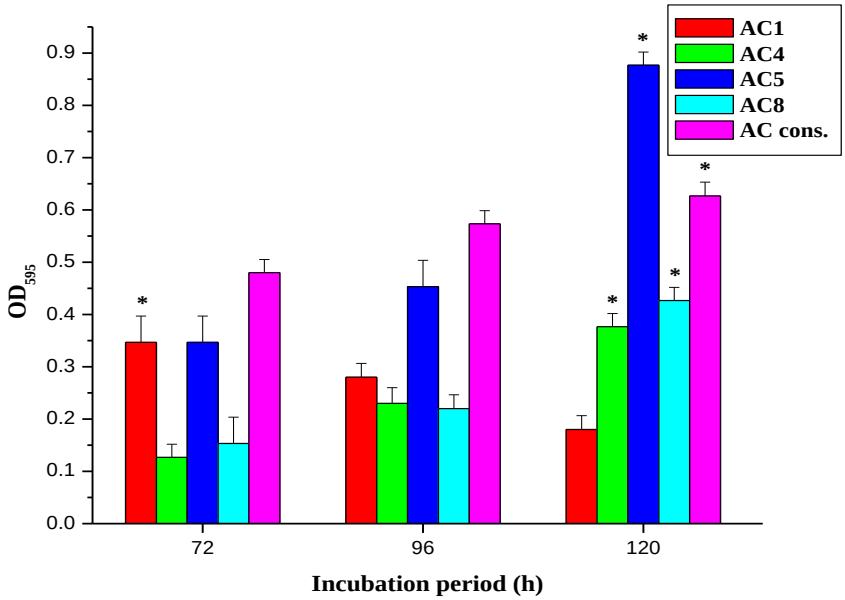
Out of the different incubation periods, the incubation period of 120 h supported highest biofilm formation by the AC consortium and its individual isolates (Figure 5.9(c)). At a significance level of 0.05 (ANOVA), *B. petrii* AC1 (p-value < 0.01) showed a statistically significant biofilm formation after 72 h; while *B. licheniformis* AC4, *S. subterranea* AC5 and *P. stutzeri* AC8 (p-value < 0.001), and the AC consortium (p-value < 0.05) showed the same after 120 h. Therefore, 3 %, 24 h old inoculum and an incubation period of 120 h were used for the further experiments.


AC1
AC4
AC5
AC8
AC cons.
OD595
Inoculum size (%)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
1
2
3
4

(a)


AC1
AC4
AC5
AC8
AC cons.
OD595
Inoculum age (h)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
0.55
0.60
0.65
12
24
48
72

(b)



(c)

Figure 5.9. Influence of (a) inoculum size, (b) inoculum age and (c) incubation period on biofilm forming ability of the AC consortium and its individual isolates (asterisk indicates that the biofilm formation is statistically significant at a significance level of 0.05). Error bars represent standard deviation from the mean, n = 3.

5.3.3. Biofilm forming ability of AC consortium and its members in optimized AMS medium

The AMS medium was optimized completely so as to support highest biofilm formation by the AC consortium and its individual isolates. Thus, the optimized AMS medium composed of (per liter): potassium acetate, 8.0 g; NH_4Cl, 1.0 g; phosphate buffer (KH_2PO_4 + Na_2HPO_4), 25.0 ml; $MgSO_4$, 1.5 g; $CaCl_2$, 0.4 g; $FeCl_3$, 6.0 mg; trace element solution, 0.5 ml, pH, 6.8. Biofilm forming ability of the isolates of AC consortium increased considerably in the modified AMS medium (Figure 5.10) as compared to the original AMS medium (Figure 5.3). The biofilm forming ability of *B. petrii* AC1 increased by 2.5 fold, that of *B. licheniformis* AC4 by 1.44 fold, that of *S. subterranea* AC5 by 3.01 fold, that of *P. stutzeri* AC8 by 1.22 fold and that of AC consortium by 2.04 fold. Among the 4 isolates, *B. licheniformis* AC4 showed highest growth in suspended mode, while *S. subterranea* AC5 showed highest growth in biofilm mode (Figure 5.10).

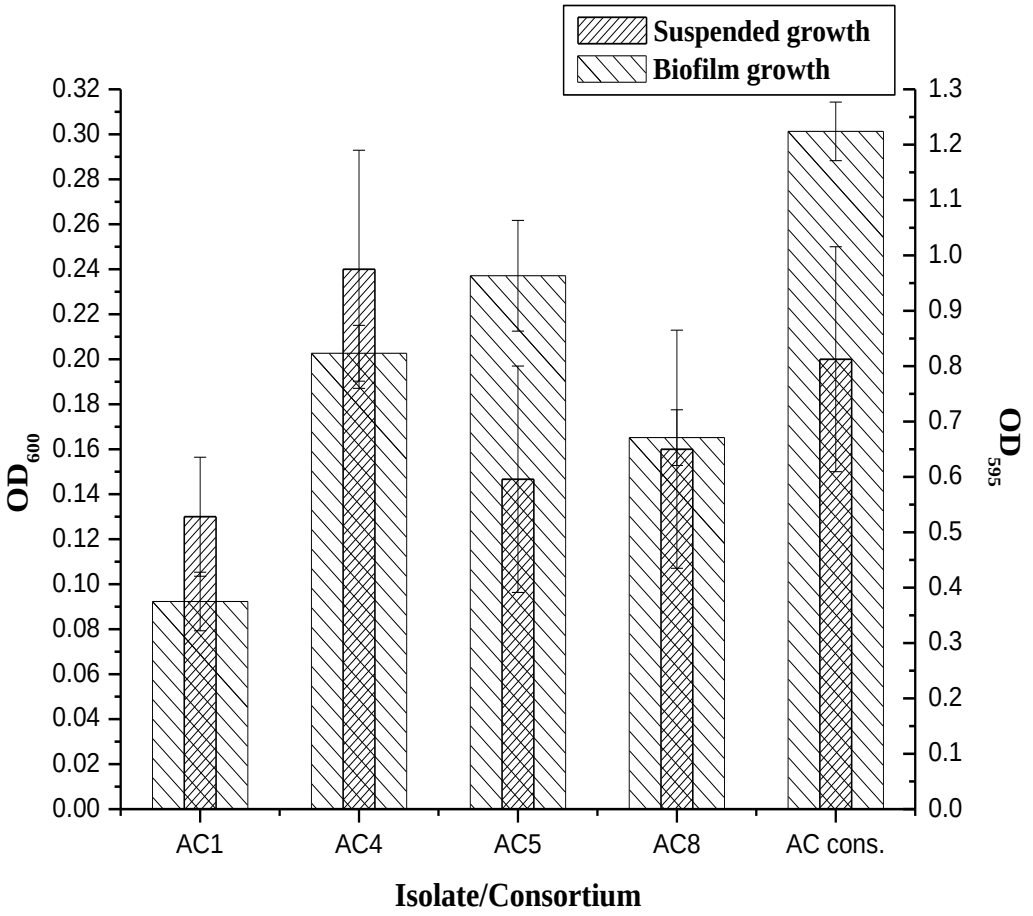


Figure 5.10. Biofilm forming ability of the AC consortium and its individual isolates in optimized AMS medium using microtiter plate biofilm assay. Error bars represent standard deviation from the mean, n = 3.

5.3.4. Biofilm forming ability of the AC consortium and its individual isolates in industrial effluent

The application of the biofilm forming ability of the isolates of AC consortium was carried out in DNR effluent using microtiter plate biofilm assay. As a result, it was observed that the individual isolates as well as AC consortium formed a strong and dense biofilm in the DNR effluent (Figure 5.11). The DNR effluent, being a complex medium, supported higher growth of the isolates of AC consortium as compared to the synthetic AMS medium. It was also noted that all the 4 individual isolates as well as AC consortium showed higher growth in biofilm mode as compared to the suspended mode. Hence, the biofilm of AC consortium was used for biotreatment of DNR effluent in the MBBR studies.

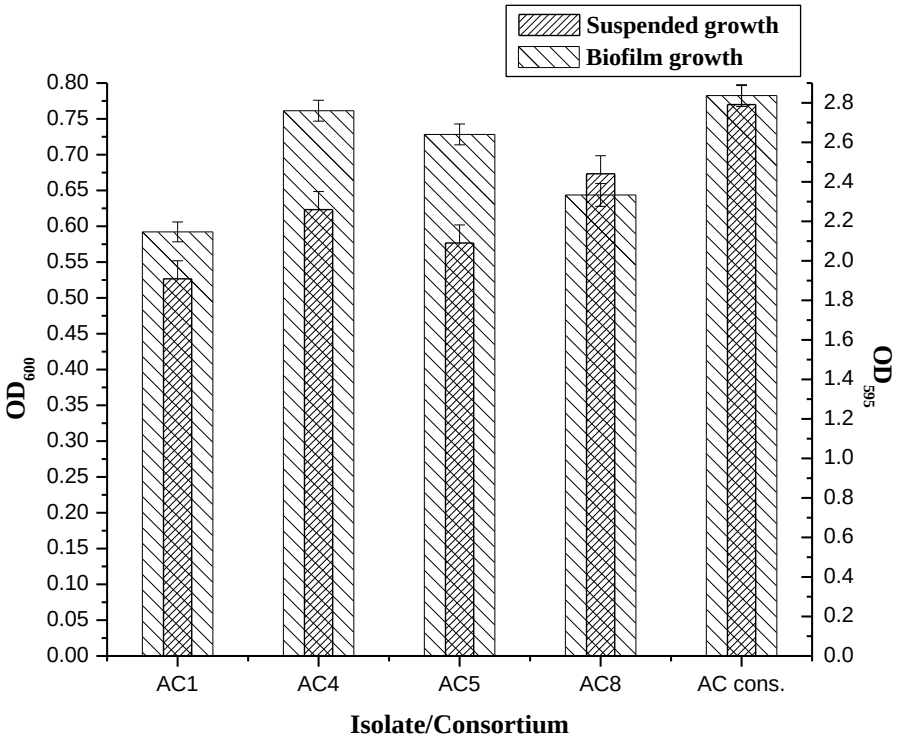


Figure 5.11. Biofilm forming ability of the AC consortium and its individual isolates in DNR effluent. Error bars represent standard deviation from the mean, n = 3.

