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 xenobiatics selected for the studies have been divided into 2 types: soluble and insoluble xenobiatics. 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
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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
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(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 °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) KNO₃, 1.0 g; K₂HPO₄, 1.0 g; MgSO₄·7H₂O, 0.2 g; CaCl₂·2H₂O, 0.02 g; MnCl₂·4H₂O, 0.002 g; NaMoO₄·2H₂O, 0.001 g; FeSO₄·7H₂O, 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 °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-amyI 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.
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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 (OD600) of the isolates of AC consortium in MM2 medium containing 1 % (v/v) MTBE supplemented with: (a) MgSO4.7H2O, 0.2 g/l; CaCl2.2H2O, 0.02 g/l; MnCl2.4H2O, 0.002 g/l, NaMoO4.2H2O, 0.001 g/l, and FeSO4.7H2O, 0.05 g/l; (b) MgSO4.7H2O, 0.2 g/l; (c) CaCl2.2H2O, 0.02 g/l; (d) MnCl2.4H2O, 0.002 g/l; (e) NaMoO4.2H2O, 0.001 g/l, (f) FeSO4.7H2O, 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 Mg2+, 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 Mg2+ 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): K2HPO4, 1.0 g; KNO3, 1.0 g; Yeast extract, 0.5 g, and MgSO4, 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 °C for 3 min and programmed at 10 °C/min to 160 °C. The injector and detector temperatures were 230 °C and 250 °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) MgSO₄·7H₂O, 0.2 g/l; (b) CaCl₂·2H₂O, 0.02 g/l; (c) MnCl₂·4H₂O.
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0.002 g/l; (d) NaMoO$_4$.2H$_2$O, 0.001 g/l; (e) FeSO$_4$.7H$_2$O, 0.05 g/l; (f) no cations and (g) MgSO$_4$.7H$_2$O, 0.2 g/l; CaCl$_2$.2H$_2$O, 0.02 g/l; MnCl$_2$.4H$_2$O, 0.002 g/l; NaMoO$_4$.2H$_2$O, 0.001 g/l, FeSO$_4$.7H$_2$O, 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 °C, which was maintained for 2 min, increased by 20 °C/min to 90 °C and then increased by 40 °C/min to 260 °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 °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 ºC for 3 min and programmed at 10 ºC/min to 160 ºC. 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 ºC and 230 ºC 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): K₂HPO₄, 1.0 g; KN₀₃, 1.0 g; Yeast extract, 0.5 g; MgSO₄, 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 ºC 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 ºC 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

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incubating at 37 °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 °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 1-2052, 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 OD₆₀₀); moderate growth on methylamine (0.07 – 0.11 OD₆₀₀), and poor growth on methacrylic acid and epichlorohydrin (0.03 – 0.06 OD₆₀₀) (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 OD₆₀₀) (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.
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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.
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.
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.
Chapter 3: Biodegradation of xenobiotics by the AC consortium

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

**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.](image)

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.

![Graph showing effect of different cations on growth of the AC consortium and its individual isolates on TBA.](image)

**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 Co²⁺ had a stimulatory effect on the growth of B. cepacia CIP 1-2052 and its ability to degrade TBA. However, in the present study, it was observed that Mg²⁺, Ca²⁺, Mn²⁺, Fe²⁺ 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 1-2052 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.
Chapter 3: Biodegradation of xenobiotics by the AC consortium

### Table 3A.1: TBA Utilization by the AC Consortium and Its Individual Isolates

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Sample</th>
<th>Retention time (min)</th>
<th>Area (nl<em>μV</em>sec)</th>
<th>TBA conc. (g/l)</th>
</tr>
</thead>
<tbody>
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<td>Control</td>
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<td>61453140</td>
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</tr>
<tr>
<td>b</td>
<td><em>B. petrii</em> AC1</td>
<td>0.61</td>
<td>3773684</td>
<td>0.47</td>
</tr>
<tr>
<td>c</td>
<td><em>B. licheniformis</em> AC4</td>
<td>0.61</td>
<td>4209816</td>
<td>0.53</td>
</tr>
<tr>
<td>d</td>
<td><em>S. subterranea</em> AC5</td>
<td>0.61</td>
<td>4282192</td>
<td>0.54</td>
</tr>
<tr>
<td>e</td>
<td><em>P. stutzeri</em> AC8</td>
<td>0.61</td>
<td>3882741</td>
<td>0.49</td>
</tr>
<tr>
<td>f</td>
<td>AC consortium</td>
<td>0.61</td>
<td>3755994</td>
<td>0.47</td>
</tr>
</tbody>
</table>

**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.](image-url)
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](image)

**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 cynisi*, 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 C₂H₂Cl₂. 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
Chapter 3: Biodegradation of xenobiotics by the AC consortium

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 °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 °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$_2$O, 0.2 g/l; (b) CaCl$_2$.2H$_2$O, 0.02 g/l; (c) MnCl$_2$.4H$_2$O, 0.002 g/l; (d) NaMoO$_4$.2H$_2$O, 0.001 g/l; (e) FeSO$_4$.7H$_2$O, 0.5 g/l; (f) no cation and (g) MgSO$_4$.7H$_2$O, 0.2 g/l; CaCl$_2$.2H$_2$O, 0.02 g/l; MnCl$_2$.4H$_2$O, 0.002 g/l; NaMoO$_4$.2H$_2$O, 0.001 g/l, and FeSO$_4$.7H$_2$O, 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$_2$HPO$_4$, 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 °C, which was maintained for 2 min, increased by 20 °C/min to 90 °C, and then increased by 40 °C/min to 260 °C. The injector and detector temperatures were 230 °C and 250 °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 °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) MgSO₄·7H₂O, 0.2 g/l; (b) CaCl₂·2H₂O, 0.02 g/l; (c) MnCl₂·4H₂O, 0.002 g/l; (d) NaMoO₄·2H₂O, 0.001 g/l; (e) FeSO₄·7H₂O, 0.05 g/l; (f) no cations, and (g) MgSO₄·7H₂O, 0.2 g/l; CaCl₂·2H₂O, 0.02 g/l; MnCl₂·4H₂O, 0.002 g/l; NaMoO₄·2H₂O, 0.001 g/l, FeSO₄·7H₂O, 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 °C, which was maintained for 2 min, then increased by 20 °C/min to 90 °C and further increased by 40 °C/min to 260 °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 °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 °C. The temperature program used was 3 min at 50 °C followed by 10 °C/min to 250 °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
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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\textsubscript{600}) (Figure 3B.2). However, the AC consortium and its individual isolates showed maximum utilization of DCE (0.33 – 0.52 OD\textsubscript{600}). Growth of the isolates and AC consortium was moderate in DCB and 3-chloroaniline (0.13 – 0.35 OD\textsubscript{600}), whereas low in dibromoethane and TCE (0.06 – 0.21 OD\textsubscript{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.](image)

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.

![Graph showing DCE biodegradation by the AC consortium and its individual isolates.](image)

**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$_2$HPO$_4$, 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.
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.

![Graph](image-url)

**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.
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<table>
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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.

![Graph showing CE biodegradation by the AC consortium and its individual isolates.](image)

**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](image_url)

**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](image_url)

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](image-url)

**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).

![Graph showing CE utilization by AC consortium and its individual isolates](image)

**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.
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**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.
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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


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


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