5.3.5. MBBR studies

Biofilm reactors have been successfully used in wastewater treatment (Nicolella *et al*., 2000; Schmidt *et al*., 2004). In these industrial biofilm reactors, cell mass concentration as high as 30 – 40 g/l could be maintained (Nicolella *et al*., 2000). In properly designed MBBRs, the whole reactor volume is active, with no dead space or short circuiting (Rusten *et al*., 2006). As a result of superior efficiency, biofilm reactors are being used throughout the world with a number of full scale applications for industrial and wastewater treatment (Qureshi *et al*., 2005). High sludge densities are difficult to maintain in wastewater treatment plants, while MBBR has no such difficulty of biomass maintenance. Thus, having established the biofilm forming ability of AC consortium on synthetic substrata of microtiter plates and then carrier matrix using the modified AMS medium and later the effluent itself, biotreatment ability of the AC consortium was checked using MBBR. In this chapter, the MBBR was introduced with an aim to upgrade the suspended growth reactor. Performance of the MBBR was assessed when upgradation was done in the same tank previously used for the suspended growth process.

5.3.5.1. Treatability studies

Treatability studies carried out in the suspended growth reactor studies had already established the amenability of all the 4 industrial effluents, viz. DNR, ECO, COR and UPL, to biotreatment by the AC consortium (Chapter 4 section 4.3.1). Here, the treatability studies were mainly conducted to assess the COD reduction potential of the biomass of the isolates of AC consortium, when immobilized on the carriers. The treatability studies were carried out at flask level. The AC consortium reduced the initial COD of DNR effluent from 1600 mg/l to below detection limit in 108 h (Figure 5.12). Thus, it can be implied that the biofilm produced by the AC consortium was effective in treating the DNR effluent. Hence, MBBR could be used at a larger scale for biotreatment of DNR effluent using the AC consortium.

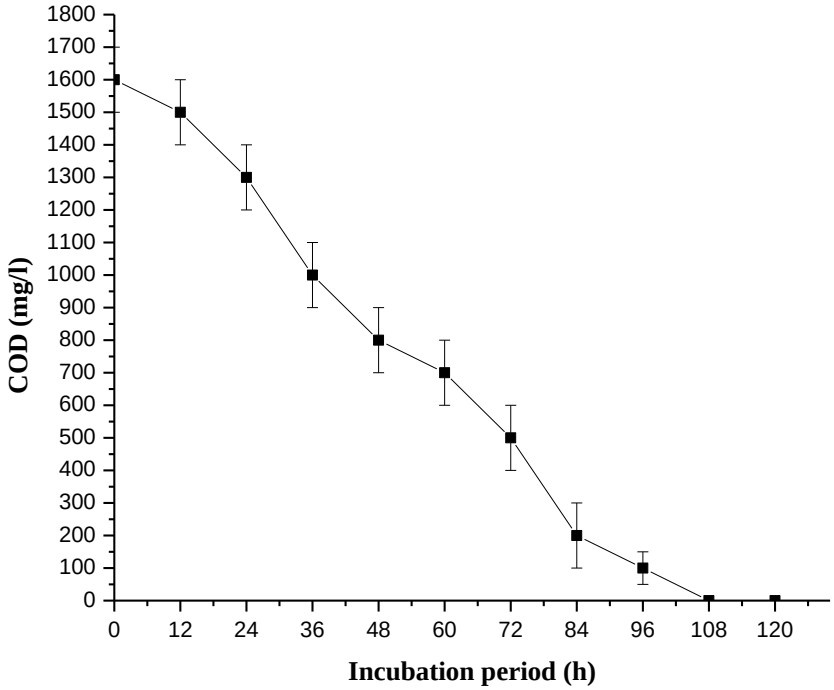


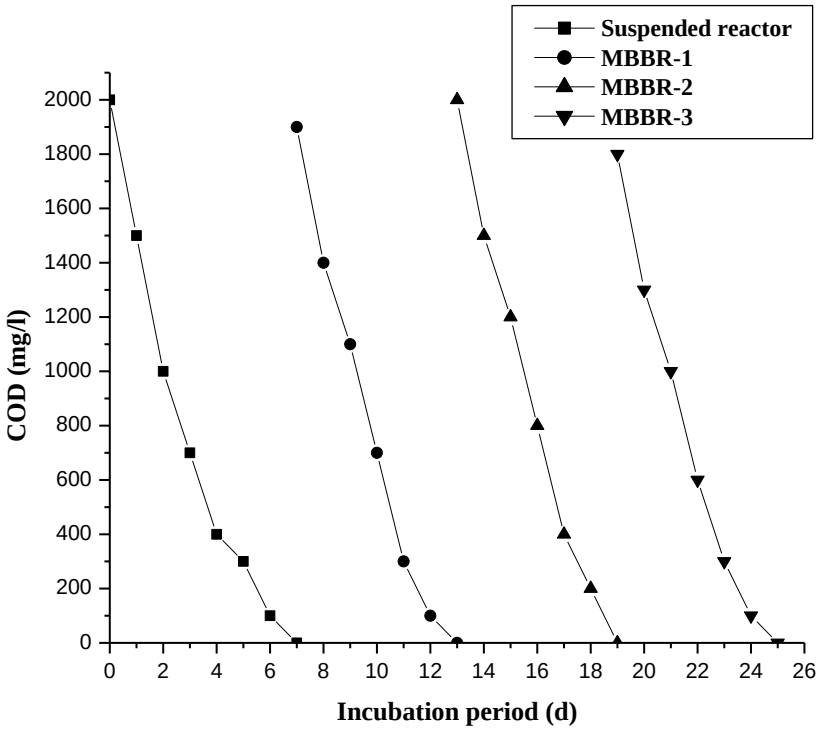
Figure 5.12. Biotreatment of DNR effluent by the AC consortium grown on biofilm carriers at flask level. Error bars represent standard deviation from the mean, n = 3.

5.3.5.2. Biotreatment of DNR effluent by the AC consortium using MBBR in batch mode

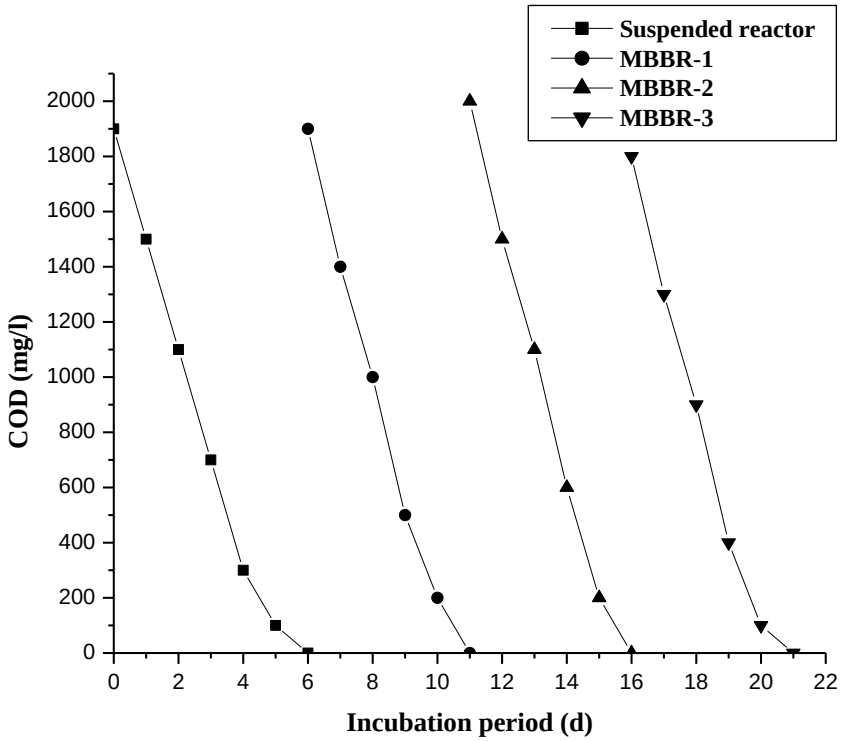
MBBR was set up in the same 6 l reactor used for the suspended growth reactor studies. Significantly, the same reactor tank was converted to MBBR, which is a major advantage of the process (Gapes and Keller, 2009). Bioreactors with up to 60

% of the working volume occupied by carriers have been studied and shown to perform effectively (Maurer *et al*., 2001; Welander *et al*., 1998). Hence, the MBBR was first filled with 60 % (v/v) biofilm carriers. The MBBR was designed so as to ensure uniform environmental conditions for each biofilm carrier. The reactor was run in a suspended mode for the first 7 d till the development of a mature biofilm on the carriers. It was observed that the AC consortium reduced the initial COD of the DNR effluent from 2000 mg/l to below detection limit in 7 d (Figure 5.13(a)). Thereafter, all the suspended sludge was discarded from the reactor in order to study the function of the biofilm attached on the biofilm carriers. The DNR effluent was changed in the reactor at an interval of 6 d for 3 consecutive runs. It was observed that the AC consortium reduced the initial COD of the DNR effluent from 1900 mg/l to below detection limit in 6 d for 3 runs (Figure 5.13(a)). 100 % COD reduction by AC consortium was obtained in 7 d in the suspended reactor, while the same was obtained in 6 d in the MBBR with a COD removal rate of 316.67 mg/l/d. The biomass obtained on the biofilm carriers was 802 mg/l and that suspended in the reactor was 3721 mg/l.

Further, in order to improve the performance of MBBR using the minimum quantity of biofilm carriers, the MBBR was first filled with 30 % (v/v) biofilm carriers in the next set of experiments. The reactor was run in a suspended mode for the first 6 d till the development of a mature biofilm on the carriers. It was observed that the AC consortium reduced the initial COD of the DNR effluent from 1900 mg/l to below detection limit in 6 d (Figure 5.13(b)). Thereafter, all the suspended sludge was removed from the reactor. The DNR effluent was changed in the reactor at an interval of 5 d for 3 consecutive runs. It was observed that the AC consortium reduced the initial COD of the DNR effluent from 1900 mg/l to below detection limit in 5 d for 3 runs (Figure 5.13(b)). 100 % COD reduction by the AC consortium was obtained in 6 d in the suspended reactor, while the same was obtained in 5 d in the MBBR with a higher COD removal rate of 380 mg/l/d, as compared to that obtained in the MBBR with 60 % (v/v) biofilm carriers. The biomass obtained on the biofilm carriers was 1320 mg/l and that suspended in the reactor was 3217 mg/l. Thus, it was concluded that the MBBR supported higher biodegradation of the DNR effluent than the suspended reactor.



(a)



(b)

Figure 5.13. Biotreatment of DNR effluent by the AC consortium using MBBR with (a) 60 % (v/v) and (b) 30 % (v/v) biofilm carriers. Run 1: Suspended reactor, Run 2: MBBR-1, Run 3: MBBR-2 and Run 4: MBBR-3

High shear stress from water circulation and carrier collision helps control biofilm development and stabilizes the reactor system (van Loosdrecht *et al*., 1995). The ideal biofilm in the moving bed process is thin and evenly distributed over the surface of the carrier; hence, excess sludge is not accumulated in the reactor (Rusten *et al*., 2006). Dupla *et al*. (2006) optimized a modified downflow MBBR for seawater denitrification in terms of its operating conditions (45^o retention grid inclination and a 30 % filling ratio) and choice of carrier, resulting in one of the highest reported volumetric and surface specific rates (27 $gN/m^2/d$) for a submerged MBBR without backwashing. In the MBBR, there is a combination of suspended and attached biomass. The robustness observed when starvation and shock loads are introduced into the moving bed may be due to the presence of both, attached and suspended biomass that take part in complementary tasks (Moreno-Andrade *et al*., 2009). Similar kind of robustness to shock loads was shown by the MBBR using AC consortium. Moreover, the biomass on the carriers worked efficiently on 3 recycles of the biofilm carriers. The washout of active biomass was avoided due to the fixed biofilm on the carriers. Also, in the case of industrial effluents where biomass growth is slow, the suitability of MBBR is more emphasized.

5.3.5.3. Biotreatment of different industrial effluents by the AC consortium using MBBR in continuous mode

The MBBR (Figure 5.14) was run in a continuous mode for 120 d to treat the 4 selected industrial effluents consecutively. The carriers containing the biofilm of AC consortium were transferred into the MBBR, thus confirming that biotreatment of the industrial effluents was majorly carried out by the biofilm and not the sludge. Different effluents were used in each consecutive run performed by the same biofilm carriers of AC consortium. The initial COD of the DNR effluent of 1700 mg/l was reduced to below permissible limit when run continuously for 60 d, followed by the COD reduction of the ECO effluent from 1300 mg/l to below permissible limit for the next 20 d, further followed by the COD reduction of the COR effluent from 1100 mg/l to below permissible limit for the next 20 d and lastly the COD reduction of the UPL effluent from 1400 mg/l to below permissible limit for the last 20 d in continuity (Figure 5.15). The biofilm of AC consortium adapted to the continuous mode of MBBR for the first 15 d, thereafter the MBBR ran in a steady state for the remaining time period with higher COD removal rate, even with the change of effluents. In all the 4 cases, the COD was maintained at below permissible limit through most of the reactor run, as can be seen in Figure 5.15.

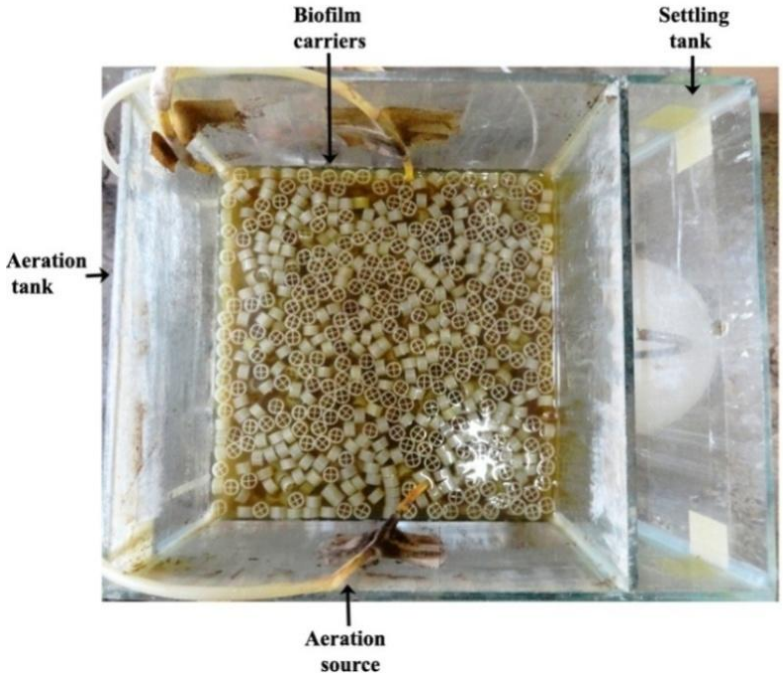


Figure 5.14. A MBBR developed for biotreatment of different industrial effluents by the AC consortium

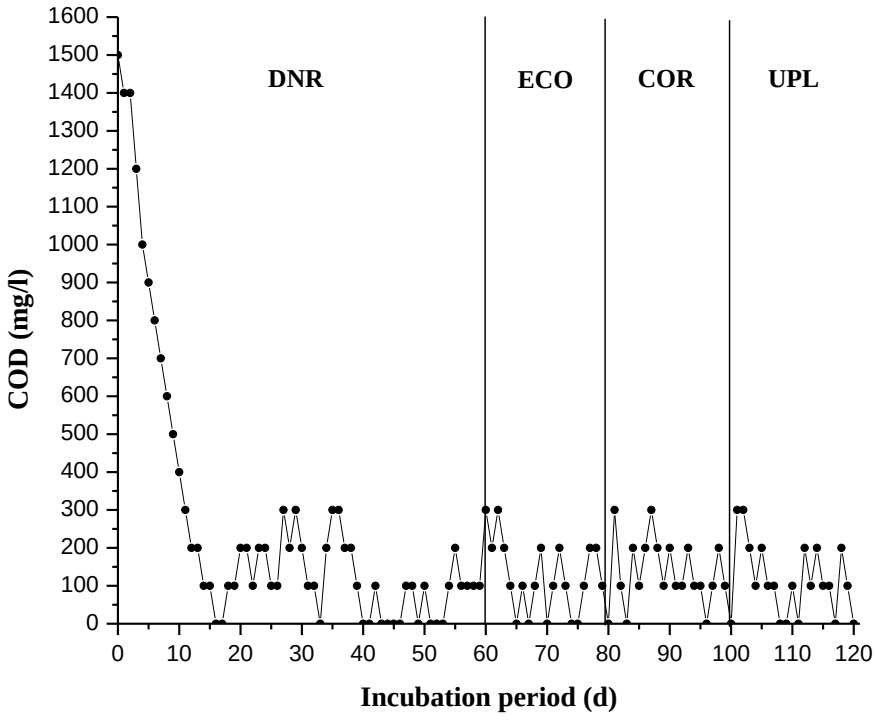


Figure 5.15. COD reduction of DNR, ECO, COR and UPL effluents by the AC consortium using continuous MBBR

The first step to biodegrade an industrial effluent is the acclimatization of the microorganisms. In the present study, the MBBR was subjected to shock loading of 1700 mg/l of DNR effluent, 1300 mg/l of ECO effluent, 1100 mg/l of COR effluent and 1400 mg/l of UPL effluent in a sequential manner. The organic loading rates of DNR, ECO, COR and UPL effluents were 0.41, 0.31, 0.26 and 0.34 kg COD/m^3/d respectively, which were found to be comparable among the 4 effluents. The COD removal rates for DNR, ECO, COR and UPL effluents were 113.33, 260, 366.67 and 175 mg/l/d respectively. Thus, the MBBR showed great robustness against shock loadings, as was also reported by Moreno-Andrade *et al*. (2009).

Jing *et al*. (2009) showed that the coking-plant wastewater could be effectively treated using moving bed biofilm sequencing batch reactor (MBBSBR) with 92.9 % of COD removal efficiency at an organic loading rate of 0.449 kg COD/m^3/d. In comparison, the AC consortium showed 100 % COD removal efficiency of different kinds of effluents at different organic loading rates. Thus, the MBBR utilizing AC consortium could be effectively used for the treatment of different kinds of industrial effluents.

In order to confirm biofilm formation on the carrier matrix, ESEM analysis was carried out at the end of the experiment. This analysis showed that the AC consortium grew well and formed a prominent biofilm on the carrier (Figure 5.16).

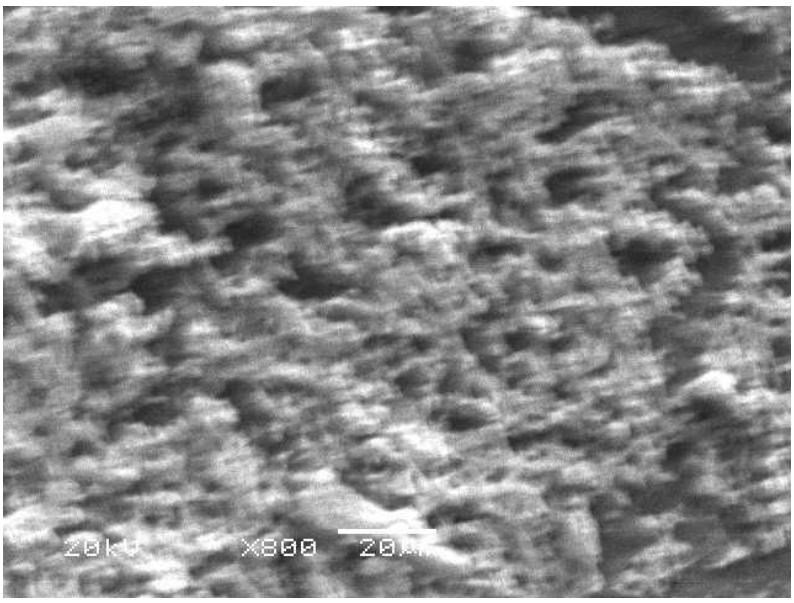


Figure 5.16. ESEM analysis of the Kaldnes type K1 biofilm carrier showing a mature biofilm formation

Table 5.2 shows the COD reduction ability of AC consortium in suspended reactor and MBBR. It was observed that the time taken by AC consortium for 100 % COD reduction of DNR effluent was the same in both the reactors. The reason of this slow COD reduction in MBBR was a high organic loading rate and a new environment exerted on the biofilm, since the MBBR was initiated with the DNR effluent, indicating that the biofilm attached on the carriers required acclimatization to the DNR effluent after it was subjected to a new environment. Thereafter, the COD reduction ability of the biofilm of AC consortium increased gradually and, hence, 100 % COD reduction of ECO, COR and UPL effluents was obtained in very less time in MBBR as compared to the suspended reactor. The reason for this higher COD reduction ability of the AC consortium was use of the same biofilm carriers for treating all the 4 effluents successively in MBBR, as opposed to the use of a fresh inoculum of suspended bacteria for treating all the 4 effluents individually in suspended reactor. Therefore, the bench scale operation of MBBR has led to an efficient approach of using the AC consortium for biotreatment of a variety of industrial effluents, as it showed an improvement in the performance of the bioreactor in terms of COD reduction.

Table 5.2. COD reduction performance of AC consortium in suspended reactor and MBBR

Effluent	100 % COD reduction (d)	
	Suspended reactor	MBBR
DNR	15	15
ECO	9	5
COR	10	3
UPL	10	8

After analyzing the potential of the AC consortium for wastewater treatment in an activated sludge process, the biotreatment potential of AC consortium was studied using MBBR. The isolates of AC consortium showed a complete biofilm formation when present as a consortium. The AC consortium showed the ability to form biofilm on glass, polystyrene and polyethylene surfaces. Biofilm forming ability of the AC consortium was enhanced by media optimization. The optimized medium was then used to develop the biofilm of AC consortium on Kaldnes type K1 biofilm carriers to be used for MBBR. The MBBR studies showed efficient biotreatment of 4 different kinds of industrial effluents by the biofilm formed by AC consortium. Hence, the AC consortium can be effectively applied for biotreatment of various kinds of industrial effluents using MBBR.

5.4. References

Andreottola, G., Foladori, P., Ragazzi, M., 2000. Upgrading of a small wastewater treatment plant in a cold climate region using a moving bed biofilm reactor (MBBR) system. *Water science and technology*, 41, 177-185.

Barbera, M.J., Mateo, E., Monkaityte, R., Constanti, M., 2011. Biodegradation of methyl *tert*-butyl ether by newly identified soil microorganisms in a simple mineral solution. *World Journal of Microbiology and Biotechnology*, 27, 813-821.

Bonomo, L., Pastorelli, G., Quinto, E., Rinaldi, G., 2000. Tertiary nitrification in pure oxygen moving bed biofilm reactors. *Water science and technology*, 41, 361-368.

Dupla, M., Comeau, Y., Parent, S., Villemur, R., Jolicoeur, M., 2006. Design optimization of a self-cleaning moving-bed bioreactor for seawater denitrification. *Water research*, 40, 249-258.

Gapes, D.J., Keller, J., 2009. Impact of oxygen mass transfer on nitrification reactions in suspended carrier reactor biofilms. *Process Biochemistry*, 44, 43-53.

Jadhav, T.S., Faldu, N.J., Patel, P., Narolkar, S.N., Nerurkar, A.S., 2005. *Alcaligenes faecalis* kw-A biofilm for denitrification of nitrate-rich effluent. *Indian journal of experimental biology*, 43, 542-547.

Jing, J., Feng, J., Li, W., Xu, Y., 2009. Removal of COD from coking-plant wastewater in the moving-bed biofilm sequencing batch reactor. *Korean Journal of Chemical Engineering*, 26, 564-568.

Johnson, C., Page, M., Blaha, L., 2000. Full scale moving bed biofilm reactor results from refinery and slaughter house treatment facilities. *Water science and technology*, 41, 401-407.

Lin, Y.-H., 2008. Kinetics of nitrogen and carbon removal in a moving-fixed bed biofilm reactor. *Applied Mathematical Modelling*, 32, 2360-2377.

van Loosdrecht, M.C.M., Eikelboom, D., Gjaltema, A., Mulder, A., Tijhuis, L., Heijnen, J.J., 1995. Biofilm structures. *Water science and technology*, 32, 35-43.

Maurer, M., Fux, C., Graff, M., Siegrist, H., 2001. Moving-bed biological treatment (MBBT) of municipal wastewater: denitrification. *Water science and technology*, 43, 337-344.

Moreno-Andrade, I., Buitron, G., Vargas, A., 2009. Effect of starvation and shock loads on the biodegradation of 4-chlorophenol in a discontinuous moving bed biofilm reactor. *Applied biochemistry and biotechnology*, 158, 222-230.

Nicolella, C., van Loosdrecht, M., Heijnen, S.J., 2000. Particle-based biofilm reactor technology. *Trends in Biotechnology*, 18, 312-320.

Qureshi, N., Annous, B.A., Ezeji, T.C., Karcher, P., Maddox, I.S., 2005. Biofilm reactors for industrial bioconversion processes: employing potential of enhanced reaction rates. *Microbial Cell Factories*, 4, 24.

Rusten, B., Hellstrm, B., Hellstrm, F., Sehested, O., Skjelfoss, E., Svendsen, B., 2000. Pilot testing and preliminary design of moving bed biofilm reactors for nitrogen removal at the FREVAR wastewater treatment plant. *Water science and technology*, 41, 13-20.

Rusten, B., Eikebrokk, B., Ulgenes, Y., Lygren, E., 2006. Design and operations of the Kaldnes moving bed biofilm reactors. *Aquacultural engineering*, 34, 322-331.

Schmidt, J., Batstone, D., Angelidaki, I., 2004. Improved nitrogen removal in upflow anaerobic sludge blanket (UASB) reactors by incorporation of Anammox bacteria into the granular sludge. *Water Science & Technology*, 49, 69-76.

Srinandan, C.S., Jadav, V., Cecilia, D., Nerurkar, A.S., 2010. Nutrients determine the spatial architecture of *Paracoccus* sp. biofilm. *Biofouling*, 26, 449-459.

Tomar, M., 1999. Quality assessment of water and wastewater. CRC press, Florida, USA.

Welander, U., Henrysson, T., Welander, T., 1998. Biological nitrogen removal from municipal landfill leachate in a pilot scale suspended carrier biofilm process. *Water research*, 32, 1564-1570.

Whittenbury, R., Phillips, K.C., Wilkinson, J.F., 1970. Enrichment, isolation and some properties of methane-utilizing bacteria. *Journal of General Microbiology*, 61, 205-218.

Chapter 6

Summary

Characterization of a methylotrophic bacterial consortium and its potential in treatment of industrial effluents

1. Construction of a bacterial consortium for COD reduction of industrial effluents and characterization of its individual isolates

- 118 aerobic, heterotrophic, mesophilic bacterial isolates were obtained from the samples collected from a fertilizer company, domestic sewage treatment plant (STP) and common effluent treatment plant (CETP).
- The isolates were screened on the basis of fusel oil (mainly containing methanol) utilization. 24 isolates showing higher growth on fusel oil were selected for the further studies.
- The 24 selected isolates were further screened on the basis of their growth in DNR effluent containing fusel oil (1 %, v/v). The isolates showing higher growth were selected to prepare 2 mixed bacterial consortia, viz. FW consortium consisting of the isolates FTE3, FTE8, FTE15, FTE22 and WAS2, and AC consortium consisting of the isolates AC1, AC4, AC5 and AC8.
- AC consortium, FW consortium, DNR A sludge, DNR B sludge and Wadi activated sludge were screened for their biotreatment potential. The AC consortium showed the maximum biotreatment potential and, hence, was selected for the further studies.
- The growth curves and specific growth rates of all the 4 isolates of AC consortium were found to be comparable, implying that they grew together in the form of AC consortium.
- The isolates of AC consortium were able to grow on methanol as a sole carbon source and they showed higher methanol utilization ability when present together as compared to their individual activities.
- The ability of the isolates of AC consortium to use methanol was also determined during their growth in DNR effluent by gas chromatography. More than 95 % methanol biodegradation was obtained using isolates AC1 (99 %), AC4 (99 %), AC5 (99 %) and AC8 (100 %), confirming their methylotrophic nature.
- PCR amplification of the partial *mxaF* gene, encoding the methanol dehydrogenase enzyme in methylotrophs, confirmed the presence of about 550 bp sized partial *mxaF* gene in all the isolates of AC consortium.

- The isolates of AC consortium were identified on the basis of their biochemical characters and 16S rRNA gene sequencing. Thus, isolate AC1 was identified as *Bordetella petrii* AC1, isolate AC4 as *Bacillus licheniformis* AC4, isolate AC5 as *Salmonella subterranea* AC5 and isolate AC8 as *Pseudomonas stutzeri* AC8.
- Phylogenetic analysis of the isolates of AC consortium indicated that *B. petrii* AC1 was phylogenetically most closely related to *B. petrii* AJ870969, *B. licheniformis* AC4 to *B. licheniformis* EU256500, *S. subterranea* AC5 to *S. subterranea* AY373829 and *P. stutzeri* AC8 to *P. stutzeri* GU339239.
- Differential carbon substrate utilization pattern of the isolates of AC consortium confirmed their facultative methylotrophic nature. Among the 10 single carbon substrates provided as sole carbon source, *B. petrii* AC1 was the best of all the 4 isolates in that it showed growth on 8 out of 10 substrates, while *B. licheniformis* AC4, *S. subterranea* AC5 and *P. stutzeri* AC8 could grow on 7 out of 10 substrates. All the isolates showed growth on methanol and formaldehyde. Methyl amine, methyl bromide, methyl chloride and methyl fluoride were utilized by at least 3 of the 4 isolates. Among the multi carbon substrates tested for sole carbon sources, ethanol, N-propanol, isopropanol, N-butanol and fusel oil were growth substrates for all the isolates of AC consortium; while 2-butanol, glucose, fructose and L-glutamate served as substrates for at least 3 out of the 4 members of AC consortium.
- The metabolic diversity of the isolates of AC consortium, further studied in terms of their ability to utilize a variety of industrially important alcohols, demonstrated that while the AC consortium and its individual members could grow on all the alcohols tested, *B. petrii* AC1, *P. stutzeri* AC8 and AC consortium showed higher utilization of methanol, ethanol, 1-propanol and 2-propanol; *B. licheniformis* AC4 showed higher utilization of methanol and ethanol; and *S. subterranea* AC5 showed higher utilization of methanol, ethanol and 1-propanol as compared to other alcohols tested.
- The biodegradation ability of the AC consortium was further checked on different carbon substrates and xenobiotics in terms of their growth and COD reduction. It was observed that out of the 15 substrates used for this study, the AC consortium showed 100 % degradation of acetate, succinate and pyruvate; methanol, ethanol, propanol, 2-propanol, 2-butanol and acetone were maximally utilized at > 50 %;

whereas butanol, formaldehyde, *tert*-amyl alcohol, benzene, xylene and toluene were utilized at < 50 %.

- ❖ The methanol dehydrogenase present in the isolates of AC consortium was in the range of 0.16 - 0.56 units/ml with the specific activity in the range of 0.48 - 0.87 units/mg.
- ❖ Methanol tolerance of the isolates of AC consortium, when analyzed, showed that the isolates of AC consortium could tolerate methanol up to 1.2 g/l, which is a highly toxic concentration of methanol, thus, implying that the AC consortium could be used for treatment of industrial effluents containing high concentrations of methanol.

2. Biodegradation of xenobiotics by the AC consortium

2A. Methyl *tert*-Butyl Ether (MTBE) biodegradation by the AC consortium

- ❖ The isolates of AC consortium could grow on all the soluble xenobiotics selected. Among the soluble xenobiotics tested, *B. petrii* AC1 showed higher utilization of 2-chloroethanol (CE), MTBE, trimethylamine hydrochloride (TMAH) and allyl chloride; *B. licheniformis* AC4 of *tert*-amyl methyl ether (TAME), MTBE and allyl chloride; *S. subterranea* AC5 of MTBE; *P. stutzeri* AC8 of MTBE and allyl chloride, and AC consortium of CE, TAME, MTBE, TMAH and allyl chloride.
- ❖ MTBE was maximally utilized by AC consortium and its individual isolates. Hence, MTBE was selected for the further xenobiotic biodegradation studies.
- ❖ MTBE biodegradation ability of the AC consortium and its individual isolates, when checked, indicated that the AC consortium was more effective than its individual members in reducing the COD of MTBE containing medium from 700 mg/l to below detection limit in 120 h, indicating its higher potential to biodegrade MTBE.
- ❖ The effect of different cations of MM2 medium, viz., Mg^{2+}, Ca^{2+}, Mn^{2+}, Na^{+} and Fe^{2+} on MTBE utilization by the isolates of AC consortium showed that Mg^{2+} at 0.3 g/l was statistically significant for maximum utilization of MTBE by AC consortium and its individual isolates, except *S. subterranea* AC5 that showed maximum growth on MTBE at the Mg^{2+} concentration of 0.2 g/l.

- All the AC isolates showed maximum growth at the MTBE concentration of 4.5 g/l, whereas the AC consortium showed maximum growth at higher MTBE concentration of 7.0 g/l.
- The AC consortium and *P. stutzeri* AC8 showed similar and maximum growth on MTBE as compared to the other isolates, viz. *B. petrii* AC1, *B. licheniformis* AC4 and *S. subterranea* AC5 individually. Elimination of *P. stutzeri* AC8 from the AC consortium drastically affected the growth of AC consortium and thereby MTBE utilization.
- The growth and COD reduction ability of the AC consortium and its individual isolates in the optimized MM2 medium increased as compared to that obtained in the original medium. The GC analysis showed that the initial MTBE concentration of 7.4 g/l was reduced to 0.13 g/l by *B. petrii* AC1, 0.14 g/l by *B. licheniformis* AC4, 0.15 g/l by *S. subterranea* AC5, 0.12 g/l by *P. stutzeri* AC8 and 0.12 g/l by AC consortium.
- The AC consortium could also grow effectively on TBA, the first metabolic intermediate of MTBE, and reduce its COD to below detection limit, indicating its potential to biodegrade TBA.
- The MM2 medium supplemented with the cations, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+} and Na^{+}, at 4 % concentration significantly enhanced the growth of AC consortium and its individual members on TBA.
- *B. petrii* AC1 and *B. licheniformis* AC4 showed maximum growth on TBA at the concentration of 7.8 g/l and 5.4 g/l respectively, while *S. subterranea* AC5, *P. stutzeri* AC8 and AC consortium showed maximum growth on TBA at the concentration of 3.9 g/l.
- TBA utilization by the individual isolates and AC consortium showed considerable increase in terms of their growth and COD reduction on optimization of MM2 medium as compared to that without optimization. The GC analysis showed that the initial TBA concentration of 7.75 g/l was reduced to 0.47 g/l by *B. petrii* AC1, 0.53 g/l by *B. licheniformis* AC4, 0.54 g/l by *S. subterranea* AC5, 0.49 g/l by *P. stutzeri* AC8 and 0.47 g/l by AC consortium in the optimized medium.
- Detection of metabolic intermediates of MTBE on its biodegradation by the AC consortium, analyzed using GC-MS, indicated that MTBE was completely

utilized by AC consortium and neither TBA nor TBF were accumulated during its biodegradation.

- The AC consortium reduced the initial COD of the MTBE containing synthetic effluent from 650 mg/l to below detection limit in 5 h at flask level, from 950 mg/l to below detection limit in 78 h at batch reactor level and from 1000 mg/l to below detection limit in 10 d at continuous reactor level. Hence, the AC consortium has a strong potential for treatment of MTBE containing effluents.

2B. 1,2-Dichloroethane (DCE) biodegradation by the AC consortium

- The AC consortium and its individual members could utilize all the insoluble xenobiotics tested in their vapor phase, except 4-chloroaniline where growth of all the isolates and AC consortium was lowest. However, the AC consortium and its individual isolates showed maximum utilization of DCE and, hence, DCE was selected for the further xenobiotic degradation studies.
- The AC consortium could grow on DCE as well as reduce the COD of DCE containing medium from 750 mg/l to below detection limit in 120 h.
- Out of all the cations, viz. Mg^{2+}, Ca^{2+}, Mn^{2+}, Na^{+} and Fe^{2+}, tested, Mg^{2+} at the concentration of 0.5 g/l was statistically significant for the maximum utilization of DCE by the individual isolates and AC consortium.
- Out of the different concentrations of DCE tested, DCE at 12.5 g/l was statistically significant for maximum growth of the individual isolates and AC consortium.
- As compared to the AC consortium, the combinations in which one of its members was eliminated showed a sizeable decrease in their growth on DCE.
- Enhancement in DCE utilization by the individual isolates and AC consortium was obtained in terms of their growth and COD reduction on media optimization. The GC analysis showed that the initial DCE concentration of 12.56 g/l was reduced to 6.06 g/l by *B. petrii* AC1, 6.97 g/l by *B. licheniformis* AC4, 5.58 g/l by *S. subterranea* AC5, 5.48 g/l by *P. stutzeri* AC8 and 4.97 g/l by AC consortium in the optimized medium.
- All the isolates and AC consortium could also grow on and biodegrade CE, the first metabolic intermediate of DCE.

- Out of all the nutrient conditions tested, it was shown that the addition of Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+} and Na^{+} together at 4 % concentration in MM2 medium had a statistically significant effect on the growth of AC consortium and its individual members on CE.
- *B. petrii* AC1, *S. subterranea* AC5, *P. stutzeri* AC8 and AC consortium showed maximum growth on CE at 6.0 g/l, while *B. licheniformis* AC4 showed maximum growth on CE at 8.4 g/l.
- CE utilization by the individual isolates and AC consortium was enhanced in terms of their growth and COD reduction on media optimization. The GC analysis showed that the initial CE concentration of 8.4 g/l was reduced to 0.44 g/l by *B. petrii* AC1, 0.35 g/l by *B. licheniformis* AC4, 0.35 g/l by *S. subterranea* AC5, 0.46 g/l by *P. stutzeri* AC8 and 0.34 g/l by AC consortium in the optimized medium.
- The detection of metabolic intermediates of DCE on its biodegradation by the AC consortium, analyzed using GC-MS, indicated that DCE was completely utilized by AC consortium and CE was not accumulated during its biodegradation.

3. Biotreatment of different industrial effluents using AC consortium

- The biodegradability indices of all the 4 effluents selected for the biotreatment studies, viz. DNR effluent obtained from a fertilizer company, ECO effluent from common effluent treatment plant, COR effluent from a pesticide company and UPL effluent from a chemical company, were in the range of 0.3 – 0.6.
- The AC consortium, used as a special microbial seed for biotreatment of these 4 effluents, showed statistically significant COD reduction of DNR effluent from 770 mg/l to below detection limit in 8 h, of ECO effluent from 1000 mg/l to below detection limit in 9 h, of COR effluent from 1600 mg/l to below detection limit in 12 h and of UPL effluent from 1600 mg/l to below detection limit in 8 h in the flask level treatability studies.
- In the flask level treatability studies, the AC consortium showed 100 % COD reduction of DNR effluent in 8 h, ECO effluent in 9 h, COR effluent in 12 h and UPL effluent in 8 h, as compared to 48, 50, 50 and 56 % COD reduction of DNR, ECO, COR and UPL effluents respectively in 12 h by the indigenous microflora of the respective industrial effluents.

- The AC consortium showed statistically significant COD reduction of DNR effluent from 1600 mg/l to below detection limit in 60 h, of ECO effluent from 950 mg/l to below detection limit in 72 h, of COR effluent from 1850 mg/l to below detection limit in 96 h and of UPL effluent from 1900 mg/l to below detection limit in 72 h at batch reactor level.
- In the batch reactor, the AC consortium showed 100 % COD reduction of DNR effluent in 60 h, ECO effluent in 72 h, and COR and UPL effluents in 96 h, as compared to 56, 68, 57 and 57 % COD reduction of DNR, ECO, COR and UPL effluents respectively by their indigenous microflora.
- The environmentally important parameters like BOD, pH, MLSS, MLVSS, SVI and F/M ratio of all the 4 effluents were also estimated for their efficient biotreatment by the AC consortium. BOD of the effluents was reduced in the range of 20 – 27 mg/l, pH in the range of 7.0 – 7.6, MLSS and MLVSS in the range of 103 – 132 mg/l, SVI in the range of 198 - 208 ml/g and F/M ratio in the range of 0.03 – 0.05.
- The AC consortium showed statistically significant COD reduction of DNR effluent from 1300 mg/l to below detection limit in 15 d, of ECO effluent from 1100 mg/l to below detection limit in 9 d, of COR effluent from 2000 mg/l to below detection limit in 10 d and of UPL effluent from 1800 mg/l to below detection limit in 10 d at continuous reactor level.
- In the continuous reactor, the AC consortium showed 100 % COD reduction of DNR effluent in 15 d, ECO effluent in 9 d, and COR and UPL effluents in 10 d, as compared to 62, 73, 65 and 67 % COD reduction of DNR, ECO, COR and UPL effluents respectively by their indigenous microflora.
- The desirable BOD value of 30 mg/l for discharge of effluents was obtained at the end of each reactor study; the pH obtained was in the fixed range of 5.5 – 9.0; the MLSS and MLVSS values were reduced approximately to 100 mg/l; the SVI obtained was approximately 100 ml/g, and the F/M ratio was lower than the stipulated value of 0.04.
- One-way ANOVA performed for the comparison of difference in % COD reduction obtained in control and AC consortium seeded flask, batch and continuous reactors showed a p-value of 0.005, indicating that there was a significant difference in the observed means of % COD reduction in all the 3 reactors at 95 % level of significance.

- The reactor studies were further scaled up to a pilot scale 2000 l continuous reactor level with DNR effluent. Here, the AC consortium reduced the COD of DNR effluent from about 1300 mg/l to below permissible limit and maintained it over a period of 120 d.
- The desirable MLSS and MLVSS values of 100 mg/l were obtained by the AC consortium for most of the reactor run. Hence, the AC consortium is an efficient microbial seed for biotreatment of DNR effluent at a larger scale.

4. Development of a bench scale Moving Bed Biofilm Reactor (MBBR) for COD reduction of industrial effluents by AC consortium

- Biofilm forming ability of the isolates of AC consortium was checked on a microscopic glass slide. All the isolates showed different stages of biofilm formation on microscopic examination of the glass slides. It was observed that the AC isolates formed a complete biofilm when present as a consortium.
- *B. licheniformis* AC4 and *P. stutzeri* AC8 showed highest biofilm formation, while *B. petrii* AC1 showed least in AMS medium using microtiter plate biofilm assay. The AC consortium showed a comparable biofilm formation to *B. licheniformis* AC4 and *P. stutzeri* AC8.
- Biofilm forming ability of the AC consortium, when checked on commercially available Kaldnes type K1 biofilm carriers in DNR effluent, showed that the AC consortium formed a strong biofilm on these carriers. Biofilm formation was higher on the inner surfaces of the biofilm carriers as compared to their outer surface.
- The methanol concentration of 0.5 % along with 0.01 % yeast extract added as a growth factor showed better growth and biofilm formation of the individual isolates and AC consortium.
- Out of potassium acetate, sodium succinate and methanol used as the carbon sources, potassium acetate at the concentration of 8 g/l was statistically significant for highest biofilm formation by the AC consortium and its individual isolates.
- Out of NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$ used as the nitrogen sources, NH_4Cl at the concentration of 1 g/l was statistically significant for highest biofilm formation by the AC consortium and its individual isolates.

- Out of KH_2PO_4 + Na_2HPO_4, KH_2PO_4 + K_2HPO_4, Na_2HPO_4 and KH_2PO_4 used as the phosphorus sources, KH_2PO_4 + Na_2HPO_4 at the concentration of 25 ml/l was statistically significant for highest biofilm formation by the AC consortium and its individual isolates.
- Out of the different concentrations of macronutrients, $CaCl_2$ at the concentration of 0.4 g/l, $MgSO_4$ at the concentration of 1.5 g/l and $FeCl_3$ at the concentration of 6 mg/l were statistically significant for highest biofilm formation by the AC consortium and its individual isolates.
- 3 %, 24 h old inoculum after an incubation period of 120 h supported highest biofilm formation by the AC consortium and its individual isolates.
- The optimized AMS medium composed of (per liter): potassium acetate, 8.0 g; NH_4Cl, 1.0 g; phosphate buffer (KH_2PO_4 + Na_2HPO_4), 25.0 ml; $MgSO_4$, 1.5 g; $CaCl_2$, 0.4 g; $FeCl_3$, 6.0 mg; trace element solution, 0.5 ml, pH, 6.8. The biofilm forming ability of the AC consortium and its individual isolates increased considerably in the optimized AMS medium as compared to the original AMS medium.
- The individual isolates as well as the AC consortium formed a strong and dense biofilm in the DNR effluent on a microtiter plate.
- The AC consortium reduced the initial COD of DNR effluent from 1600 mg/l to below detection limit in 108 h at flask level MBBR.
- 100 % COD reduction by AC consortium was obtained in 7 d in the suspended reactor, while the same was obtained in 6 d in the MBBR filled with 60 % (v/v) biofilm carriers, with the COD removal rate of 316.67 mg/l/d. The biomass obtained on the biofilm carriers was 802 mg/l and the biomass suspended in the reactor was 3721 mg/l.
- 100 % COD reduction was achieved by the AC consortium in 6 d in the suspended reactor, while the same was obtained in 5 d in the MBBR filled with 30 % (v/v) biofilm carriers, with a higher COD removal rate of 380 mg/l/d, as compared to that obtained in the MBBR with 60 % (v/v) biofilm carriers. The biomass obtained on the biofilm carriers was 1320 mg/l and the biomass suspended in the reactor was 3217 mg/l.
- The COD was maintained at below permissible limit in DNR, ECO, COR and UPL effluents during the course of MBBR run in a continuous mode for 120 d. The initial COD of the DNR effluent of 1700 mg/l was reduced to below

permissible limit when run continuously for 60 d, followed by the COD reduction of the ECO effluent from 1300 mg/l to below permissible limit for the next 20 d, further followed by the COD reduction of the COR effluent from 1100 mg/l to below permissible limit for the next 20 d and lastly the COD reduction of the UPL effluent from 1400 mg/l to below permissible limit for the last 20 d in continuity.

- The MBBR was subjected to shock loading of 1700 mg/l DNR effluent, 1300 mg/l ECO effluent, 1100 mg/l COR effluent and 1400 mg/l UPL effluent. The organic loading rates of DNR, ECO, COR and UPL effluents were 0.41, 0.31, 0.26 and 0.34 kg COD/m^3/d respectively. The COD removal rates for DNR, ECO, COR and UPL effluents were 113.33, 260, 366.67 and 175 mg/l/d respectively.
- ESEM analysis of the biofilm carrier showed that the AC consortium grew and formed a prominent biofilm on the carrier.
- The time taken by AC consortium for 100 % COD reduction of DNR effluent was the same in both suspended reactor and MBBR. Thereafter, the COD reduction ability of the biofilm of AC consortium increased gradually and, hence, 100 % COD reduction of ECO, COR and UPL effluents was obtained in very less time in MBBR as compared to the suspended reactor. Hence, different kinds of industrial effluents could be effectively treated by the AC consortium using MBBR.

Chapter 7
Conclusion

Conclusion

Industrial effluents have high COD, mainly contributed by toxic chemicals. COD enhancement is environmentally hazardous and needs to be addressed by developing a special microbial seed for reducing the COD of industrial effluents to below permissible limit. Methylotrophs are the bacteria which grow on compounds with no C-C bonds like methane, methyl halides, methanol, methylamine, methyl sulfides and formate as sole source of carbon and energy. They are known to biodegrade many toxic compounds including xenobiotics. Therefore, methylotrophic bacteria were selected for development of a consortium suitable as a microbial seed for biotreatment of diverse industrial effluents.

In order to construct a methylotrophic bacterial consortium for reducing the COD of industrial effluents, samples were collected from different wastewater treatment plants. 118 aerobic, heterotrophic bacterial isolates were obtained from different sources. Screening of the isolates on the basis of fusel oil (mainly containing methanol) utilization followed by screening on the basis of growth in DNR effluent finally resulted in a 4 membered mixed bacterial AC consortium which showed maximum biotreatment potential of the DNR effluent.

The isolate AC1 was identified as *Bordetella petrii* AC1, isolate AC4 as *Bacillus licheniformis* AC4, isolate AC5 as *Salmonella subterranea* AC5 and isolate AC8 as *Pseudomonas stutzeri* AC8 based on their phenotypic characteristics and 16S rRNA gene sequence analysis. *B. licheniformis* and *P. stutzeri* have been reported as methylotrophic in nature, but, this is the first report of methylotrophic *B. petrii* and *S. subterranea*. Phylogenetic analysis of the isolates of AC consortium showed that *B. petrii*, *B. licheniformis*, *S. subterranea* and *P. stutzeri* are widely distributed in nature.

All the 4 isolates of AC consortium were able to utilize methanol as the sole source of carbon and their methanol utilization ability increased when they were present together as a consortium. The presence of *mxaF* gene, the gene encoding methanol dehydrogenase, in the isolates of AC consortium further confirmed their methylotrophic nature. Methanol dehydrogenase activities of the isolates of AC consortium were higher than those reported. The isolates of AC consortium could tolerate highly toxic concentrations of methanol, implying that the AC consortium could be used for treatment of industrial effluents containing high concentrations of methanol. Furthermore, the isolates of AC consortium could grow on a variety of

single and multi carbon substrates. Additionally, they were also able to utilize different industrially important alcohols. Thus, the members of AC consortium were found to be facultative methylotrophs showing a broad metabolic diversity.

The biodegradation ability of the AC consortium was further checked on different carbon substrates and xenobiotics and it was observed that it showed highest biodegradation of methanol and ethanol, among the alcohols, and of acids like acetate, succinate and pyruvate. Even the xenobiotics tested, viz. benzene, xylene, toluene and *tert*-amyl alcohol, were appreciably degraded by the AC consortium, implying that the AC consortium had a broad biodegradation potential. Hence, it could be concluded that *B. petrii* AC1, *B. licheniformis* AC4, *S. subterranea* AC5 and *P. stutzeri* AC8 comprising the AC consortium were found to possess the potential of being applied for wastewater treatment.

Considering the biodegradation potential of the AC consortium, it was further used for biodegradation of both soluble and insoluble xenobiotics. Out of all the soluble xenobiotics tested, methyl *tert*-butyl ether (MTBE) was the best growth substrate for AC consortium and its individual isolates. The AC consortium was more effective than its individual members in COD reduction of MTBE containing medium, indicating its higher potential to biodegrade MTBE. The effect of different cations on MTBE biodegradation showed that Mg^{2+} at 0.3 g/l enhanced the MTBE biodegradation ability of the AC consortium and its individual isolates. Increasing concentrations of MTBE had a stimulatory effect on the isolates of AC consortium as they were able to grow on higher concentrations of MTBE, i.e. 7 g/l. The isolates of AC consortium contributed together towards MTBE degradation and absence of any 1 of them affected its performance. Optimization of different parameters led to enhancement in the MTBE biodegradation ability of the AC consortium and its individual isolates. The GC analysis implied that all the members of AC consortium contributed equally towards efficient MTBE biodegradation.

tert-Butyl alcohol (TBA) is an obligatory intermediate of MTBE, whose degradation can be a limiting step in MTBE metabolism. The isolates of AC consortium showed efficient TBA utilization which was higher by the AC consortium than its individual isolates. Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+} and Na^{+} present together at 4 % concentration in the nutrient medium showed a statistically significant effect on TBA biodegradation by the AC consortium and its individual isolates. 3.9 g/l TBA supported maximum growth of AC consortium and its individual isolates. After

optimization of different parameters, the TBA utilization ability of the AC consortium was gradually enhanced to show up to 94 % TBA biodegradation.

Absence of the metabolic intermediates of MTBE, viz. TBA and *tert*-butyl formate (TBF), on MTBE biodegradation by the AC consortium analyzed by GC-MS implied its complete biodegradation by the AC consortium. The AC consortium degraded MTBE containing synthetic effluent completely at a higher rate of 292 mg/l/d and 100 mg/l/d in batch and continuous reactors respectively as compared to those reported, demonstrating that it can serve as an effective microbial seed for biotreatment of MTBE containing industrial effluents.

The AC consortium and its individual members could grow on and utilize all the insoluble xenobiotics, but showed highest utilization of 1,2-dichloroethane (DCE). The isolates of AC consortium could biodegrade DCE effectively with Mg^{2+} at 0.5 g/l supporting highest DCE utilization by the AC consortium and its individual isolates. The AC consortium showed statistically significant growth on DCE at a high concentration of 12.5 g/l. Also, it was observed that the presence of all the isolates in the consortium was important for effective biodegradation of DCE. A marked enhancement in DCE biodegradation was reported by AC consortium and its individual isolates on optimization of different parameters. Up to 60 % DCE biodegradation was obtained using the AC consortium and its individual isolates in 120 h, implying that all the members of AC consortium were efficient in DCE biodegradation.

Just as TBA degradation might be the limiting step in MTBE metabolism, 2-chloroethanol (CE, the first metabolic intermediate of DCE) degradation might as well be the limiting step in DCE metabolism. Hence, the CE degradation capacity of the AC consortium showed that this was not the limiting step in DCE biodegradation and the AC consortium possessed the ability to degrade DCE beyond CE. The supplementation of Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+} and Na^{+} in the MM2 medium at 4 % concentration had a stimulatory effect on the growth of AC consortium and its members on CE. In comparison to the previous reports, the isolates of AC consortium degraded CE at a higher concentration of 8.4 g/l. A considerable increase in CE biodegradation by the AC consortium and its individual isolates was noted after optimization of different parameters. Up to 96 % CE degradation was obtained using the individual isolates and AC consortium in 120 h, implying that all the members of AC consortium contributed equally towards efficient CE biodegradation. No

metabolic intermediates of DCE, like CE, were accumulated on its biodegradation by the AC consortium, implying its complete biodegradation. The results obtained with the degradation studies of MTBE, TBA, DCE and CE indicate that the AC consortium showed promising potential for effective bioremediation of xenobiotics.

The biodegradability indices of DNR, COR, UPL and ECO effluents obtained from fertilizer, pesticide and chemical companies, and common effluent treatment plant respectively indicated that they required a special microbial seed for their efficient biotreatment. Suitability of the AC consortium as an efficient microbial seed for biotreatment of these 4 effluents proved the feasibility of the process at flask level, indicating that the AC consortium was suitable to biodegrade the carbon substrates and other oxidizable matter from all the selected effluents of diverse nature, since it showed 100 % COD reduction of all the 4 effluents. As all the 4 effluents selected for the study contained a variety of pollutants, the treatability studies proved the versatility of the AC consortium.

As compared to the indigenous microflora of the respective effluents, the AC consortium showed significant COD reduction of the 4 effluents when the process was scaled up to a 6 l batch reactor, demonstrating the potential of the AC consortium as a special seed for treatment of these effluents. Apart from COD, other environmentally important parameters like BOD, pH, MLSS, MLVSS, SVI and F/M ratio of all the 4 effluents at the end of the reactor run were also maintained below the permissible limit by the AC consortium for suitable discharge of the effluents. It was observed that the AC consortium was responsible for efficiently reducing the COD of DNR, ECO, COR and UPL effluents to below detection limit in minimum time period when the process was further scaled up to a 6 l continuous reactor. Estimation of other environmentally important parameters like BOD, pH, MLSS, MLVSS, SVI and F/M ratio of DNR, ECO, COR and UPL effluents at the end of the reactor run also suggested their efficient biotreatment by the AC consortium. Thus, the AC consortium treated 4 different kinds of industrial effluents within shorter duration than that reported in literature, implying its broad potential to treat various kinds of industrial effluents. One-way ANOVA implied that COD reduction by the AC consortium was significant in all the 4 effluents and the COD reduction ability of the AC consortium differed significantly for the 4 effluents at flask, batch and continuous reactor levels. The AC consortium also showed efficient biotreatment of DNR effluent in a pilot scale 2000 l continuous reactor run for 120 d.

The Moving Bed Biofilm Reactor (MBBR) has several advantages over suspended processes, the most important one being no need of sludge recycle. An equally important advantage is that the existing facility can be upgraded to MBBR without any modifications in the reactor design. Hence, the work was undertaken to upgrade the suspended growth activated sludge process to an attached growth MBBR using the AC consortium. Biofilm forming ability of the isolates of AC consortium, when checked, showed that the AC isolates formed a complete biofilm when present as a consortium on a microscopic glass slide. The isolates of AC consortium also showed biofilm forming ability on a microtiter plate. The AC consortium further showed efficient biofilm formation on commercially available Kaldnes type K1 biofilm carriers, which were used for the subsequent MBBR studies.

Methanol concentration was optimized and yeast extract was added as a growth substrate to the nutrient medium so as to enhance the biofilm forming ability of the individual isolates as well as AC consortium. Out of different carbon, nitrogen and phosphorus sources screened, potassium acetate, NH_4Cl and KH_2PO_4 + Na_2HPO_4 were statistically significant for biofilm formation by the AC consortium and its individual isolates. The optimized AMS medium, composed of potassium acetate, 8 g/l; NH_4Cl, 1 g/l; KH_2PO_4 + Na_2HPO_4, 25 ml/l; $CaCl_2$, 0.4 g/l; $MgSO_4$, 1.5 g/l, and $FeCl_3$, 6 mg/l, supported highest biofilm formation by the AC consortium and its individual isolates. 3 % 24 h old inoculum of AC consortium and its individual isolates when incubated for 120 h showed highest biofilm formation. The biofilm forming ability of *B. petrii* AC1, *B. licheniformis* AC4, *S. subterranea* AC5, *P. stutzeri* AC8 and AC consortium increased by approximately 2-fold on media optimization.

On analyzing the biofilm forming ability of the isolates of AC consortium in DNR effluent, it was observed that all 4 individual isolates as well as AC consortium showed higher growth in biofilm mode as compared to the suspended mode in a microtiter plate. These studies formed the basis for checking the biotreatment potential of AC consortium using MBBR.

It was observed that the carriers containing biofilm formed by the AC consortium were effective in treating the DNR effluent in the flask level treatability studies. Therefore, it was envisaged that MBBR could be used at a larger scale for biotreatment of DNR effluent using the AC consortium. The first set of MBBR experiments carried out using 60 % biofilm carriers concluded that the MBBR

supported comparable biodegradation as a suspended reactor. The subsequent experiments showed that 30 % biofilm carriers improved the MBBR, supporting higher biodegradation of the DNR effluent than the suspended reactor. Even though biomass on the carriers was less as compared to the suspended reactor, it proved to be more efficient in COD removal, emphasizing the fact that thinner biofilms are more efficient in biotreatment.

The AC consortium effectively reduced the COD of DNR, ECO, COR and UPL effluents to below permissible limit consecutively for a period of 120 d. In spite of subjecting the MBBR to shock loading of the 4 effluents, high COD removal rates were obtained by the AC consortium. The robustness observed when shock loads were introduced into the MBBR might be due to the presence of both, attached and suspended biomass that took part in complementary tasks. 100 % COD reduction of the 4 effluents was obtained in very short period in the MBBR as compared to the suspended reactor since the same biofilm carriers were used for treating all the 4 effluents successively in MBBR, as opposed to the use of a fresh inoculum of suspended bacteria for treating all the 4 effluents individually in suspended reactor. Hence, the MBBR with all its advantages was found to be more efficient in biotreatment of the various industrial effluents than the suspended reactor.

In conclusion, the 4 membered AC consortium consisting of diverse methylotrophic bacteria, viz. *B. petrii* AC1, *B. licheniformis* AC4, *S. subterranea* AC5 and *P. stutzeri* AC8, showed efficient biodegradation of hazardous xenobiotics like MTBE, TBA, DCE and CE. It was effective in biotreatment of different kinds of industrial effluents, containing the waste products of fertilizer, pesticide and chemical companies, using activated sludge process and more so using MBBR. Therefore, the AC consortium holds promise in treatment of effluents containing a broad spectrum of pollutants generated from diverse chemical industries.

Chapter 8

Publications and presentations

8.1. List of publications

1. **Krushi Hingurao** and Anuradha Nerurkar. A novel methylotrophic bacterial consortium for treatment of industrial effluents. **In communication.**

2. **Krushi Hingurao** and Anuradha Nerurkar. Biodegradation of methyl *tert*-butyl ether and its intermediate, *tert*-butyl alcohol, by a novel methylotrophic bacterial consortium. **Manuscript in preparation.**

3. **Krushi Hingurao** and Anuradha Nerurkar. Upgradation of an activated sludge process by a novel methylotrophic bacterial consortium using a moving bed biofilm reactor (MBBR). **Manuscript in preparation.**

8.2. List of presentations

1. **Krushi Hingurao** and Anuradha Nerurkar (2009) Development of a methylotrophic bacterial consortium for treatment of industrial effluent, at 50th Annual Conference of Association of Microbiologists of India, organized by National Chemical Laboratory, Pune, India.

2. **Krushi Hingurao**, Payal Shah, Kintu Vankar and Anuradha Nerurkar (2010) Characterization of a methylotrophic bacterial consortium for application in Moving Bed Biofilm Reactor (MBBR), at 79th Annual Meeting of the Society of Biological Chemists (India), organized by Indian Institute of Science, Bangalore, India.

3. **Krushi Hingurao**, Shivangi Dhebar and Anuradha Nerurkar (2012) Characterization of a methylotrophic bacterial consortium and its potential in treatment of industrial effluents, at Regional Science Congress, jointly organized by The Maharaja Sayajirao University of Baroda, Vadodara and Indian Science Congress Association (Baroda Chapter), India.